UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

Departamento de Química Analítica

Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional

Programa de doctorado oficial en Química

SCREENING DE VEGETALES Y SUS DERIVADOS COMO FUENTES DE COMPUESTOS BIOACTIVOS: CARACTERIZACIÓN ANALÍTICA, PURIFICACIÓN, Y EVALUACIÓN DE SU ACTIVIDAD *IN VITRO* FRENTE A ENFERMEDADES RELACIONADAS CON EL SÍNDROME METABÓLICO

SCREENING OF VEGETABLES AND DERIVATIVES AS SOURCES OF BIOACTIVE COMPOUNDS: ANALYTICAL CHARACTERIZATION, PURIFICATION, AND EVALUATION OF THEIR *IN VITRO* BIOACTIVITY AGAINST METABOLIC SINDROME AND RELATED DISORDERS

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para optar al grado de:

Doctor Internacional por la Universidad de Granada

Tesis doctoral dirigida por:

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Granada, 2016

Editor: Universidad de Granada. Tesis Doctorales
Autora: Cecilia Jiménez Sánchez
ISBN: 978-84-9125-950-3
URI: http://hdl.handle.net/10481/44030

Esta tesis doctoral ha sido realizada gracias a una beca predoctoral concedida por el Ministerio de Educación, Cultura y Deporte (FPU AP2010-2793) 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 UGR, así como de empresas interesadas en los resultados de la investigación.

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Que el trabajo que se presenta en esta tesis doctoral con el título:

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Y para que así conste, expido y firmo el presente certificado en Granada a 26 de Mayo de 2016.

The will to win, the desire to succeed,

the urge to reach your full potential...

these are the keys that will unlock

the door to personal excellence.

Confucius

Agradecimientos

En primer lugar me gustaría dar las gracias a mis directores de tesis, Alberto Fernández Gutiérrez y Jesús Lozano Sánchez. Alberto, muchas gracias por permitirme formar parte de esta gran familia, acogerme con cariño en este centro, por la confianza depositada en mí desde el principio. Y por supuesto, por tu excelente labor como Director del CIDAF, que ha hecho posible que hoy este trabajo vea la luz. Jesús, muchas gracias por toda tu dedicación, tu paciencia, todas esas horas a mi lado, ayudando siempre en que lo he necesitado. Durante esta etapa me he formado, y he madurado científicamente, y como persona. Gran parte del mérito es vuestro, muchas gracias.

A Antonio Segura, por su apoyo, por su excelente labor como coordinador científico, por velar por la gran familia que es el CIDAF y por su excelente trabajo en este centro. Debo dar las gracias también a Javier Valverde, sin vuestro trabajo, el CIDAF no sería una realidad.

Como no, a todos y cada uno de mis compañeros del CIDAF. A los que están en el centro, y a los ya se marcharon. A los que estuvieron más tiempo con nosotros, y a los que pasaron menos: Celia, María del Mar, Nassima, Patri, Arancha, Ana Gómez, Ana López, Mari Luz, Sandra, Hakim, Vito, Geovanny, Eli, Álvaro, Lidia, Rosa, Jesús, Isa, David Arráez, David Melguizo, Fran, Amani, Ihsan, Ibrahim, Salva, Cristina, Natasa, Cristiano, Edira, Sonda, Xiao Qin, Reham, Elaheh. De uno u otro modo he compartido cosas con cada uno de vosotros, os doy las gracias porque habéis hecho que mi experiencia en el CIDAF sea inolvidable. Me llevo un trocito de cada uno de vosotros. Y a los que acaban de llegar, les deseo lo mejor.

A los compañeros del grupo FQM-297 y del Departamento de Química Analítica, por la ayuda brindada siempre que ha sido necesario.

A mis chicas del IBMC de la Universidad Miguel Hernández de Elche: Mariló, Almu, Vero, Luz, Sara, Noelia, y Teresa. Por la ayuda prestada, en especial a Mariló, por ser mi mentora en cultivos. Por ser todas unas grandes compañeras, trabajadoras, serviciales, atentas, por hacerme (hacernos) sentir parte del grupo. Os voy a echar muchísimo de menos. A María y a

Quique, por ayudar con su sabiduría y buen criterio en todo, siempre con una sonrisa. A Maite, una mujer a la que admiro y aprecio. Y a Vicente Micol, un investigador ejemplar, por permitirme formar parte de su gran equipo, atender mis demandas burocráticas con cariño, y darme la oportunidad de formarme en el campo que tanto he deseado, una experiencia que me ha cambiado la vida.

A Nuria Martí y Domingo Saura, de la Unidad Mixta IBMC – FMC FoodTech, de la Universidad Miguel Hernández de Elche, por disponibilidad y labor en el estudio del zumo de caqui. Gracias.

I would also like to thank Peter Winterhalter for receiving me at Food Chemistry Institute of the Technische Universität of Braunschweig, and for his hospitality. Thanks to my colleagues Miriam, Eva, Margret, Mariana, Stefanie, Philipp, Marcela. Thanks for your help in the lab, and to make me feel like at home.

A mi familia, por su amor incondicional. Por su apoyo, por estar siempre ahí, pase lo que pase. Alberto, Laura, espero que encontréis vuestro camino y lleguéis muy alto.

A mi futuro marido, Aitor. Por haberme transmitido esa pasión por lo que hacemos, por haberme enseñado a mirar alto. Por haber hecho que crea en mí. Eres mi felicidad. Porque a tu lado, todo es perfecto.

A mi buena amiga María Marsilio, gracias por tu colaboración desinteresada en el diseño de esta tesis. Tienes mucho talento.

A las personas que, aunque no aparecen aquí con nombres y apellidos, han estado presentes de alguna forma durante el desarrollo de este trabajo.

A todos,

Muchas gracias

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Abbreviations

3,4-DHPEA-EA, oleuropein aglycone

3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone

4CL, 4-coumaroyl:CoA-ligase

ADP, adenosine diphosphate

AICAR, 5-Aminoimidazole-4-carboxamide ribonucleotide

AMP, adenosine monophosphate

AMPK, adenosine monophosphate -activated protein kinase

ANR, anthocyanidin reductase

ANS, anthocyanidin synthase

ATP, adenosine triphosphate

BCS, bovine calf serum

BPC, base peak chromatogram

C3H, *p*-coumarate-3-hydroxylase

C4H, cinnamate-4-hydroxylase

CaMKKβ, Calcium/calmodulin-dependent protein kinase 2

CCC, counter-current chromatography

CE, capillary electrophoresis

CHI, chalcone isomerase

CHS, chalcone synthase

CPC, centrifugal partition chromatography

DAD, Diode Array Detector

DEX, dexamethasone

DFR, dihydroflavonol reductase

DMEM, Dulbecco's modified Eagle's medium

EIC, extracted-ion chromatogram

ESI, electrospray ionization

ESI, electrospray Ionization

EVOO, extra-virgin olive oil

EVOO-PE, extra-virgin olive oil-phenolic extract

F3H, flavanone 3-hydroxylase

FA, fatty acid

FBS, fetal bovine serum

FDA, Food and Drug Administration

FID, flame ionization detector

FLS, flavonol synthase

FS, flavone synthase

FUFOSE, European Commission Action on Functional Food Science in Europe

FVE, Flash-vacuum expansion

GC, gas chromatography

HCT, hydroxycinnamoyl transferase

HHP, high-hydrostatic-pressure

HILP, high-intensity light pulses

HPC, high-precision calibration

HPLC, high performance liquid chromatography

HSCCC, high speed counter-current chromatography

IBMX, 3-isobutyl-1-methylxanthine

IFS, isoflavone synthase

ILSI, Life Science Institute

LAR, leucoanthocyanidin reductase

LC, liquid chromatography

LKB1, liver kinase 1

MH, microwave heating

MS, mass spectrometry

MS^2, tandem mass spectrometry

mTOR, mammalian target of rapamycin

MUFA, monounsaturated fatty acid

NMR, nuclear magnetic resonance

OH, ohmic heating

PAL, phenylalanine ammonia-lyase

pAMPK phospho adenosine monophosphate-activated protein kinase

PASS, prediction of activity spectra for substances

PEF, pulsed electric fields

PTFE, politetrafluoroetileno

QTOF, quadrupole time-of-flight

RFEF, radiofrequency electric fields

RRLC, rapid resolution liquid chromatography

SC-CO_2, supercritical carbon dioxide

SPE, solid phase extraction

Thr172, Threonine172

TOF, time-of-flight

UPLC, ultra performance liquid chromatography

US, power ultrasound

UV-Vis, ultraviolet-visible

WHO, World Health Organization

Objectives

The overall objective of this PhD dissertation is the screening, evaluation of bioactivity, purification of plant compounds with functional properties, and the establishment of the impact of technological processing as a strategy in the development of functional foods. This broad objective is subdivided into three main sub-goals:

1. In the first, the aim is to expand knowledge of the bioactive fraction of extracts derived from green asparagus (*Asparagus officinalis*) and marula bark (*Sclerocarya birrea*). This experimental work is based on the analytical potential of the platform QTOF-RP-HPLC-ESI/MS^2. The results of this characterization are meant to enable an in-depth examination of the metabolic profile of plant sources known to have healthy properties attributed by the nutritional epidemiology as well as by numerous *in vivo* and *in vitro* scientific studies.

2. Secondly, the goal is to assess the bioactivity of extracts leaf of olive (*Olea europaea*) and the potential of different methodologies for isolating and purifying bioactive compounds from extra-virgin olive oil and olive-leaf extracts. For this, the phenolic profile of the above-mentioned extracts will be exhaustively characterized by RP-HPLC-ESI-TOF/MS. Regarding extra-virgin olive oil, the aim will be to optimize the purification of decarboxymethyl, oleuropein aglycone, a compound with demonstrated anticancer bioactivity, by countercurrent chromatography (HSCCC) and high-performance liquid chromatography (HPLC), both of these at semi-preparative scale, for later comparison of the two methodologies. With regards to olive leaf, the *in vitro* anti-obesity potential of both the full extract and fractions purified are evaluated by semi-preparative HPLC in a hypertrophic adipocite model.

3. The third and final partial sub-goal is the evaluation of the effect that different alternatives of the manufacturing process of fruit juices have on their content of bioactive compounds. This entails a review of the state of the art and scientific knowledge about the effect of each of these technologies has on the composition of bioactive compounds. Finally, experimental pilot plant design is planned

with different combinations of fruit-juice processing technologies as a strategy to preserve/increase the content of bioactive compounds of the final product. Persimmon (*Diospyros kaki*) is used as vegetable source, and the effect of different treatment combinations on the final composition of juice is assessed by HPLC-DAD-ESI-TOF/MS.

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El objetivo general de la presente Tesis Doctoral es el **screening, evaluación de la bioactividad y purificación de compuestos de origen vegetal** con propiedades funcionales, así como el establecimiento del impacto del **procesado tecnológico como estrategia en el desarrollo de alimentos funcionales**. Este amplio objetivo, se va a subdividir en tres grandes objetivos parciales:

1. En el primero se pretenderá profundizar en el conocimiento de la fracción bioactiva de extractos obtenidos a partir de espárrago verde *(Asparagus officinalis)* y corteza de marula *(Sclerocarya birrea)*. Para ello el trabajo experimental a llevar cabo estará basado en el potencial analítico que ofrece la plataforma RP- HPLC -ESI- QTOF/ MS^2. Los resultados de caracterización podrán permitir profundizar en el perfil metabólico de ambas fuentes vegetales que presentan propiedades saludables atribuidas por la epidemiología nutricional así como por numerosos estudios científicos con ensayos *in vivo* e *in vitro*.

2. En el segundo objetivo parcial se tratará de evaluar la bioactividad de extractos de aceite de oliva virgen extra y hoja de olivo *(Olea europaea)* así como el potencial de diferentes metodologías para el aislamiento y purificación de compuestos bioactivos. Para tal fin, se realizará una caracterización exhaustiva del perfil de compuestos fenólicos de los extractos de las mencionadas matrices mediante RP-HPLC-ESI-TOF/MS. Con respecto al aceite de oliva, se pretende optimizar la purificación de la decarboximetil oleuropeina aglicona, compuesto con bioactividad anticancerígena demostrada mediante cromatografía en contracorriente (HSCCC) y

cromatografía líquida de alta resolución (HPLC), ambas a escala semi-preparativa, para posterior comparación de la aplicación de ambas técnicas en la purificación del citado compuesto. En el caso particular de la hoja de olivo, se evaluará el potencial *in vitro* frente a modelos de obesidad tanto del extracto completo como de las fracciones purificadas mediante HPLC semi-preparativa.

3. El tercer y último objetivo parcial plantea evaluar el efecto que diferentes alternativas del proceso de elaboración de los zumos tienen en el contenido en compuestos bioactivos. Para ello se procederá a una revisión del estado de la técnica y del conocimiento científico sobre el efecto que cada una de estas etapas tienen sobre la composición en compuestos bioactivos. Finalmente, se desarrollarán en planta piloto diferentes combinaciones de tecnologías de elaboración de zumos como estrategias para preservar/incrementar el contenido en compuestos bioactivos del producto final. Como fuente vegetal se utilizará el caqui (*Diospyros kaki)* y se estudiará el efecto de las diferentes combinaciones de tratamiento sobre la composición final de zumo obtenido mediante HPLC–DAD–ESI-TOF/MS.

Summary

Today's society is characterized by a growing understanding of the diet-health relationship, demanding a diet based on natural, safe and functional products. Therefore, different branches of science have focused on the development of these functional foods, which provide a variety of beneficial physiological effects beyond the accepted nutritional benefits. Functional foods are composed of a series of compounds responsible for such functions, which are called bioactive compounds. Among these, phenolic compounds comprise one of the families of bioactive compounds that have aroused the greatest interest, as evidenced by the growing number of scientific publications that highlight their healthy effects on different areas of nutrition and medicine.

This context gave rise to the present PhD dissertation entitled "Screening of Vegetables and Derivatives as Sources of Bioactive Compounds: Analytical Characterization, Purification, and Evaluation of Their in vitro Bioactivity Against Metabolic Syndromes and Related Disorders". In the practical work for the dissertation, the following plant sources were selected: green asparagus, marula bark, extra-virgin olive oil, olive leaf, and persimmon juice. This dissertation has been divided into two sections: the Introduction section and the experimental part.

The Introduction section reviews the literature on the bioactive compounds present in the plant matrixes under study, with a special focus on phenolic compounds. A description is included of the analytical techniques used for characterization, isolation and purification of bioactive compounds, as well as bioactivity assays and functional-food formulation strategies.

The experimental part presents the results of this dissertation and has been divided into three blocks:

Block I is dedicated to the screening of bioactive compounds (mainly phenolic compounds) in the shoots of green asparagus (*Asparagus officinalis*), and the bark of the African marula tree (*Sclerocarya birrea*). Chapter 1 describes the application of high-resolution liquid chromatography

in reverse-phase tandem mass spectrometry (RP-HPLC-ESI-QTOF/MS^2) for the analytical characterization of a hydroalcoholic extract of green asparagus. In Chapter 2, the potential is demonstrated for the same analytical methodology to evaluate the metabolic profile of different hydro-alcoholic extracts with anti-diabetic properties of the marula bark.

Block II is focused on the development of analytical methodologies aimed at isolating and purifying bioactive compounds for the exploration of their biological activity against the pathologies of modern society. In Chapter 3, the optimization of the purification of a secoiridoid present in a phenolic extra-virgin olive-oil extract obtained by solid phase extraction (SPE) is described. For this, a comparative study was made between high-performance liquid chromatography (HPLC) coupled to time-of-flight mass spectrometry (TOF-MS) and countercurrent chromatography (HSCCC) coupled to ultraviolet-visible spectroscopy (UV-Vis) with a diode array detector (DAD), both at a semi-preparative scale. Part of this work was conducted at the Institute of Food Chemistry at the Technical University of Braunschweig (Germany). Chapter 4 demonstrates the potential of the olive leaf as a source of bioactive compounds with application in metabolic syndrome-related diseases. An analytical characterization by RP-HPLC-ESI-TOF/MS of a solid-liquid olive leaf extract has been performed, and its anti-obesity potential has been evaluated using an *in vitro* murine hypertrophic adipocyte model. Then, the phenolic compounds present in the extract were fractionated by semi-preparative HPLC-TOF/MS to determine which compound/s could be responsible for reducing the accumulation of intracellular lipids through AMPK-dependent mechanisms. Part of this work was performed at the Institute of Molecular and Cell Biology at Miguel Hernández University of Elche.

Block III concerns the study of different fruit-juice processing strategies and their effect on the content of bioactive compounds of the final product. Chapters 5 and 6 include a literature review on alternatives to conventional heat treatment aimed at microbiological and enzyme inactivation

as well as increased production efficiency. Next, the impact of these alternatives on the physicochemical, rheological, and organoleptic properties of the juice as well as the phytochemical content is reviewed in greater detail. Finally, Chapter 7 ends this block and the dissertation by evaluating the effect that different technological combinations applied in the extraction of persimmon juice (clarification, reduction of astringency, flash-vacuum expansion, centrifugation, and pasteurization) exert on the composition of the hydroalcoholic extracts by HPLC-DAD-ESI-TOF/MS. This work was done in collaboration with the Department of Agrifood Technology, Joint Unit, F.M.C. Food-Tech University Miguel Hernández of Elche.

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La sociedad actual se caracteriza por tener, cada vez más, un mayor conocimiento de la relación que existe entre la dieta y la salud, por lo que suele demandar una alimentación basada en productos naturales, seguros y que sean también funcionales. Es por ello que diferentes ramas de la ciencia se han ocupado en el desarrollo de estos alimentos funcionales, que además de proporcionar nutrientes suficientes para satisfacer los requerimientos metabólicos de un individuo, producen una serie de efectos fisiológicos beneficiosos, más allá de los beneficios nutricionales aceptados. Los alimentos funcionales se caracterizan por tener en su composición una serie de compuestos que son responsables de tales funciones, a los que se denominan compuestos bioactivos. Entre todos ellos, los compuestos fenólicos son una de las familias de compuestos bioactivos que han despertado un mayor interés como lo demuestra el creciente número de publicaciones científicas que ponen de manifiesto sus efectos saludables en los diferentes ámbitos de la nutrición y la medicina.

Esta memoria recoge los resultados obtenidos durante la realización de la tesis doctoral titulada “Screening de vegetales y sus derivados como fuentes de compuestos bioactivos: caracterización analítica, purificación, y evaluación de su actividad in vitro frente a enfermedades

relacionadas con el síndrome metabólico". Para los trabajos se seleccionaron las siguientes fuentes vegetales: *Asparagus officinalis, Sclerocarya birrea, Olea europaea,* y *Diospyros kaki*, quedando dividida la memoria en dos secciones: introducción y parte experimental.

La **INTRODUCCIÓN** incluye una revisión bibliográfica acerca de los compuestos bioactivos presentes en las matrices vegetales objeto de estudio, con especial interés en los compuestos fenólicos. También se incluye una descripción de las técnicas analíticas utilizadas en la caracterización, aislamiento y purificación de compuestos bioactivos, así como de los ensayos de bioactividad y de estrategias de formulación de alimentos funcionales.

La **PARTE EXPERIMENTAL**, expone los resultados obtenidos en el desarrollo de la presente tesis doctoral, y se ha dividido en tres bloques:

El bloque I se ha dedicado al screening de compuestos bioactivos (principalmente compuestos fenólicos) en la parte aérea de espárrago verde (*Asparagus officinalis*), y la corteza del árbol africano de la marula (*Sclerocarya birrea*). En el capítulo 1 de este bloque se describe la aplicación de cromatografía líquida de alta resolución en fase reversa acoplada a espectrometría de masas en tándem (RP- HPLC -ESI- QTOF/ MS^2) en la caracterización analítica de un extracto hidroalcohólico de espárrago. En el capítulo 2, se demuestra el potencial de la misma metodología analítica para evaluar el perfil metabólico de distintos extractos con propiedades antidiabéticas de la corteza de la marula.

El bloque II se ha centrado en el desarrollo de metodologías analíticas dirigidas al aislamiento y purificación de compuestos bioactivos para la exploración de su actividad biológica frente a patologías de la sociedad actual. En el capítulo 3, primero de este bloque, se describe la optimización de la purificación de un secoiridoide presente en un extracto fenólico de aceite de oliva obtenido mediante extracción en fase sólida (SPE). Para ello se ha realizado un estudio comparativo entre la cromatografía líquida de alta resolución (HPLC) acoplada a espectrometría

de masas de tiempo de vuelo (TOF-MS) y la cromatografía en contracorriente (HSCCC) acoplada a espectroscopía ultravioleta-visible (UV-Vis), ambas a escala semi-preparativa. Parte de este trabajo se realizó en el Instituto de Química de los Alimentos de la Universidad Técnica de Braunschweig (Alemania). En el capítulo 4, se demuestra el potencial de la hoja del olivo como fuente de compuestos bioactivos con aplicación en patologías relacionadas con el síndrome metabólico. Se ha llevado a cabo una caracterización analítica mediante RP-HPLC-ESI-TOF/MS de un extracto sólido-líquido de hoja de olivo y se ha evaluado su potencial *in vitro* en un modelo hipertrófico adipocitario murino. A continuación, los compuestos fenólicos presentes en el extracto se fraccionaron mediante HPLC-TOF-MS a escala semi-preparativa para establecer qué compuesto/os podrían ser los responsables de la reducción de la acumulación de lípidos intracelulares a través de mecanismos dependientes de AMPK. Parte de este trabajo se realizó en el Instituto de Biología Molecular y Celular de la Universidad Miguel Hernández de Elche.

El bloque III se ha dirigido al estudio de diferentes estrategias de elaboración de zumos y su efecto en el contenido en compuestos bioactivos. En los capítulos 5 y 6 se ha incluido una revisión bibliográfica sobre las alternativas al tratamiento térmico convencional destinadas a inactivación microbiológica, enzimática, y al aumento en el rendimiento de la producción. A continuación se ha desarrollado con mayor nivel de detalle el efecto de estas alternativas en las propiedades fisicoquímicas, reológicas, y organolépticas de los zumos así como en el contenido en fitoquímicos. Para finalizar este bloque y la memoria, en el capítulo 7 se ha evaluado el efecto de distintas combinaciones tecnológicas aplicadas en la extracción del zumo de caqui (clarificación, reducción de la astringencia, expansión súbita, centrifugación, y pasteurización) sobre la composición de los extractos hidro-alcohólicos mediante HPLC–DAD–ESI-TOF/MS.

Este trabajo se realizó en colaboración con el Departamento de Tecnologia Agroalimentaria, Unidad Mixta, F.M.C. Food-Tech de la Universidad Miguel Hernández de Elche.

Introduction

The link between food and health has been documented since Antiquity. In the 5th Century BC, Hippocrates asserted the now famous phrase "let food be thy medicine and medicine be thy food", unaware of how pertinent his tenet would remain after 2500 years. However, it was not until the 19th and 20th Century that scientific evidence started to demonstrate a valid link between food and health thanks to epidemiolgy, biology, biochemistry, and animal experimentation. This led to the concept of balanced diet, as a result of a century of research in nutrition as a consequence of the discovery of nutrients and their requirements for the development, growth, and maintenance of the body. At the turn of the 21st century, the industrialized world faced new challenges, i.e. an enormous increase in the costs of health care, longer life expectancy, improved scientific knowledge, development of new technologies, and major changes in lifestyles. As a consequence, nutrition scientists wish to rise to these new challenges and have embraced the idea of "optimal nutrition", which focuses on optimizing the quality of the daily diet in terms of its content of nutrients and non-nutrients as well as other food properties that favor the maintenance of health. In this context, a new generation of concepts and products has emerged such as functional food or nutraceuticals, among others (Doyon & Labrecque, 2008).

Although functional food is a recent concept that originated in Japan in 1991, it was not until 1999 that the European Commission Action on Functional Food Science in Europe (FUFOSE) became actively involved when a large number of the most prominent European experts in nutrition and related sciences were coordinated by the International Life Science Institute (ILSI Europe) to reach a consensus on "Scientific concepts of functional foods in Europe". Because functional food is a concept rather than a well-defined group of food products, the FUFOSE consensus document proposed a working definition. A food can be regarded as "functional" if it is satisfactorily demonstrated to benefit one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant either to an improved state of health and well-being and/or to a

reduction of disease risk. Functional foods must remain foods, and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. They are not pills or capsules, but part of a normal dietary pattern (Diplock *et al.,* 1999).

Instead, a nutraceutical has been defined as a dietetic supplement that provides a concentrated form of a presumably bioactive agent from a food, presented in a non-food matrix, and used to enhance health in dosages that exceed those that could be derived from normal food (Barnes & Prasain, 2005). According to this definition, the main difference between a nutraceutical and a functional food is the form and the dosage. While a functional food must remain food, a nutraceutical could be presented in a pharmacological form such as tablets, pills, syrups etc. and therefore it could deliver the bioactive component in a higher dose than found in functional food. Whether they are used for treating or preventing disease remains a matter of debate. Besides the differences between them, their development shares a common structured approach. This process involves different stages, which are diagrammed in Figure 1.

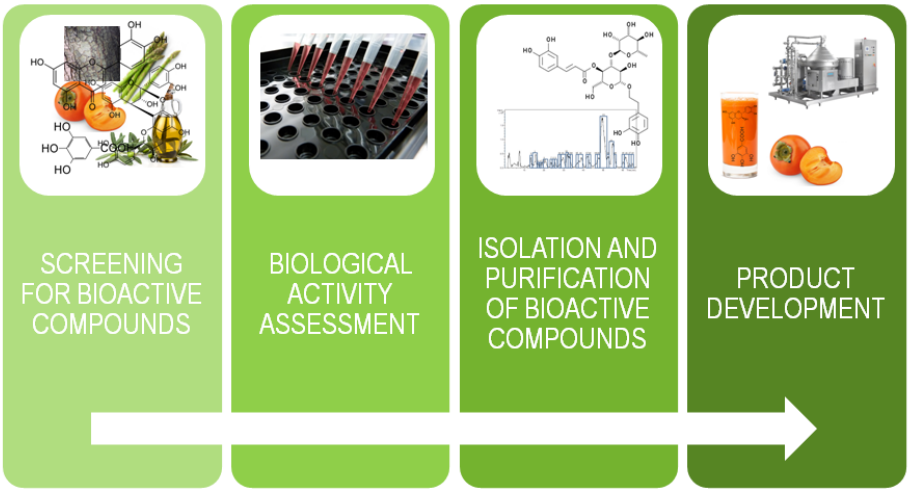

Figure 1. Basic scheme of functional food and nutraceuticals development process.

1. Screening for bioactive compounds

The first step in the functional food and nutraceutical development process involves the screening for possible bioactive analytes in the target source. A bioactive compound is a compound that exerts an effect on a living organism, tissue or cell. These could come from any of the kingdoms of nature, although the present doctoral dissertation is focused on bioactive compounds from the plant kingdom.

1.1. Bioactive compounds of plant origin

Throughout history, several types of plants have been used in traditional medicine to treat diseases in humans as well as animals. This ability has been associated, at least partly, to the secondary metabolites or phytochemicals they contain, which have been demonstrated to exert a wide variety of biological activities. There is a broad variety of bioactive compounds with diverse structures and biological activities, some of which are listed in Figure 2.

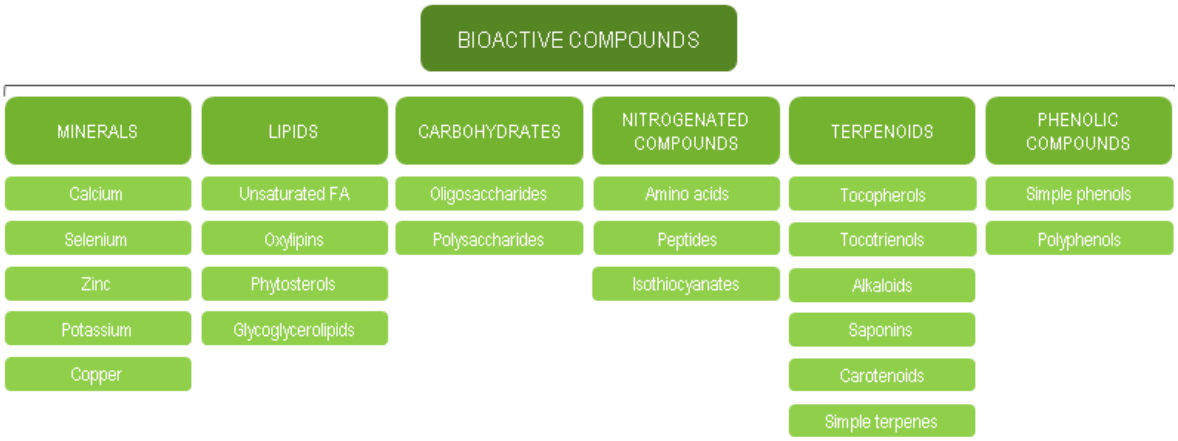

Figure 2. Classification of some bioactive compounds.

Plants have been demonstrated to be a rich source of bioactive compounds with a large variety of chemical structures. Several of these compounds are found to have important functions in the

living plants. Among them, phenolic compounds are one of most widely studied chemical families due to their broad distribution in the plant kingdom, diversity, and biological activity.

There are more than 8000 known structures of phenolic compounds, although they generally present an aromatic ring with one or more hydroxyl substituents. There are several classes and subclasses of phenolic compounds, some of which are illustrated in Figure 3.

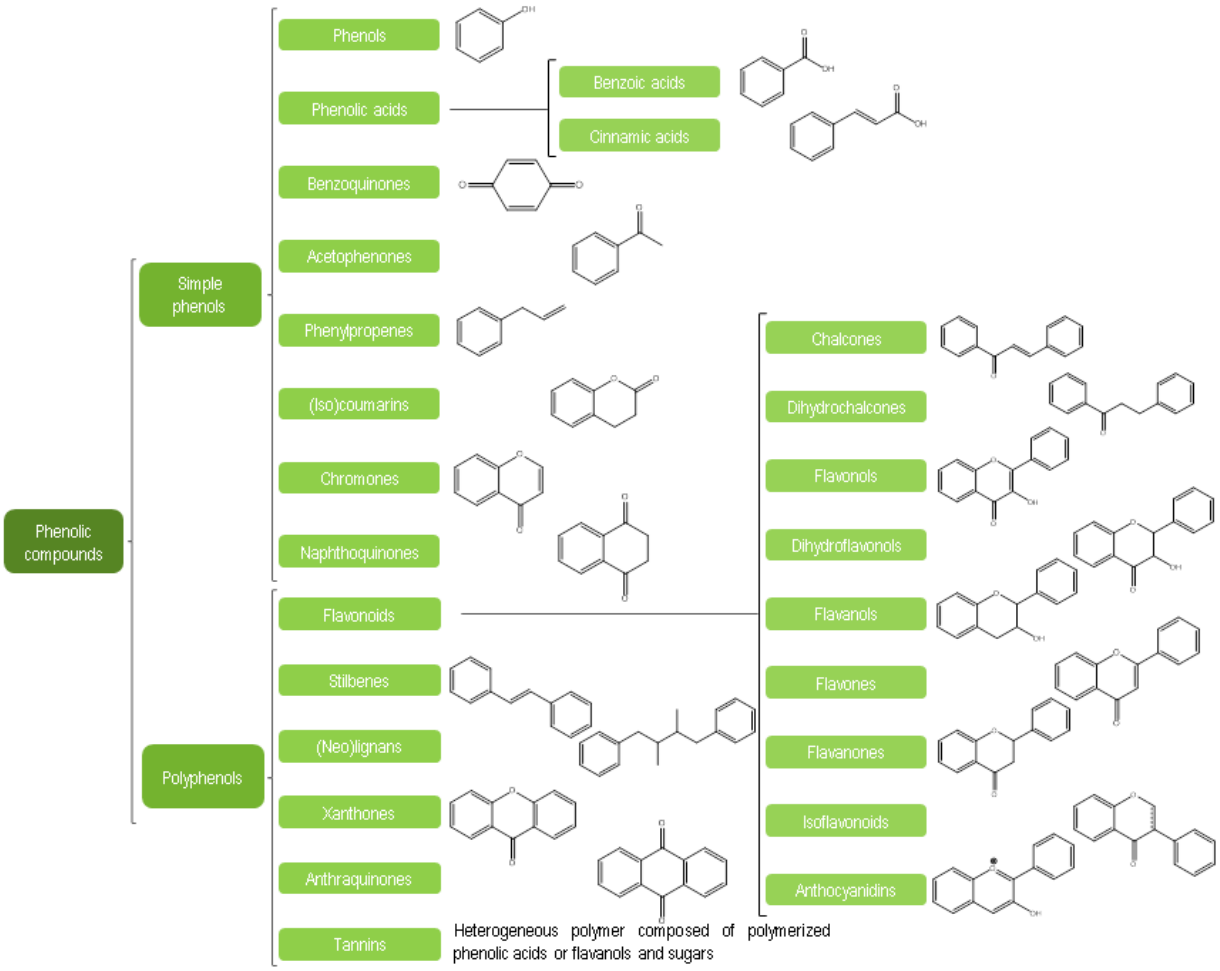

Figure 3. Classification of most phenolic compounds.

As can be seen in the above figure, phenolic compounds are divided into simple phenols, which are formed by one phenolic structure, and polyphenols, which include more than one in their structure. Phenolic compounds are normally conjugated to one or more sugar residues linked to

their hydroxyl groups, or even to the aromatic backbone. These sugars can be preferably glucose, but also xylose, rhamnose, galactose, or arabinose, as well as glucuronic or galacturonic acids, among others. Conjugations with other compounds such are carboxylic and organic acids, amines, lipids, and even other phenolic compounds are also common (Bravo, 1998; Tsao, 2010).

Plant phenolic compounds arise biogenetically from the shikimate/phenylpropanoid pathway, which directly provides phenylpropanoids, or the 'polyketide' acetate/malonate pathway, this being capable of producing simple phenols, or both of them (Quideau *et al.,* 2011) (Figure 4). These pathways produce a bewildering array of monomeric and polymeric structures of large structural diversity.

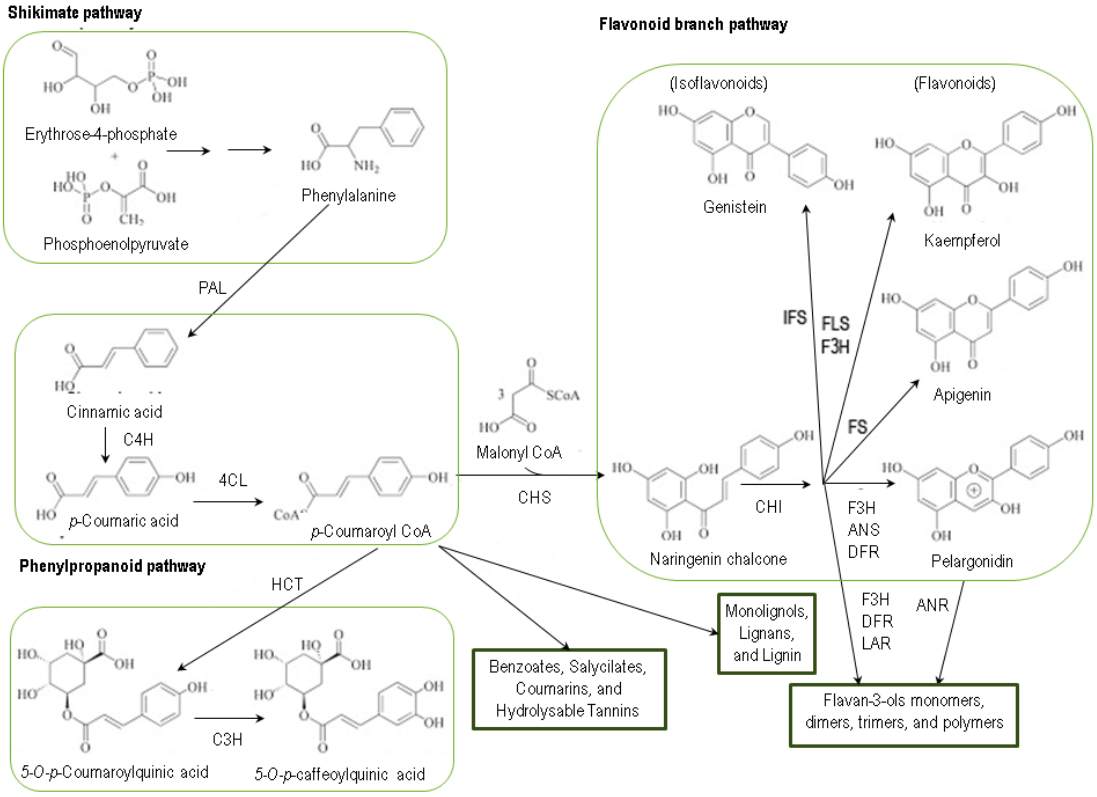

Figure 4. Most phenolic compounds biosynthesis pathways. PAL, phenylalanine ammonia-lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; HCT, hydroxycinnamoyl transferase; C3H, *p*-coumarate-3-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; ANS, anthocyanidin synthase; DFR, dihydroflavonol reductase; FS, flavone synthase; FLS, flavonol synthase; F3H, flavanone 3-hydroxylase; IFS, isoflavone synthase; ANR, anthocyanidin reductase; LAR, leucoanthocyanidin reductase. Adapted from Cheynier *et al.,* 2013.

Given that this structural variet fulfills a very broad range of physiological roles in plants, these molecules appear to be involved in diverse biological activities. Indeed, there is epidemiological evidence linking a diet rich in phenolic compounds with reduced incidences of several pathologies such as coronary heart disease, cancer, and various chronic diseases (Margetts & Buttriss, 2008). In an attempt to establish the molecular mechanisms underlying these beneficial effects, several studies conducted *in vivo* and *in vitro* worldwide that have supported shed light on such hypotheses. Specifically, Figure 5 shows some of the action mechanisms and their respective biological activities reported to date. Due to their functional potential, the scientific interest in phenolic compounds of plant origin has intensified in recent years.

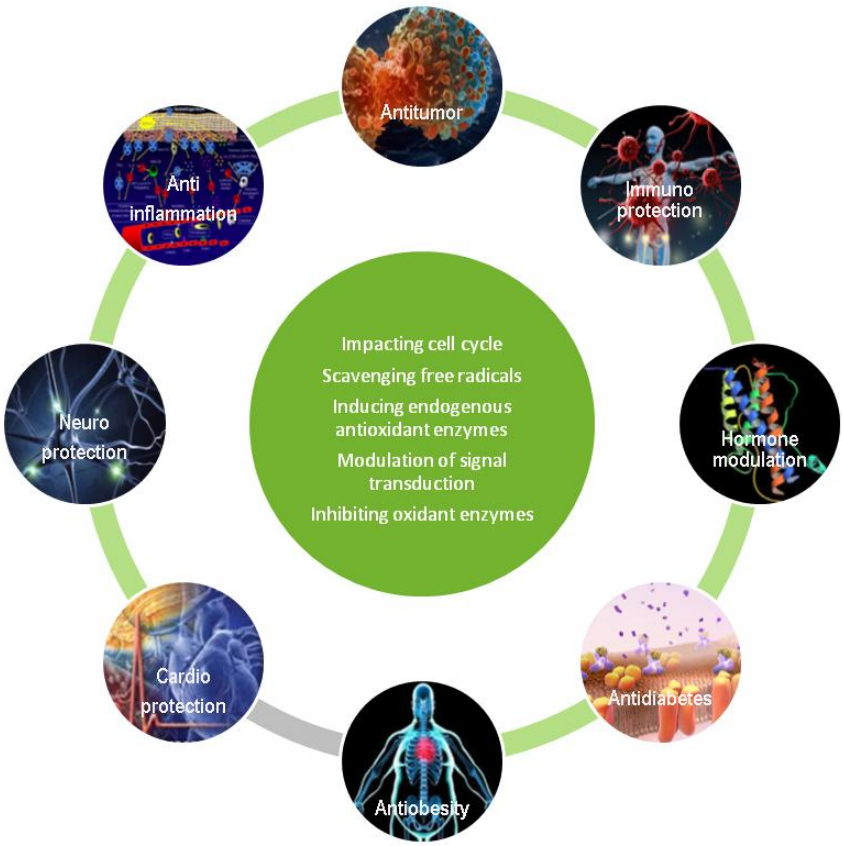

Figure 5. The main biological activities attributed to phenolic compounds. Adapted from (Li et al., 2014; Han et al., 2007).

1.2. Plant sources of phenolic compounds

Classical plantl sources of bioactive compounds with bioactive properties include wine by-products (Del Pino-García *et al.,* 2016), rosemary *(Rosmarinus officinalis)* (Santana-Méridas *et al.,* 2014), and lemon verbena *(Lippia citriodora)* (Herranz-López *et al.,* 2015), just to name a few. In the research for this dissertation, four different vegetal matrixes were selected to deal with the full process of the functional-food development process (Figure 6).

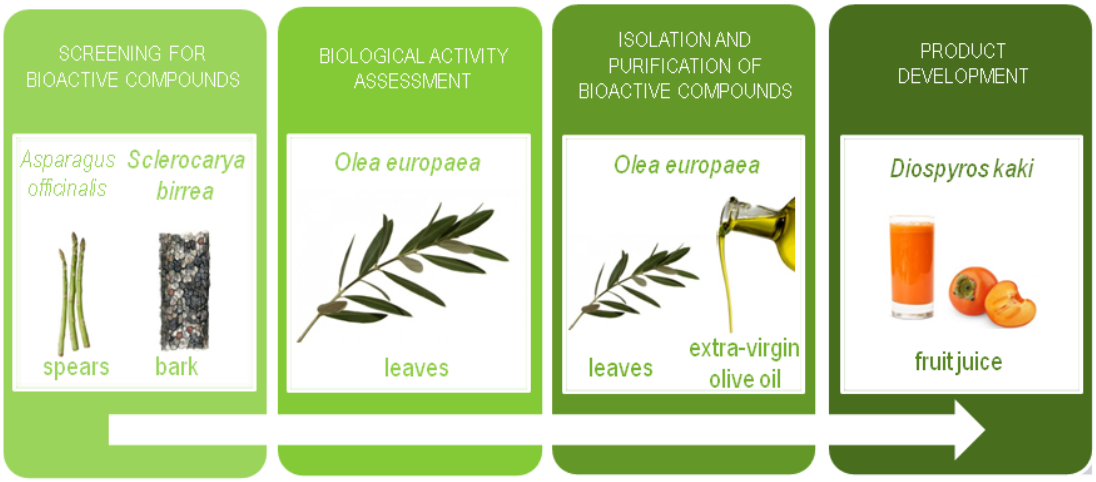

Figure 6. Matrixes selected, according to the functional-food development process for which they have been selected.

Asparagus officinalis

Asparagus or green asparagus, scientific name *Asparagus officinalis*, is a spring vegetable, a feathery-looking flowering perennial plant. Only early shoots, called asparagus spears, are commonly harvested, as once the buds start to open, the spears quickly turn fibrous (Figure 7). It is native to most of Europe, northern Africa, and western Asia, and is widely cultivated as a vegetable crop.

Green asparagus has recently received increasing research interest because of its important nutritional and medicinal effects, such as antidiabetic, liver-protective, or antioxidant properties (Lee *et al.,* 2014; Zhong *et al.,* 2015).

While water makes up 93% of asparagus composition, it is a good source of vitamins B_6, C, E, and K, thiamin, riboflavin, and folic acid, as well as minerals such as calcium, magnesium, and zinc. In addition, it is an excellent source of dietary fiber. Phytochemical investigations have revealed that asparagus contains flavonoids, specifically di-and tri-glycosides of quercetin, kaempferol, and isorhamnetin (Fuentes-Alventosa *et al.,* 2008); saponins (Wang *et al.,* 2003); and hydroxycinnamic acids (Rodríguez *et al.,* 2005).

Figure 7. Young Asparagus officinalis spears.

Sclerocarya birrea

Commonly known as the marula, it is a medium-sized dioecious tree, indigenous to the miombo woodlands of southern Africa, the Sudano-Sahelian range of West Africa, and Madagascar (Figure 8).

The fruit, which is about the size of a small plum, is pale-yellow when ripe, highly aromatic with a sweet-sour taste. The fruit is rich in soluble phenolics (derivatives of hydrolyzable tannins, catechins, and hydroxycinnamic acid), minerals, and high vitamin C content (of 168 mg/100 g)

(Borochov-Neori *et al.,* 2008). In Africa, it is used to make the cream liqueur Amarula, it is ground into a frozen puree to make in juice blends, and it serves as an ingredient in cosmetics.

The bark is known for its ethnotherapeutic uses in South African traditional medicine. It has traditionally been used to treat diarrhea, dysentery, proctitis, stomach ailments, ulcers, inflammation, arthritis, hypertension, skin diseases, fever, malaria, and diabetes mellitus *(Gondwe et al.,* 2008; Ojewole, 2006; Ojewole, 2003; van Wyk *et al.,* 2002). Reports in the biomedical literature have indicated the presence of medicinally-major chemical constituents in the plant, notably gallotannins, flavonoids, steroids (including β-sitosterol), coumarins, triterpenoids, sesquiterpene hydrocarbons, ascorbic acid, oleic, myristic, stearic acids, and amino acids (Iwu, 1993; van Wyk *et al.,* 2002). Still, a large amount of chemical information remains unknown and there are no reports available regarding its phenolic profile.

Figure 8. *Sclerocarya birrea* (marula) tree.

Olea europaea

Commonly known as olive, it is a dense, medium-sized evergreen tree (Figure 9), which produces one of the most important crops of the Mediterranean basin. In fact this tree constitutes one of the foundations of the agriculture of this region as the source of olive oil. In 2013, world production of virgin olive oil was 2.8 million tonnes, Spain producing 39% of world production (Food and

Agricultural Organization of the United Nations, Statistics Division., 2013). The most cultivated cultivars depend on the final use of the olive, i.e. whether for eating or making olive oil. For table olives, the most common variety is Manzanilla, with a high pulp:stone ratio, low oil content, and good properties for technological processing. For olive-oil production, the varieties with the best oil content and yield, and particular organoleptic properties, include such cultivars as Picual, Hojiblanca, Arbequina, Cornezuelo, Cornicabra, although there are hundreds of varieties throughout the Mediterranean region.

Extra-virgin olive oil is the product of pressing whole olives, exclusively by mechanical means. Extra-virgin olive oil contains monounsaturated, polyunsaturated, and saturated fatty acids mainly in the form of esters with glycerol (triacylglycerols), which constitute more than 98% of the total olive-oil content. Important minor components in olive oil include sterols, hydrocarbons, volatile compounds, terpenols and terpenic acids, and phenolic compounds (e.g. phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavonoids). Phenolic compounds are present mostly in aglycone form, as a result of the activity of many enzymes that are released during the pressing and malaxation steps of oil production (Dais & Hatzakis, 2013; Talhaoui *et al.,* 2016). The literature has revealed that extra-virgin olive oil might exert anticancer, cardioprotective, neuroprotective, gastroprotective, hepato-protective, anti-diabetes, anti-obesity, radioprotective, antioxidant, and anti-inflammatory effects (Hassen *et al.,* 2015; Scoditti *et al.,* 2014; Whayne, 2014).

Olive fruit contains an appreciable concentration, 1–3% of fresh pulp weight, of hydrophilic compounds (mostly hydroxytyrosol glucoside and verbascoside), which are known to possess multiple biological properties such as antioxidant, anticarcinogenic, antiinflammatory, antimicrobial, antihypertensive, antidyslipidemic, cardiotonic, laxative, and antiplatelet (Ghanbari *et al.,* 2012; Talhaoui *et al.,* 2016). Other important compounds present in olive fruit are pectin, organic acids, and pigments.

Due to the potential of the phenolic compounds present in the olive fruit and oil, several studies have been sought alternative sources of these compounds, such as the olive leaves. It is estimated that olive leaves might account for up to 5% of harvested olives weight, being a cheap raw material, which can be used as a source of high-value-added products. Olive leaves have long been known for their therapeutic and medicinal properties, being used in both traditional and modern medicine. Traditionally, olive leaves have been used to treat febrile symptoms of malaria, while recent investigations have suggested important antioxidant, anti-inflammatory, anti-atherogenic, antidiabetic, and antimicrobial activities, and even possible anti-cancer effects (Herrero *et al.,* 2011; Zhang *et al.,* 2014; Zun-Qiu *et al.,* 2015). These beneficial properties have been attributed in part to their phenolic content, including secoiridoids, flavones, hydroxycinnamic acids, flavonols, flavan-3-ols, and substituted phenols (Laguerre *et al.,* 2009; Meirinhos *et al.,* 2005).

Figure 9. Olea europaea tree (top), Olea europaea leaves and olive fruit (bottom left), and olive oil (bottom right).

In the present doctoral dissertation, extra-virgin olive oil has been employed for extraction and purification of compounds with demonstrated bioactivity using different isolation techniques, while

olive leaves have been used for purifying bioactive fractions, and bioactivity assessment against current pathologies such as obesity.

Diospyros kaki

Known as persimmon, this tree is grown worldwide, with 90% of the total in China, Japan, and Korea (Figure 10). Its fruit is from yellow to reddish-orange, sweet, slightly tangy with a soft to occasionally fibrous texture. In general, there are two types of persimmon varieties: astringent and non-astringent. Astringent varieties such as the Japanese Hachiya or the Spanish "Rojo Brillante" contain very high levels of soluble tannins, which are reduced over the shelf-live. The fruit is not edible in its crisp, firm state, and it tastes best when allowed to soften after harvest. Ripening can be accelerated by adding ethylene gas to the atmosphere in which the fruit is stored. Other chemicals are used commercially to artificially speed up or delay ripening. Examples include alcohol and carbon dioxide, which change tannin into the insoluble form. Non-astringent varieties such as Fuyu, are not actually free of tannins as the term suggests but rather are far less astringent before ripening and lose more of their tannic quality sooner. Non-astringent persimmons may be consumed when still very firm and remain edible when very soft.

The health-promoting potential of persimmon includes its effectiveness against free-radical production, hypercholesterolemia, diabetes mellitus, cancer, skin disorders, and hypertension, among ailments (Butt *et al.,* 2015).

In addition, persimmons have nutritional value due to their high content in vitamins, carotenoids, organic acids, as well as phenolic compounds such as *p*-coumaric acid, catechin, epicatechin, epigallocatechin, and proanthocyanidins; also they are a good source of fiber (González *et al.,* 2015).

The persimmon fruit is also used for production of persimmon juice, which is emerging now in the global juice market. This juice has shown strong antioxidant activities that might be correlated with high phenolic acid and catechin contents (Lee *et al.,* 2012).

Figure 10. Diospyros kaki tree and fruit before harvest.

This fruit has been employed in the present doctoral dissertation to cover the last link in the functional-food production process. Specifically, an evaluation was made concerning the impact of different fruit-juice processing technologies on the phytochemical content of the juice, mainly phenolic compounds, through analytical characterization.

2. Analytical characterization

As mentioned above, phenolic compounds present a series of functional properties that have attracted the interest of the scientific community. The need to delve into the knowledge of these compounds has made analytical characterization a tool for the qualitative and/or quantitative identification of the components present in a specific sample, thereby enabling the screening for bioactive compounds in plant sources. The analytical procedure typically consists of a number of equally important steps for previous operations which include sampling, sample treatment, and extraction, analysis, and data processing (Figure 11).

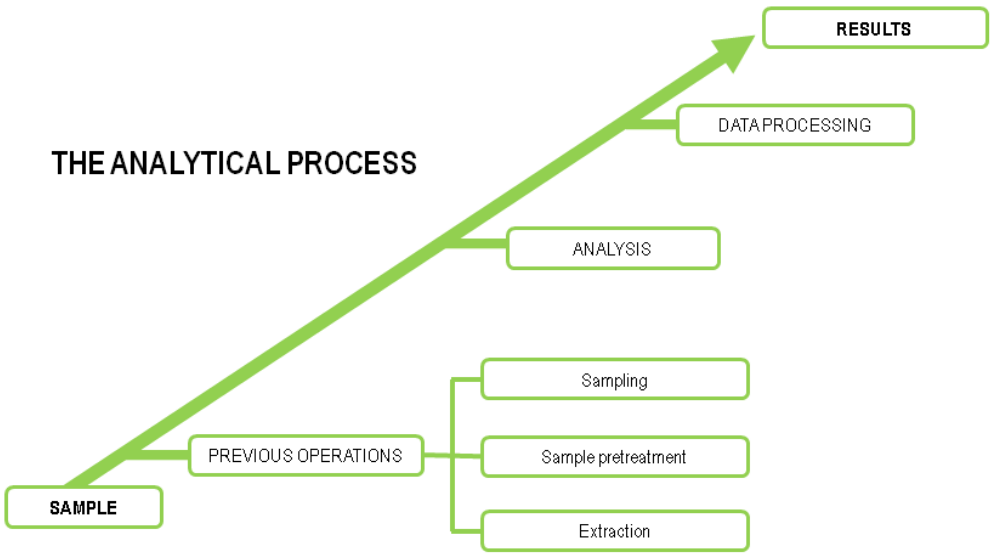

Figure 11. The analytical process used for charaterization approaches.

2.1. Previous operations

It bears stressing that the previous operations largely determine the quality of the results found and are the main source of systematic errors and random lack of precision of analytical methodologies. These operations must guarantee a quantitative recovery of analytes, avoiding contamination and providing matrix isolation as far as possible, in order to reduce potential interference and matrix effects during the measurement stage (Armenta *et al.,* 2015).

Sampling

The content in plant analytes may vary markedly among cultivars depending on their genetic background and on other factors, such as cultural practices (conventional, organic) development stage, growing conditions (climate, temperature), and post-harvest management and processing. Therefore, careful sampling is essential in order to guarantee the representativity of the pool analyzed. Samples must be collected without contamination, and properly handled for the

analytical results to be meaningful, minimizing the loss of compounds of interest during the transportation and preservation of the samples.

Sample pretreatment

Samples generally require other pretreatments before extraction to reduce sample size and to avoid loss of bioactive compounds from the plant material during storage, such as dehydration. Vegetable matter can be dehydrated by different methods such as natural drying (drying in the shade), hot-air drying, or freeze-drying. Natural drying and hot-air drying usually have lower costs, but freeze-drying is generally better to preserve the quality of plant parts, because of the use of low temperatures (Pinela *et al.,* 2011). Regarding freeze-drying, the literature has revealed that freezing at -20ºC, followed by two successive drying steps at 0.5 mbar and 0.1 mbar, preserves the total phenolic content in red tomatoes (Georgé *et al.,* 2011). Other authors comparing freeze-drying with other drying methods have reported that freeze-dried cocoa beans (when dried in the freeze drier at -30ºC for 24 h and -50ºC for 6 h) contained significantly more phenolic compounds than did samples dried in the sun and at 80ºC in the oven (Hii *et al.,* 2009).

Another pretreatment commonly applied to vegetal samples is the reduction of the particle size, as this ensures proper contact between the sample and the solvent used in the subsequent extraction process. A wide variety of equipment is available for this purpose, from scissors to mills and grinders etc.. It is necessary to take into account the time between this process and the extraction, as the reduction of the particle size would allow the contact between the analytes of interest and enzymes present within the tissues, thereby resulting in enzymatic degradations. Similarly, reducing the particle size of the sample would favor the contact between the analytes and other factors such as oxygen and temperature, which could also accelerate degradation reactions.

Extraction

Extraction is one of the most important steps of the previous operations, its outcome will determine the release of analytes from the vegetable matrix into the medium, and this in turn allows the quantitative/qualitative determination of the extract (Garcia-Salas *et al.,* 2010). Generally, it is a separation process where the distribution of the analyte (the bioactive compounds) between the matrix and the extractant is made in order to arrive at the appropriate distribution coefficient, with the ultimate goal being the elimination or reduction of potential matrix interferences and analyte preconcentration (Luthria, 2008). As the polarities of bioactive compounds vary significantly and it is difficult to develop a single method for optimum extraction of all compounds, optimization of the extraction procedure is essential for an accurate assay.

The literature has revealed many procedures for extracting bioactive compounds from vegetal matrixes, solvent and sorbent extraction being the most commonly used (Figure 12). Among solvent-extraction methodologies, a number of novel techniques which are less time and solvent consuming are emerging in this field. Nevertheless, solid-liquid extraction still plays a central role in the extraction of phenolic compounds from solid matrixes, as it is cheap and easy method, and therefore has been used in the present doctoral dissertation. The solvents most frequently used are alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate. However, there are cases in which components of very polar nature (e.g. phenolic acids) could result in no extraction using pure organic solvents, and thus it is highly recommendable to use of water-alcohol or water-acetone mixtures (Rodŕiguez-Delgado *et al.,* 2001). Thus, phenolic compounds have been successfully extracted from plant matrixes such as bean, kale, potato, green onion, and others, using mixtures of methanol:water (80:20) (v/v) (Garcia-Salas *et al.,* 2010).

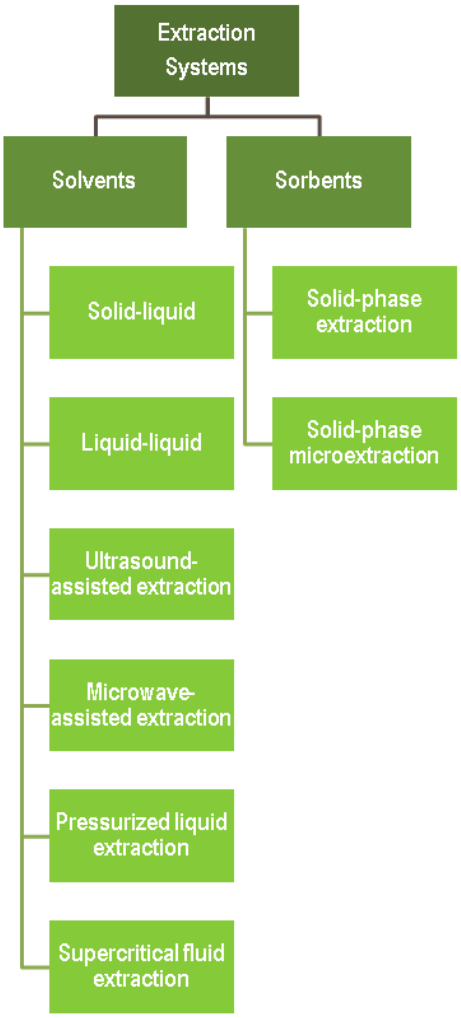

Figure 12. General classification of extraction systems based on solvents and sorbents.

In the case of liquid samples, solid-phase extraction (SPE) has resolved many of the problems associated with liquid-liquid extraction, such as incomplete phase separations, less-than-quantitative recoveries, or use of large quantities of organic solvents (Peysson & Vulliet, 2013; Fumes *et al.*, 2015). SPE uses the affinity of the dissolved or suspended solutes on a liquid (matrix) for a solid (sorbent) through which the liquid is made to pass. As a result, the analyte/s of interest are retained onto the sorbent and are collected by washing with an appropriate solvent (Figure 13). There is a huge variety of sorbents that are able to separate the analytes according to their chemical properties.

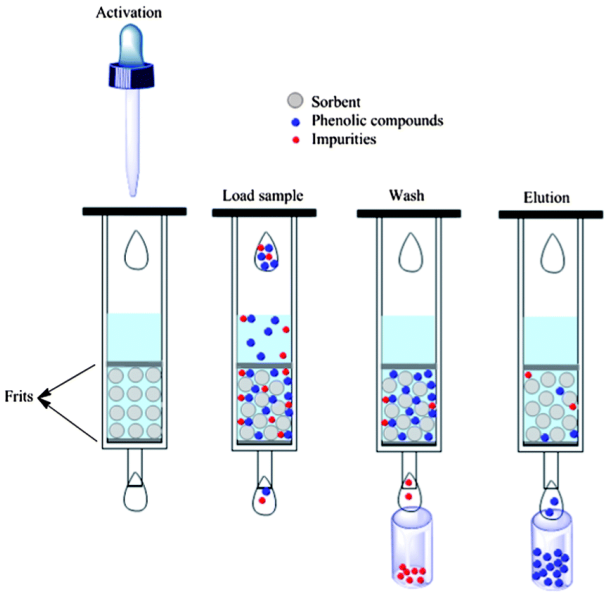

Figure 13. Procedure for Solid Phase Extraction (SPE) (Su et al., 2014).

Some of the variants of the method include: reversed-phase SPE, which involves a polar/moderately polar matrix and a non-polar sorbent; normal-phase SPE, which involves the utilization of a polar sorbent with non-polar analytes; ionic-exchange SPE, in which the compounds of interest are charged in solution; and adsorption SPE in which the analytes are adsorbed onto modified sorbents (Supelco, 1998), among others.

Due to the high variety of sorbents available, SPE might be used in a variety of matrixes, such as olive oil, one of the matrixes studied in the present doctoral dissertation. Comparative studies between different extraction systems for olive oil including liquid-liquid extraction have shown that normal-phase diol-bonded silica has shown large recoveries of the phenolic fraction (Gómez Caravaca *et al.,* 2005). Other studies have confirmed that the use of this type of stationary phase constitutes a rapid and simple methodology while allowing good recoveries of gallic acid, hydroxytyrosol, tyrosol, vanillic acid, caffeic acid, syringic acid, gibberellic acid, *p*-coumaric acid, ferulic acid, oleuropein, luteolin, and apigenin (Gilbert-López *et al.,* 2014). In some cases, a recovery of 95% was achieved (Alarcón-Flores *et al.,* 2012).

2.2. Analytical determination of phenolic compounds

Traditionally, total phenolic analysis has been carried out by spectrophotometric methods that rely on the reaction of the phenolic compounds functional groups with a reagent, such as Folin-Ciocalteu. This reagent includes phosphomolybdic and phosphowolframic acids that are reduced by the functional groups of phenolic compounds in basic media, producing a series of blue oxides with a color intensity proportional to the amount phenolic compounds present in the sample. The main advantage of this technique is its simplicity and speed. However, it presents the disadvantage of not allowing individual identification or quantification of the compounds.

In this sense, separation techniques have emerged that allow qualitative and quantitative information regarding individual components of the sample under study. Today, continuous techniques are generally used, meaning that the compounds are detected on-line after the separation process. These techniques can be divided into two groups: non-chromatographic such as capillary electrophoresis (CE), and chromatographic such as gas chromatography (GC), or liquid chromatography (LC).

CE presents a series of advantages such as the capability of handling small sample volumes, higher separation efficacy, and a shorter analysis time (Carrasco-Pancorbo *et al.,* 2006). The most commonly employed detectors have been UV-Vis or diode array (DAD) (Gómez Caravaca *et al.,* 2005). The latter coupling with mass spectrometry detectors has enhanced the potential of CE (Nevado *et al.,* 2009), although it presents more difficulties than with other separation techniques, which together with difficult automation make this methodology less suitable for analyzing phenolic compounds.

GC has been widely employed for the characterization of these compounds (Bajoub *et al.,* 2016; Bittencourt *et al.,* 2015). The most commonly used stationary phase has been fused silica capillary columns (Rohloff, 2015). The first detector employed was the flame ionization detector

(FID), although in later years applications with mass spectrometry detectors have arisen (Capriotti *et al.,* 2015). This technique presents the advantage of having greater resolving power, a larger linear range, and more stability than in LC applications. However, a major drawback of GC in the analysis of phenolic compounds is the need for a previous derivatization step. Most of these reactions consist on the formation of trimethylsilyl derivatives. The main problem of the derivatization reactions is than they can occur incompletely, with the appearance of an array of different chemical species coming from the same compound. This complicates the identification and quantification of the analytes (Carrasco-Pancorbo *et al.,* 2005).

However, to date, LC is still the most commonly used separation technique for the determination of phenolic compounds of plant origin, as it combines high resolution, efficacy, and versatility {{1324 Motilva,Maria-José 2013}}. For the analysis of phenolic compounds by LC, reversed-phase partition chromatography, which implies an apolar stationary phase and a polar mobile phase, has been mostly used. For the chromatographic separation the most commonly employed stationary phase has been C18, with lengths ranging between 15 and 25 cm of chromatographic bed, and a packing particle size of 1.8 to 5 μm (Ignat *et al.,* 2011). As the mobile phase, acidified water and organic solvents are used as eluents A and B, respectively. The pH of the mobile phase is normally kept below 3.0 by the addition of a small amount of formic, acetic, or trifluoroacetic acid (between 0.05 and 0.2%, or in some case 5%) (Motilva *et al.,* 2013). Proper pH control of the mobile phase is essential, as it influences the pK_a of the compounds, affecting their retention into the column. Phenolic compounds with similar polarity are separated most effectively by gradient elution, by using methanol and acetonitrile or methanol/acetonitrile mix as an organic eluent B. Normally, acetonitrile is preferred due to the lower pressure reached than methanol (Motilva *et al.,* 2013).

Detectors used in LC can be very diverse in nature, but undoubtedly the detection system most used, with an exponential rise in its applications, is mass spectrometry (MS). A reflection of this has been the growing number of scientific publications that have appeared in recent years using this analytical methodology (Abu-Reidah *et al.,* 2015; Amessis-Ouchemoukh *et al.,* 2014; Taamalli *et al.,* 2015). Together with the information generated with other detection systems such as UV-Vis spectroscopy and nuclear magnetic resonance (NMR), it allows an almost unequivocal identification of the compounds of interest. The analytical potential of this coupling has increased with the progress made in High Performance LC (HPLC) equipment such as supporting higher working pressure, which has permitted working with smaller particle-size columns and, thus, improved chromatographic resolution of complex mixtures. The instrumentation used in this doctoral dissertation includes liquid-chromatography equipment that incorporates these improvements and their link to UV-Vis spectroscopy and MS detectors. Therefore they will be described in more detail in the following sections.

2.3. Instrumentation used for determining phenolic compounds

High-performance LC (HPLC) has been the technique of choice when the chromatographic separation is performed in columns of 1.5 to 4.5 mm internal diameter (i.d.), 3 to 30 cm long, with a particle size of 3 to 40 µm, and a flow rate of 0.2 to 0.5 mL/min (Desmet *et al.,* 2006). Different commercial devices are available on the market for this technique. Examples are the RRLC called (Rapid Resolution Liquid Chromatography, trademark of Agilent Technologies), which allows working pressures of up to 600 bar and UPLC (Ultra Performance Liquid Chromatography, registered Waters Corporation) that can work with pressures of up to 1,000 bar. Table 1 summarizes the main characteristics of the LC equipment based on the column size and flow rate. The equipment used in the present doctoral dissertation was RRLC, which is described next.

	UPLC/RRLC	HPLC	Micro LC	Capillary LC	Nano LC
Column i.d. (mm)	1.5-4.5	1.5-4.5	0.8	0.18-0.32	0.075-0.1
Column length (cm)	3-15	3-30	5-25	5-25	5-15
Column particle size (μm)	>2	3-40	3-5	3-5	3-5
Flow rate	0.2-5 mL/min	0.2-2.5 mL/min	10-100 μL/min	1-10 μL/min	0.1-1 μL/min

Table 1. Some parameters of the different LC techniques, classified according to the chromatographic column parameters and flow rates used.

The equipment used is composed of a basic series of components: pump/s, mixer, injector, chromatographic column, thermostatic oven, detector, and data-processing system (Figure 14). Below is a description of the major advances incorporated to each case.

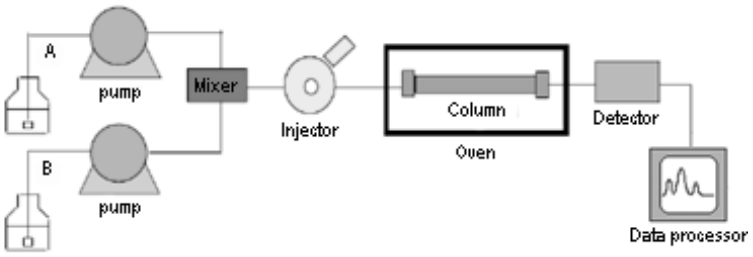

Figure 14. Basic scheme of the RRLC equipment.

Pumping systems provide adequate pressure to allow the solvent system to reach sufficient flow. Mobile-phase deposits are equipped with filters that eliminate impurities present in the mobile phase such as suspended particles, which could damage pumps and the injection system, and could cause plugging of the columns. In addition, the pumping system includes a degasser to remove dissolved gases that could form bubbles inside the column and in the detection system. These bubbles could cause widening of bands and could interfere with the detection. However, the main advances in pumping systems have been directed at achieving more accurate

reproducibility of flow by reducing or eliminating the pulses and greater ability to work at high pressures, allowing the use of more packaged and smaller particle-size columns.

The injector allows the introduction of a quantity of sample to be analyzed in the chromatograph. Current instrumentation has injection systems based on rotary valves with sample loops, which allow the sample to be introduced into the column without depressurizing the system. These sample loops have different capacities and make it possible to inject small and accurate volumes, avoiding the band widening which accompanies the overloading of the column, and enabling high reproducibility between injections. In addition, the system has the option to perform injector cleaning between one analysis and another, thereby reducing the possibility of contamination or "carry-over".

The chromatographic column contains the stationary phase, where the analytes are separated. The particle size is directly related to the efficiency of the separation, columns of 5-µm particle size being frequently used in conventional HPLC equipment. Smaller particles offer a larger surface area, increasing the number of theoretical plates and thus allowing better separation and achieving higher resolution. Furthermore, working with smaller-particle-size columns would allow the use of larger workflows and thereby would reduce the analysis time without compromising the efficiency of the chromatographic separation. However, in conventional HPLC equipment it is practically impossible to work with columns having a particle size of less than 3 µm, as the overpressure generated exceeds the maximum value tolerated by the system. RRLC equipment incorporates improvements in the pumping system that have increased the maximum working pressure to values of up to 600 bars. This enables the use of stationary phases with smaller particle size (<2 µm) (Cunliffe *et al.,* 2007). Additionally, a precolumn with the function of removing suspended matter or contaminants of the mobile phase or sample is usually placed between the injection system and the column.

To ensure greater reproducibility in chromatographic separations, the RRLC equipment has a thermostatic oven compartment which controls temperature, as this strongly influences the separation.

The RRLC equipment has a diode array detector (DAD) in series with the chromatographic column, offering multiple wavelength and full spectral online detection. DAD is particularly useful to detect bioactive compounds with characteristic chromophores. The multiple conjugated bonds that phenolic compounds possess in their structure make them chromophores showing specific absorption bands in the UV-vis region. Several molecules present this behavior, and thus this detector is considered universal. Although this detection system is not equipped to provide structural information and therefore it does not allow the unequivocal identification of the analytes, it is able to delimit the chemical family to which the analytes belong, as each chemical family presents characteristic absorption bands (Wolfender, 2009) (Table 2).

Chemical family	UV absorption bands (nm)
Benzoic acids	270-280
Cinnamic acids	305-325
Coumarins	220-230 310-350
Chalcones	220-230 340-390
Dihydrochalcones	~220 ~280
Flavonols	220-270 350-380
Flavones	220-270 330-350
Flavanols	270-280
Flavanones	270-295
Isoflavones	245-270 300-340
Anthocyanidins	240-280 450-560

Table 2. Characteristic absorption bands specific to each family of phenolic compounds.

To be able to carry out the coupling between a liquid-based technique such as LC and a mass spectrometer that works in the gas-phase, an adequate ionization system that acts as an interface is needed. **Electrospray ionization** (ESI) has been used, as it is effective for polar, or labile compounds such as phenolic compounds. In this case, the analyte solution coming from the LC passes through a hypodermic needle or stainless-steel capillary. A very high voltage (2–6 kV) is applied to the tip of the metal capillary relative to the surrounding source-sampling cone or heated capillary (typically located at 1–3 cm from the spray needle tip). This strong electric field causes the dispersion of the sample solution into an aerosol of highly charged electrospray droplets (Figure 15). A coaxial sheath gas (dry N_2) flow around the capillary results in better nebulization. This gas flow also helps to direct the spray emerging from the capillary tip towards the mass spectrometer. The charged droplets diminish in size by solvent evaporation, assisted by the flow of nitrogen (drying gas). Finally the charged analytes are released from the droplets, some of which pass through a sampling cone or the orifice of a heated capillary (kept in the interface of atmospheric pressure and the high vacuum) into the analyzer of the mass spectrometer, which is held under high vacuum. The heated capillary (100–300ºC) causes the complete desolvation of the ions passing through it. In positive ion mode (when the spray nozzle is kept at positive potential) the charging generally occurs via protonation, but in negative ion mode (when the spraying nozzle is kept at negative potential) charging occurs via deprotonation of the analyte (Banerjee & Mazumdar, 2012).

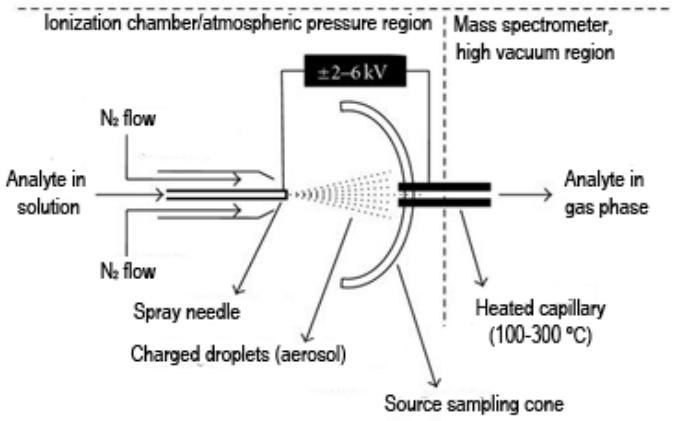

Figure 15. Schematic representation of the ESI interface.

After the interface, the ions are directed into the mass analyzer, enabling the separation, detection, and quantification of the target analytes with a high degree of sensitivity and selectivity, and providing information on their molecular mass. There is a variety of mass analyzers, including time-of-flight (TOF), and quadrupole time-of-flight (QTOF). Both were used in the present doctoral dissertation, and are described next.

The mass analyzer **time-of-flight** (TOF) separates the ions according to different speeds in its interior according to their *m/z*. First, the ions are extracted from the ionization chamber and accelerated into the flight tube by an electrostatic field which gives them high kinetic energy. The higher their *m/z* the faster they will fly and vice versa. The greater the length of the tube, the better the resolution between ions of different *m/z* (there will be a greater separation of ions per time) (Verentchikov *et al.,* 1994) (Figure 16).

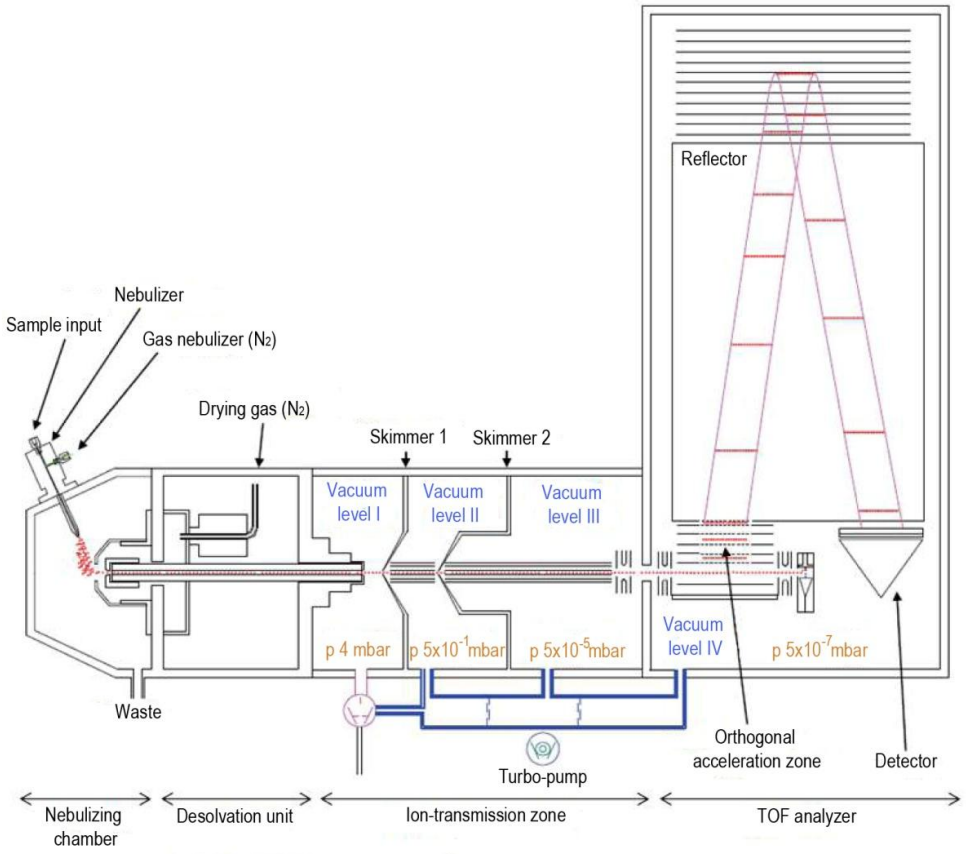

Figure 16. Outline of the inside of a TOF.

Ions formed in the spray chamber pass through the desolvation unit, which separates the area under atmospheric pressure and the first high-vacuum region, and comprises a drying gas heater and a glass capillary. This unit connects to the ion-transmission zone, which consists of three high-vacuum modules separated by two skimmers. In both vacuum levels II and III, a hexapole transfers ions to the area of greatest vacuum. At the end of the transmission area, three lenses focus and direct these ions. Finally, the TOF analyzer itself consists of three areas. In the first part, the orthogonal acceleration zone, two lenses accelerate ions into the flight tube by applying an intermittent electric field. Ions will fly along the flight tube to reach the second zone, the reflector (also known as a reflectron), making it possible to correct the dispersion in the kinetic energy of the ions and thus increasing resolution. The last TOF analyzer component is the

electron impact detector, which is a series of high voltage plates that transform the impact of ions into electrical signals (Bruker Daltonics, 2005). One of the main attributes of TOF instrument is its accurate mass measurement and isotopic distribution, which give the elemental composition of ions and can be used to identify unknown compounds (Chen *et al.,* 2011).

The other mass analyzer used in the doctoral dissertation has been the **quadrupole time-of-flight** (QTOF), which is similar to the TOF analyzer, with the difference that a quadrupole is introduced. This element allows the selection of certain ions which are then fragmented within a collision cell using a collision gas (typically N_2). The fragmented ions are separated in the TOF tube according to their *m/z* in the same way as described for the TOF analyzer. In addition, the skimmers are replaced by ion funnels (funnel-shaped concentric rings) to prevent loss of ions during ion transmission and thereby increase sensitivity (Figure 17).

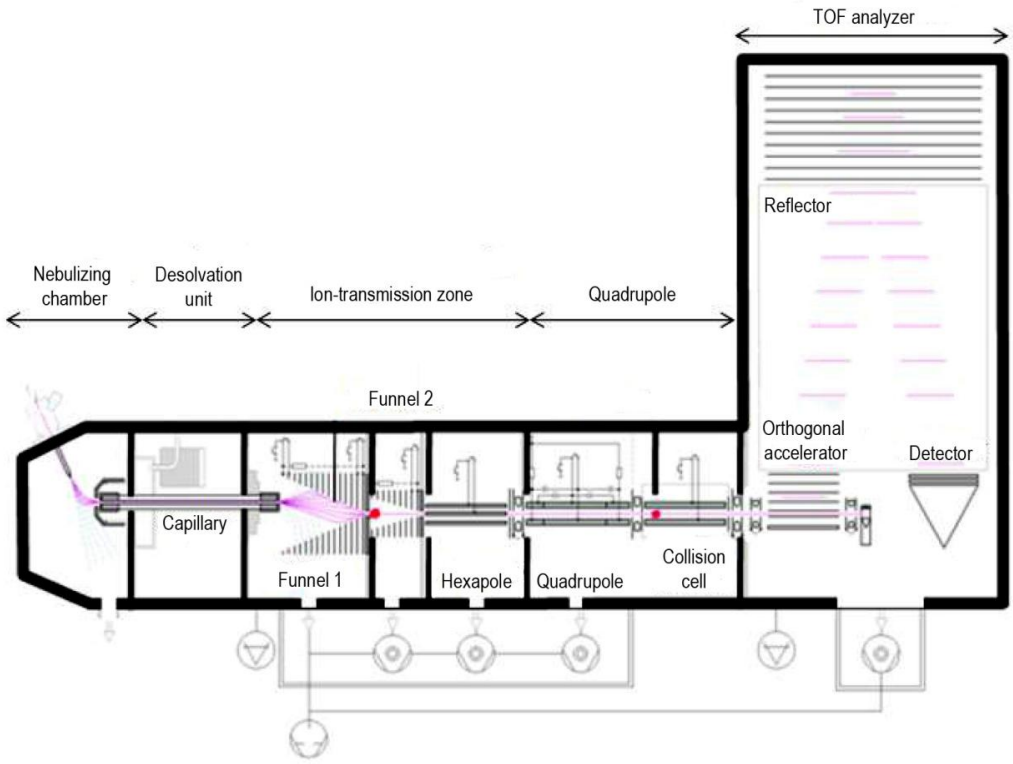

Figure 17. Outline of the inside of a QTOF.

Apart from the features of the TOF, the QTOF analyzer allows MS/MS experiments. These induce ion fragmentation that can offer valuable structural information by producing various characteristic fragment ions, which together with their elemental compositions provided by the TOF analyzer can boost confidence concerning the origin of the ion. The product ions with accurate masses provide additional structural information which is useful for discriminating between possible isomeric structures, making elucidation feasible. Normally the identification or elucidation of the analytes by TOF and QTOF is performed to a great extent with the assistance of reference compounds, and with previous knowledge of the chemical constituents in plant extracts available in the literature and in databases.

3. Isolation and purification of bioactive compounds

As the chemical profile and biological effects of vegetal extracts are being elucidated, the isolation and purification of their individual compounds attracts interest aimed at the evaluation of the biological activity of these individual components. When target compounds are not available in the form of commercial standards, labor-intensive purification procedures are required. In addition, the structural diversity and complexity of these molecules make their chemical synthesis unprofitable.

Generally, the separation of target products includes the extraction, the analytical characterization of the extract, the purification at semi-preparative or preparative scale, and finally the analytical characterization of the purified fractions to check their purity. As the extraction and analytical characterization have been described previously, this section focuses on the purification procedure itself.

The use of adsorption and desorption onto non-ionic macroporous resins has proved to be an efficient technique in phenolic compounds isolation, as it has high adsorption capacity, low operational expense and easy regeneration of the adsorbent (Xu *et al.,* 2000). Yet it is tedious,

time consuming, and usually requires multiple steps resulting in low recovery rates of analytes (Kicel *et al.,* 2015). Therefore, these types of resins are often used for prepurification or prefractionation prior to separation with other more efficient techniques such as HPLC or high-speed counter-current chromatography (HSCCC) (Li *et al.,* 2016). These techniques, which have seen an explosion of interest and have been successfully applied to the separation of bioactive compounds in the past decades, have been used in the present doctoral dissertation, and are described next.

3.1. Semi-preparative High-Performance Liquid Chromatography

The main difference between analytical and preparative HPLC is the objective of the separation. The aim of an analytical HPLC run is the qualitative and quantitative determination of a multicomponent mixture. For a preparative HPLC run, the objective is to isolate and purify a valuable compound/s present in a multicomponent mixture (Table 3).

Analytical HPLC	Preparative HPLC
Sample goes from detector waste	Sample goes from detector into fraction collector
Goal: Identification and/or Quantification of compounds	**Goal:** Isolation and/or purification of compounds

Table 3. Definition of analytical and preparative HPLC.

Although the size of the instrumentation or the amount of mobile phase pumped through the system in preparative HPLC differs with respect to analytical HPLC (Table 4), it is not what determines a preparative HPLC experiment, but rather the much higher amount of sample applied to the stationary phase. In analytical chromatography the sample amounts applied to the column are typically in the µg range but can be lower. The mass ratio of compound to stationary

phase on the column is less than 1:100000. The applied sample volume is also usually much smaller than the column volume (< 1:100) (Huber & Majors, 2007).

	Analytical HPLC	Preparative HPLC
Column internal diameter (mm)	4.6	20
Flow rate (mL/min)	0.8	15
Consumption of solvent (mL/h)	48	900
Injection amount (µg)	0.250	5000 mg
Injection volume (µL)	20 µL	400 µL

Table 4. Comparison of analytical vs. preparative HPLC, according to some operational parameters.

The term semi-preparative is often used to refer to a smaller scale than preparative, although there is no consensus on the threshold between semi- and preparative scales. Below, the semi-preparative scale will be referred to.

For a semi-preparative HPLC method to be created, an optimization at analytical scale is required to avoid wasting of solvents and sample, and scale-up calculations or trial-and-error measurements are necessary to perform the scaling-up to the semi-preparative scale.

In terms of mobile-phase composition, the one evaluated in analytical chromatography can be transferred to the preparative scale. However, it is important to consider certain factors such as spectroscopic characteristics of the solvents used as eluents, volatility to be easily removed from the collected fractions, viscosity that does not increase the pressure in the system, and a high degree of purity to reduce the presence of contaminants in the collected fractions. In parallel with analytical chromatography, the application of multistep gradients can facilitate the separation of compounds, yet we must consider the peak broadening caused by increasing the amount of sample injected into the column and enlarging the particle size of the stationary phase. As with

the analytical scale, the solvents systems used for phenolic compounds separations normally consist of an aqueous eluent and an organic eluent (mainly methanol or acetonitrile). Usually, an acid is added to the solvents, acetic, formic, or trifluoroacetic acids being the most commonly used. With regard to flow rates, values of 5-15 mL have been successfully applied to purify a great number of phenolic compounds (Mai & Glomb, 2013; Xu *et al.,* 2010; He & Liu, 2008; Valls *et al.,* 2009).

With respect to the column, particle size is an important parameter for analytical and semi-preparative HPLC. Generally, the smaller particle size allows greater efficiency and permits the use of shorter columns to increase separation speed. In semi-preparative chromatography, the particle size is important but, since higher working flow rates are required, the smaller and more expensive particles of 1.8- and 3.5-μm average diameters used in analytical columns would imply extremely higher pressures and are not normally used in larger-scale semi-preparative columns. Column particle size and dimensions are dictated by the amount of material per injection that the technician desires to inject. In semi-preparative HPLC, it has to be taken into account that if more than a certain amount of sample is injected into the column, the peaks becomes unsymmetrical, and show strong tailing/fronting. This effect is called concentration overloading. Since this effect depends on the compounds, the loadability of the chromatographic system column has to be determined for each semi-preparative HPLC experiment. As with the analytical scale, columns chosen for the purification determination of phenolic compounds are almost exclusively reversed-phase columns, composed of a C18 stationary phase, but in this case with greater internal diameter, and a particle size ranging from 5 to 10 μm (Valls *et al.,* 2009). The three important parameters used to judge the result of a semi-preparative run are product purity, yield, and throughput. (Huber & Majors, 2007).

HPLC plays a dominant role in the purification of bioactives from vegetal sources, as it is a technique that enables high-resolution chromatographic separations. On the other hand, HPLC columns have limited loading capacity, requiring several analyses to be able to collect significant amounts of sample. Therefore, alternative technologies have emerged such as high-speed countercurrent chromatography (HSCCC).

3.2. Semi-preparative high-speed counter-current chromatography

Counter-current chromatography (CCC) is a liquid chromatography technique that uses two immiscible liquid phases and no solid support. One liquid acts as the stationary phase and the other as the mobile phase. There are two modes of centrifugal force CCC, hydrostatic and hydrodynamic. In the hydrostatic mode, the column is spun about a central axis. These devices are marketed under the commercial name centrifugal partition chromatography (CPC). The dynamic mode is the one used in this doctoral dissertation and the one that will be referred to further on. It is often called high-speed CCC (HSCCC) and relies on the Archimedes' screw force in a helical coil to make the separation (Berthod *et al.,* 2009). This centrifugal force is used to hold the liquid stationary phase in place, which is first pumped to entirely fill the coil. Next, the liquid mobile phase is pushed through it from the opposite end of the coil, quickly moving through the coil, leaving a large volume of the other stationary phase in it, and causing the partitioning of the analytes between the two immiscible liquid phases.

The selection of the two-phase solvent system for the target compound(s) is the most important step in HSCCC, where searching for a suitable two-phase solvent system may be estimated as 90% of the entire work. Two elution modes can be chosen: *head-to-tail* and *tail-to head*. This term indicates how to introduce the mobile phase (upper or lower) into the rotating coil (from the head or the tail). Of four combinations, only two elution modes should be used in HSCCC, i.e., either the lower phase from the head towards the tail or the upper phase from the tail towards the head

(Ito, 2005). As in HPLC, analytical procedures are first developed to proceed to the scaling-up to semi-preparative and preparative scales, but with the advantage that the scaling-up is linear in HSCCC.

HSCCC operates under gentle conditions and enables non-destructive isolation even of labile natural compounds. Due to the absence of any solid stationary phase, adsorption losses are minimized and 100% sample recovery is guaranteed. The liquid nature of the stationary phase means that the compounds retained in this phase can be easily recovered by pumping this phase out. This is an important feature for samples containing phenolic compounds, which tend to get irreversibly adsorbed in HPLC columns (Cao *et al.,* 1999).

A large choice of biphasic solvent systems is available in the literature, and sometimes several systems can be suitable for the purification of the same compound. Solvent systems composed of an organic and an aqueous phase are the most popular in conventional HSCCC. For instance, the Arizona liquid system, which combines n-heptane/ethyl acetate/methanol/water in different possible proportions, and the HemWat system which combines n-hexane/ethyl acetate/methanol/water are widely used for the purification of natural products and especially of phenolic compounds. Typical preparative separations of phenolic compounds use flow rates ranging from 5-10 mL/min, and load between 100-1000 mg of sample (Hubert *et al.,* 2013; de Beer *et al.,* 2015; Kicel *et al.,* 2015). The undeniable applicability of this separation technique has led to an enormous number of papers on the fractionation and isolation of various compounds from plant sources (Valls *et al.,* 2009). However, its low theoretical plates of the separation column (Tong *et al.,* 2015), makes this technique less suitable when purifying complex mixtures of compounds of similar nature. For that reason, a pre-separation step, or combination of HPLC and HSCCC are frequently used for isolation of phenolic compounds from complex mixtures (Kicel *et al.,* 2015).

3.3. Instrumentation used for phenolic compounds isolation.

The HPLC equipment used is composed of a basic series of components: pump/s, mixer, injector, chromatographic column, detector, fraction collector, and data-processing system (Figure 18).

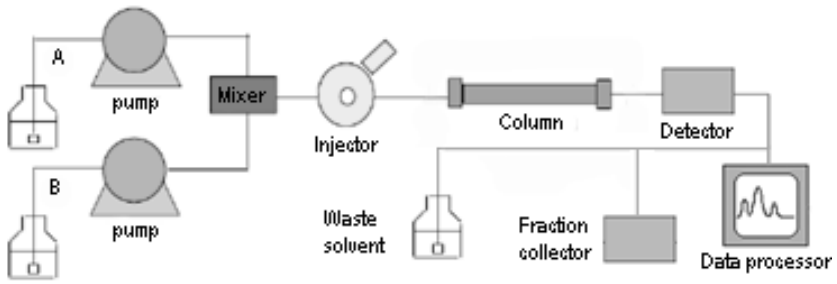

Figure 18. Basic scheme of the semi-preparative HPLC equipment.

The semi-preparative, multi-solvent pumps are able to accommodate flow rates ranging from 0.5mL/min to 50 mL/min at pressures up to 8,700 psi (600 bar) to pump the mobile phase towards the system. The injection module allows the introduction of the sample, and can be configured with sample loops of different capacities, depending on the needs of the application, featuring a continuous flow-path design, minimizing pressure spikes when switching between load and injection while running at high flow rates. The semi-preparative column allows the separation of the compounds to finally arrive to the detector. In a semi-preparative system, to monitor the chromatographic separation of the mixture components, as well as the collection of fractions, different types of detectors can be used. The semi-preparative HPLC equipment includes a UV-Vis detector that will aid in monitoring the separated compounds according to their specific UV-Vis absorption bands. However, the coupling in series with a mass spectrometry detector was of great utility thanks to the implementation of a flow splitter that divides the flow out of the column into two volumes: one larger that is directed to the fraction collector and one smaller that enters the make-up flow that is directed to the detector, allowing the monitoring of each individual compound eluting from the column. Finally, a fraction collector is synchronized to

the HPLC equipment to collect the fractions of interest. These normally operate by time (specifying a time window), by drop (specifying a fraction volume), or by slope (in which the collector monitors a detector signal and identifies peaks that surpass a specific slope, or signal threshold). Figure 19 shows a semi-preparative HPLC equipment coupled to a ESI-TOF Mass Spectrometer.

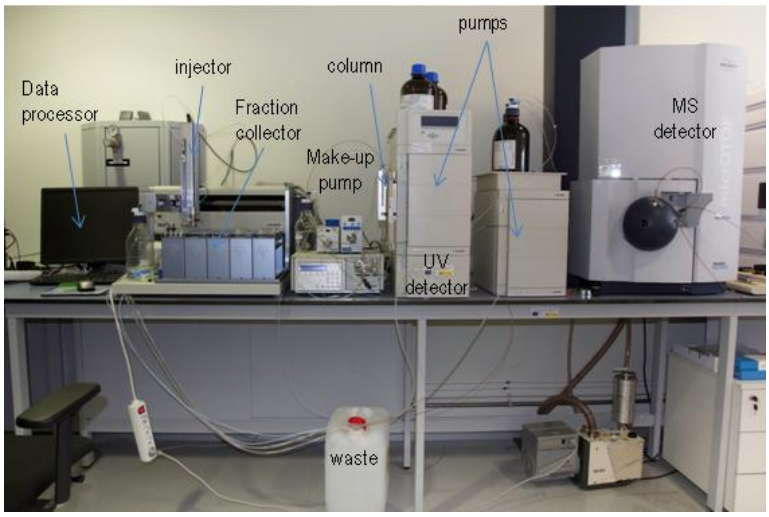

Figure 19. Semi-preparative HPLC equipment coupled to a ESI-TOF Mass Spectrometer.

The equipment used for the isolation of bioactive analytes was composed of two solvent reservoirs with two pumps, an injection valve, the coil, or HSCCC centrifuge, the detector, fraction collector, and computer system (Figure 20).

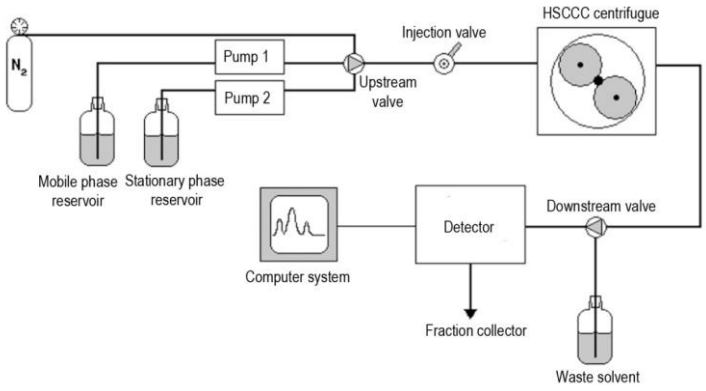

Figure 20. Basic scheme of the HSCCC equipment.

The HSCCC centrifuge (the column) is a coil of Teflon tubing wound in multiple layers on a bobbin, in such a way that there are two identical bobbins mounted on a single rotor. The equipment contains a small, interchangeable method-development (analytical) column and a semi-preparative column. The choice of columns is based upon the desired sample loading, as the larger the column the greater the sample capacity. Fluid flow through the bobbins is serial, eliminating the need for balancing. The rotor is contained in a temperature-regulated cabinet. The speed of rotation is controlled by a self-contained electronics module and is variable between 200 and 1600rpm. A UV-Vis detector is connected to the system to monitor the compounds of interest, which is synchronized to a fraction collector (Figure 21).

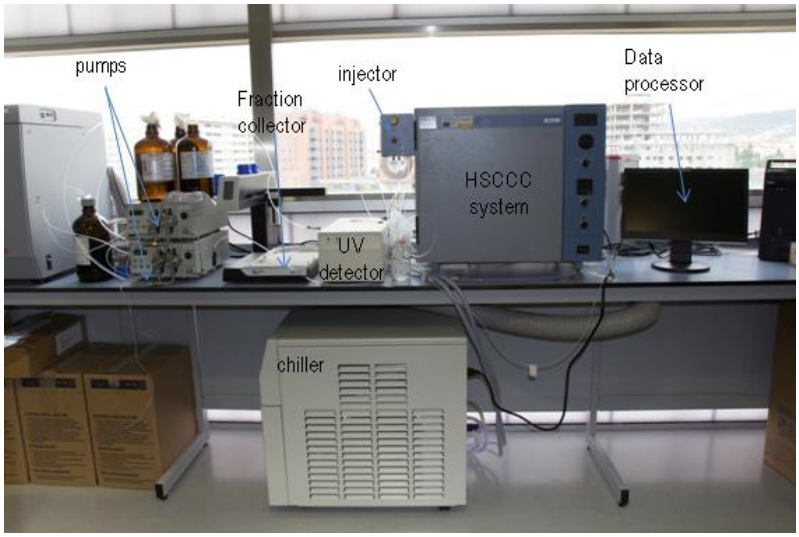

Figure 21. Semi-preparative HSCCC equipment.

4. Assessment of the potential biological activity

As Functional Food science is based on the way in which specific nutrients and food components positively affect target functions (biological responses) in the human body, this biological activity needs to be evaluated. The biological activity of phytochemicals might be assessed by nutritional epidemiology, a field of medical research that studies the relationship between nutrition and

health. Basic nutritional epidemiological methods rely on careful observation and use of valid comparison of groups to identify risk factors for disease and targets for preventive healthcare. Epidemiological studies include case series, case control, and cohort studies. Specifically, the epidemiological observation that consumption of specific foods, such as fruits and vegetables is strongly associated with reduced risk of cancer and other chronic diseases, has led to the hypothesis that specific metabolites or plant-derived phytochemicals may be responsible for the observed preventive action (Jansen *et al.,* 2004; Key *et al.,* 2004; González *et al.,* 2006; Sala-Vila *et al.,* 2015; Zhu & Prince, 2015; Shen *et al.,* 2015). This epidemiological observation has directed global policy decisions to encourage the intake of fruits and vegetables.

In addition to nutritional epidemiology, the biological activity of certain phytochemicals might be evaluated using *in vivo* and *in vitro* experimental designs. *In vitro* studies are performed with microorganisms, cells or biological molecules outside their normal biological context. By contrast, *in vivo* studies are conducted in animals, humans, and whole plants. Specifically, *in vitro* cellular models have been used in the present doctoral dissertation.

4.1. Obesity and the role of AMPK in energy homeostasis

Although the list of the possible biological activities attributed to phenolic compounds is huge, the present doctoral dissertation is dedicated to the study of the molecular mechanisms involved in obesity. Obesity has global significance, given that, according to the World Health Organization, 39% of adults over 18 years old were overweight in 2014, (WHO, January 2015), thus worldwide efforts aimed to prevent and alleviate this disorder are essential.

Obesity is a multifactorial complex disease influenced by lifestyle, behavior, and environmental as well as genetic factors. It results from an energy imbalance due to excess caloric intake relative to energy expenditure; the latter primarily reflects sedentary lifestyle and lack of physical activity. Other factors such as genetic susceptibility, family history, and gene-environment interactions all

contribute to the development of obesity (Bouchard, 2010). This disease is defined by excess adipose mass and adipose tissue expansion, which occurs through adipocyte hypertrophy and hyperplasia (Siriwardhana *et al.,* 2013). Adipose tissue has a vital energy-storage function, and has only been progressively recognized over the past two decades as a key endocrine organ with active metabolism (Kalupahana *et al.,* 2012). Indeed, adipose endocrine function is critical to overall energy balance and homeostasis with adipocyte-derived pro- and anti-inflammatory adipokines playing key roles. When the production and secretion of proinflammatory adipokines prevail, systemic inflammation, insulin resistance, and obesity-related metabolic disorders arise. (Wang *et al.,* 2014).

Recently, AMP-activated protein kinase (AMPK) has been revealed to be an important regulator of cell-energy homeostasis. The AMPK complex is an evolutionally conserved serine/threonine heterotrimer kinase complex consisting of α, β, and γ subunits. AMPK can be considered to be a multipurpose switch, which under energy depletion ignites processes aimed at ATP production, while inhibiting ATP-consuming processes (Figure 22).

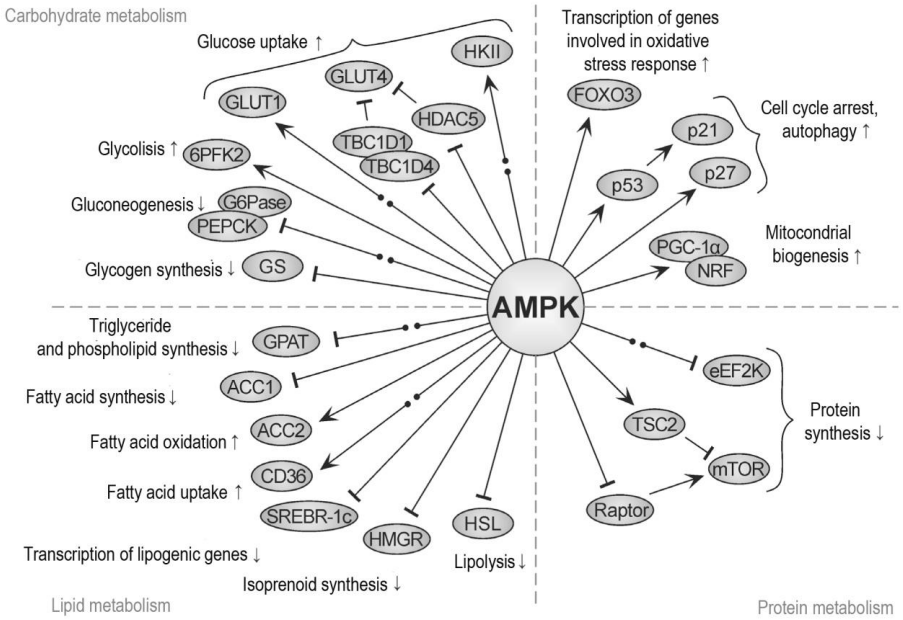

Figure 22. AMPK in various biochemical processes. A gap in the line indicates an unknown mechanism of action.

The switching is performed by acute phosphorylation of key enzymes in major branches of metabolism including fatty acid synthesis, protein synthesis, carbohydrate metabolism, and at the same time, by phosphorylation of transcription factors ensuring long-term regulatory effects. AMPK is not only involved in carbohydrate, lipid, and protein metabolism, but it is also responsible for regulating mitochondrial biogenesis, cell growth, and proliferation, cell polarity, apoptosis, and autophagy. AMPK plays a major role in hormonal signaling, being a central node of signaling pathways. AMPK can regulate the endocrine system, and at the same time, its activity is regulated by a number of hormones and cytokines (adipokines) such as leptin, interleukin 6, resistin, ghrelin, and adiponectin. In addition, AMPK controls appetite through a neuroendocrine system that makes it a key regulator of energy metabolism at the whole-body level (Novikova *et al.,* 2015).

A significant number of natural compounds, and specifically phenolic compounds, are known to alleviate obesity-related disturbances in hypertrophic adipocytes through AMPK-dependent mechanisms, such as verbascoside and other *Lemon verbena* phenolic compounds (Herranz-López *et al.,* 2015), esculetin (Kim & Lee, 2015), theaflavin-3,3'-digallate (Ko *et al.,* 2015), or trans-cinnamic acid (Kopp *et al.,* 2014), to cite just a few. Such structural diversity of natural activators suggests that these compounds do not interact directly with AMPK but activate kinase complex in an indirect manner: 1) via increasing the intracellular AMP:ATP ratio mostly by reducing the ATP concentration (which can be achieved by inhibiting Complex I of the respiratory chain, inhibiting mitochondrial functions) or via inhibition of AMP metabolizing enzymes (Kulkarni *et al.,* 2011); and 2) via activation or upregulation of upstream kinases (Novikova *et al.,* 2015).

This key role of AMPK in energy homeostasis makes it an attractive target in the search for bioactive compounds that could provide ingredients for the development of new functional products aimed at preventing and/or alleviating the detrimental effects of metabolic diseases.

4.2. The 3T3-L1 cell line, an *in vitro* obesity model

The best characterized cell line for studying the differentiation and metabolic disturbances in the adipose tissue is 3T3-L1. This is a 3T3 subline (Swiss albino mouse) with a fibroblast-like morphology in a proliferation state, but under appropriate conditions the cells differentiate into an adipocyte-like phenotype, reaching a confluence and contact-inhibition state. This process is called adipogenesis.

The differentiation into adipocytes in cell culture is induced by treating fibroblasts with high-glucose medium containing insulin and transcription factors (dexamethasone [DEX] and 3-isobutyl-1-methylxanthine [IBMX]). The adipogenesis process includes a noticeable change in the cell morphology (they increase in size and take on a rounded morphology), the halting of the proliferation, and changes in gene expression and lipid storage (they start accumulating lipids as cytoplasmic droplets, which increase both in number and in size at subsequent times from differentiation) (Figure 23). Normally, 18-20 days after differentiation maintained with high-glucose medium containing insulin, they become hypertrophic adipocytes, a cell model characterized by high cytoplasmatic lipid accumulation, insulin resistance, and oxidative stress, reasonably similar to the adipose tissue of an obese person.

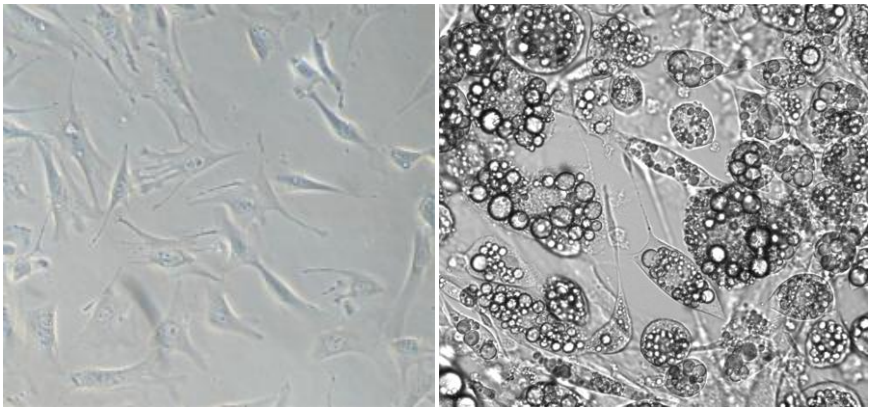

Figure 23. Microphotograph 20x taken with Cytation 3. Left: fibrobasts with typical tapering morphology. Right: hypertrophic adipocytes with typical rounded morphology and cytoplasmatic lipid droplets.

With the aim of evaluating the anti-obesity effects of the olive-leaf extract and purified fractions, we measured the intracellular lipid content and/or AMPK modulation.

4.3. Quantification of intracellular lipid content

The quantification of cytoplasmatic lipid accumulation in 3T3-L1 hypertrophic adipocytes, which is exacerbated in the hypertrophic state, is frequently measured by dye-labeled assays, which are inexpensive and easy to handle, such as Nile Red or Oil Red O (Figure 24).

Oil Red O is a fixative-based dye, which can be used only on fixed samples. It presents the advantage that it can be visualized with conventional bright-field microscopy. On the other hand, it is very sensitive to preparation conditions, requiring fresh solving of powder and filtering, which is time consuming and leads to less consistent results (Elle *et al.,* 2010).

Alternatives to these classical dyes have been developed and include the cell-permeable lipophilic fluorophore Nile Red, which has been used in the present doctoral dissertation. This dye can be used in either live or fixed samples (Daemen *et al.,* 2016), and it is visualized with fluorescence microscopy. This molecule is an uncharged heterocyclic molecule and thus is quite soluble in organic solvents and lipids, but relatively insoluble in water. It acts as a hydrophobic probe—that is, its fluorescence maxima vary depending on the relative hydrophobicity of the surrounding environment. Staining with Nile Red has several advantages over other fat stains such as Oil Red O. First, the dye is applied in aqueous medium, avoiding the dilemma imposed by most fat stains that must be dissolved in organic solvents which may also dissolve the lipids that the dye is intended to stain. Second, Nile Red fluoresces only in the presence of a hydrophobic environment, whereas in aqueous media the fluorescence of the dye is quenched (Fowler & Greenspan, 1985).

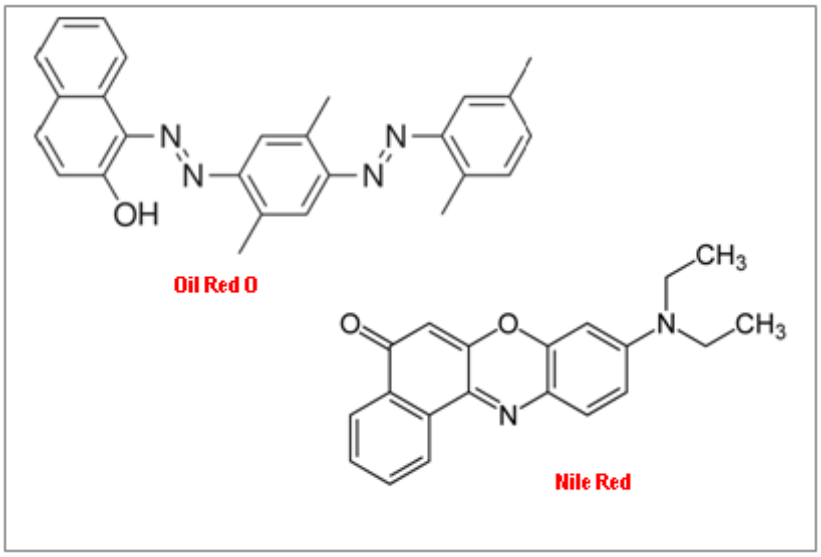

Figure 24. Oil Red O (up left) and Nile Red (bottom right) molecular formula.

4.4. Immunofluorescence for measurement of AMPK modulation

Immunofluorescence is a technique that uses the specificity of antibodies to their antigen and makes use of fluorophores to visualize the location of the antibodies with fluorescence microscopy. There are two types of immunofluorescence techniques, primary (or direct) and secondary (or indirect).

Primary, or direct, immunofluorescence uses a single, primary antibody, chemically linked to a fluorophore. The primary antibody recognizes the target molecule (antigen) and binds to a specific region called the epitope. The attached fluorophore can be detected via fluorescent microscopy, which, depending on the messenger used, will emit a specific wavelength of light when excited. Direct immunofluorescence, although somewhat less common, has notable advantages over the secondary (indirect) procedure. The direct attachment of the messenger to the antibody reduces the number of steps in the procedure, saving time and reducing non-specific background signal. This also limits the possibility of antibody cross-reactivity and possible mistakes throughout the process. However, since the number of fluorescent molecules that can

be bound to the primary antibody is limited, direct immunofluorescence is substantially less sensitive than indirect immunofluorescence and may result in false negatives. Direct immunofluorescence also requires the use of much more primary antibody, which is extremely expensive (Robinson *et al.,* 2001).

Secondary, or indirect, immunofluorescence is the technique used in the present doctoral dissertation to measure AMPK modulation. It uses two antibodies; the unlabeled first (primary) antibody specifically binds the target molecule, and the secondary antibody, which carries the fluorophore, recognizes the primary antibody and binds to it (Figure 25). Multiple secondary antibodies can bind a single primary antibody. This protocol is more complex and time-consuming than the primary (or direct) protocol above, but provides signal amplification by increasing the number of fluorophore molecules per antigen, and allows more flexibility because a variety of different secondary antibodies and detection techniques can be used for a given primary antibody (Robinson *et al.,* 2001).

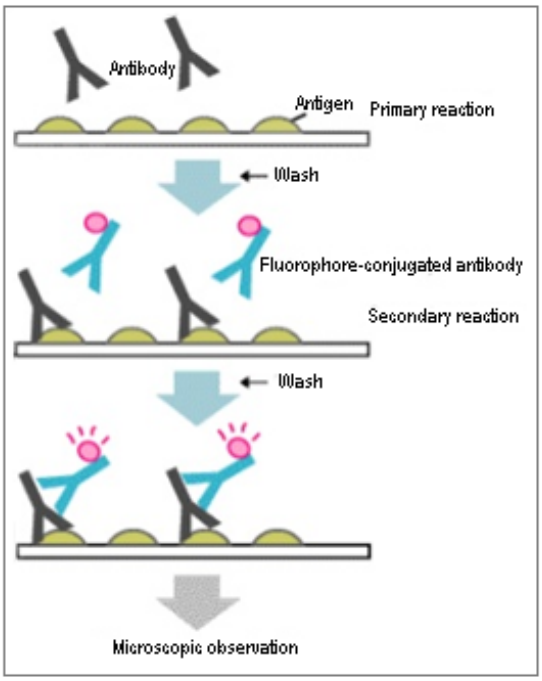

Figure 25. Basic scheme of indirect immunofluorescence.

4.5. Instrumentation for intracellular lipid quantification and measurement of AMPK modulation

For the quantification of intracellular lipid and the measurement of AMPK modulation, the instrument Cytation™ 3 was used (Figure 26). It is a cell-imaging multi-mode microplate reader that combines automated digital microscopy and conventional microplate detection including fluorescence. This design provides rich phenotypic cell information with well-based quantitative data. The microscopy module provides high-quality cellular and sub-cellular imaging in fluorescence, or bright field. The multi-mode microplate reader incorporates variable bandwidth monochromator optics and high-sensitivity filter-based detection optics, designed to achieve excitation and emission of the specific fluorophores.

The ability to perform both conventional quantitative fluorescence measurements and cell imaging is of great usefulness in cell biology, as a single instrument platform can process workflows that would traditionally require multiple instruments and software interfaces.

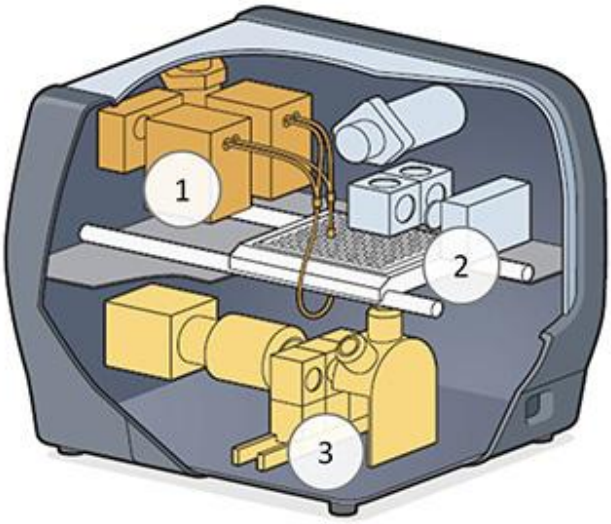

Figure 26. Basic scheme of the Cytation 3 Imaging Multi-Mode Reader. 1: Monochromator optics, 2: filter optics, 3: imaging optics.

5. Product development

For the development of Functional Food, the most widely applied methods involve: the removal of a harmful component; the addition of a beneficial compound that is not naturally present in the target food, or that is already found in concentrations that do not meet the standard recommendations; or the modification of the technological process to be able to preserve, or to increase the content in bioactive compounds.

A wide range of food products of plant origin suitable to be developed as functional food is the juice sector. The fruit-juice production process includes a series of basic steps consisting of: cleaning, sorting, and inspection of the fruit; astringency removal when the fruit tannin content is very high; crushing and juicing; pressing; clarification, which typically involves enzyme-catalyzed depectinization and fining; filtration of solid residues; and a thermal processing that normally consists of a pasteurization step that will allow fruit-juice conservation (Figure 27).

Figure 27. Basic scheme for fruit-juice production.

In the last few years, research has been devoted to finding innovative food-processing techniques to enhance fruit-juice production with a minimum of changes in the nutritional and organoleptic properties, maintaining or improving the content in bioactive compounds. These new technologies

are described in detail in Chapters 5 and 6 of the present doctoral dissertation, and a practical case is discussed in Chapter 7.

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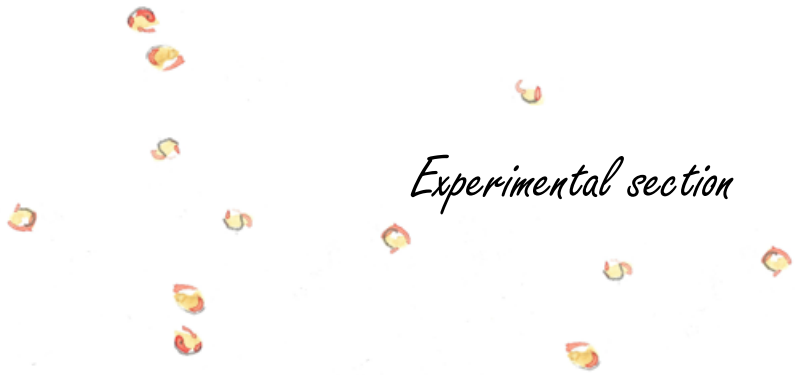

Experimental section

Contents block I

Screening for bioactive compounds

Chapter 1

Journal of Food Composition and Analysis 46 (2016) 78–87

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Original Research Article

Comprehensive, untargeted, and qualitative RP-HPLC-ESI-QTOF/MS^2 metabolite profiling of green asparagus (*Asparagus officinalis*)

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ARTICLE INFO

Article history:
Received 15 July 2015
Received in revised form 12 November 2015
Accepted 21 November 2015
Available online 24 November 2015

Keywords:
RP-HPLC-ESI-QTOF/MS^2
Tandem mass spectrometry
Asparagus officinalis
Asparagaceae
Metabolic profile
Polyphenols
Flavonoids
Oxylipins
Food analysis
Food composition

ABSTRACT

Asparagus officinalis (green asparagus) is a well-known health-promoting vegetable crop, now widely consumed all over the world. Because health-promoting characteristics in food are increasingly demanded and included in the purchase decision by the discriminating consumer, the investigation of the metabolic profile is of great interest for its revalorization. In this study, a reverse-phase high-performance liquid chromatography coupled to electrospray quadrupole time-of-flight mass spectrometry (RP-HPLC-ESI-QTOF/MS^2) methodology was applied for the comprehensive profiling of polar and semi-polar metabolites from a hydromethanolic green asparagus extract. A total of 94 compounds were tentatively identified, belonging to different chemical classes such as organic acids, amino acids, peptides and derivatives, polyphenols (hydroxycinnamic acids, flavonols, lignans, and norlignans), oxylipins, and others. Among them, 74 are reported for the first time in this vegetable. This methodology demonstrated to be one of the tools of choice for the metabolite profiling of plant extracts.

1. Introduction

Asparagus officinalis (green asparagus), is a well known health-promoting vegetable crop. Some scientific research has provided evidence that indicates this crop may exert hypocholesterolemic and hepatoprotective effects (Vázquez-Castilla *et al.,* 2013) as well as possessing antitumor (Bousserouel *et al.,* 2013), hypotensive and renal protective (Sanae & Yasuo, 2013) and anti-diabetic (Hafizur *et al.,* 2012) properties. Because health-promoting characteristics in food are increasingly demanded and included in purchasing decisions by the discriminating consumer, investigating the metabolic profile as a first step in establishing the beneficial effects associated with a specific product is of great interest for its revalorization (Fuentes-Alventosa *et al.,* 2008). However, only a few phytochemical investigations, now outdated, have been reported, revealing that *Asparagus officinalis* contains flavonoids, specifically di-and tri-glycosides of quercetin,

kaempferol, and isorhamnetin (Fuentes-Alventosa *et al.,* 2008); saponins (Wang *et al.,* 2003), and hydroxicinnamic acids (Rodríguez *et al.,* 2005). Still, a very large percentage remains unknown and needs to be identified before their relation to health can be fully understood.

In this regard, untargeted profiling methods have shown the potential of probing the entire metabolic space, including substances that are currently unknown (or at least unidentified) at the time of measurement, trying to identify analytic features of all detectable compounds (Schuhmacher *et al.*, 2013). Among the methods used to determine small polar and semipolar metabolites, the most widely used are based on reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) or tandem MS with atmospheric-pressure ionization techniques, i.e. electrospray ionization (ESI). The hybrid mass analyser quadrupole-time-of-flight (QTOF) provides excellent mass accuracy over a wide dynamic range, and measurements of the true isotope pattern that elucidates the molecular formulas of unknown metabolites with a high degree of reliability (Allwood *et al.*, 2012). This instrument also performs tandem MS, with which more detailed structural information can subsequently be gained, above all when standard compounds are not available, becoming the tool of choice for the metabolite profiling of vegetables (Abu-Reidah *et al.*, 2012). Therefore, the main objective of this study was to evaluate the metabolic profile of a hydromethanolic *Asparagus officinalis* extract employing a HPLC–ESI–QTOF/MS^2 approach, using this methodology as a starting point for elucidating some structural information based on the suggested molecular formula, information provided by MS/MS fragmentation, and the literature.

2. Materials and methods.

2.1. Chemicals and apparatus.

All chemicals were of analytical reagent grade and used as received. Methanol used for the extraction was purchased from Panreac (Barcelona, Spain). Acetic acid and acetonitrile for HPLC

were purchased from Fluka and Sigma-Aldrich (Steinheim, Germany), respectively. Solvents were filtered using a Solvent Filtration Apparatus 58061 (Supelco, Bellefonte, PA, USA). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). Authentic standards were used for comparison of the retention time, MS, and MS2 data with the compounds identified. Quercetin-3-O-rutinoside (rutin), 3-caffeoylquinic acid (chlorogenic acid), quinic acid, and quercetin-3-β-D-glucoside were purchased from Sigma-Aldrich (San Luis, USA); and kaempferol-3-Orutinoside (nicotiflorin) from Extrasynthese (Lyon, France).

2.2. Samples.

Green asparagus samples were collected over the spring of the year 2014. About 1 to 1.5 kg of fresh edible sprouts was crushed, frozen at −25 °C and placed on a lyophiliser shelf (Christ Alpha 1–2 LD Freeze dryer, Shropshire, UK) that was precooled to −50 °C for 1 h at 1 mbar. Then, the sample was ground with an ultra-centrifugal mill Retsch ZM200 (Haan, Germany). The resulting powder was kept at −18 °C until analysed.

2.3. Extract preparation.

The extraction was conducted as follows: 500 mg of dried green asparagus was placed in a test tube. Then, 15 mL methanol/water (80:20 v/v) was added and the sample was sonicated for 20 min and then centrifuged at 984 ×g for 10 min. This value was kept at 37-40ºC in order to avoid any degradation of metabolites. The supernatant was removed, evaporated, and reconstituted in 2 mL methanol/water (80:20 v/v). Finally, the extract solution was filtered through a 0.2 PTFE µm micro-filter (Millipore, Bedford, MA, USA) before analysis (Morales-Soto *et al*., 2014).

2.4. Analytical characterisation.

Separation of metabolites from the hydromethanolic extracts was performed on an Agilent 1200 series Rapid Resolution LC (Agilent Technologies, CA, USA) consisting of a vacuum degasser,

an auto-sampler and a binary pump. This instrument was equipped with an Agilent Zorbax C18 column (4.6 × 150 mm, 1.8 µm) from Agilent Technologies. Acidified water (0.5% acetic acid, v/v) and acetonitrile were used as eluents A and B, respectively. The following multi-step linear gradient was applied: 0 min, 0% B; 20 min, 20% B; 30 min, 30% B; 40 min, 50% B; 50 min, 75% B; 60 min, 100% B. The initial conditions were held for 10 min, according to previous research developed in our laboratory (Fernández-Arroyo *et al.*, 2010). The flow rate was set at 0.8 mL/min throughout the gradient. The effluent from the HPLC column was split using a T-type splitter before being introduced into the mass spectrometer (ratio 1:3). Thus, in this study the flow that arrived in the MS detector move at 0.2 mL/min to ensure stable spray. The column temperature was maintained at 25 °C and the injection volume was 10 µL. The MS analysis was performed using the microTOF-Q™ (Bruker Daltonik, Bremen, Germany), which was coupled to the HPLC system. The Q-TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative ion mode. External mass-spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% acetic acid) in quadratic high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to the identification. The optimum values of the source and transfer parameters were established according to previous research in our laboratory (Abu-Reidah *et al.*, 2012). Briefly, parameters for analysis were set using negative ion mode with spectra acquired over a mass range from *m/z* 50 to 1100. The optimum values of the ESI-MS parameters were: capillary voltage, + 4.0 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; nubilizing gas pressure, 29 psi; collision RF, 150 Vpp; transfer time 70 µs, and pre-pulse storage, 5 µs. Moreover, 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 collision gas.

The acquisition in automated MS/MS mode was of one precursor ion. The MS and MS/MS data were processed through Data Analysis 4.0 software (Bruker Daltonik), which gave a list of possible elemental formulas by using the Generate Molecular Formula Editor.

3. Results and Discussion.

The compounds were assigned by taking into account their elution order and comparing MS and MS^2 data (accurate mass, isotopic distribution, fragmentation patterns of the compounds detected) with respect to green asparagus compounds reported in the literature and in existing online public databases such as Metlin, Massbank or SciFinder Scholar, and pure standards confirmation of some compounds. The HPLC-ESI-Q-TOF/MS^2 base peak chromatogram (BPC) corresponding to negative polarity to illustrate the positions of the peaks over the course of the chromatographic run is shown in supporting information. A total of 94 compounds were tentatively identified, belonging to different chemical classes such as organic acids; amino acids, peptides and derivatives; phenolic compounds (hydroxycinnamic acids, flavonoids, stilbenes, lignans and norlignans); oxylipins, glyceroglycolipids, and others. Table 5 list the retention time, experimental and calculated *m/z*, molecular formula, mass error (ppm), *mσ* values, main fragment ions detected in MS/MS spectra, and the proposed tentative identification for the 94 metabolites.

Peak	RT (min)	*m/z* experimental (M-H)	Molecular formula	*m/z* calculated (M-H)	Error (ppm)	mSigma value	Fragments	Proposed compound
				Organic acids				
1	2.038	195.0507	$C_6H_{12}O_7$	195.0510	1.7	19.3	-	Gluconic acid
3	2.256	191.0566	$C_7H_{12}O_6$	191.0561	4.2	26.4	127(17)	Quinic acid*
4	2.407	133.0141	$C_4H_6O_5$	133.0142	1.3	4.2	115(100)	Malic acid
44	26.089	187.0976	$C_9H_{16}O_4$	187.0976	0.0	7.2	125(100)	Nonanedioic acid (azelaic acid)
				Amino acids, peptides and derivatives				
2	2.063	131.0471	$C_4H_8N_2O_3$	131.0462	-6.9	86.3	114(100), 76(14)	L-asparagine
5	4.893	130.0870	$C_6H_{13}O_2$	130.0874	2.4	7.3	-	L-(iso)leucine
6	5.044	180.0667	$C_9H_{11}NO_3$	180.0666	-0.4	16.1	119(100), 163(47), 134(14)	L-tyrosine
7	5.363	292.1395	$C_{12}H_{23}NO_7$	292.1402	2.3	5.1	130(100)	L-(iso)leucine-hexose
9	7.529	245.1140	$C_{10}H_{18}N_2O_5$	245.11463	1.2	38.4	116(100), 128(68)	L-glutamic acid-L-valine
10	7.730	164.0774	$C_9H_{11}NO_2$	164.0717	2.0	11.6	147(100)	L-phenylalanine
11	7.982	326.1235	$C_{15}H_{21}NO_7$	326.1245	3.3	61.9	164(100)	L-phenylalanine-hexose
12	9.224	259.1306	$C_{11}H_{20}N_2O_5$	259.1299	-2.5	32.1	130(66), 255(100), 215(31)	L-glutamic acid-L-leucine isomer
15	10.333	316.1508	$C_{13}H_{23}N_3O_6$	316.15514	1.9	18.6	254(100), 223(87), 298(86), 187(50), 197(49), 146(37), 143(30), 283(20),	L-glutamic-L-alanine-L-valine
16	11.122	203.0829	$C_{11}H_{12}N_2O_2$	203.0826	1.6	17.9	116(100), 142(28)	L-tryptophan

18	12.147	259.1300	$C_{11}H_{20}N_2O_5$	259.1299	-0.1	11.1	130(100), 128(52), 197(23)	L-glutamic acid-L-leucine isomer
21	14.214	263.1392	$C_{14}H_{20}N_2O_3$	263.1401	3.4	16.9	115(100)	L-Valine-L-phenylalanine
31	18.999	372.2121	$C_{24}H_{27}N_3O$	372.2081	-10.6	23.6	173(100), 130(86), 277(85)	L-(iso)leucine-L-(iso)leucine-L-glutamic acid
45	27.820	406.1938	$C_{15}H_{29}N_5O_8$	406.1943	1.3	105.9	376(100), 164(58), 277(18), 332(15)	L-serine-L-serine-L-serine-L-lysine
47	30.039	583.2841	$C_{24}H_{40}N_8O_9$	583.2845	0.8	10.3	450(37), 523(10)	L-proline-L-proline-L-threonine-L-arginine-L-aspartic acid
				Phenolic compounds				
Phenolic acids								
14	9.677	329.0894	$C_{14}H_{18}O_9$	329.0878	-4.7	60.2	167(100)	Vanillic acid hexose
17	11.609	353.0875	$C_{16}H_{18}O_9$	353.0878	0.8	59.2	191(100), 179(77), 135(23)	3-caffeoylquinic acid (chlorogenic acid)*
19	13.610	355.1044	$C_{16}H_{20}O_9$	355.1035	-2.7	41.1	193(100), 134(82), 149(76)	Feruloyl hexose isomer
20	13.861	337.0928	$C_{16}H_{18}O_8$	337.0929	0.4	15.5	163(100), 119(21), 191(16)	3-*p*-coumaroylquinic acid
22	14.264	341.0875	$C_{15}H_{18}O_9$	341.0878	0.8	20.7	179(100), 135(10)	Caffeoyl hexose
23	14.398	353.0876	$C_{16}H_{18}O_9$	353.0878	0.7	37.0	191(100)	5-caffeoylquinic acid (neo-chlorogenic acid)/1-caffeoylquinic acid
24	15.137	367.1029	$C_{17}H_{20}O_9$	367.1035	1.5	1.0	193(100), 134(14)	3-feruloylquinic acid
25	16.279	355.1036	$C_{16}H_{20}O_9$	355.1035	-2.7	41.1	193(100), 149(18)	Feruloyl hexose isomer
26	17.438	355.1029	$C_{16}H_{20}O_9$	355.1035	1.7	34.4	193(100), 295(71), 175 (64), 235(51)	Feruloyl hexose isomer
28	17.992	367.1049	$C_{17}H_{20}O_9$	367.1035	-3.9	36.7	173(100), 193(27)	4-feruloylquinic acid isomer
30	18.781	367.1027	$C_{17}H_{20}O_9$	367.1035	2.2	20.3	173(100), 193(28)	4-feruloylquinic acid isomer

35	21.351	267.0883	$C_{13}H_{16}O_6$	267.0874	-3.5	16.4	252(100), 175(59), 149(21)	Feruloyl glycerol
57	36.610	383.1138	$C_{21}H_{20}O_7$	383.1136	-0.3	20.3	163(100), 145(24), 119(17), 219(15)	Dicoumaroyl glycerol
58	36.996	413.1233	$C_{22}H_{22}O_8$	413.1242	0.2	0.9	193(100), 163(53), 175(13), 145(11)	Coumaroylferuloyl glycerol
59	37.332	443.1340	$C_{23}H_{24}O_9$	443.1348	1.7	14.5	193(98), 175(16), 249(15)	Diferuloyl glycerol
Flavonoids								
27	17.606	593.1494	$C_{27}H_{30}O_{15}$	593.1512	3.0	11.0	473(6)	Apigenin-6-8-di-C-glucoside (vicenin 2)
34	20.729	771.1981	$C_{33}H_{40}O_{21}$	771.1989	1.1	4.0	-	Quercetin glucosyl rutinoside
36	21.855	625.1395	$C_{27}H_{30}O_{17}$	625.1410	2.5	30.8	299(20)	Quercetin diglucoside
37	21.906	609.1454	$C_{27}H_{30}O_{16}$	609.1461	1.2	6.4	301(6)	Quercetin-3-O-rutinoside(rutin)*
38	22.863	785.2146	$C_{34}H_{42}O_{21}$	785.2146	0.0	19.8	315(7)	Isorhamnetin glucosyl rutinoside
39	22.896	463.0881	$C_{21}H_{20}O_{12}$	463.0882	0.3	22.3	300(100), 301(74)	Quercetin-3-β-D glucoside*
40	23.031	533.1662	$C_{26}H_{30}O_{12}$	533.1664	0.5	24.1	353(100), 341(70)	Noricaritin-O-glucoside (amurensin)
41	23.955	593.1504	$C_{27}H_{30}O_{15}$	593.1512	1.3	4.0	285(16)	Kaempferol-3-O-rutinoside (nicotiflorin)*
42	24.325	623.1609	$C_{28}H_{32}O_{16}$	623.1618	1.4	4.5	315(22)	Isorhamnetin-O-rutinoside (narcissin)
43	24.963	447.0930	$C_{21}H_{20}O_{11}$	447.0933	0.7	22.0	284(69), 285(40)	Kaempferol glucoside
46	28.391	489.1073	$C_{23}H_{22}O_{12}$	489.1038	-7.0	29.9	285(100)	Kaempferol-O-acetylglucoside
48	30.341	635.1610	$C_{29}H_{32}O_{16}$	635.1618	1.2	11.2	285(100)	Kaempferol-O-acetylrutinoside
50	31.937	593.1313	$C_{30}H_{26}O_{13}$	593.1301	-2.0	21.1	285(38)	Kaempferol-coumaroyl glucoside

51	32.492	301.0356	$C_{15}H_{10}O_7$	301.0354	0.8	5.9	151(100), 179(61)	Quercetin
Stilbenes								
29	18.177	447.1651	$C_{23}H_{28}O_9$	447.1661	2.1	25.4	285(100), 225(36)	Trimethoxystilbene hexose
Lignans and Norlignans								
33	20.275	523.2186	$C_{26}H_{36}O_{11}$	523.2185	-0.2	17.8	361(100)	Secoisolariciresinol hexose
66	42.304	251.1081	$C_{17}H_{16}O_2$	251.1078	-1.4	10.9	235(13)	Hinokiresinol
Oxylipins								
49	31.668	345.2271	$C_{18}H_{34}O_6$	345.2283	3.5	29.2	343(91), 283(87)	Dihydroxy-octadecanedioic acid
53	34.912	327.2182	$C_{18}H_{32}O_5$	327.2177	-1.6	1.5	211(76), 229(55), 171(11)	Trihydroxy-octadecadienoic acid isomer
54	35.400	327.2176	$C_{18}H_{32}O_5$	327.2177	0.3	5.9	-	Trihydroxy-octadecadienoic acid isomer
55	35.820	327.2174	$C_{18}H_{32}O_5$	327.2177	1.0	7.6	-	Trihydroxy-octadecadienoic acid isomer
56	36.342	329.2236	$C_{18}H_{34}O_5$	329.2233	-0.7	5.7	211(55), 229(41), 171(19)	Trihydroxy-octadecaenoic acid isomer
60	38.441	329.2332	$C_{18}H_{34}O_5$	329.2233	0.4	1.9	211(22), 199(25)	Trihydroxy-octadecaecaenoic acid isomer
61	38.659	329.2331	$C_{18}H_{34}O_5$	329.2233	0.8	9.4	201(35), 171(24)	Trihydroxy-octadecaecaenoic acid isomer
62	39.180	329.2331	$C_{18}H_{34}O_5$	329.2233	0.9	1.9	199(18), 211(17)	Trihydroxy-octadecaecaenoic acid isomer
63	40.238	329.2332	$C_{18}H_{34}O_5$	329.2233	0.5	2.1	211(13), 199(16)	Trihydroxy-octadecaecaenoic acid isomer
64	41.733	309.2064	$C_{18}H_{30}O_4$	309.2071	2.5	85.0	251(100), 291(34), 223(18), 195(15)	15-hydroperoxy-octadecatrienoic acid
65	42.119	329.2345	$C_{18}H_{34}O_5$	329.2333	-3.6	15.2	-	Trihydroxy-octadecaecaenoic acid isomer

67	42.925	311.2224	$C_{18}H_{32}O_4$	311.2228	1.4	6.8	223(100), 291(16), 195(12)	13-hydroperoxy-octadecadienoic acid
68	43.262	311.2224	$C_{18}H_{32}O_4$	311.2228	1.4	6.8	183(96), 241(44), 293(36), 223(27), 213(23), 127(20)	10-hydroperoxy-octadecadienoic acid
69	43.497	309.2071	$C_{18}H_{30}O_4$	309.2071	0.1	95.1	197(100), 209(88), 251(49), 171(44), 291 (40), 225(35), 223(35), 181(13)	11-hydroperoxy-octadecatrienoic acid
71	44.403	311.2232	$C_{18}H_{32}O_4$	311.2228	-1.4	16.5	171(100), 185(38), 293(38), 139(22), 201(17)	9-hydroperoxy-octadecadienoic acid
72	44.924	313.2385	$C_{18}H_{34}O_4$	313.2384	-.03	0.8	183(79), 129(33), 295(24)	12,13-dihydroxy-octadecenoic acid
73	45.394	313.2383	$C_{18}H_{34}O_4$	313.2384	0.5	4.0	201(100), 171(26), 295(11)	9,10-dihydroxy-octadecenoic acid
74	45.528	311.2234	$C_{18}H_{32}O_4$	311.2228	1.3	73.4	211(96), 293(72), 197(559, 199(32), 181(26), 201(19), 129(18), 183(15), 169(12), 193(12), 212(11), 171(10)	12-hydroperoxy-octadecadienoic acid
75	46.200	311.2226	$C_{18}H_{32}O_4$	311.2228	-0.5	8.8	171(76), 211(45), 293(44), 197(23), 201(20), 139(17), 185(14), 199(10)	9-hydroperoxy-octadecadienoic acid
76	46.938	291.1991	$C_{18}H_{28}O_3$	291.1966	-8.6	42.8	247(23), 165(17)	12-oxo-phytodienoic acid
78	47.677	293.2126	$C_{18}H_{30}O_3$	239.2122	-1.5	1.6	275(100), 171(40), 121(20)	Hydroxy-octadecatrienoic acid isomer
79	48.080	293.2126	$C_{18}H_{30}O_3$	239.2122	-1.5	50.2	275(68), 195(52), 223(50), 171(11)	Hydroxy-octadecatrienoic acid isomer
80	49.171	295.2286	$C_{18}H_{32}O_3$	295.2279	-2.6	20.8	277(100), 195(68)	13-hydroxy-octadecadienoic acid
81	49.910	295.2289	$C_{18}H_{32}O_3$	295.2279	-3.5	6.1	277(98), 171(28), 195(27)	Hydroxy-octadecadienoic acid isomer
82	50.396	295.2289	$C_{18}H_{32}O_3$	295.2279	-1.6	3.0	-	Hydroxy-octadecadienoic acid isomer
83	51.001	293.2126	$C_{18}H_{30}O_3$	239.2122	-1.5	15.6	113(15), 195(9)	Oxo-octadecadienoic acid isomer
84	51.202	293.2127	$C_{18}H_{30}O_3$	239.2122	-1.7	18.0	113(15), 195(9)	Oxo-octadecadienoic acid isomer
85	51.941	293.2128	$C_{18}H_{30}O_3$	239.2122	-1.5	13.9	185(12),	Oxo octadecadienoic acid isomer
86	52.546	295.2299	$C_{18}H_{32}O_3$	295.2279	7.0	1.4	-	Hydroxy-octadecadienoic acid isomer

88	53.118	295.2290	$C_{18}H_{32}O_3$	295.2279	-3.8	4.2	277(42), 195(36)	13-hydroxy-octadecadienoic acid
89	53.336	295.2299	$C_{18}H_{32}O_3$	295.2279	-6.7	7.6	277(100), 171(46)	9-hydroxy-octadecadienoic acid
90	54.580	291.1970	$C_{18}H_{28}O_3$	291.1966	-1.6	4.9	211(100), 193(70), 97(44)	12-oxo-octadecatrienoic acid
91	55.960	293.2125	$C_{18}H_{30}O_3$	239.2122	-1.1	1.1	193(65), 99(20)	Hydroxy-octadecatrienoic acid isomer
92	56.381	293.2130	$C_{18}H_{30}O_3$	239.2122	-2.6	2.0	-	Hydroxy-octadecatrienoic acid isomer/ oxo-octadecadienoic acid isomer
93	56.751	277.2179	$C_{18}H_{29}O_2$	277.2173	2.1	7.6	-	9,12,15-octadecatrienoic acid (linolenic acid)
94	59.039	279.2336	$C_{18}H_{32}O_2$	279.2330	-2.3	1.7	-	9,12-octadecadienoic acid (linoleic acid)
					Glyceroglycolipids			
70	43.899	675.3589	$C_{33}H_{56}O_{14}$	675.3597	1.2	11.8	397(100), 415(43), 277(36)	Linoleoyldigalactopyranosyl glycerol (Gingerglycolipid A)
77	47.202	513.3069	$C_{27}H_{46}O_9$	513.3069	-0.0	6.4	277(100), 253(22)	Linoleoylgalactopyranosyl glycerol
					Other compounds			
8	6.924	282.0836	$C_{10}H_{13}N_5O_5$	282.0844	2.7	12.6	150(100)	Guanosine
13	9.342	218.1032	$C_9H_{17}NO_5$	218.1034	0.7	188.0	146(100)	Pantothenic acid
32	19.906	148.9741	$C_4H_6O_2S_2$	148.9736	-3.4	8.5	104(100)	Asparagusic acid
52	34.056	349.1879	$C_{16}H_{30}O_8$	349.1868	-3.1	15.9	187(100)	Tyrosol glucoside (rhodioloside D)
87	52.949	205.1605	$C_{14}H_{21}O$	205.1598	3.7	8.6	-	Di-tert-butylphenol

Table 5. Reverse-phase high-performance liquid chromatography coupled to electrospray quadrupole time-of-flight mass spectrometry (HPLC-ESI-TOF/MS2) data on the compounds identified. *Compounds identified by comparison with pure standard.

3.1. Organic acids.

A total of four organic acids were tentatively identified in the green asparagus extract. These strongly influence the organoleptic properties of vegetables and plants, being responsible for sourness or acidity (Gómez-Romero *et al.*, 2010); specifically, gluconic (peak 1), quinic (peak 3), malic (peak 4), and azelaic (peak 44) acids. The fragmentation patterns of these compounds generated mainly neutral losses of a molecule of water, such as with malic acid, of the carboxylic residue followed by dehydration as with azelaic acid, or of the carboxylic residue followed by dehydration but requiring the migration of two hydrogens from the remaining portion of the molecule, as was the case with quinic acid, which was confirmed by the relevant standard. No previous reports on the presence of organic acids in *Asparagus officinalis* were found.

3.2. Amino acids, peptides and derivatives.

Besides proteins, peptides as well as amino acids have been associated with a variety of biological functions in plants. In the case of plant peptides, they have not received much attention compared to other food sources and other plant metabolites, probably because they are not abundant, making their detection difficult (Farrokhi *et al.*, 2008).

Five free amino acids, Asn (2), Leu/Ile (5), Tyr (6), Phe (10), and Trp (16), were detected in asparagus samples. In general, neutral losses of NH_3 from the amino-terminal (Asn, Phe), in some cases followed by loss of CO_2 from the carboxy-terminal (Tyr, Trp) were observed independent of their chain substitution, except Leu/Ile, which was not fragmented under our MS/MS conditions. In the case of Trp, its characteristic indole group (*m/z* 116) was the most abundant fragment generated after sequential neutral losses of NH_3, CO_2 and C_2H_2, as reported by Abu-Reidah *et al.*, (2013). In some cases such as Tyr, they produced immonium ions [R–CH=NH2]-, where R is the residue of the amino acid, after the loss of H_2O and CO. It is noteworthy that dipeptides and oligomers with up to five residues were also detected, which were

proposed as Glu,Val (9), Glu,Leu/Ile (12 and 18), Glu,Ala,Val (15), Val,Phe (21), Glu,Ile/leu,Ile/leu (31), Ser,Ser,Ser,Lys (45), and Pro,Pro,Thr,Arg,Asp (47). Please note that the positions of the amino acids along the peptidic chain could not be established. Their fragmentation occurred at the peptidic bond (amide bond), with preferential cleavage of bonds adjacent to acidic amino acids. For example, the oligopeptide at *m/z* 583 (48), characterized by a sequence composed of the amino acids Pro, Pro, Thr, Arg and Asp, produced its main ion at *m/z* 450, due to release of the aspartic acid. This is in accordance with Qin J., (1995), who also observed this preferential cleavage using MALDI-ion trap mass spectrometer experiments. Losses of H_2O, CO_2 and NH_3 were detected in some cases. Furthermore, Amadori compounds derived from the condensation of the amino acids Ile/leu, and Phe with reducing sugars (hexoses) formed in food via Maillard reactions were also present in asparagus (7, and 11, respectively). Their fragmentation gave rise to ions at *m/z* 130, and 164, respectively, corresponding to loss of the hexose moiety. In this regard, although freeze-drying is performed under reduced temperature and high pressure, initial Maillard reaction products have previously been found in other vegetables after freeze-drying (Iswaldi *et al.*, 2013). Still, this initial Maillard stage does not cause serious physical and flavour changes in comparison to advanced stages of Maillard reaction browning (Hui, 2006).

3.3. Phenolic compounds

A total of 32 compounds belonging to the phenolic class were identified, namely 15 phenolic acids, 14 flavonoids, 1 stilbene, and 2 lignans and norlignans.

Among phenolic acids, 15 compounds belonging to the hydroxycinnamic acid subclass were detected, while there was only one belonging to the hydroxybenzoic acid subclass. It has been reported that substantial amounts of hydroxycinnamic acids are intimately associated with plant cell walls and can have a significant impact on their mechanical properties (Jaramillo *et al.*, 2007). These compounds consisted of different hexose, quinic, and glycerol derivatives of caffeic, ferulic,

and coumaric acids. Hexose derivatives, namely feruloyl hexose (19, 25, and 26), and caffeoyl hexose (22), produced fragments matching their corresponding hydroxycinnamic acids under our MS/MS conditions after loss of the hexosyl residue (162u). Quinic acid derivatives of caffeic, ferulic, and coumaric acids are called chlorogenic acids. In the asparagus samples, two compounds with the same molecular ion were found at *m/z* 353. They were tentatively assigned as caffeoylquinic acids (compounds 17 and 23). Compound 17 gave the same MS^2 base peak at *m/z* 191 due to the loss of caffeic acid and a relatively intense secondary ion at *m/z* 179, allowing the assignment of 3-caffeoylquinic acid (chlorogenic acid) as reported by Clifford *et al.*, (2003). This assignment was confirmed by injection of the authentic standard. In addition, compound 23 showed the same MS^2 base peak at 191, while the secondary ion at *m/z* 179 was undetectable. According to the same authors (Clifford *et al.*, 2005), this fragmentation pattern is indicative of 1-, or 5-caffeoylquinic acids, these being distinguishable only by their retention times. Given that only one of those compounds was found in our samples, no differentiation between position 1- or 5- was possible and, therefore, compound 23 was tentatively assigned as 1-caffeoylquinic acid/5-caffeoylquinic acid. Likewise, the fragmentation pattern allowed us to distinguish between isomers at *m/z* 367, identified as feruloylquinic acids. The first eluting isomer (15.1 min) produced the base MS^2 peak at *m/z* 193, characteristic of 3-feruloylquinic acid (24), while isomers eluting at 17.9, and 18.7 min produced this peak at *m/z* 173, this being indicative of 4-feruloylquinic acid isomers (28 and 30). A quasimolecular ion $[M–H]^-$ at *m/z* 337 was found for peak 20. Fragmentation of this compound yielded a position specific fragment at *m/z* 163, corresponding to loss of the quinic acid radical, which is characteristic of 3-*p*-coumaroylquinic acid. This assignment based on the specific fragmentation pattern has been previously described by Clifford *et al.*, (2003). Figure 28 represents the MS/MS spectrum of selected chlorogenic acids, together with their respective structures and fragmentation sites. Among these compounds, there is

previous evidence only of ferulic acid and its dimers in asparagus spears, which have been reported to be involved in postharvest textural changes through the formation of polysaccharide-lignin cross-links (Rodríguez *et al.*, 2005). Four chromatographic peaks corresponded to one or two molecules of ferulic and/or coumaric acid esterified to a glycerol backbone, namely feruloyl glycerol (35), dicoumaroyl glycerol (57), coumaroyl feruloyl glycerol (58), and diferuloyl glycerol (59). Their MS spectra showed $[M - H]^-$ at *m/z* 267, 383, 413, and 443, respectively. The compound at *m/z* 267 (35) presented its main fragment at *m/z* 252, indicative of loss of the methyl residue proceeding from the methoxyl group of the feruloyl residue. The compound at *m/z* 383 (57) showed its major fragment at *m/z* 163, formed upon loss of a coumaric acid molecule. The compounds at *m/z* 413 (58), and 443 (59) showed the MS^2 base peak at *m/z* 193 [M-H-feruloyl-CO]-. These molecules also presented other minor fragments, produced as a result of fragmentation within the bonds adjacent to the oxygen involved in the ester link between the glycerol moiety and the ferulic/coumaric acid molecules, in some cases requiring migration of two hydrogen atoms. These unconventional phenolic compounds have previously been isolated from aerial parts of green asparagus (Jang *et al.*, 2004). One compound belonging to the hydroxybenzoic acid subclass was detected at *m/z* 329, namely vanillic acid hexose (14). It produced a parent ion at *m/z* 167 due to loss of the hexosyl moiety.

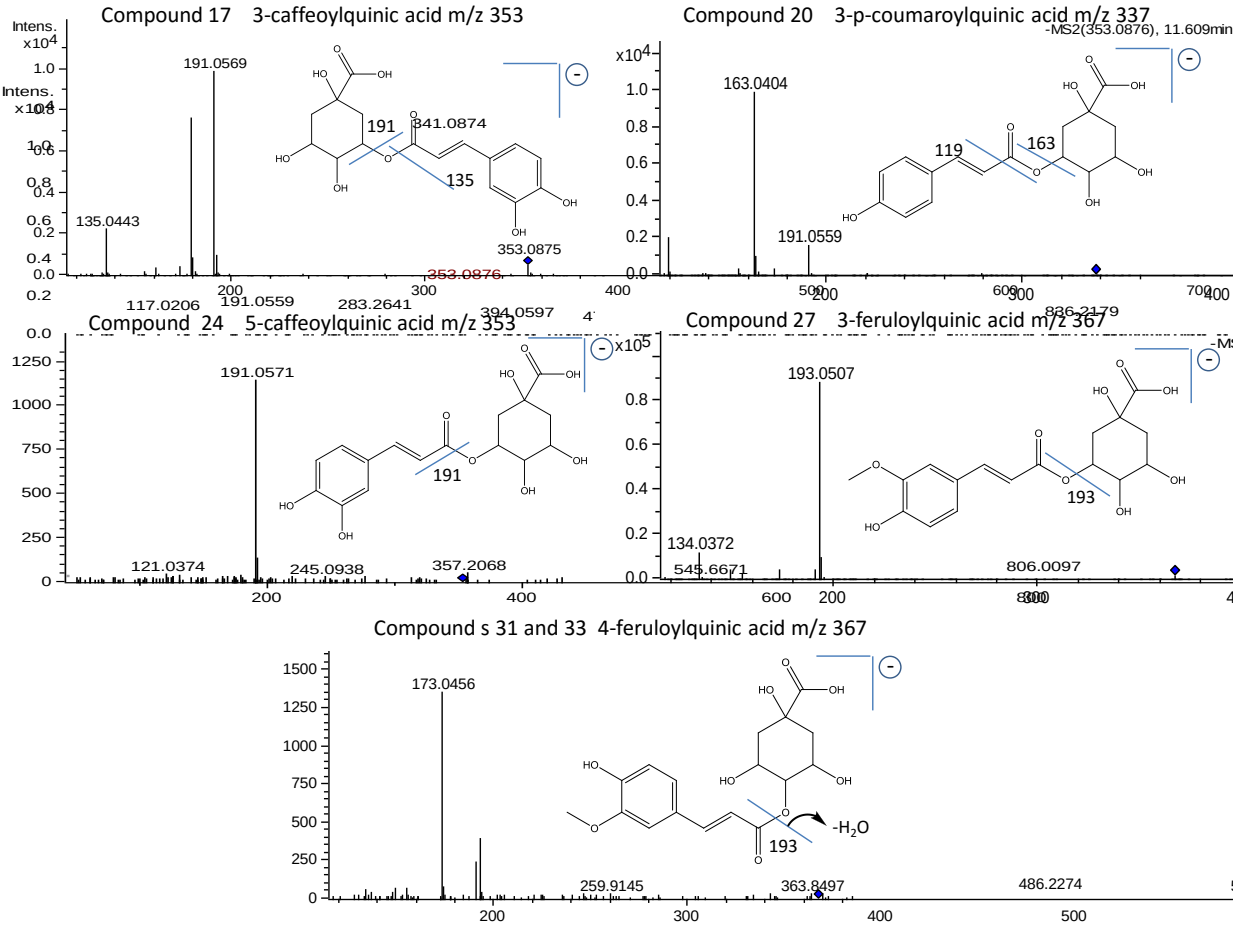

Figure 28. Tandem mass spectrometry (MS^2) spectra of selected hydroxybenzoic acids in negative ion mode and proposed fragmentation sites.

Among the flavonoids, our results showed that 13 flavonoids belonged to the flavonol subclass and 1 to the flavone subclass. Flavonols include molecules of quercetin, kaempferol, and isorhamnetin linked to sugars. Their fragmentation involves cleavage at the glycosidic *O*-linkage with a concomitant H-rearrangement leading to elimination of the monosaccharide residue and providing the aglycone $[Y_0]^+$ ion (Abad-García *et al.*, 2009). The type of monosaccharide can be determined by taking into account the mass difference between the *m/z* values of the quasi-molecular and $[Y_0]^+$ ions. In this way, differences of 162 Da are related to the loss of a glucosyl residue; 146 Da, a rhamnosyl; 308 Da, a rutinosyl (glucose plus rhamnose); 470 Da, a glucosyl rutinosyl; 192 Da, an acetylglucosyl; 350 Da, an acetylrutinosyl; and finally, 308 Da, a coumaroyl

glucosyl. Therefore, the aglycone ions corresponding to quercetin (*m/z* 301), kaempferol (*m/z* 285), and isorhamnetin (*m/z* 315) were observed as major peaks of these compounds in the MS/MS spectra. Consequently, these compounds were tentatively identified as: quercetin glucosyl rutinoside (34)(*m/z* 771), quercetin diglucoside (36)(*m/z* 625), quercetin-3-O-rutinoside (37)(*m/z* 609), isorhamnetin glucosyl rutinoside (38)(*m/z* 785), quercetin-3-β-D-glucoside (39)(*m/z* 463), kaempferol-3-O-rutinoside (41)(*m/z* 593), isorhamnetin-O-rutinoside (42)(*m/z* 623), kaempferol-glucoside (43)(*m/z* 447), kaempferol-O-acetylglucoside (46)(*m/z* 489), kaempferol-O-acetylrutinoside (48)(*m/z* 635), kaempferol-coumaroyl glucoside (50)(*m/z* 593), and quercetin (51)(*m/z* 301). It is noteworthy that quercetin and kaempferol glucosides showed both the aglycone fragment, at *m/z* 301 and 285, respectively, and the product ion resulting after homolytic cleavage of the O-glycosidic bond, rendering a radical aglycone anion at *m/z* 300 and 284, respectively. The radical aglycone anion:aglycone fragment ratio suggests a certain glycosylation position. However, it cannot be used as a diagnostic tool for characterization of the glycosylation position in unknown flavonoid O-glycosides (Cuyckens & Claeys, 2005). Only the position of the sugar of compounds 37, 39, and 41 was established by comparison to the corresponding standard. Fuentes-Alventosa *et al.*, (2008) provided evidence for some of these flavonols in asparagus samples. And finally, it is remarkable that another compound was detected at *m/z* 593, with an identical molecular formula to compound 41, yet different MS/MS behaviour, allowing us to differentiate between these two molecules. This compound eluting at 17.6 min showed a MS^2 fragment ion at *m/z* 473 [M-H-120 Da]. Losses of 120 Da corresponded to cross-ring cleavages in the sugar unit. Abad-García *et al.*, (2009) reported that these losses are indicative of C-glycosides, as flavonoid *C*-glycosides need higher collision energies to fragment than do *O*-glycosides since they lack a labile bond, and the main fragmentations take place in the sugar, which has the weakest bonds in the molecule. Therefore, this compound was tentatively assigned

as apigenin-6-8-di-C-glucoside (vicenin 2)(27). Its fragmentation pattern is consistent with Gouvea *et al.*, (2012).

Among stilbenes, one deprotonated molecule was detected at *m/z* 447. It was tentatively identified as trimethoxystilbene hexose (32). Its deprotonated molecule generated a major ion at *m/z* 285 in the MS/MS spectrum, which could be attributed to loss of the hexosyl residue. Most methoxyllated stilbenes are known for exerting a more potent anticancer activity than their parent compound resveratrol (Park *et al.*, 2007).

Finally, two compounds belonging to the lignan and norlignan family were identified. A compound with a deprotonated molecule at *m/z* 523 showed a base peak at *m/z* 361 resulting from the loss of a hexosyl residue. Therefore, this compound was tentatively assigned as secoisolariciresinol hexose (33). A compound at *m/z* 251 was tentatively identified as hinokiresinol (66). It showed a major fragment at *m/z* 235 [M-H-CH_4], which is in accordance with Kim *et al.*, (2014), who performed HPLC-QTOF analyses in *Anemarrhena asphodeloides*.

3.4. Oxylipins

Oxylipins are bioactive metabolites derived from the oxygenation of polyunsaturated fatty acids. They can be found, in plants, as free compounds or esterified with other molecules such as phospholipids or galactolipids (Ghanem *et al.*, 2012). They are formed after release of the free fatty acids from triglycerides due to the effect of lipolytic enzymes. Many of these oxidized lipids or oxylipins are isomers, which make their identification a challenging task. LC–MS–MS currently is the most powerful tool because of its specificity and sensitivity (Ludovici *et al.*, 2014).

In our study, 9,12,15-Octadecatrienoic (linolenic) acid as well as 9,12-octadecadienoic (linoleic) acid were detected at *m/z* 277 and 279, respectively (93, and 94), as well as many other oxidized metabolites derived from them. Specifically, two isomers of hydroperoxides of octadecadienoic

acid at *m/z* 309 (64, and 69), and five isomers of hydroperoxides of octadecatrienoic acid at *m/z* 311 (67, 69, 71, 74, and 75) were detected. The MS^2 spectra of hydroperoxides produced the major fragment due to breakage of the weakest bond in the molecule, which is the one in the vicinity of the functional group, followed by loss of a molecule of water. More concretely, hydroperoxy-linolenic acid isomers produced the MS^2 fragments at *m/z* 251 [M-H-C_3H_5-H_2O]-, and 197 [M-H-C_7H_{11}-H_2O]-, while hydroperoxy-linoleic acid isomers produced them at *m/z* 223 [M-H-C_4H_6O-H_2O]-, 183 [M-H-$C_7H_{12}O_2$]-, 171 [M-H-C_9H_{14}-H_2O]-, and 211 [M-H-$C_6H_{12}O$]-. This allowed us to assign the peroxide position. Interestingly, other minor fragment ions were produced within the alkyl chain. These fragments have been attributed to charge-remote fragmentations. This type of fragmentation might occur in closed-shell ions of long-chain alkyl compounds such as fatty acids holding a charge (i.e. the carboxylate end), no matter how the molecule is charged, occurring at sites that are physically removed from the location of the charge (Wang *et al.*, 2013). These normally contain an ion series with an inter-peak spacing of 14u, representing cleavages of consecutive C-C single bonds in the fatty acid chain (Perret *et al.*, 2004). This technique is usually employed on instruments at high collisional energy, producing a unique pattern of product ions from which its molecular structure is revealed. However, certain chain fragmentations have also been achieved in MS/MS instruments at collision energies of 20–60 eV (Cheng & Gross, 2000; Wang *et al.*, 2013). Therefore, this aided us in assigning the position of the hydroperoxide, but assigning the position of the double bonds was not possible. Figure 29 illustrates an example of the fragmentation pattern proposed for one of these compounds.

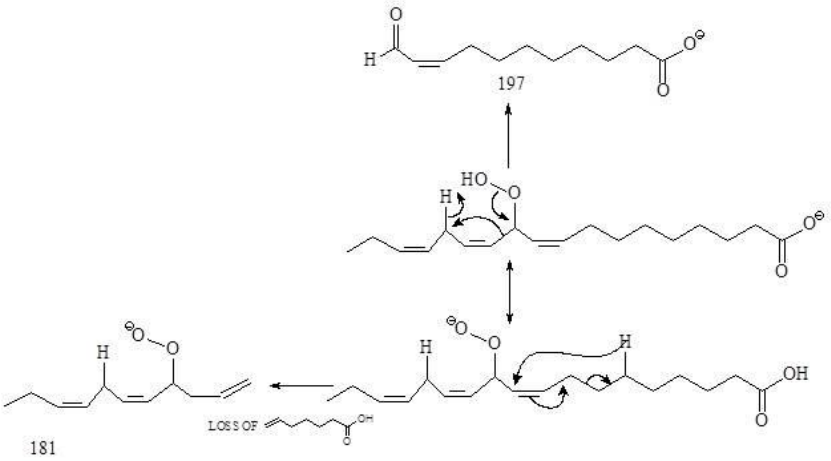

Figure 29. Fragmentation pattern proposed for compound 69 (11-hydroperoxy-octadecatrienoic acid isomer).

In addition, six compounds at *m/z* 293 with a similar molecular formula ($C_{18}H_{30}O_3$) were detected. This formula coincided with isomers of hydroxyloctadecatrienoic acids and oxooctadecadienoic acid. The assignment of one or the other was based on the observation of distinguishing MS^2 fragments. On the one hand, compounds with characteristic fragments at *m/z* 171, 195, or 193 were characterised as hydroxyoctadecatrienoic acids (78, 79, and 91), as suggested by Ludovici *et al.*, (2014). On the other hand, compounds with characteristic fragments at *m/z* 185, and 113 were characterised as keto-octadecadienoic acids (83, 84, and 85) according to the information provided by Strassburg et al., (2012), who recorded the fragmentation pattern of these metabolites through a MS/MS methodology. Similar criteria were followed to assign the structures of compounds detected at *m/z* 295. Observation of the structure-specific fragment ion at *m/z* 171 allowed the assignment of 9-hydroxy-octadecadienoic acid (89), while an ion at *m/z* 195 allowed the assignment of 13-hydroxy-octadecadienoic acids (80, and 88) (Masoodi *et al.*, 2008). When this assignment was unclear, the general name hydroxy-octadecadienoic acid was given (81, 82, and 86).

Moreover, two isomers of dihydroxy-octadecenoic were present in the asparagus samples (72, and 73). Ludovici *et al.*, (2014) suggested that MS^2 fragmentation can discriminate between the 12,13-, and the 9,10- positions of the hydroxyl residues, a fragment at *m/z* 183 being indicative of the 12,13- position, a fragment at *m/z* 201 of the 9,10- position, and a fragment at *m/z* 171 of the 8,13-position. This fragmentation pattern is in accordance with our findings. We found fragments at *m/z* 183, and 201, which allowed us to discriminate between positions 12,13- (compound 72), and 9, 12- (compound 73) of the two hydroxyl groups of the molecule. Further on, six isomers of trihydroxy-octadecaenoic acid were detected at *m/z* 329 (56 60, 61, 62, 63, and 65), and three of trihydroxy-octadecadienoic acid at *m/z* 327 (53, 54, and 55). Major fragments were produced in the vicinity of the hydroxyl group, producing MS^2 base peaks at *m/z* 211 [M-H-$C_6H_{14}O_2$}-, 201 [M-H-$C_8H_{15}O$}-, and 199 [M-H-$C_7H_{14}O_2$}- for trihydroxy-octadecaenoic acids, and at *m/z* 211 [M-H-$C_6H_{12}O_2$}- for trihydroxy-octadecadienoic acids. While the MS^2 fragmentation allowed us to assign these compounds, MS^2 fragments are not specific for one particular structure, as different structures share the same fragments. Therefore, assigning the positions of the functional groups and double bonds within the structure was not possible.

Compound 76 was identified as 12-oxo-phytodienoic acid. It is a cyclopentenone oxilipin belonging to the jasmonate subclass. It showed a molecular ion at *m/z* 291 and presented the base peak at *m/z* 247, due to loss of the carboxylic residue, while a minor product ion at *m/z* 165 arose from the cyclopentenone ring plus the five-carbon side chain, but required the migration of two hydrogens from the remaining portion of the molecule, according to the hypothesis of fragmentation reported by *Bao et al.*, (2014).

Finally, compound 90 was characterized as oxo-octadecatrienoic acid. It showed the MS^2 base peak fragment at *m/z* 211 [M-H-C_6H_8]- due to a fragmentation in the vicinity of the keto group, this

being indicative of the 12-oxo position, and at *m/z* 193 [M-H-$C_6H_{11}O$]- owing to a C-C breakage of the carbon backbone, due to charge-remote fragmentations.

Figure 30 shows the MS^2 spectra of selected oxylipins and proposed fragmentation sites. As far as we know, this is the first time that the oxylipin profile has been described in *Asparagus officinalis;* only their precursors 9,12,15-octadecatrienoic acid (linolenic acid), and 9,12-octadecadienoic acid (linoleic acid) have previously been reported in this vegetable (Jang et al., 2004).

3.5. Glyceroglycolipids

Glyceroglycolipids consist of hydrophilic carbohydrate groups and hydrophobic fatty acid moieties that bind to a glycerol moiety. They are known to be some of the major components of biomembranes and have been considered to play important roles in these structures, such as enhancing membrane stability (Chapman *et al.*, 1983). Two compounds belonging to this chemical class were tentatively identified in asparagus samples at *m/z* 675 and *m/z* 513, comprised of a molecule of linolenic acid linked to the glycerol moiety as well as to a molecule of digalactose in the case of compound 70 (linoleoyldigalactopyranosyl glycerol or gingerglycolipid A), and to a molecule of galactose in the case of compound 77 (linoleoylgalactopyranosyl glycerol). These molecules may break within the bonds adjacent to the ester between the glycerol and the fatty acid. Glyceroglycolipids are widely distributed in edible plants such as cereals, legumes, vegetables, and fruits (Jiang *et al.*, 2014). Yet to date, this is the first evidence of the presence of glycerolipids in *Asparagus officinalis*.

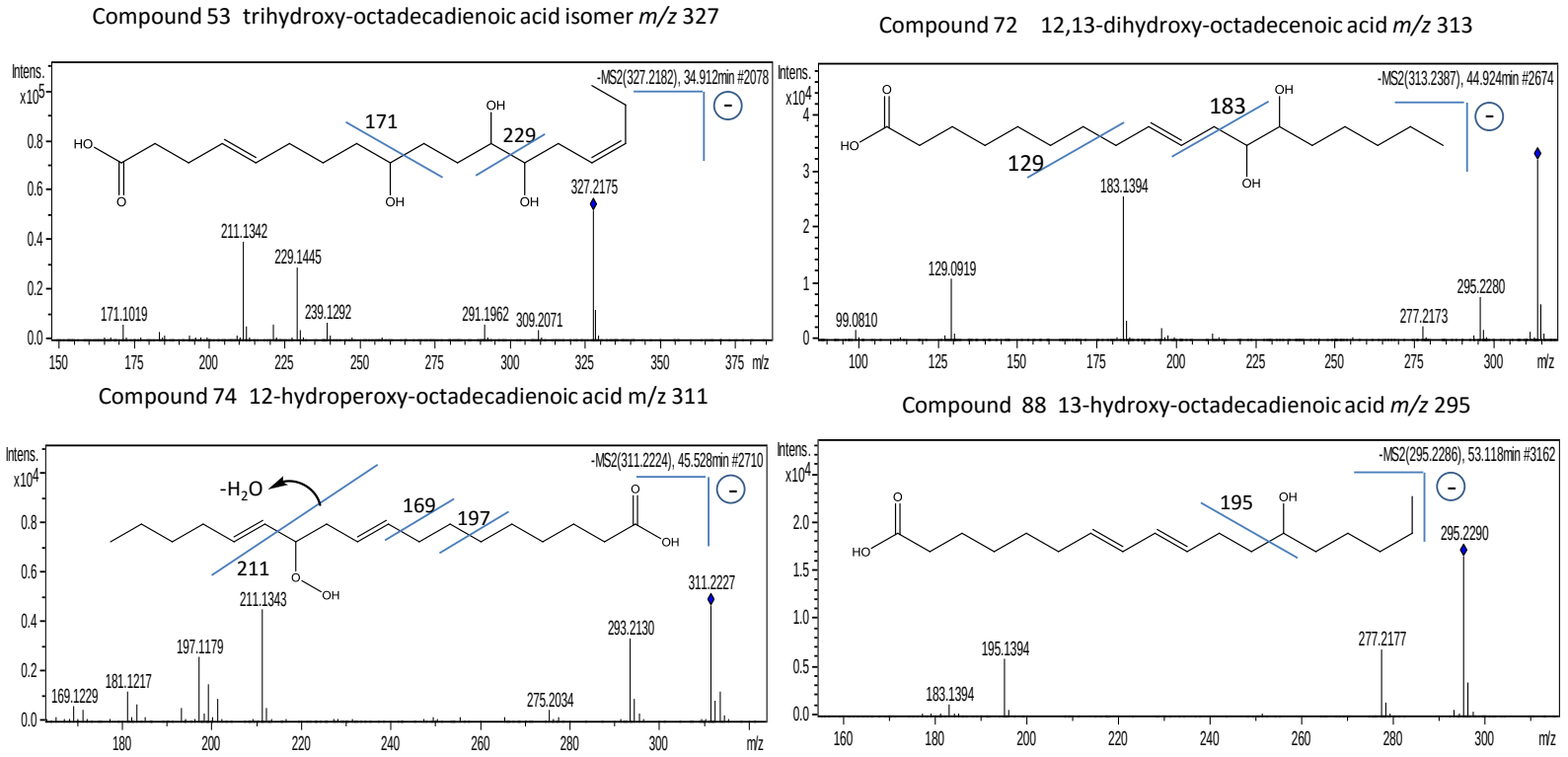

Figure 30. Tandem mass spectrometry (MS^2) spectra of selected oxylipins in negative ion mode and proposed fragmentation sites.

3.6. Other compounds

This section includes the compounds that were not classified in the previous sections. Specifically, one nucleoside, one vitamin, one organosulfur compound, one monoterpene, and one alkyphenol were identified. The nucleoside was tentatively characterized as guanosine (8), whose corresponding most prominent fragment ion at *m/z* 150 was generated by breakage of the glycosidic bond and loss of the ribose moiety ($C_5H_8O_4$). The characterization of the vitamin as pantothenic acid (13) was based on the presence of the fragment at *m/z* 146 $[M\text{-}H\text{-}CO\text{-}CO_2]^-$, as described by Rodríguez-Pérez *et al.*, (2013). The compound at *m/z* 148 was tentatively assigned as the organosulfur compound asparagusic acid (1,2-dithiolane-4-carboxylic acid) (32). This compound appears unique to asparagus and, while apparently innocuous toxicologically to man, is the most probable culprit responsible for the curious excretion of odorous urine following asparagus ingestion (Mitchell & Waring, 2014). The compound that presented a deprotonated molecule at *m/z* 349 showed a major fragment at *m/z* 187 due to loss of a glucosyl residue. It was therefore identified as tyrosol glucoside (rhodioloside D) (52). Rhodiolosides are monoterpene glycosides, which were isolated from the roots of *Rhodiola rosea* (Crassulaceae) for the first time in 2006 (Ma *et al.*, 2006), from which they receive their name. Finally, the compound showing a molecular ion at *m/z* 205 was tentatively assigned as di-tert-butylphenol (87). Although this alkyl phenol did not undergo fragmentation under our MS conditions, it has been previously described in asparagus volatile oil (Kang et al., 2010), which justifies this assignment.

4. Conclusion

A total of 94 compounds were tentatively identified, belonging to different chemical classes such as organic acids; amino acids, peptides and derivatives; phenolic compounds (hydroxycinnamic acids, flavonols, lignans and norlignans); oxylipins; glyceroglycolipids, and others. Among them, 74 are reported for the first time in *Asparagus officinalis*. However, agronomic and environmental

conditions are known to influence the metabolite profiling of vegetables and therefore, the possibility of finding different compounds in another batch of the same vegetable should be borne in mind. MS detection enabled the tentative identification of compounds with similar retention times, proving adequate specificity for the analysis. Therefore, the methodology applied was demonstrated to be a tool of choice for metabolite profiling of plant extracts, providing excellent MS efficiency, and enabling us to elucidate some structural features as a starting point prior to NMR and spectroscopy studies.

5. Acknowledgements

This work was supported by the projects BFU2014-52433-C3-2-R and AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as by P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are also grateful for the scholarship FPU AP2010-2793 and to Professor Fong-Fu Hsu (Washington University School of Medicine, USA) for his assessment on fragmentation of oxylipins.

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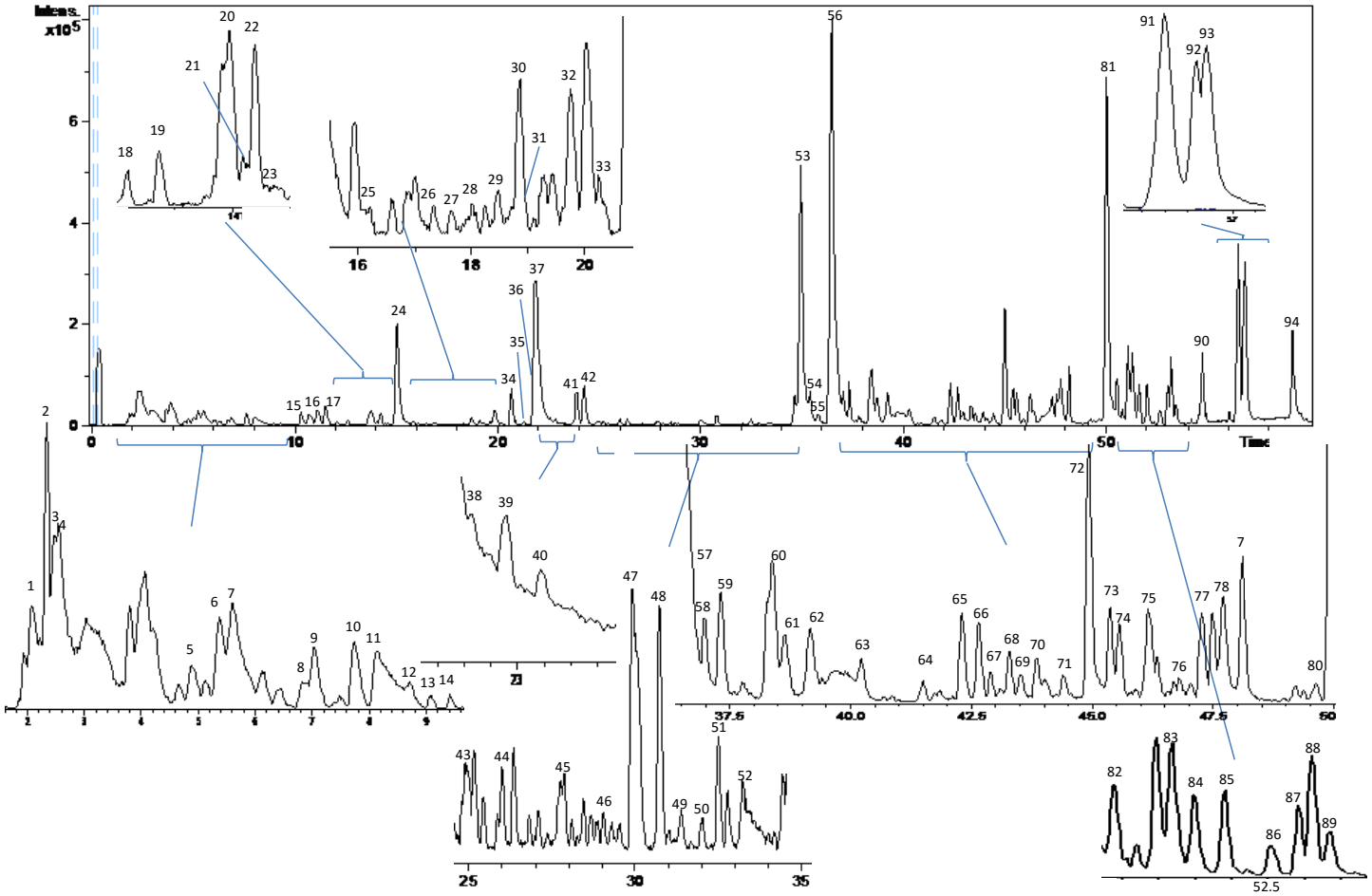

Figure S1. Base peak chromatogram (BPC) obtained by HPLC-ESI-QTOF/MS^2 in negative ion mode of the green asparagus extract.

Chapter 2

Industrial Crops and Products 71 (2015) 214–234

Contents lists available at ScienceDirect

Industrial Crops and Products

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RP-HPLC–ESI–QTOF/MS^2 based strategy for the comprehensive metabolite profiling of *Sclerocarya birrea* (marula) bark

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ARTICLE INFO

Article history:
Received 6 November 2014
Received in revised form 9 January 2015
Accepted 30 January 2015
Available online 23 March 2015

Keywords:
RP-HPLC–ESI–QTOF/MS^2
Tandem mass spectrometry
Sclerocarya birrea
Metabolite profiling
Proanthocyanidins
Polyphenols

ABSTRACT

In this research, a non-selective extraction procedure followed by a RP-HPLC–ESI–QTOF/MS^2 method was applied to evaluate the metabolic profile of these extracts, allowing the identification of a total of 95 compounds belonging to the chemical classes of organic acids, polyphenols, fatty acid derivatives, and others, most of these being identified for the first time in these extracts. These proved far richer in polyphenols, and more specifically in proanthocyanidins. This methodology successfully detected from monomers up to dimers of (epi)catechin, (epi)gallocatechin, and (epi)afzelechin units with one or two galloyl residues. A very high degree of galloylation was found, which may serve in the bioactivity attributed to these extracts. The chromatographic method had sufficient resolving power to separate up to six isomeric forms of several compounds, and the structure of some of these isomeric compounds has been elucidated. Therefore, the methodology applied proved useful for the metabolite profiling of *Sclerocarya birrea* stem–bark extracts, providing essential information that could be used to explain the plethora of ethnotherapeutic properties and pharmacological actions that have been attributed to this African tree.

1. Introduction

Diabetes mellitus has become one of the leading public-health challenges in the world. The most recent data from the International Diabetes Federation show that there are now 382 million people with diabetes, and predict that by 2035 this number will climb to almost 600 million worldwide (Alberti and Zimmet, 2014, Zimmet *et al.*, 2014). Herbal remedies are widely used to treat type 2 diabetes mellitus. Such plants include a tree indigenous Southern Africa, *Sclerocarya birrea* (Anacardiaceae), which has traditionally been used for stem-bark decoctions as medication for this disease (Mariod and Abdelwahab, 2012, Ojewole, 2003, Ojewole *et al.*, 2010). Some reports have attempted to establish the fundamentals for the ethnomedicinal uses of this plant. *In vitro* studies have shown that *Sclerocarya birrea* extracts increase glucose uptake in C2C12, 3T3-

L1, and HepG2 cells. Furthermore, *in vivo* studies have shown that various extracts of *Sclerocarya birrea* may improve glucose homeostasis in diabetic rats by the stimulation of insulin-secreting cells (Ojewole, 2003 and 2004, Ndifossap *et al*., 2010), as well as exert reno- and cardio-protective effects in diabetes mellitus (Gondwe *et al*., 2008). However, only general compositional analysis, now outdated, have been reported, revealing that these barks may contain chemical compounds such as gallotannins, flavonoids, alkaloids, steroids (including β-sitosterol), coumarins, triterpenoids, sesquiterpene hydrocarbons, ascorbic acid, oleic, myristic, stearic acids, and amino acids (Iwu, 1993, van Wyk *et al*., 2002). Still, a very large percentage remains unknown and needs to be identified before their relation to health can be fully understood.

Among the methods used to determine small polar molecules, the most widely used are based on reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to mass spectrometry (MS) or tandem MS with atmospheric-pressure ionization techniques, i.e. electrospray ionization (ESI). The hybrid mass analyzer quadrupole-time-of-flight (QTOF) provides excellent mass accuracy over a wide dynamic range and measurements of the true isotope pattern that elucidates the molecular formula of unknown metabolites with a high degree of reliability, taking into account the seven golden heuristic and chemical rules for selecting elemental compositions (Allwood *et al*., 2012). This instrument also performs tandem MS, with which more detailed structural information can subsequently be gained, above all when standard compounds are not available. Furthermore, QTOF provides high selectivity by the extracted-ion chromatogram (EIC) mode when there are overlapping peaks, where spectrophotometric detection could be limited. Thus, HPLC coupled to highly sensitive and high-resolution MS, such as QTOF, enables the separation and detection of minor compounds that could co-elute and be underestimated vs. major ions, these not being identified or even detected by older

methodologies (Abu-Reidah *et al*., 2013a). Our previous studies have demonstrated that RP-HPLC-ESI-QTOF-MS^2 is well suited to the untargeted characterization of plants, enabling a wide overview of their composition (Abu-Reidah *et al*., 2013b, Quirantes-Piné *et al*., 2013, Rodríguez-Pérez *et al*., 2013). Therefore, the main objective of this study was to evaluate the metabolic profile of four selected *Sclerocarya birrea* stem-bark extracts for which the anti-diabetic properties have been demonstrated (Ndifossap *et al*., 2010, Dimo *et al*., 2007, Fotio *et al*., 2009), although their chemical constituents have never been characterized, and therefore the scientific basis of their anti-diabetic properties remains to be established for the potential development of nutraceuticals and industrial cultivation of this African tree.

2. Materials and methods

2.1. Chemicals and apparatus

All chemicals were of analytical reagent grade. Methanol and dicloromethane were purchased from Panreac (Barcelona, Spain) and acetic acid from Fluka and Sigma-Aldrich (Steinheim, Germany). Double-deionized water with conductivity lower than 18.2MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Samples

Sclerocarya birrea stem−barks were provided by the Medicinal Plants Research Group of the Laboratory of Pharmacognosy and Botanics of the Faculty of Medicine, Pharmacy, and Odontology (University of Cheikh Anta DIOP, Dakar, Sénégal), in 2013. Stem barks were cut from the tree manually from different parts of the trunk and transported in darkness. They were air dried and then ground with an ultra-centrifugal mill Retsch ZM200 (Haan, Germany) to a final fineness < 40 µm. The resulting *Sclerocarya birrea* stem-bark powder was kept in darkness until used.

2.3. Extract preparation

The extraction conditions of four representative extracts used to test the bioactivity of *Sclerocarya birrea* stem bark extracts were reproduced. Extract 1 was obtained by maceration of 1 kg powdered stem bark in 3 L of distilled water for 48 h (Ndifossap *et al*., 2010); extract 2 by maceration of 500 g powdered stem bark in 2 L distilled water for one h (Fotio *et al*., 2009); extract 3 by maceration of 2 kg powdered stem bark in methanol for 72 h (Fotio *et al*., 2009); extract 4 by maceration of 2 kg powdered stem bark in a mixture methanol:dicloromethane (1:1) for one week (Dimo *et al*., 2007). Full conditions of the extractions are reported in literature. All extracts were centrifuged for 15 min at 3500 rpm and the supernatant was collected. Finally, the extract was filtered through a 0.2 µm syringe filter and conserved at -20°C prior to analysis. Three replicates were prepared per sample.

2.4. Analytical characterization

The *Sclerocarya birrea* extracts were analytically characterized by high-performance liquid chromatography coupled to electro-spray quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF/MS). The HPLC-ESI-QTOF/MS method was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump, and a diode-array detector (DAD). The chromatographic separation was performed in a Zorbax Eclipse Plus RP-C18 analytical column 150 x 4.6 mm i.d., 1.8 µm particle size (Agilent Technologies, Palo Alto, CA, USA). The flow rate was 0.80 ml/min, and the temperature of the column was maintained at 25°C. The mobile phase used was water with 0.25% acetic acid as eluent A, and methanol as eluent B. The total run time was 27 min using the following multistep linear gradient: 0 min, 5%B; 7 min, 35%B; 12 min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5% B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis, according to previous research developed

in our laboratory (Lozano-Sánchez *et al*., 2010). The injection volume in the HPLC was 20 µl. The compounds separated were monitored in sequence first with DAD (240, 280 and 350 nm) and then with a mass-spectrometry detector. The MS analysis was performed using the microTOFQ™ (Bruker Daltonik, Bremen, Germany), which was coupled to the HPLC system. At this stage, the use of a splitter was required for the coupling with the MS detector, as the flow arriving to the QTOF detector had to be 0.2 mL/min in order to ensure reproducible results and stable spray. The QTOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative ion mode. External mass-spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of acetic) in quadratic high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to the identification. The optimum values of the source and transfer parameters were established according to previous research in our laboratory (Abu-Reidah *et al*., 2012). The acquisition in automated MS/MS mode was of one precursor ion. The MS and MS/MS data were processed through Data Analysis 4.0 software (Bruker Daltonik), which gave a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula. The accuracy threshold for confirmation of elemental compositions was established at 5 ppm for most of the compounds.

3. Results

3.1. Nonselective extraction of the different Sclerocarya birrea stem-bark extracts

Figure 31a shows the HPLC-ESI-Q-TOF/MS BPC (base peak chromatogram) corresponding to negative polarity for the four extracts. Although most of the compounds were present in all extracts, in general the water extractions seemed to extract more polar compounds which eluted at the beginning of the chromatographic run such as (epi)gallocatechin-(epi)gallocatechin dimer (prodephinidin B2) isomers. No qualitative differences were detected among them; however sharper peaks appeared in the longer extraction in comparison with the same peaks. By contrast, methanol and dicloromethane extracted compounds with less polarity such as pentamethoxystilbene or eriodictyol glucoside isomers. This difference was appreciated at the end of the run (from minute 15 on), while in the water extractions this zone appears to be almost flat while in methanol (3) and methanol:dicloromethane (4) extracts, several peaks are shown.

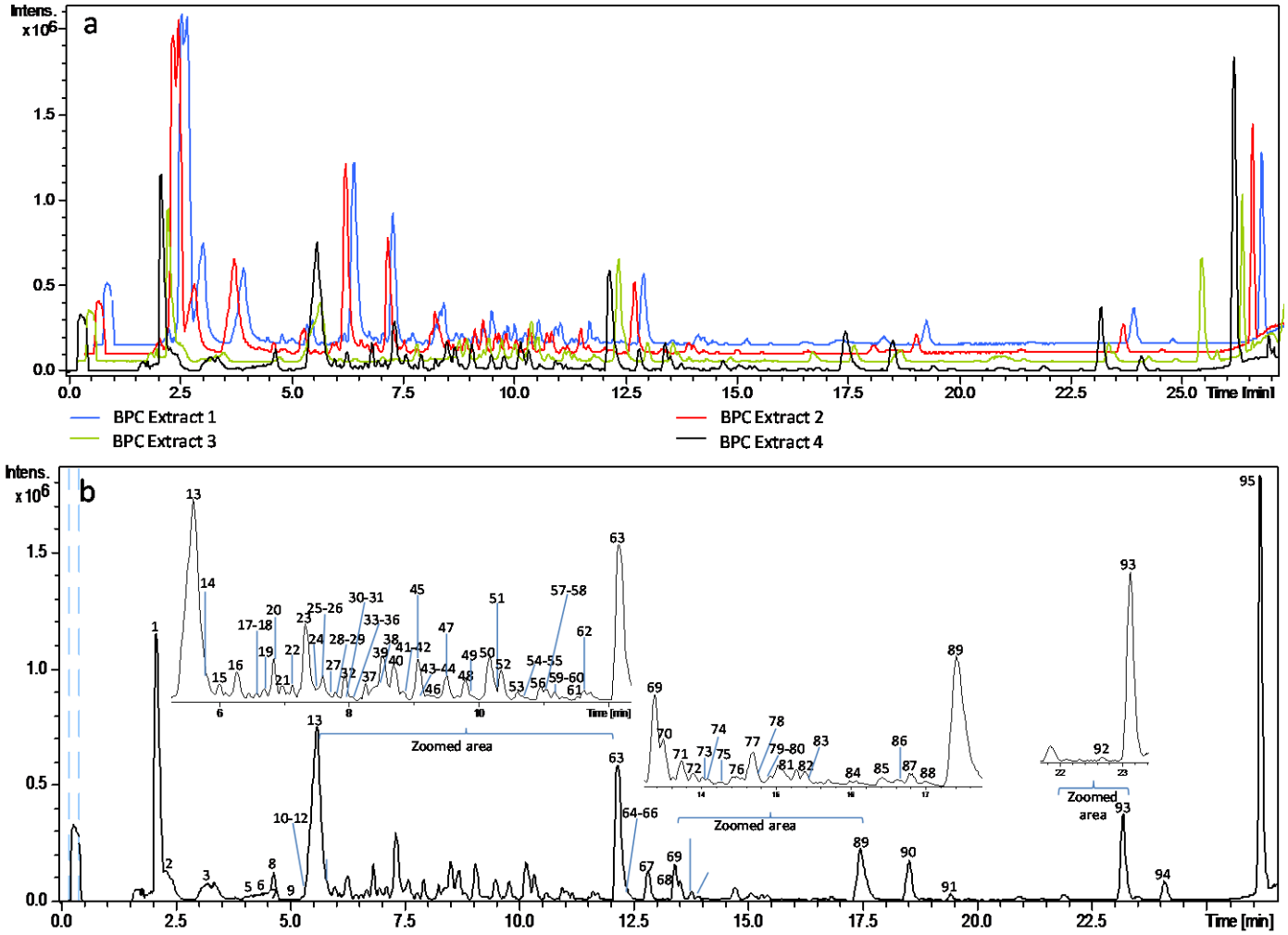


Figure 31. Comparison of the base peak chromatograms (BPC) obtained by HPLC-ESI-QTOF/MS in negative ion mode of the four Sclerocarya birrrea stem bark extracts. (a), and Base peak chromatograms (BPC) obtained by HPLC-ESI-QTOF/MS in negative ion mode of the representative (methanol:dichloromethane, 1:1) of *Sclerocarya birrrea* stem bark extract 4 (b).

Neutral loss	Da
CO	28
CO_2	44
CH_3	15
H_2O	18
$C_8H_8O_4$	168
$C_6H_{12}O_6$	180
$C_6H_{10}O_5$	162
$C_4H_8O_4$	120
$C_3H_5O_3$	90
$C_2H_4O_2$	60
$C_6H_7O_3$	126
$C_7H_4O_4$	152
$C_7H_5O_5$	170
CHO	29
$C_8H_8O_3$	152
$C_8H_8O_4$	168
$C_8H_8O_2$	136

Table 6. Main neutral losses produced in the MS^2 experiments.

3.2. HPLC-ESI-QTOF/MS as a power tool for metabolic profiling

Figure 31b shows the HPLC-ESI-Q-TOF/MS BPC (base peak chromatogram) of a representative extract (methanol:dichloromethane) to illustrate the position of the peaks over the chromatographic run. The compounds were assigned while taking into account the retention times, and by comparing MS and MS^2 data (accurate mass, isotopic distribution, and fragmentation pattern in negative mode) of the compounds detected with respect to the *Sclerocarya birrea* compounds reported in the literature and in existing online public databases such as Metlin, Massbank or SciFinder Scholar. MS fragmentation allowed us to establish neutral losses related to functional groups and substitutions, as well as the molecular formula of the product ions for the unambiguous identification of the precursor ion. A list of neutral losses exhibited in this research is presented in Table 6. Thus, a list with the molecular ions ([M-H]-) found in the different extracts with their retention time, observed and calculated *m/z*, molecular formula, mass error (ppm), *mσ* values, main fragment ions detected in MS/MS, the proposed tentative identification, and references is presented (Table 7).

Pk	RT (min)	*m/z* experimental (M-H)	Molecular formula (M-H)	*m/z* calculated (M-H)	Score	Error (ppm)	mSigma value	Ms² Fragments *	Proposed compound	Extracts
1	1.995	191.0574	$C_7H_{12}O_6$	191.0561	100	-7.0	11.4	**127** [M–H–CO–2H_2O]-	Quinic acid	1, 2, 3, 4
2	2.463	133.0143	$C_4H_6O_5$	133.142	100	-0.3	3.5	**115** [M–H–H_2O]-	Malic acid	1, 2, 3, 4
3	3.367	191.0206	$C_6H_8O_7$	191.197	100	-4.6	3.3	**111** [M–H–CO_2–2H_2O]-	Citric acid	1, 2, 3, 4
4	4.308	205.0365	$C_7H_{10}O_7$	205.0354	100	-5.5	6.3	**143** [M–H_2–CO_2–CH_3]- 111[M–H–CO_2–2H_2O–CH_3]-	Methyl citric acid	1, 2, 3
5	4.405	359.0957	$C_{15}H_{20}O_{10}$	359.0984	100	7.6	9.3	-	Syringic acid glucoside isomer	1, 2, 3, 4
6	4.455	117.0189	$C_4H_6O_4$	117.0193	100	3.5	3.7	**99** [M–H–H_2O]-	Succinic acid	1, 2, 3, 4
7	4.527	609.1292	$C_{30}H_{26}O_{14}$	609.1250	100	5.5	35.5	305 [M–H–$C_{15}H_{13}O_7$]- **423** [M–H–$C_8H_8O_4$–H_2O]- 441 [M–H–$C_8H_8O_4$]-	(Epi)gallocatechin-(epi)gallocatechin (prodephinidin B2) isomer	1, 2
8	4.907	331.0663	$C_{13}H_{16}O_{10}$	331.0671	100	2.3	3.2	151 [M–H–$C_6H_{12}O_6$]- **169** [M–H–$C_6H_{10}O_5$]- 211 [M–H–$C_4H_8O_4$]- 241 [M–H–$C_3H_5O_3$]- 271 [M–H–$C_2H_4O_2$]-	Galloyl glucoside isomer	1, 2, 3, 4
9	5.242	359.0971	$C_{15}H_{20}O_{10}$	359.0984	100	3.7	19.0	197 [M–H–$C_6H_{10}O_5$]- **179** [M–H–$C_6H_{12}O_6$]-	Syringic acid glucoside isomer	1, 2, 3, 4
10	5.543	331.0658	$C_{13}H_{16}O_{10}$	331.0671	100	3.9	23.6	**169** [M–H–$C_6H_{10}O_5$]- 211[M–H–$C_4H_8O_4$]- 241 [M–H–$C_3H_5O_3$]- 271[M–H–$C_2H_4O_2$]-	Galloyl glucoside Isomer	1, 2, 3, 4
11	5.644	493.1168	$C_{19}H_{26}O_{15}$	493.1199	100	4.3	3.7	169 [M–H–2$C_6H_{10}O_5$]- **331** [M–H–$C_6H_{10}O_5$]-	Galloyl diglucoside	1, 2, 3, 4
12	5.769	609.1298	$C_{30}H_{26}O_{14}$	609.1250	100	1.7	338.9	305[M–H–$C_{15}H_{13}O_7$]- **423** [M–H–$C_8H_8O_4$–H_2O]- 441 [M–H–$C_8H_8O_4$]-	(Epi)gallocatechin-(epi)gallocatechin (prodephinidin B2) isomer	1, 2
13	5.828	169.0148	$C_7H_6O_5$	169.0142	100	-3.3	6.5	**125** [M–H–CO_2]-	Gallic acid	1, 2, 3, 4

14	6.280	609.1235	$C_{30}H_{26}O_{14}$	609.1250	100	2.4	6.9	305 [M–H–$C_{15}H_{13}O_7$]- **423** [M–H–$C_8H_8O_4$–H_2O]- 441 [M–H–$C_8H_8O_4$]-	(Epi)gallocatechin-(epi)gallocatechin (prodephinidin B2) isomer	1, 2, 3, 4
15	6.330	593.1280	$C_{30}H_{26}O_{13}$	593.1301	100	3.5	18.8	125 [M–H–$C_{15}H_{12}O_7$-$C_6H_9O_3$]- **289** [M–H–$C_{15}H_{12}O_7$]- 303 [M-H-290 Da] 407 [M–H–$C_8H_8O_4$–H_2O]- 425 [M–H–$C_8H_8O_4$]- 467 [M–H–$C_6H_7O_3$]-	(Epi)gallocatechin-(epi)catechin (prodelphinidin B3) isomer	1, 2, 3, 4
16	6.531	343.0665	$C_{14}H_{16}O_{10}$	343.0671	100	1.8	16.6	**169** [M–H–$C_7H_{11}O_5$]- 191 [M–H– $C_7H_4O_4$]-	Galloylquinic acid	1, 2, 3, 4
17	6.867	483.0782	$C_{20}H_{20}O_{14}$	483.0780	100	-0.3	11.1	125 [M–H–$C_6H_{10}O_5$-$C_7H_4O_4$-CO_2]- **169** [M–H–$C_6H_{10}O_5$-$C_7H_4O_4$]- 271 [M–H–$C_7H_4O_4$-$C_2H_4O_2$]-	Digalloyl glucoside isomer	4
18	6.899	593.1292	$C_{30}H_{26}O_{13}$	593.1301	100	1.4	11.3	125 [M–H–$C_{15}H_{12}O6$–C_6H9O4]- **305** [M–H–$C_{15}H_{12}O6$]- 423 [M–H–$C_8H_8O_3$–H_2O]- 441 [M–H–$C_8H_8O_3$]- 467 [M–H–$C_6H_7O_3$]-	(Epi)catechin-(epi)gallocatechin isomer	1, 2, 3, 4
19	7.067	761.1333	$C_{37}H_{30}O_{18}$	761.1359	100	3.4	29.9	423 [M–H–$C_7H_4O_4$–$C_8H_8O_4$–H_2O]- 441 [M–H–$C_7H_4O_4$–$C_8H_8O_4$]- 591 [M–H–$C_7H_6O_5$]- **609** [M–H–$C_7H_4O_4$]-	(Epi)gallocatechin-(epi)gallocatechin-3'-O-gallate (prodelphinidin B3-3'-O-gallate)/ (epi)gallocatechin-3-O-gallate-(epi)gallocatechin (prodelphinidin B3-3-O-gallate) isomer	1, 2, 3, 4
20	7.150	305.0661	$C_{15}H_{14}O_7$	305.0667	100	2.0	9.7	**125** [M–H–$C_9H_9O_4$] 137 [M–H–$C_8H_8O_4$] 165 [M–H–140 Da] 167 [M–H– $C_7H_6O_3$] 179 [M–H–$C_6H_6O_3$] 219 [M–H–86 Da] 261 [M–H–CO_2]	(Epi)gallocatechin isomer	1, 2, 3, 4
21	7.184	153.0188	$C_7H_6O_4$	153.0193	100	3.7	5.8	**109** [M–H–CO_2]-	Protocatechuic acid isomer	1, 2, 3, 4

22	7.268	167.0339	$C_8H_8O_4$	167.0350	100	6.7	7.7	**123** [M–H–CO_2]-	Vanillic acid	1, 2, 3, 4
23	7.401	761.1367	$C_{37}H_{30}O_{18}$	761.1359	100	2.0	51.3	591 [M–H– $C_7H_4O_4$–H2O]- **609** [M–H– $C_7H_4O_4$]-	(Epi)gallocatechin-(epi)gallocatechin-3'-O-gallate (prodelphinidin B3-3'-O-gallate)/ (epi)gallocatechin-3-O-gallate-(epi)gallocatechin isomer	1, 2, 3, 4
24	7.520	527.1405	$C_{23}H_{28}O_{14}$	527.1406	100	0.2	12.3	169 [M–H–$C_9H_{10}O_6$-$C_6H_8O_4$]- **313** [M–H–$C_9H_{10}O_6$]-	Guaiacylglycerol glucoside gallate isomer	4
25	7.587	577.1336	$C_{30}H_{26}O_{12}$	577.1351	100	2.7	9.3	125 [M-H-$C_{15}H_{12}O_6$–$C_9H_9O_3$]– **289** [M–H–$C_{15}H_{12}O_6$]– 407 [M–H–$C_8H_8O_3$–H_2O]– 425 [M–H–$C_8H_8O_3$]–	(Epi)catechin-(epi)catechin (proanthocyanidin B2)	1, 2, 3, 4
26	7.653	301.2932	$C_{13}H_{18}O_8$	301.0929	100	-0.9	13.3	**139** [M–H–$C_6H_{10}O_5$]-	(Iso)tachioside	1, 2, 3, 4
27	7.721	527.1417	$C_{23}H_{28}O_{14}$	527.1406	100	-2.0	34.5	-	Guaiacylglycerol glucoside gallate isomer	4
28	7.736	593.1289	$C_{30}H_{26}O_{13}$	593.1301	100	2.0	13.0	125 [M–H–$C_{15}H_{12}O_7$-$C_6H_9O_3$]- 16 [M-H-424 Da]– 177 [M-H-416 Da]– **289** [M–H–$C_{15}H_{12}O_7$]- 303 [M-H-290] 407 [M–H–$C_8H_8O_4$–H_2O]- 425 [M–H–$C_8H_8O_4$]- 467 [M–H–$C_6H_7O_3$]-	(Epi)gallocatechin-(epi)catechin (prodelohinidin B3) isomer	1, 2, 3, 4
29	7.837	761.1367	$C_{37}H_{30}O_{18}$	761.1359	100	-0.9	17.3	**423** [M–H– $C_7H_4O_4$–$C_8H_8O_4$–H_2O]- 591 [M–H– $C_7H_6O_5$]- 609 [M–H– $C_7H_4O_4$]-	(Epi)gallocatechin-(epi)gallocatechin-3'-O-gallate (prodelphinidin B3-3'-O-gallate)/ (epi)gallocatechin-3-O-gallate-(epi)gallocatechin isomer	1, 2, 3, 4
30	8.004	325.0556	$C_{14}H_{14}O_9$	325.0565	100	2.9	26.0	**169** [M–H– $C_7H_9O_4$]-	Galloyl shikimic acid	1, 2, 3, 4
31	8.021	745.1417	$C_{37}H_{30}O_{17}$	745.1410	100	-0.9	16.5	169 [M–H– $C_{30}H_{25}O_{12}$]- 289 [M–H–$C_7H_6O_5$–$C_{15}H_{12}O_6$]- 425 [M–H– $C_7H_4O_4$–$C_8H_8O_4$]- 575 [M–H– $C_7H_6O_5$]-	(Epi)gallocatechin3-O-gallate-(epi)catechin isomer	1, 2, 3, 4

								593 [M–H– $C_7H_4O_4$]-		
32	8.255	153.0190	$C_7H_6O_4$	153.0193	100	2.4	4.1	**109** [M–H–CO_2]-	Protocatechuic acid isomer	1, 2, 3, 4
33	8.321	527.1426	$C_{23}H_{28}O_{14}$	527.1406	100	3.7	5.1	169 [M–H–$C_9H_{10}O_6$-$C_6H_8O_4$]- **313** [M–H–$C_9H_{10}O_6$]-	Guaiacylglycerol glucoside gallate isomer	1, 2, 3, 4
34	8.389	645.1300	$C_{26}H_{30}O_{19}$	645.1309	100	1.4	4.8	169 [M–H–$C_6H_{12}O_6$-$C_6H_9O_4$-$C_7H_4O_4$]- 331 [M–H–$C_6H_{10}O_5$-$C_7H_6O_5$]- **483** [M–H–$C_6H_{10}O_5$]-	Digalloyl diglucoside (humarain)	1, 2, 3, 4
35	8.423	593.1278	$C_{30}H_{26}O_{13}$	593.1301	100	3.8	11.0	125 [M–H–$C_{15}H_{12}O6$–$C_6H_9O_4$]- **305** [M–H–$C_{15}H_{12}O6$]- 423 [M–H–$C_8H_8O_3$–H_2O]- 441 [M–H–$C_8H_8O_3$]- 467 [M–H–$C_6H_7O_3$]-	(Epi)catechin-(epi)gallocatechin isomer	1, 2, 3, 4
36	8.490	359.0967	$C_{15}H_{20}O_{10}$	359.0984	100	4.7	17.5	**197** [M–H–$C_6H_{10}O_5$]-	Syringic acid glucoside isomer	1, 2, 3, 4
37	8.540	285.0615	$C_{12}H_{14}O_8$	285.0616	100	0.4	22.5	109 [M–H–CO_2-$C_5H_8O_4$]- **153** [M–H–$C_5H_8O_4$]-	Dihydroxybenzoic acid xyloside	1, 2, 3, 4
38	8.540	483.0765	$C_{20}H_{20}O_{14}$	483.0780	100	3.2	19.3	169 [M–H–$C_6H_{10}O_5$-$C_7H_4O_4$]- **271** [M–H–$C_7H_4O_4$-$C_2H_4O_2$]- 313 [M–H–$C_7H_4O_4$]- 331 [M–H–$C_7H_6O_5$]- 439 [M–H–CO_2]-	Gallic acid-galloyl-glucoside	1, 2, 3, 4
39	8.545	913.1464	$C_{44}H_{34}O_{22}$	913.1469	100	0.5	7.8	423 [M–H–$C_7H_4O_4$– $C_7H_6O_5$–$C_8H_8O_4$]– 573 [M–H–$C_7H_4O_4$– $C_7H_4O_4$]– 591[M–H–$C_7H_4O_4$– $C_7H_6O_5$]– **743** [M–H–$C_7H_6O_5$]– 761[M–H–$C_7H_4O_4$]–	(Epi)gallocatechin-3-O-gallate-(epi)gallocatechin-3'-O-gallate	1, 2, 3, 4
40	8.590	745.1383	$C_{37}H_{30}O_{17}$	745.1410	100	3.6	29.4	169 [M–H– $C_{30}H_{25}O_{12}$]- 305 [M–H–$C_7H_6O_5$–$C_{15}H_{12}O_5$]- 441 [M–H– $C_7H_4O_4$-$C_8H_8O_3$]- 575 [M–H– $C_7H_6O_5$–H_2O]-	(Epi)catechin-3-O-gallate-(epi)gallocatechin isomer	1, 2, 3, 4

								593 [M–H– $C_7H_4O_4$]-	
41	8.925	745.1407	$C_{37}H_{30}O_{17}$	745.1410	100	0.4	20.2	169 [M–H– $C_{30}H_{25}O_{12}$]- 287 [M-H-458 Da] - 305 [M–H–$C_7H_6O_5$–$C_{15}H_{12}O_5$]- **423** [M–H– $C_7H_4O_4$-$C_8H_8O_3$-H_2O]- 441 [M–H– $C_7H_4O_4$-$C_8H_8O_3$]- 575 [M–H– $C_7H_6O_5$]- **593** [M–H– $C_7H_4O_4$]-	(Epi)gallocatechin3-O-gallate-(epi)catechin / (Epi)gallocatechin-(epi)catechin-3'-O-gallate / (Epi)catechin3-O-gallate-(epi)gallocatechin / (Epi)catechin-(epi)gallocatechin-3'-O-gallate isomer	1, 2, 3, 4
42	9.193	305.0662	$C_{15}H_{14}O_7$	305.0667	100	1.5	18.2	**125** [M–H–$C_9H_9O_4$] 137 [M–H–$C_8H_8O_4$] 165 [M–H–140 Da] 167 [M–H– $C_7H_6O_3$] 179 [M–H– $C_6H_6O_3$] 219 [M–H–86 Da] 261 [M–H–CO_2]	(Epi)gallocatechin isomer	1, 2, 3, 4
43	9.276	745.1403	$C_{37}H_{30}O_{17}$	745.1410	100	0.9	33.7	169[M–H– $C_{30}H_{25}O_{12}$]- 289 [M–H–$C_7H_6O_5$–$C_{15}H_{12}O_6$]- **407** [M–H– $C_7H_4O_4$– $C_8H_8O_4$ –H_2O] 441 [M–H– $C_7H_4O_4$-$C_8H_8O_3$]- 593 [M–H– $C_7H_4O_4$]-	(Epi)gallocatechin3-O-gallate-(epi)catechin / (Epi)gallocatechin-(epi)catechin-3'-O-gallate / (Epi)catechin3-O-gallate-(epi)gallocatechin / (Epi)catechin-(epi)gallocatechin-3'-O-gallate isomer	1, 2, 3, 4
44	9.377	729.1422	$C_{37}H_{30}O_{16}$	729.1461	100	5.4	8.4	287 [M–H– $C_7H_4O_4$-$C_{15}H_{13}O_6$]- 407 [M–H– $C_7H_4O_4$-$C_8H_8O_3$-H_2O]- 425 [M–H– $C_7H_4O_4$-$C_8H_8O_3$]- 559 [M–H– $C_7H_6O_5$]- **577**[M–H– $C_7H_4O_4$]-	(Epi)catechin-(epi)catechin-3'-O-gallate	1, 2, 3, 4
45	9.427	289.0712	$C_{15}H_{14}O_6$	289.0718	100	1.8	5.5	109 [M–H–180 Da]– 123 [M–H–166 Da]– 151 [M–H–$C_7H_6O_3$]– 221[M–H–68 Da]–	(Epi)catechin isomer	1, 2, 3, 4

								245[M–H–CO_2]–		
46	9.477	137.0234	$C_7H_6O_3$	137.0244	100	7.2	4.9	**108** [M–H–CHO]-	Protocatechuic aldehide	1, 2, 3, 4
47	9.678	897.1508	$C_{44}H_{34}O_{21}$	897.1520	100	1.3	16.1	169 [M–H–$C_{37}H_{29}O_{16}$]– 407 [M–H–$C_7H_6O_5$–$C_7H_6O_4$–$C_8H_8O_4$]– 577 [M–H–$C_7H_4O_4$–$C_8H_8O_4$]– 727 [M–H–$C_7H_6O_5$]– **745** [M–H– $C_7H_4O_4$]–	(Epi)gallocatechin-3-O-gallate-(epi)catechin-3'-O-gallate isomer	1, 2, 3, 4
48	9.946	897.1499	$C_{44}H_{34}O_{21}$	897.1520	100	2.3	26.2	169 [M–H–$C_{37}H_{29}O_{16}$]– 423 [M–H–$C_7H_6O_5$–$C_7H_4O_4$–$C_8H_8O_3$]– 557 [M–H–$C_7H_6O_5$–$C_7H_6O_5$]– **727** [M–H–$C_7H_6O_5$]– 745 [M–H–$C_7H_4O_4$]–	(Epi)catechin-3-O-gallate-(epi)gallocatechin-3'-O-gallate isomer	1, 2, 3, 4
49	9.966	729.1501	$C_{37}H_{30}O_{16}$	729.1461	100	-5.5	7.8	289 [M–H– $C_7H_4O_4$-$C_{15}H_{11}O_6$]- **407** [M–H– $C_7H_6O_5$-$C_8H_8O_3$]- 577 [M–H– $C_7H_4O_4$]- 451 (152-126 [M–H–$C_7H_4O_4$-$C_6H_7O_3$]-	(Epi)catechin-3-O-gallate-(epi)catechin isomer	1, 2, 3, 4
50	10.348	457.0760	$C_{22}H_{18}O_{11}$	457.0776	100	3.6	3.6	**169** [M–H–$C_{15}H_{13}O_6$]- 305 [M–H–$C_7H_4O_4$]- 161 [M–H–$C_6H_6O_3$]-	(Epi)gallocatechin 3-O-gallate isomer	1, 2, 3, 4
51	10.465	713.1502	$C_{37}H_{30}O_{15}$	173.1512	100	1.4	76.4	-	(Epi)afzelechin-(epi)catechin-3'-O-gallate/ (epi)catechin-(epi)afzelechin-3'-O-gallate/ (epi)catechin-3-O-gallate -(epi)afzelechin isomer	1, 2, 3, 4
52	10.482	881.1560	$C_{44}H_{34}O_{20}$	881.1571	100	1.2	18.3	169 [M–H–$C_{37}H_{29}O_{15}$]- 287 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_{15}H_{12}O_6$]- 407 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_8H_8O_3$]- 559 [M–H– $C_7H_4O_4$–$C_7H_6O_5$]– 577 [M–H– $C_7H_4O_4$–$C_7H_4O_4$]– 711[M–H–$C_7H_6O_5$]–	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate (procyanidin B2-3,3' di-O-gallate) isomer	1, 2, 3, 4

								729 [M–H– $C_7H_4O_4$]–		
53	10.699	881.1602	$C_{44}H_{34}O_{20}$	881.1571	100	-3.6	25.0	169 [M–H–$C_{37}H_{29}O_{15}$]- 287 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_{15}H_{12}O_6$]- 407 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_8H_8O_3$]- 559 [M–H– $C_7H_4O_4$–$C_7H_6O_5$]– 577 [M–H– $C_7H_4O_4$–$C_7H_4O_4$]– 711[M–H–$C_7H_6O_5$]– **729** [M–H– $C_7H_4O_4$]–	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate (procyanidin B2-3,3' di-O-gallate) isomer	1, 2, 3, 4
54	10.783	603.1335	$C_{28}H_{28}O_{15}$	603.1355	100	3.3	11.0	169 [M–H–$C_{21}H_{23}O_{10}$]– 289 [M-H-$C_7H_4O_4$ –$C_6H_{10}O_6$]– 331 [M–H–$C_{15}H_{12}O_5$]– **451** [M–H–$C_7H_4O_4$]–	(Epi)catechin-3-O-glucoside-gallate	1, 2, 3, 4
55	10.967	497.0912	$C_{21}H_{22}O_{14}$	497.0937	100	4.9	22.7	169 [M–H–$C_{14}H_{17}O_9$]– **313** [M–H–$C_8H_9O_5$]– 453 [M–H–CO_2]–	Methoxy gallic acid glucoside gallate	1, 2, 3, 4
56	11.124	289.0737	$C_{15}H_{14}O_6$	289.0718	100	-6.7	8.1	109 [M–H–180 Da]– 123 [M–H–166 Da]– 151 [M–H–$C_7H_6O_3$]– 221[M–H–68 Da]– **245**[M–H–CO_2]– 247 [M–H–42 Da]–	(Epi)catechin isomer	1, 2, 3, 4
57	11.185	457.0770	$C_{22}H_{18}O_{11}$	457.0776	100	1.4	5.1	**169** [M–H–$C_{15}H_{13}O_6$]- 305 [M–H–$C_7H_4O_4$]- 161 (126) [M–H–$C_6H_6O_3$]-	(Epi)gallocatechin-3-O-gallate isomer	1, 2, 3, 4
58	11.201	449.1075	$C_{21}H_{22}O_{11}$	449.1089	100	3.1	11.9	259 [M–H–190 Da]- 269 [M–H–$C_6H_{12}O_6$]- **287** [M–H–$C_6H_{10}O_5$]-	Eriodictyol-O-glucoside isomer	1, 2, 3, 4
59	11.419	881.1532	$C_{44}H_{34}O_{20}$	881.1571	100	4.3	28.7	**169** [M–H–$C_{37}H_{29}O_{15}$]- 287 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_{15}H_{12}O_6$]- 303 [M–H–578 Da]-	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate (procyanidin B2-3,3' di-O-gallate) isomer	1, 2, 3, 4

								407 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_8H_8O_3$]- 577 [M–H– $C_7H_4O_4$–$C_7H_4O_4$]– 729 [M–H– $C_7H_4O_4$]–		
60	11.493	745.1379	$C_{37}H_{30}O_{17}$	745.1410	100	4.2	33.4	169[M–H– $C_{30}H_{25}O_{12}$]- 289[M–H–$C_7H_6O_5$–$C_{15}H_{12}O_6$]- **423** [M–H– $C_7H_4O_4$-$C_8H_8O_3$-H_2O]- 44 [M–H– $C_7H_4O_4$-$C_8H_8O_3$]- 575 [M–H– $C_7H_6O_5$]- 593 [M–H– $C_7H_4O_4$]-	(Epi)gallocatechin3-O-gallate-(epi)catechin/ (Epi)gallocatechin-(epi)catechin-3'-O-gallate/ (Epi)catechin3-O-gallate-(epi)gallocatechin/ (Epi)catechin-(epi)gallocatechin-3'-O-gallate isomer	1, 2, 3, 4
61	11.653	495.1120	$C_{22}H_{24}O_{13}$	495.1144	100	4.9	16.6	313 [M–H–$C_9H_{11}O_4$]– **451** [M–H–CO2]–	Galloyl glucosyl dihydroxy methoxyacetophenone	1, 2, 3, 4
62	11.754	897.1482	$C_{44}H_{34}O_{21}$	897.1520	100	4.2	39.7	169 [M–H–$C_{37}H_{29}O_{16}$]– 423 [M–H–$C_7H_6O_5$– $C_7H_4O_4$-$C_8H_8O_3$]– 575 [M–H– $C_7H_6O_5$-$C_7H_4O_4$]– **727** [M–H–$C_7H_6O_5$]– 745 [M–H– $C_7H_4O_4$]–	(Epi)catechin-3-O-gallate-(epi)gallocatechin-3'-O-gallate isomer	1, 2, 3, 4
63	12.323	441.0822	$C_{22}H_{18}O_{10}$	441.0827	100	1.2	16.1	**169** [M–H–$C_{15}H_{13}O_5$]– 289 [M–H–$C_7H_4O_4$]–	(Epi)catechin-3-O-gallate isomer	1, 2, 3, 4
64	12.423	497.1297	$C_{22}H_{26}O_{13}$	497.1301	100	2.8	20.5	169 [M–H–$C_{15}H_{21}O_{10}$]– **313** [M–H–C_9H13O_6]–	Trimethoxyphenyl glucoside gallate	1, 2, 3, 4
65	12.624	601.1293	$C_{28}H_{26}O_{15}$	601.1199	100	1.0	63.7	169 [M–H–$C_6H_{10}O_5$–$C_{15}H_{11}O_5$]– 449 [M–H–$C_7H_4O_4$]– **475** [M–H–126 Da]–	Eriodictyol-O-glucoside-O-gallate isomer	1, 2, 3, 4
66	12.624	629.0814	$C_{28}H_{22}O_{17}$	629.0784	100	-4.7	20.9	169 [M–H–$C_{21}H_{17}O_{12}$]–	Trigalloyl shikimic acid	1, 2, 3, 4
67	12.959	441.0819	$C_{22}H_{18}O_{10}$	441.0827	100	-1.9	7.3	**169** [M–H–$C_{15}H_{13}O_5$]– 289 [M–H– $C_7H_4O_4$]–	(Epi)catechin 3-O-gallate isomer	1, 2, 3, 4
68	13.528	441.0852	$C_{22}H_{18}O_{10}$	441.0827	00	-5.7	42.1	**319** [M–H–$C_7H_6O_3$]– 169 [M–H–$C_{15}H_{13}O_5$]–	(Epi)catechin 3-O-gallate isomer	1, 2, 3, 4
69	13.560	881.1616	$C_{44}H_{34}O_{20}$	881.1571	100	-5.1	21.0	287 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_{15}H_{12}O_6$]-	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate (procyanidin B2-3,3' di-O-gallate) isomer	1, 2, 3, 4

								407 [M–H–$C_7H_4O_4$-$C_7H_4O_4$-$C_8H_8O_3$]- 559 [M–H– $C_7H_4O_4$–$C_7H_6O_5$]– 577 [M–H– $C_7H_4O_4$–$C_7H_4O_4$]– 711[M–H–$C_7H_6O_5$]– **729** [M–H– $C_7H_4O_4$]–		
70	13.661	729.1503	$C_{37}H_{30}O_{16}$	729.1461	100	-5.7	9.2	289 [M–H– $C_7H_4O_4$-$C_{15}H_{11}O_6$]- **407** [M–H– $C_7H_4O_4$-$C_8H_8O_3$-H_2O]- 577 [M–H– $C_7H_4O_4$]- 451 [M–H–$C_7H_4O_4$- $C_6H_7O_3$]-	(Epi)catechin-3-O-gallate-(epi)catechin isomer	1, 2, 3, 4
71	13.915	897.1495	$C_{44}H_{34}O_{21}$	897.1520	100	2.7	71.8	169 [M–H–$C_{37}H_{29}O_{16}$]– 423 [M–H–$C_7H_6O_5$– $C_7H_4O_4$-$C_8H_8O_3$]– 557 [M–H–$C_7H_6O_5$- $C_7H_6O_5$]– 575 [M–H–$C_7H_6O_5$-$C_7H_4O_4$]– **727** [M–H–$C_7H_6O_5$]– 745 [M–H– $C_7H_4O_4$]–	(Epi)catechin-3-O-gallate-(epi)gallocatechin-3'-O-gallate isomer	3, 4
72	13.982	593.0936	$C_{29}H_{22}O_{14}$	593.0937	100	0.2	24.7	169 [M–H–$C_{15}H_{13}O_4$]– 271 [M–H–$C_7H_4O_4$–$C_7H_6O_5$]– **423** [M–H–$C_8H_8O_3$–H_2O]- 441 [M–H–$C_8H_8O_3$]-	(Epi)catechin di-O-gallate	3, 4
73	14.048	425.0935	$C_{22}H_{18}O_9$	425.0937	100	0.4	97.8	169 [M–H–$C_{15}H_{13}O_4$]– 255 [M–H–$C_7H_6O_5$]– **273** [M–H–$C_7H_4O_4$]–	(Epi)afzelechin-O-gallate	1, 2, 3, 4
74	14.283	479.0853	$C_{21}H_{20}O_{13}$	479.0831	100	-4.5	34.9	**316** [M–2H–C6H11O5]–	Myricetin glucoside	1, 2, 3, 4
75	14.300	897.1539	$C_{44}H_{34}O_{21}$	897.1520	100	2.1	10.2	**169** [M–H–$C_{37}H_{29}O_{16}$]– 727 [M–H–$C_7H_6O_5$]–	(Epi)gallocatechin-3-O-gallate-(epi)catechin-3'-O-gallate/ (Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate isomer	3, 4
76	14.616	173.0820	$C_8H_{14}O_4$	173.0819	100	0.3	5.4	127 [M–H–H_2O-CO]-	Hydroxynonanoic acid (suberic acid) isomer	1, 2, 3, 4

77	14.785	881.1554	$C_{44}H_{34}O_{20}$	881.1571	100	1.9	14.3	559 [M–H– $C_7H_4O_4$– $C_7H_6O_5$]– **711**[M–H–$C_7H_6O_5$]– 729 [M–H– $C_7H_4O_4$]–	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate (procyanidin B2-3,3' di-O-gallate) isomer	3, 4
78	14.817	541.1321	$C_{27}H_{26}O_{12}$	541.1351	100	5.6	117.6	169 [M–H– $C_{14}H9O3$– C6H10O_4]– 227 [M–H– $C_7H_4O_4$– $C_6H_{10}O_5$]– **313** [M–H– $C_{14}H_9O_3$]–	Trihydroxystilbene glucosyl-O-gallate	1, 2, 3, 4
79	15.003	865.1599	$C_{44}H_{34}O_{19}$	865.1622	100	2.6	33.9	169 [M–H–$C_{37}H_{29}O_{16}$]– 271 [M–H– $C_7H_4O_4$– $C_7H_4O_4$– $C_{15}H_{14}O_6$]– 289 [M–H– $C_7H_4O_4$– $C_7H_4O_4$–$C_{15}H_{12}O_5$]– 423 [M–H–442 Da] 441 [M–H–424 Da] 543 [M–H– $C_7H_4O_4$–$C_7H_6O_5$]– 695 [M–H–$C_7H_6O_5$]– **713** [M–H– $C_7H_4O_4$–$C_7H_6O_5$]–	(Epi)afzelechin-3-O-digallate-(epi)catechin/ (epi)catechin-(epi)afzelechin-3'-O-digallate	3, 4
80	15.102	713.1510	$C_{37}H_{30}O_{15}$	173.1512	100	0.3	25.7	169 [M–H–$C_{30}H_{25}O_{10}$]– 271 [M–H–$C_7H_4O_4$– $C_{15}H_{14}O_6$]– **289** [M–H–$C_7H_4O_4$– $C_{15}H_{12}O_5$]– 407 [M–H– $C_7H_6O_5$– $C_8H_8O_2$]– 441 [M–H–272 Da]– 561 [M–H–$C_7H_4O_4$]– 543 [M–H–$C_7H_6O_5$]–	(Epi)afzelechin3-O-gallate-(epi)catechin isomer	1, 2, 3, 4
81	15.331	617.1191	$C_{28}H_{26}O_{16}$	617.1148	100	-1.3	71.2	169[M–H–$C_6H_{10}O_5$-$C_{15}H_{11}O_6$-]- 303 [M–H–$C_7H_4O_4$-$C_6H_{10}O_5$-]- **465** [M–H–$C_6H_{10}O_5$]-	Dihydroquercetin-O-glucosyl-O-gallate	3, 4
82	15.504	673.2116	$C_{33}H_{38}O_{15}$	673.2138	100	3.3	16.2	**169** [M–H–$C_{20}H_{25}O_6$– $C_6H_8O_4$]-	Galloyl glucosyl dihydrodehydrodiconiferyl alcohol	1, 2, 3, 4

83	15.520	435.1272	$C_{21}H_{24}O_{10}$	435.1297	100	5.6	24.0	**315** [M–H– $C_4H_8O_4$]- 345 [M-H-$C_3H_5O_3$]-	Phloretin-C-glucoside (nothofagin)	1, 2, 3, 4
84	16.272	463.0875	$C_{21}H_{20}O_{12}$	463.0882	100	-0.7	15.9	137 [M–H–326 Da]- 300 [M–H–163 Da]- 301 [M–H– $C_6H_{10}O_5$]- 419 [M–H–CO_2]-	Quercetin glucoside	3, 4
85	16.526	881.1546	$C_{44}H_{34}O_{20}$	881.1571	100	2.8	21.3	169 [M–H–$C_{37}H_{29}O_{15}$]- 711[M–H–$C_7H_6O_5$]– **729** [M–H– $C_7H_4O_4$]–	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate (procyanidin B2-3,3' di-O-gallate) isomer	3, 4
86	16.761	601.1201	$C_{28}H_{26}O_{15}$	601.1199	100	-0.4	13.4	**179** [M–H–422 Da]– 169 [M–H–$C_6H_{10}O_5$– $C_{15}H_{11}O_5$]– 269 [M–H–$C_7H_4O_4$–$C_6H_{12}O_6$]–	Eriodictyol-O-glucoside-O-gallate isomer	3, 4
87	16.895	329.1359	$C_{19}H_{22}O_5$	329.1394	11.35	10.6	66.1	269 [M–H–3CH_3]- 299 [M–H–2CH_3]- **314** [M–H–CH_3]-	Pentamethoxystilbene	3, 4
88	17.164	449.1077	$C_{21}H_{22}O_{11}$	449.1089	100	-2.7	60.8	151 [M–H–298 Da]- **287** [M–H–$C_6H_{10}O_5$]-	Eriodictyol-O-glucoside isomer	3, 4
89	17.534	300.9992	$C_{14}H_6O_8$	300.9990	100	-0.6	5.3	283 [M–H–H2O]- 273 [M–H–CO]- 257 [M–H–CO2]- **229** [M–H–C11H2O6]- 201 [M–H–100]- 185 [M–H–116]-	Ellagic acid	1, 2, 3, 4
90	18.617	187.0976	$C_9H_{16}O_4$	187.0976	100	-0.2	6.8	**125** [M-H-H_2O-CO_2]- 143 [M-H-CO_2]- 169 [M-H-H_2O]-	Nonanedioic acid (azelaic acid)	1, 2, 3, 4
91	19.508	173.1180	$C_9H_{18}O_3$	173.1183	100	2.0	73.5	**127** [M–H–H_2O-CO]-	Hydroxynonanoic acid (suberic acid) isomer	1, 2, 3, 4
92	22.796	867.1386	$C_{43}H_{32}O_{20}$	867.1414	100	3.3	23.1	169 [M–H–$C_{36}H_{27}O_{15}$]- 527 [M–H–$C_7H_6O_5$–$C_7H_6O_5$]- **697** [M–H–$C_7H_6O_5$]- 715 [M–H–$C_7H_4O_4$]-	Theaflavin 3,3'di-O-gallate	3, 4
93	23.271	201.1140	$C_{10}H_{18}O_4$	201.1132	100	-4.0	4.7	**183** [M–H–H_2O]-	Decanedioic acid (sebacic acid)	1, 2, 3, 4

94	24.181	187.1355	$C_{10}H_{20}O_3$	187.1340	100	-8.2	17.1	**141** [M–H–H_2O-CO]-	Hydroxydecanoic acid (hydroxycapric acid)	1, 2, 3, 4
95	26.133	215.1300	$C_{11}H_{20}O_4$	215.1289	100	-5.4	12.7	**197** [M–H–H_2O]- 153 [M–H–H_2O–CO_2]-	Undecanedioic acid	1, 2, 3, 4

Table 7. HPLC-ESI-TOF-MS data of the compounds identified.

*Bold type figures indicate the base peak.

As a result, a total of 95 tentative metabolites belonging to different chemical classes (organic acids; phenolic compounds belonging to the hydroxybenzoic acids, flavonoids, and stilbenes subclass; fatty acid derivatives; and others) were identified, for which the peak number was assigned according to the overall elution order. Most of them were reported for the first time in *Sclerocarya birrea* extracts, as existing information is very scarce.

4. Discussion

4.1. Nonselective extraction of the different Sclerocarya birrea stem-bark extracts

Matrixes of plant origin contain thousands of diverse metabolites of varying polarities and concentrations, making it difficult to develop a single method for optimum extraction of all metabolites. Within this context, we tried to reproduce the different extraction conditions employed in previous *in vivo* studies testing the bioactivity of *Sclerocaria birrea* stem -bark extracts, covering different ranges of polarity. We used solvents that can successfully extract polar and semi-polar metabolites of great interest. The use of different extraction procedures has enabled us to extract metabolites of a wider polarity range, from organic acids to fatty acid derivatives, and therefore to accomplish a more complete characterization of bioactive *Sclerocarya birrea* stem-bark extracts.

4.2. HPLC-ESI-QTOF/MS as a power tool for metabolic profiling

The chromatographic method showed sufficient resolving power to separate up to six isomeric forms of several compounds, and the structures of some of these isomeric compounds were elucidated as a starting point based on the prior data. In agreement with literature (Abu-Reidah *et al*., 2013a, Iswaldi *et al*., 2013, Gómez-Romero *et al*., 2010), a certain tendency in the elution order of the compounds related to their chemical-structure class was observed, appearing in the following order of increasing retention time (RT), and thus hydrophobicity: organic acids, phenolic acids, flavonoids and other phenolic compounds, and fatty acid derivatives. Within the same

chemical class, the retention time decreases as more hydroxyl groups have the structure, e.g. gallocatechin eluted earlier than catechin; if the molecule contains apolar subtituents, such as methoxy groups, the retention time increases, e.g. trihydroxystilbene glucosyl-O-gallate eluted earlier than pentamethoxystilbene; if the structure contains sugars, the molecule elutes earlier than the respective aglycone, e.g. galloyl glucoside elutes earlier than gallic acid; and regarding hydrocarbon chains, the longer they are, the later they elute from the column, this being illustrated very well by observing the elution order of fatty acid derivatives: nonanedioic acid followed by decanedioic acid, and finally followed by undecanedioic acid.

4.2.1. Organic acids

A total of five organic acids were tentatively identified in all the extracts. These strongly influence the organoleptic properties of vegetables and plants, being responsible of sourness or acidity (Gómez-Romero *et al*., 2010): specifically, quinic (peak 1) (*m/z* 191), malic (peak 2) (*m/z* 133), and citric acids (peak 3) (*m/z* 191), together with a methyl derivative of citric acid (peak 4) (*m/z* 205), and the dicarboxylic acid succinic acid (peak 6) (*m/z* 117). Fragmentation patterns of these compounds generated mainly neutral losses of CO_2 from carboxylic group and/or H_2O, but also CH_3 from the methylated derivative, and CO from quinic acid.

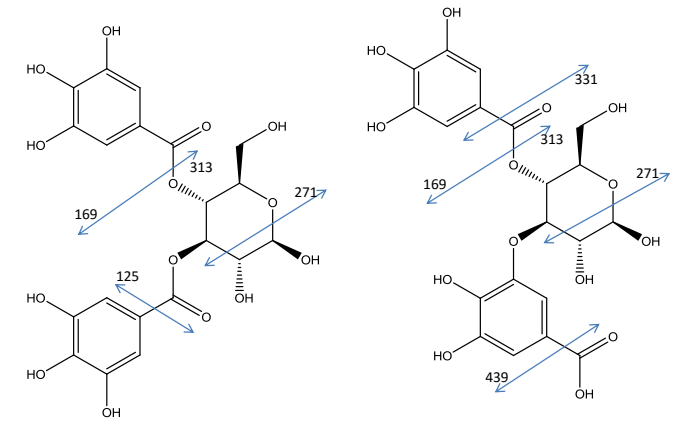
4.2.2. Phenolic compounds

A total of 76 compounds belonging to the class phenolic compounds were identified, namely 19 hydroxybenzoic acids, 55 flavonoids, and 2 stilbenes. These molecules play an important role for antioxidative defense systems from oxidative stress in plants (Osawa, 1999).

4.2.2.1. Hydroxybenzoic acids

Among the hydroxybenzoic acids, the precursor ion at *m/z* 169 (compound 13), yielding a product ion at *m/z* 125 by neutral loss of a CO_2 group, could be identified as gallic acid. Several

combinations of gallic acid and glucose were present, such as galloyl glucoside (8, and 10), galloyl diglucoside (11), digalloyl glucoside (17), gallic acid galloyl glucoside (38), and digalloyl diglucoside (34), which were detected at *m/z* 331, 493, 483, 483, and 645 respectively. These compounds exhibited neutral losses of glucose [M-H-$C_6H_{12}O_6$]-, glucosyl [M-H-$C_6H_{10}O_5$]-, and galloyl [M-H-$C_7H_4O_4$]- residues. The product ions at *m/z* 271, 241, and 211 that appeared in the mass spectra were due to cross-ring cleavages in the glucose unit (Santos *et al*., 2013, Da Silva *et al*., 2011). The existence and intensity of fragmentation ions that differed for each peak with same molecular formula were due to different isomeric forms that differed in their elution behaviour. More specifically, compound at *m/z* 483 corresponding to molecules with one gallic acid and one glucose unit presented two isomers. The presence of the ion at *m/z* 125 in the first eluting isomer (17) is indicative of the trihydroxybenzoic moiety of the gallic acid, while the presence of the ion at *m/z* 439 in the second isomer (38) is indicative of the loss of the carboxylic moiety of gallic acid. These fragments indicate that in the first isomer, gallic acid moieties are bonded to glucose via an ether linkage through the carboxylic acid group (digalloyl glucoside), while in the second isomer, one gallic acid is bonded to glucose via an ether linkage through one hydroxyl group (gallic acid galloyl glucose) (Figure 32) (Da Silva *et al*., 2011, Meyers *et al*., 2006).



Digalloyl glucoside

Gallic acid digalloyl glucoside

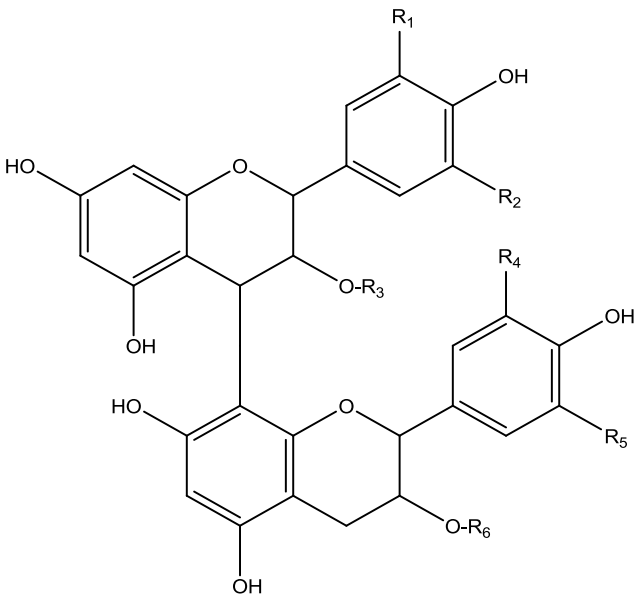
Figure 32. Possible sites of fragmentation of the isomers at m/z 483. Adapted from Meyers *et al.,* (2006).

These galloyl derivatives are abundant phenolic compounds in stem bark and wood of other trees such as eucalyptus (Santos *et al*., 2013) or yellow fir (Wang *et al*., 2013). Other isomeric structures were detected at *m/z* 359 (5, and 9), these being identified as syringic acid glucoside isomers, yielding losses of glucose [M-H-180 Da]- and glucosyl residues [M-H-162 Da]-. The compound that showed a deprotonated molecule at *m/z* 343 (16) was identified as galloyl quinic acid, as it exhibited losses of the galloyll [M-H-$C_7H_4O_4$]- and the quinic acid residues [M-H-$C_7H_{11}O_5$]-. Simple forms such as protocatechuic acid isomers (21, 32, and 46), and vanillic acid (22) released CO_2 molecules upon fragmentation. Compound 17, eluting at 17.53 min, was characterized as ellagic acid based on the [M-H]- ion at *m/z* 301, the main product ions at *m/z* 229 as a result of fragmentation of the aromatic ring, and other minor fragments resulting from losses of H_2O, CO, and CO_2. Earlier studies reported that ellagic acid is widely distributed in vegetal woods and barks (Hamzaoui *et al*., 2013, Panchal and Brown, 2013, Saha *et al*., 2012, Si *et al*., 2013).

4.2.2.2. Flavonoids

Flavonoids were the most abundant phenolic class detected in *Sclerocarya birrea* stem-bark extracts. Among these, the proanthocyanidin subclass was by far the richest. These are known as the second most abundant class of natural phenolic compounds in the vegetable kingdom, after lignin (Khanbabaee and Van Ree, 2001). They reportedly possess a variety of physiological activities, e.g. antioxidant (Bräunlich *et al*., 2013), anti-atherosclerotic (Hort *et al*., 2012), anti-allergenic (Tokura *et al*., 2005), and anticarcinogenic effects (Woo and Kim, 2013), as well as inhibition of such enzymes as alpha amylase. These beneficial effects on human health have been attributed mainly to their strong free-radical scavenging and antioxidant activities (Xu *et al*., 2006).

Among flavonoids, proanthocyanidins were the predominant subclass in *Sclerocarya birrea* extracts. Proanthocyanidins are condensed flavan-3-ols, with different degrees of polymerization: monomers; dimers up to heptamers, which are referred as oligomeric proanthocyanidins, whereas larger chains are generally referred to as polymeric proanthocyanidins or tannins (Li and Deinzer, 2007). Condensed tannins are usually linked to each other through C4-C8 or C4-C6 B-type interflavanoid bonds and sometimes, an A-type interflavanoid bond is observed when an additional ether linkage is formed between C2 and O7, leading to B-type and A-type proanthocyanidins, respectively (Rodrigues *et al*., 2007, Tala *et al*., 2013). In the present study, monomers up to B-type dimers of procyanidins were detected, which are oligomers and polymers of (epi)catechin subunits; B-type dimers of prodelphinidins, which are made up of (epi)gallocatechin units; and propelargonidins, constituted of (epi)afzelechin flavan-3-ol units. "Epi" refers to the epimer and the parentheses indicate that it could be either epimer. Their representative structures are depicted in Figure 33.



R_1	R_2	R_3	R_4	R_5	R_6	[M−H]−	Name
OH	OH	H	OH	OH	H	609	(Epi)gallocatechin-(epi)gallocatechin
OH	OH	H	H	OH	H	593	(Epi)gallocatechin-(epi)catechin
OH	OH	H	OH	OH	Gallate	761	(Epi)gallocatechin-(epi)gallocatechin-3'-O-gallate
OH	OH	Gallate	OH	OH	H	761	(Epi)gallocatechin-3-O-gallate-(epi)gallocatechin
OH	OH	H	H	OH	Gallate	745	(Epi)gallocatechin3-O-gallate-(epi)catechin
H	OH	H	H	OH	H	577	(Epi)catechin-(epi)catechin
OH	OH	Gallate	OH	OH	Gallate	913	(Epi)gallocatechin-3-O-gallate-(epi)gallocatechin-3'-O-gallate
H	OH	H	H	OH	Gallate	729	(Epi)catechin-(epi)catechin-3'-O-gallate
OH	OH	Gallate	H	OH	Gallate	897	(Epi)gallocatechin-3-O-gallate-(epi)catechin-3'-O-gallate
H	H	H	H	OH	Gallate	713	(Epi)afzelechin-(epi)catechin-3'-O-gallate
H	H	Gallate	H	OH	H	713	(Epi)afzelechin-3-O-gallate-(epi)catechin
H	OH	H	H	H	Gallate	713	(Epi)catechin-(epi)afzelechin-3'-O-gallate
H	OH	Gallate	H	H	H	713	(Epi)catechin-3-O-gallate -(epi)afzelechin
H	OH	Gallate	H	OH	Gallate	881	(Epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate
H	H	Gallate-Gallate	H	OH	H	865	(Epi)afzelechin-3-O-digallate-(epi)catechin
H	OH	H	H	H	Gallate - Gallate	865	(Epi)catechin-(epi)afzelechin-3'-O-digallate

Figure 33. Representative structure of the proanthocyanidins detected in Sclerocarya birrea extracts.

These flavan-3-ols units are sometimes esterified with gallic acid to form 3-O-gallates (Li and Deinzer, 2007). In the present work, an exceptionally high percentage of proanthocyanidins was galloylated, while some of these were also glucosylated. The degree of galloylation is biologically important, as galloyllation has been reported to inhibit cell growth, trigger cell-cycle arrest in tumor-cell lines and induce apoptosis (Salucci *et al*., 2002, Stagos *et al*., 2005), enhancing the antiproliferative capacity of polyphenolic compounds. Furthermore, studies have shown that they also offer protection by scavenging reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radicals, destroying biochemical components that are important in physiological metabolism (Cao and Li, 2004). This capacity to prevent the imbalance between

high-level oxidant exposure and antioxidant capacity, which leads to several pathological processes, may contribute to the chemopreventive effect of the gallic acid derivatives (Lizarraga *et al*., 2007). For the identification of proanthocyanidins, general fission rules were applied: a subunit (or a single ring) cannot simultaneously undergo two or more types of fragmentation involving heterocyclic ring (HRF_c) fission, retro-Diels-Alder (RDA) fission, benzofuran-forming (BFF) fission, or quinone methide (QM) fission; neutral losses occur from the [M-H]- ion through HRF_C, RDA, BFF, and BFF/H_2O fissions from (epi)afzelechin, (epi)catechin, and (epi)gallocatechin units. Following these guidelines, possible fragmentation pathways are proposed for non-galloylated proanthocyanidins in Figure 34. For galloylated proanthocyanidins neutral losses occur also through the breakage of the gallic acid or the galloyl residues, and then they follow the same fragmentation pathway as the non-galloylated ones. On the basis of these rules, the subunit sequence of some proanthocyanidins was identified, as well as the relative position of the galloyl moiety.

Three peaks were detected at *m/z* 609 in the BPC (7, 12, and 14) and were tentatively identified as procyanidin dimmers with (epi)gallocatechin units. They all presented similar fragmentation patterns with comparable intensities of daughter ions. They all produced the MS^2 base peak at *m/z* 423, which originated from an RDA fragment followed by the loss of a water molecule [M-H-168 Da-18 Da]- whereas the secondary peaks at *m/z* 441 and 305 originated from an RDA fragment [M-H-168 Da]- and from the loss of an (epi)gallocatechin unit, respectively.

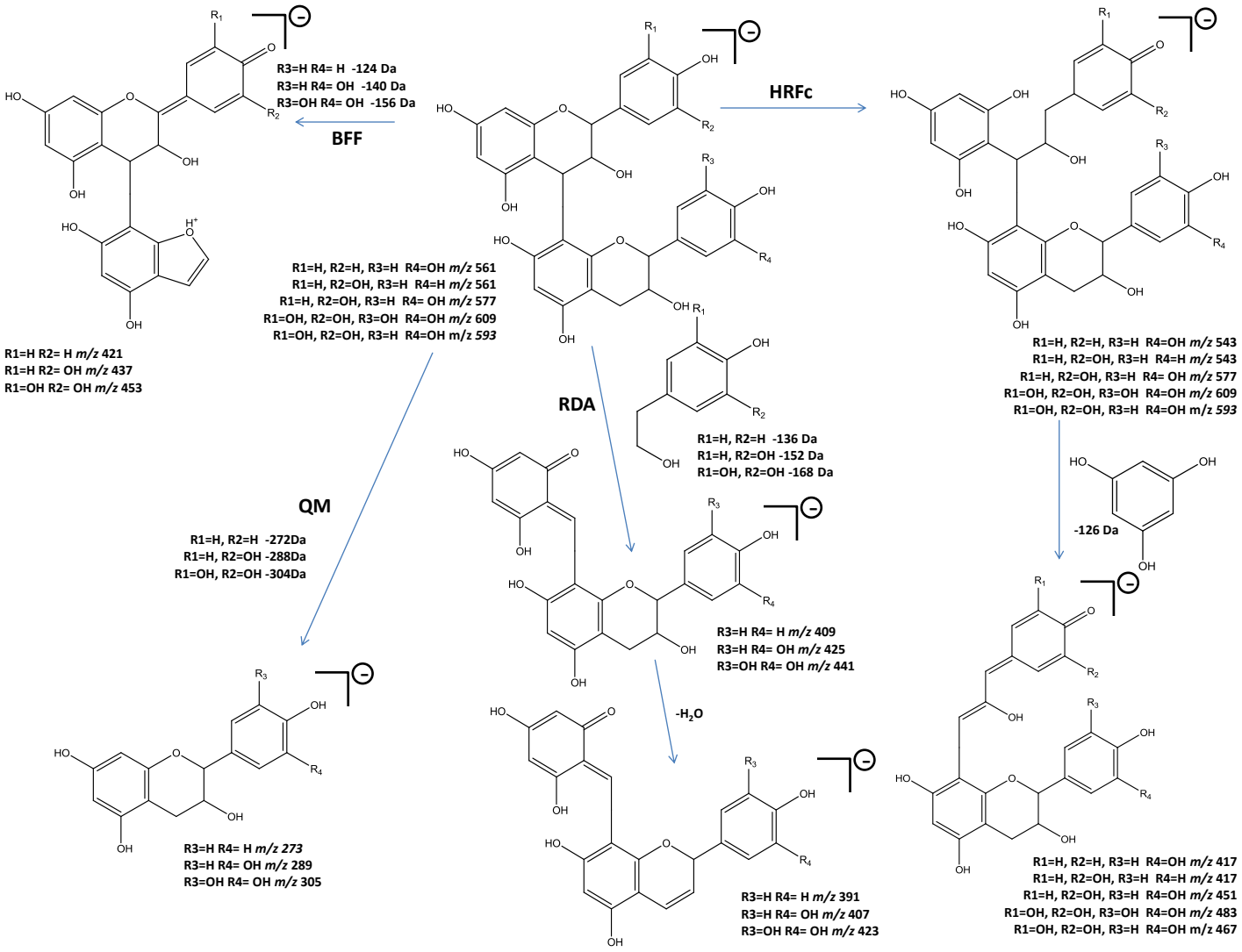


Figure 34. Proposed fragmentation pathways of non galloylated proanthocyanidins.

Four peaks were detected at *m/z* 593 in the BPC (15, 18, 28, and 35) with different elution behavior and were tentatively identified as proanthocyanidin dimers with an (epi)catechin and an (epi)gallocatechin unit. Isomers corresponding to compounds 15 and 28 presented similar fragmentation patterns with comparable intensities of daughter ions. They produced the MS^2 base peak at *m/z* 289 which originated from the cleavage of the interflavan linkage though the QM mechanism, this being indicative of an (epi)catechin as the base unit; secondary peaks at 425 [M-H-168 Da]-, and at *m/z* 407 [M-H-168 Da-H_2O]-, originated from RDA fragments which are indicative of an (epi)gallocatechin as the top unit. The RDA fragmentation on the top unit gave a fragment ion with a larger π-π hyperconjugated system, which is energetically more favorable than the RDA on the base unit (Jaiswal *et al*., 2012). From the preceding arguments, these proanthocyanidins must have (epi)gallocatechin as the top unit and (epi)catechin as the base unit. Isomers corresponding to compounds 18 and 35 produced similar fragmentation patterns with comparable intensities of daughter ions. They produced the MS^2 base peak at *m/z* 305, which originated from a QM fragment, this being indicative of an (epi)gallocatechin as the base unit; secondary peaks at *m/z* 441 [M-H-152 Da]-, and at *m/z* 423 [M-H-152 Da-H_2O]-, originated from RDA fragments, which are indicative of an (epi)catechin as the top unit. From the preceding arguments, these proanthocyanidins must have (epi)catechin as the top unit and (epi)gallocatechin as the base unit. All four isomers showed HRFc fragments at *m/z* 467 with similar intensities, as this fragment preserves the B ring on the top unit as well as the E ring on the base unit, to which the differentiating hydroxyls of (epi)catechin and (epi)gallocatechin are linked (Figure 35a).

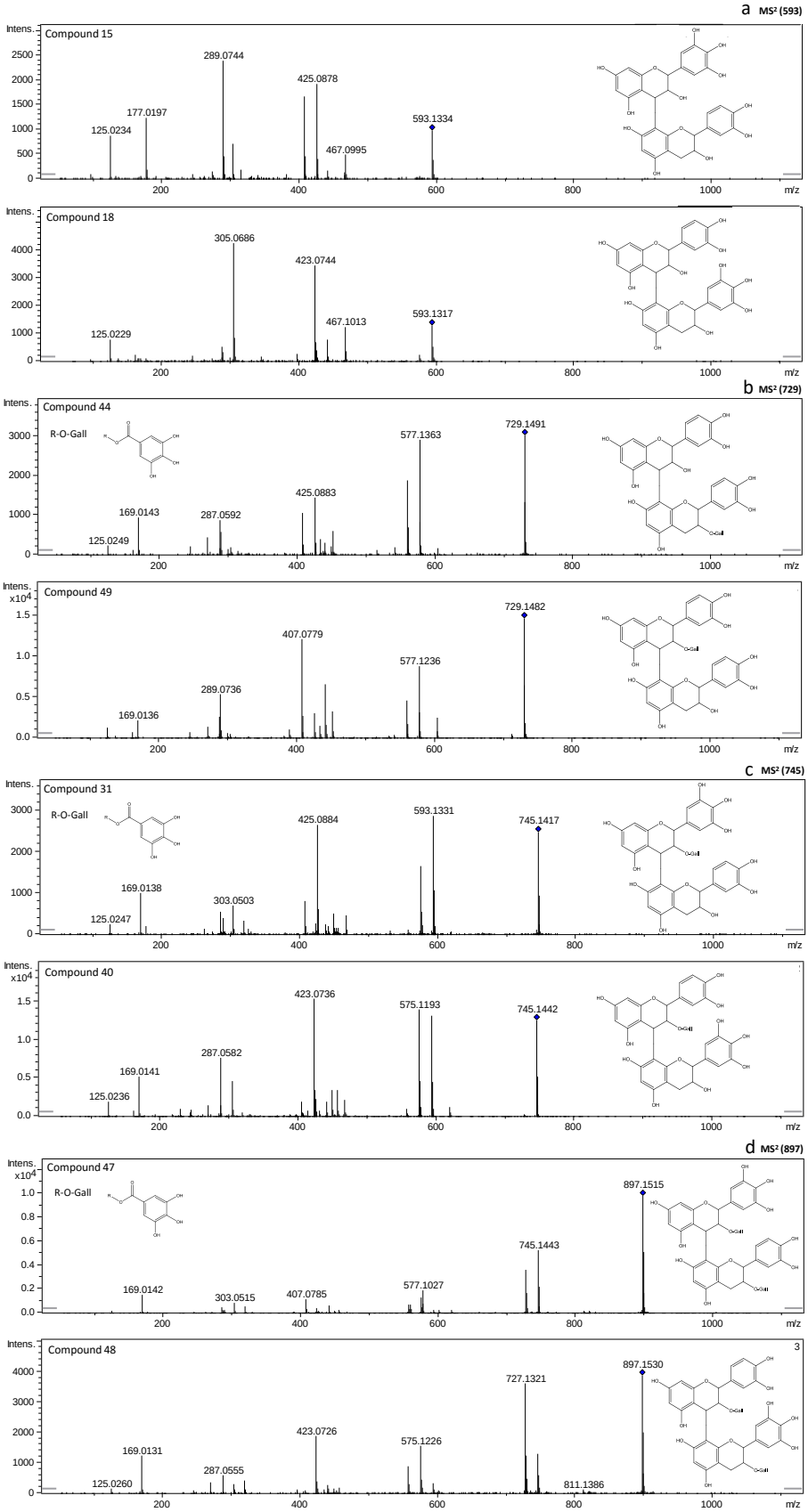


Figure 35. MS² spectra of compounds 15, and 18 (15 and 28 are similar) at *m/z* 593 (a), 44 and 49 (49 and 70 are similar) at *m/z* 745 (b), 31 and 40 at *m/z* 745 (c), and 47 and 48 (48, 62, and 75 are similar) at *m/z* 897 in negative ion mode (d).

Three peaks were detected at *m/z* 761 in the BPC (19, 23, and 29) and were tentatively identified as gallates of dimeric proanthocyanidins with (epi)gallocatechin units. They rendered product ions at *m/z* 609, characteristic of the loss of the galloyl residue [M-H-152 Da]-, at *m/z* 441, characteristic of the RDA fragmentation [M-H-152 Da-168 Da]-, at *m/z* 591, due to the loss of gallic acid [M-H-170]-, and at *m/z* 423 due to the sequential RDA fragmentation and water loss [M-H-168 Da-18 Da]-.

Two peaks at *m/z* 305 were detected in the BPC and were tentatively identified as (epi)gallocatechin isomers. These presented similar MS spectrum with the base peak at *m/z* 125, the formation mechanism of which was proposed by Miketova *et al*., (2000); and secondary peaks at *m/z* 137, 167 and 179, due to RDA, BFF, and HRFc fissions, respectively. They also showed other fragments at *m/z* 165 and 219, which have been previously described (Jaiswal *et al*., 2012).

Five isomers were detected at *m/z* 745 in the BPC (31, 40, 41, 43, and 60) and were assigned as gallates of dimeric proanthocyanidins with (epi)gallocatechin and (epi)catechin units. The relative positions of the monomeric units and the galloyl residues were established for the first two eluting isomers. The first eluting isomer (31) produced the base peak at *m/z* 593 [M-H-152 Da]-, and secondary peaks at *m/z* 575 [M-H-170 Da]-, at *m/z* 425 from an RDA fragment typical of (epi)catechin units, at *m/z* 289 from a QM fragment, and at *m/z* 169, the mechanism formation of which was proposed by Miketova *et al.*, (2000). The presence of a QM fragment at *m/z* 289 showed that the base ring is an (epi)catechin unit, and further confirmation came from the RDA fragment at *m/z* 425, which showed that the top unit is (epi)gallocatechin. The second isomer (40) produced the base peak at *m/z* 593 [M-H-152]-, and secondary peaks at *m/z* 575 [M-H-170]-, at *m/z* 441 from an RDA fragment typical of (epi)gallocatechin, and at *m/z* 305 from a QM fragment. The presence of a QM fragment at *m/z* 305 showed that the base ring is an (epi)catechin unit,

and further confirmation came from the RDA fragment at *m/z* 441, which showed that the top unit is (epi)catechin. There are two possibilities for the galloyl residue: it is attached either to the C3' of the base unit or to the C3 of the top unit. In the first case, it would favor the loss of gallic acid followed by the RDA fragment of the top unit (Jaiswal *et al*., 2012). Hence, in MS^2 base peak at *m/z* 407, [M-H-170-168 Da]- should be produced if the galloyl residue is attached to an (epi)catechin unit, and at *m/z* 423, [M-H-170-152 Da]-, if it is attached to an (epi)gallocatechin unit. In the second case, if the galloyl residue is attached to the C3 of the top unit, then it loses the galloyl residue and gallic acid and produces the MS^2 base peak at *m/z* 593 [M-H-152 Da]- and a secondary peak at *m/z* 575 [M-H-170 Da]-. From the above arguments, isomers 31 and 40 were assigned as (epi)gallocatechin-3-O-gallate-(epi)catechin and (epi)catechin-3-O-gallate-(epi)gallocatechin (Figure 35b). The rest of the isomers presented fragments at *m/z* 575 [M-H-170 Da]-, and 593 [M-H-152 Da]-, QM fragments at *m/z* 289 and 305, and RDA fragments at 407 [M-H-170 Da-168 Da]-, 423 [M-H-170 Da-152 Da]-, 441 [M-H-152 Da-152 Da]-, and 559 [M-H-168 Da-18 Da]-.

Compound 25 was detected at *m/z* 577 and was assigned as a dimeric proanthocyanidin with two (epi)catechin units. It produced the base peak at *m/z* 289, which originated from a QM fragment, and secondary peaks at *m/z* 407 [M-H-152-18 Da]- due to an RDA fragment followed by the loss of a water molecule, an RDA fragment at *m/z* 425 [M-H-152 Da]-, and at *m/z* 125, the formation mechanism of which was proposed elsewhere (Miketova *et al*., 2000).

One peak (39) was detected at *m/z* 913 and was tentatively characterized as a dimeric proanthocyanidin with two (epi)gallocatechin monomeric units and two galloyl residues. The product ion spectrum of the deprotonated molecule produced the base peak at *m/z* 743 [M-H-170 Da]- as a result of the loss of one molecule of gallic acid; and secondary peaks at *m/z* 423 [M-H-170 Da-152 Da-168 Da]- formed due to the sequential loss of gallic acid, galloyl residue, and

RDA fragmentation; at *m/z* 591 [M-H-170 Da-152 Da]- due to the loss of gallic acid and a galloyl residue; at *m/z* 573 resulting from the cleavage of the two gallic acid molecules [M-H-170 Da-170 Da]-; and at *m/z* 761 [M-H-152 Da]- due to the loss of one galloyl residue.

Three peaks were detected at *m/z* 729 in the BPC (44, 49, and 70) and were tentatively assigned as gallates of dimeric proanthocyanidins with (epi)catechin units. The first eluting isomer (44) produced the MS^2 base peak at *m/z* 577 [M-H-152 Da]-. Secondary peaks were produced at *m/z* 559 [M-H-170 Da]-, which originated from the loss of the gallic acid molecule; at *m/z* 425 [M-H-152 Da-152 Da]- from an RDA fragment after the loss of the galloyl residue; at 407 [M-H-152 Da-170 Da]- from an RDA fragment after the loss of gallic acid; and at *m/z* 287 from a QM fragment. The presence of the base peak at *m/z* 593 and a secondary peak at *m/z* 559 suggested that the galloyl residue is attached to the C3 of the top unit. This might be confirmed by the presence of the QM fragment 289 over 287. Hence this isomer was assigned as (epi)catechin-3-O-gallate-(epi)catechin. Isomers 49 and 70 produced similar MS^2 spectra, with the base peak at *m/z* 407 [M-H-152 Da-152 Da-18 Da]-; secondary peaks appeared at *m/z* 577 [M-H-152 Da]-, at *m/z* 451 [M-H-152 Da-126 Da]- originated from an HRF_C fragment, and at *m/z* 289 from a QM fragment. The presence of the MS^2 base peak at *m/z* 407 confirmed that the galloyl residue is attached to the C3' of the base unit. The presence of the fragment 287 over 289 was detected; this might be indicative of the QM fragmentation being produced on the base unit, and that the galloyl residue is attached to the base unit. Hence, it was assigned as (epi)catechin-(epi)catechin-3'-O-gallate (Figure 35c).

Two peaks showed the deprotonated molecule [M-H]- at *m/z* 289 (45, and 56). They presented similar fragmentation patterns with MS^2 ions at *m/z* 123, 149, 221, and 245 characteristics of catechin, in agreement with MS^2 experiments previously performed with QTOF (Simirgiotis & Schmeda-Hirschmann, 2010).

Five peaks were detected at *m/z* 897 in the BPC (47, 48, 62, 71, and 75). They were tentatively assigned as digallates of dimeric proanthocyanidins with (epi)catechin and (epi)gallocatechin monomeric units. Three different MS^2 fragmentation patterns were observed in the MS^2 spectra. The first eluting isomer showed the base peak at *m/z* 745 [M-H-152 Da]-, and secondary peaks at *m/z* 727 [M-H-170]-, at *m/z* 577 [M-H-152 Da-168 Da]- due to the sequential loss of the galloyl residue and an RDA fragment typical of gallocatechin units, at *m/z* 407 [M-H-170 Da-152 Da-168 Da]- due to the sequential loss of gallic acid, the galloyl residue, and RDA fragmentation typical of gallocatechins. Therefore, this isomer was tentatively identified as (epi)gallocatechin-3-O-gallate-(epi)catechin-3'-O-gallate. The second fragmentation pattern found corresponded to isomers 48, 62, and 71. Their fragmentation led to the base peak at *m/z* 727 [M-H-170 Da]-, and secondary peaks at *m/z* 423 [M-H-170 Da-152 Da-152 Da]-, typical of RDA of catechins, at *m/z* 745 [M-H-152 Da]-, and 557 [M-H-170-170 Da]-. These isomers were therefore identified as (epi)catechin-3-O-gallate-(epi)gallocatechin-3'-O-gallate (Figure 35d). The third fragmentation pattern corresponded to isomer 82, and produced only the base peak at *m/z* 169 and a secondary peak at *m/z* 727 [M-H-170 Da]-. With this information the relative positions of the monomeric units could not be tentatively assigned.

Two isomers with different eluting behaviors were detected at *m/z* 457 (50, and 57) and were successfully assigned as (epi)gallocatechin-3-O-gallate isomers. Their product ions at *m/z* 169 and 305 indicated the presence of gallic acid and (epi)gallocatechin and confirmed their assignation.

Compounds 51, and 80 were detected at *m/z* 713. The first eluting isomer did not undergo fragmentation in the MS^2 experiment, and therefore the relative positions of the monomeric units and the galloyl residue were not tentatively assigned. The second eluting isomer produced the MS^2 base peak at *m/z* 289 [M-H-152 Da-272 Da]-, which is a QM fragment from the breakage of

the interflavan linkage between the monomeric units. This fragment showed that the base ring is an (epi)catechin unit, and also that the galloyl residue is not attached to this base unit, but to the upper one. Further confirmation for the (epi)afzelechin being in the upper position came from the secondary peak at *m/z* 407 [M-H-152 Da-136 Da]- due to an RDA fragment typical of (epi) afzelechin unit.

Six isomers were detected at *m/z* 881 (52, 53, 59, 69, 77, and 85). Product ions at *m/z* 729 [M-H-152 Da]-, at *m/z* 711 [M-H-170 Da]-, at *m/z* 577 [M-H-152 Da-152 Da]-, and at *m/z* 559 [M-H-152 Da-170]- confirmed the presence of the attachment of the gallic acid molecules to the monomeric units. They also showed other product ions at *m/z* 407 [M-H-152 Da-170 Da-152 Da]-, indicative of the RDA fragmentation typical of catechin after the loss of the galloyl and gallic acid residues; and a QM fragment at *m/z* 287, showing the monomeric units to be catechins. Therefore, these isomers were tentatively identified as (epi)catechin-3-O-gallate-(epi)catechin-3'-O-gallate.

One isomer at *m/z* 425 (73) was tentatively identified as (epi)afzelechin-O-gallate. Product ions at *m/z* 169 and *m/z* 277 indicated the presence of gallic acid and (epi)afzelechin molecules, respectively, confirming this assignation.

Isomer at *m/z* 865 (79) was assigned as a digalloyl derivative of a dimeric proanthocyanidin with (epi)afzelechin and (epi)catechin units. The presence of the fragments at *m/z* 271 and at *m/z* 289 suggested that the two galloyl residues are attached to the (epi)afzelechin unit. Given that the molecular mass of the (epi)afzelechin is 274, upon ionization it loses 1 Da, upon breakage of the interflavan bond the monomeric unit loses 1 Da, and upon breakage of the bonds that link the two galloyl residues the monomeric unit loses other 2 Da, resulting in fragment at *m/z* 271. Therefore, this isomer was tentatively assigned as a (epi)afzelechin-3-O-digallate-(epi)catechin or (epi)catechin-(epi)afzelechin-3'-O-digallate.

Finally, one derivative of theaflavin was detected in the BPC. Fragments at *m/z* 537 [M-H-170-170]-, 697 [M-H-170]-, and 715 [M-H-152]- were indicative the successive losses of gallic acid and galloyl residues. Therefore, this molecule was identified as theaflavin-3,3'-di-O-gallate.

Four compounds belonging to the flavonol subclass were detected. A molecular ion at *m/z* 463 was tentatively identified as quercetin glucoside (84), based on MS/MS data. The aglycone fragment appeared at *m/z* 301 and the product ion at *m/z* 300 resulted after homolytic cleavage of the O-glycosidic bond, rendering a radical aglycone anion. The radical aglycone anion:aglycone fragment ratio suggests a certain glycosylation position. However, it cannot be used as a diagnostic tool for the characterization of the glycosylation position in unknown flavonoid O-glycosides (Cuyckens and Claeys, 2005). This fragmentation pattern was described by Hvattum and Ekeberg, (2003), and is in agreement with the results found by Sun *et al*., (2014), and Vallverdú-Queralt *et al*., (2014). One peak at *m/z* 603 (54) was tentatively assigned as quercetin-O-glucoside-gallate. It showed a major fragment at *m/z* 169 due to the loss of the glucosyl-quercetin moiety; at *m/z* 331, which can be justified by the elimination of the quercetin moiety, and indicative of the galloyl residue being attached to the glucose unit and not to the quercetin unit; at *m/z* 289, indicative of the cleavage of the glucosyl-galloyl residue; and at *m/z* 451 due to the loss of the galloyl moiety. This fragmentation pattern is in accordance to characteristic fragment mass of O-glycosyl-gallates. These compounds have been mentioned in the literature for flower buds of *Eugenia caryophyllate* (Dan *et al*., 2010). The structure of compound detected at *m/z* 479 (74) was determined to be a myricetin monosaccharide. MS data of the radical aglycone, with *m/z* 316 [A-2H]- confirmed the assignation as a glucoside of myricetin. This fragmentation pattern agrees with the results published by Chen *et al*., (2012). Moreover, one dihydroflavonol was detected at *m/z* 617 (81), which led to MS/MS ions at *m/z* 465, 303, and 169, indicating the loss of the glucosyl moiety, the glucosyl and galloyl moieties, and the glucosyl

and dihydroquercetin moieties, respectively. Therefore, it was assigned as dihydroquercetin-O-glucosyl-O-gallate. There are no records for any fragmentation pattern of this compound in the literature. However, this compound has been previously described in *Taxillus kaempferi* leaves (Konishi *et al*., 1996).

Dihydrochalcones are a class of flavonoids that lack a heterocyclic C ring. They are compounds of restricted occurrence and therefore are described as minor flavonoids (Portet *et al*., 2008). Compound 83 exhibited the deprotonated molecule at *m/z* 435, showing the base peak at *m/z* 315 [M-H-120 Da]-, and minor fragments at *m/z* 345 [M-H-90 Da]-, and 273 [M-H-162 Da]-. Losses of 120 and 90 Da correspond to cross-ring cleavages in the sugar unit. Waridel *et al*., (2001) reported that these losses are indicative of C-glycosides, as flavonoid *C*-glycosides need higher collision energies to fragment than do *O*-glycosides since they lack a labile bond, and the main fragmentations take place in the sugar, which has the weakest bonds in the molecule (Abad-García *et al*., 2009). Therefore, this compound was tentatively assigned as phloretin-c-glycoside (nothofagin). Its fragmentation pattern is consistent with Kazuno *et al*., (2005), who performed ESI-Qtrap MS^2 analyses. Other authors have demonstrated the presence of this compound in rooibos tea (Bramati *et al*., 2002).

Finally, three flavanones were characterized in *Sclerocarya birrea* extracts, eriodictyol-O-glucoside-O-gallate and eriodictyol-O-glucoside. Two isomers with different eluting behaviour were assigned as eriodictyol-O-glucoside (58, and 88). Both showed the corresponding most prominent fragment ion at *m/z* 287, which was generated by the breakage of the glycosidic bond and losing the glucose moiety. Secondary fragments were observed for the first eluting isomer at *m/z* 269, and 259, which were previously described by Qiao *et al*., (2011), using an IT mass analyzer. Finally, two other isomers were characterized as eriodictyol-O-glucoside-gallate (65, and 86), based on their molecular formula (and thus mass weight) and MS/MS spectra, since no

information related to their MS fragmentation was found in the literature. These gave rise to the presence of fragments at *m/z* 169 due to the loss of the glucosyl-eriodictyoyl moieties, at *m/z* 269 due to the cleavage of the glucose-galloyl residues, and at *m/z* 449 indicating the loss of the galloyl moiety. These compounds have been isolated from different plant sources (Wang *et al*., 2009, Zhang *et al*., 2002).

4.2.2.3. Stilbenes

Regarding the class stilbenes, two compounds eluting at 14.817 and 16.895 min were detected (78, and 87). The first eluting compound was tentatively characterized as trihydroxystilbene glucosyl-O-gallate. The [M-H]- ion at *m/z* 541 generated a major ion at *m/z* 313 in the MS/MS spectrum, which could be attributed to the loss of a neutral molecule of trihydroxystilbene. The ion at *m/z* 313 underwent a loss of a glucose unit, yielding the ion at *m/z* 169. Moreover, an ion at *m/z* 227 was also observed, resulting from the loss of the glucosyl-galloyl moieties. This fragmentation pathway was proposed by Jin et al., (2007), using ESI-QTOF/MS^2 full-scan experiments, and also compared to the one having an authentic standard. The second eluting compound was tentatively assigned as pentamethoxystilbene. This compound showed fragment ions at *m/z* 314, 299, and 269, due to successive looses of 15 Da, corresponding to the methyl residues. This compound has been shown to exert a highly potent anti-proliferative effect on different human cancer-cell lines (Pan *et al*., 2010, Horvath *et al*., 2007). Essentially, most of methoxylated resveratrol derivatives exhibit far more potent cytotoxic and pro-apoptotic activity against cancer cells than does its parent compound resveratrol (Gosslau *et al*., 2005, Park *et al*., 2007, Roberti *et al*., 2003). Methoxysilbenes have been naturally found in *Yucca gloriosa* bark (Montoro *et al*., 2008).

4.2.3. Fatty acid derivatives

Fatty acid derivatives consisted of dicarboxylic and hydroxyl fatty acids. These molecules may naturally occur as components of vegetal lipids forming part of vegetal surfaces (Kurdyukov *et al*., 2006), but they are also metabolic products of fatty acids since they originate from them by oxidation (Ding *et al*., 2013). Dicarboxylic fatty acids corresponding to deprotonated molecules at *m/z* 187, 201, and 215 showed neutral loses of H_2O, CO_2, and H_2O-CO_2; therefore they were tentatively assigned as nonanedioic (90), decanedioic (93), and undecanedioic (95) acids (Abu-Reidah *et al*., 2013a, Freire *et al*., 2002, Du *et al*., 2007). However, MS^2 analysis of hydroxylfatty acids corresponding to molecular ions at *m/z* 173, and 187 showed neutral loses of H_2O-CO, and thus, they were tentatively characterized as hydroxynonanoic (91) and hydroxydecanoic (94) acids (Rebollido-Fernandez *et al*., 2012, Isidorov *et al*., 2009).

4.2.4. Other polar compounds

Two monolignols were detected in the BPC at *m/z* 527 (24), and 673 (82). They showed fragments at *m/z* 313, and 169, indicating the presence of glucosyl-gallate and gallate residues, respectively. They were tentatively assigned as guaiacylglycerol glucoside gallate and dihydrodehydrodiconiferyl alcohol glucoside gallate. Monolignol sugars have been reported to be monomers involved in lignin synthesis. (Donaldson, 2001, Lu & Ralph, 2010). However, no information is available regarding the galloylation of these monomers. These galloylated compounds have already been isolated from pods and stem bark of different plants (Hsu *et al*., 2012, Kang *et al*., 2008, Ouyang *et al*., 2007). Moreover, mono- and tri-galloyl derivatives of shikimic acid were detected in the BPC (30, and 66). In both cases, the fragment at *m/z* 169 corresponding to the deprotonated gallic acid molecule was observed. This molecule is an important precursor in plant biosynthetic pathways. Finally, a glucosyl-gallate derivative of

methoxyacetophenone was also characterized (61). It showed the fragment at *m/z* 313 characteristic of the glucosyl-gallate residue, confirming this assignation.

5. Conclusion

This research demonstrates that the combination of a non-selective extraction procedure, the high resolving power and wide selective retention of the C18 (with particle size 1.8 µm) stationary phase using an HPLC system and the sensitive and accurate detection of the QTOF mass analyzer constitute a valuable methodology for the metabolic profiling of *Sclerocarya birrea* stem-bark extracts. Our study proved far richer in polyphenols, and more concretely in proanthocyanidins. This methodology successfully detected from monomers up to dimers of (epi)catechin, (epi)gallocatechin, and (epi)afzelechin units with one or two galloyl residues, for some of which the positions of monomers and galloyl substituents were tentatively proposed. It is important to remark that a very high degree of galloylation was observed, which may have a role in the bioactivity attributed to these extracts. The chromatographic method was sufficiently selective to separate up to six isomeric forms of several compounds, and MS detection showed adequate specificity for the analysis, enabling the separation of compounds with similar retention times. Therefore, the methodology applied is useful to characterize plant metabolites, providing excellent MS efficiency as well as to elucidating some structural information in an initial attempt prior to NMR and spectroscopy studies. This information is crucial as an initial step to establish the scientific basis of the anti-diabetic properties of *Sclerocarya birrea* stem-bark extracts for the development of nutraceuticals and therefore, the potential industrial cultivation of this African tree.

6. Acknowledgements

Authors want to thank the Medicinal Plants Research Group of the Laboratory of Pharmacognosy and Botanics of the Faculty of Medicine, Pharmacy, and Odontology (University of Cheikh Anta DIOP, Dakar, Sénégal) for providing the samples. This work was supported by the project

AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as by P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science) projects. The authors are also grateful for the scholarship FPU AP2010-2793.

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Contents block II

Bioactivity assessment of full extracts and of individual components: isolation and purification of bioactive compounds

Chapter 3

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Research Article

Full-length title: Application and Comparison of High-Speed Countercurrent Chromatography and High-Performance Liquid Chromatography in semi-preparative separation of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA), a bioactive secoiridoid from extra-virgin olive oil[†]

Running title: Application of HSCCC and HPLC for the isolation of 3,4-DHPEA-EDA

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Keywords:

Extra-virgin olive oil, isolation, *Olea Europaea*, purification, separation

Abbreviations:

3,4-DHPEA-EA, oleuropein aglycone; **3,4-DHPEA-EDA**, decarboxymethyl oleuropein aglycone; **BPC**, base peak chromatogram; **ESI**, electrospray Ionization; **EVOO**, extra-virgin olive oil; **EVOO-PE**, extra-virgin olive oil-phenolic extract; **HPLC**, high-performance liquid chromatography; **HSCCC**, high-speed counter-current chromatography; **MS**, mass spectrometry; **MUFA**, monounsaturated fatty acid; **PASS,** prediction of activity spectra for substances; **SPE**, solid-phase extraction; **TOF**, time-of-flight.

Received: November 9, 2015 / Revised: April 16, 2016 / Accepted: April 28, 2016

www.ejlst.com European Journal of Lipid Science and Technology

Abstract

This paper primarily extends research on applying and comparing high-speed countercurrent chromatography (HSCCC) and high-performance liquid chromatography (HPLC) in semi-preparative isolation of decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA), a bioactive secoiridoid, from extra-virgin olive oil (EVOO). An EVOO phenolic extract (EVOO-PE) was obtained by solid-phase extraction (SPE), and this extract was subjected to one run using the maximum loading capacity of each methodology without loss of resolution. For this sample set, the HSCCC method proved to be a more efficient approach regarding loading capacity, solvent consumption, and throughput compared with the HPLC method. However, the purity of the target from HPLC was superior to that of HSCCC, as the number of theoretical plates could not match that of HPLC. Therefore, we propose that HPLC should be used when higher purities are required, despite higher costs. In addition, HPLC is orthogonal and complementary to HSCCC, and can often provide straightforward resolution of otherwise difficult separations by HSCCC.

Practical applications: Our results indicate that the optimized HSCCC is an economic process and hence a good candidate for further scaling-up to preparative and industrial scales, as long as the chromatographic profile of the sample itself is simple enough to achieve good resolution, or a very high purity of the compound of interest is not required. For those cases, HPLC would provide superior target purity but would result in higher costs.

1. Introduction

It has been repeatedly suggested that the ability of the so-called “Mediterranean diet” (i.e. the dietary patterns found in olive-growing areas of the Mediterranean basin) to significantly reduce the incidence of atherosclerosis and cardiovascular disease and decrease the risk of several

types of human carcinomas can be attributed largely to the unique characteristics of extra-virgin olive oil (EVOO), which is a basic ingredient in the traditional Mediterranean diet (Colomer & Menéndez, 2006; Pauwels, 2011). Apart from the health benefits that can be ascribed to the monounsaturated fatty acid (MUFA) oleic acid (Escrich *et al.,* 2006), other studies have reported that much of the biological potential of EVOO is due to its minor components (Owen *et al.,* 2004). These include aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and several antioxidants. Although tocopherols and carotenes are also present, hydrophilic phenolics represent the most abundant family of bioactive EVOO compounds (Menendez *et al.,* 2013). These authors have postulated that phenolic extracts that are rich in the secoiridoids oleuropein aglycone (3,4-DHPEA-EA) and decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA) might exert an anticancer effect related to the activation of anti-aging/cellular stress-like gene signatures. Later studies have described the biological activity spectra for 3,4-DHPEA-EA and 3,4-DHPEA-EDA using PASS (Prediction of Activity Spectra for Substances) software. These reports indicate that the oleuropein derivatives 3,4-DHPEA-EA and 3,4-DHPEA-EDA exhibit two naturally occurring isomeric structures with different expected biological activity from hemiacetalic *vs.* dialdehydic forms, and that 3,4-DHPEA-EDA favors the open dialdehydic form, which appears to be more conformationally free and adaptable to interact with a suitable target (Corominas-Faja *et al.,* 2014).

Therefore, further studies are needed to evaluate *in vivo* and *in vitro* the potential therapeutic role of this EVOO secoiridoid, and it is important either to synthesize or isolate this individual compound, as chemical standards are not available. Synthesis is currently not practical and, consequently, isolation procedures must be used. Among the various kinds of separation methods, semi-preparative chromatography, especially preparative high-performance liquid chromatography (HPLC), plays a dominant role in the modern pharmaceutical engineering.

However, the semi-preparative HPLC method is a rather expensive technique, compared to traditional purification methods such as distillation, crystallization, or extraction (Tong *et al.,* 2015). Therefore, alternative technologies have emerged such as high-speed counter-current chromatography. This was developed at the end of 1970s, and since has become a popular modern technique for effective separations of components from natural products at semi-preparative and preparative scales (Ito, 2005). Since HSCCC uses a liquid stationary phase without solid support, sample loss due to irreversible adsorption onto the column is avoided. The distinctive characteristics for HSCCC lies in its preparative capacity due to its ease to be scaled up, compared with conventional HPLC, and it is especially useful for semi-preparative separations at a lower cost. Unfortunately, HSCCC has not been a mainstream technique due to its low theoretical plates of the separation column (Tong *et al.,* 2015). Semi-preparative HPLC provides a good existing solution to the resolution of such mixtures and, therefore, those two techniques were used for comparison to establish the most adequate methodology for the semi-preparative isolation of 3,4-DHPEA-EDA from EVOO in terms of efficiency, purity, and recovery to obtain an acceptable source to support biological investigations.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade and used as received. Methanol and n-hexane, reagents used for the extraction of the phenolic compounds from the olive-oil samples, were purchased from Panreac (Barcelona, Spain). Methanol, *n*-hexane, analytical grade ethyl acetate (Merck, Darmstadt, Germany), and Nanopure® (Barnstead, USA) water were used for liquid–liquid partitioning and the preparative HSCCC separation. Acetic acid from Fluka and Sigma-Aldrich (Steinheim, Germany), and methanol (Panreac, Spain) were used for preparing the mobile phase. Solvents were filtered using a solvent-filtration apparatus model 58061 (Supelco,

Bellefonte, PA, USA). Double-deionizedwater with conductivity lower than 18.2MΩwas obtained with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Extra-virgin olive oil

The extra-virgin olive oil (EVOO) used in this study was of the Arbequina olive variety of *Olea europaea* L. In November 2014, olives were collected in Seville (Spain) and processed by continuous industrial plants equipped with a hammer crusher, a horizontal malaxator, and a two-phase decanter (Metropolitan Park of Granada, Aceites Maeva S.L.). Samples were stored in bottles without headspace at room temperature and darkness before analysis.

2.3. Extra-virgin olive oil extraction

A concentrated EVOO phenolic extract (EVOO-PE) of the Arbequina olive was used in this study. The phenolic fraction was isolated from the EVOO using solid-phase extraction (SPE) with Diol-cartridges (bed weight 10g, 600 mL of tube size), following the method described by Lozano-Sánchez *et al*., (2010). Briefly, the cartridge was first conditioned with 150 mL of hexane and 150 mL of methanol. EVOO (600 g) was dissolved in *n*-hexane and loaded into the column. Then, the cartridge was washed with 150 mL of *n*-hexane. Finally, the sample was recovered by passing through 400 mL of methanol and the solvent was evaporated under vacuum and kept at -18° C.

2.4. Analytical characterization of the EVOO-PE and isolated fractions

The EVOO-PE and the isolated fractions were analytically characterized by means of high-performance liquid chromatography coupled to electrospray time-of-flight mass spectrometry (HPLC-ESI-TOF/MS) method to characterize the phenolic profile in the EVOO-PE. This was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump, and a UV-vis detector. The chromatographic separation was carried out on a Zorbax Eclipse Plus C18

analytical column (4.6 mm x 150 mm, 1.8 µm particle size). The flow rate was 0.80 mL/min, and the temperature of the column was maintained at 25°C. The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The optimal chromatographic method consisted of the following multistep linear gradient: 0 min, 5% B; 7 min, 35% B; 12min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5%B, and finally a conditioning cycle of 5 min with the same conditions for the following analysis. After 0.5 mg of the SPE dried extract were dissolved in 1 mL of methanol, 10 µL of this extract were injected per analytical run.

The compounds separated were monitored in sequence first with DAD (240 and 280 nm) and then with a mass-spectrometry detector. MS was performed using the microTOF (Bruker Daltonik, Bremen, Germany), which was coupled to the HPLC system. At this stage, the use of a splitter was required for the coupling with the MS detector as the flow which arrived to the TOF detector had to be 0.2 mL/min in order to ensure reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative-ion mode. External mass spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of acetic) in quadratic high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to phenolic compounds identification. The optimum values of source parameters were: capillary voltage of 4 kV; drying gas temperature, 190°C; drying gas flow, 9 L/min; nebulizing gas pressure, 2 bar, and end-plate offset, -0.5 kV. The values of transfer parameters were: capillary exit, -120 V; skimmer 1, -40 V; hexapole 1, -23 V, RF hexapole, 50 Vpp, and skimmer 2, -22.5 V. The source and transfer parameters were set for good sensitivity and a reasonable resolution of the mass range for compounds of interest (50-1000 *m/z*) in order to improve the ionization performance. The accurate mass data for the molecular ions were

processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental range, electron configuration, and a ring-plus double-bond equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula.

2.5. Semi-preparative HPLC conditions

Semi-preparative isolation of the 3,4-DHPEA-EDA from the EVOO-PE was achieved using a Gilson preparative HPLC system (Gilson, 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). The compounds were fractionated at room temperature. A 250 mm x 10 mm i.d., 5 µm Phenomenex RP-C18 column was used for separating the phenolic compounds. The mobile phases consisted of acetic acid 0.25% (A) and methanol (B). The following multi-step linear gradient was applied: 0 min, 5% B; 1 min, 35% B; 35 min, 45% B; 45 min, 50% B; 57 min, 55% B; 70 min, 60% B; 74 min, 95% B; 82 min, 5% B; 85 min, 5% B. The injection volume was 200 µL. The flow rate used was set at 3 mL/min.

The compounds separated were monitored with UV-Vis (240 and 280 nm). Fraction collection step consisted of mass spectrometry (MS)-based purification, determining the elution time window for collecting the target compound. MS analysis was performed using the time-of-flight mass spectrometer detector microTOF (Bruker Daltonik, Bremen, Germany) equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative-ion mode. At this stage, the use of a make-up pump and MRA splitter (model 307, Gilson, Middleton, WI, USA) was required for the coupling with the MS detector as the flow which arrived to the TOF detector had

to be 0.2 mL/min in order to ensure reproducible results and stable spray. Values of source and transfer parameters were as described in the previous section.

For semi-preparative isolation of the 3,4-DHPEA-EDA from the EVOO-PE, 100 mg of the dried extract obtained by SPE was reconstituted in 2 mL of methanol to give a concentration of 50 mg/mL. Finally, target compounds were collected and the solvent was evaporated under vacuum. The residue was weighted and dissolved with an appropriate volume of methanol at a concentration level of 100 µg/mL. The fraction was filtered through a 0.25 µm filter before the HPLC analysis to establish its purity grade.

2.6. Semi-preparative HSCCC conditions

To evaluate the suitability for semi-preparative HSCCC to isolate the target compound 3,4-DHPEA-EDA from the EVOO-PE, 2–3 mg of the dried extract obtained by SPE was dissolved in 500 µL of upper and lower phase of the pre-equilibrated biphasic HSCCC solvent systems, and were agitated in small glass vials, as described elsewhere (Ito, 2005). The settling time was recorded and 200 µL of each phase were evaporated separately under a stream of nitrogen gas. The residues were redissolved in 200 µL of methanol and analyzed using HPLC-ESI-TOF/MS. The partition ratio for a given compound was calculated as follows:

$$K = \frac{[\text{stationary phase}]}{[\text{mobile phase}]}$$

HSCCC separations were performed on a Dynamic Extractions Spectrum instrument (Slough, UK) which was fitted with both an analytical-scale column with a volume of 25 mL, an I.D. of 0.8 mm, and a semi-preparative-scale column with a volume of 130 mL, and an I.D. of 1.6 mm. Cooling was provided by a Neslab ThermoFlex 1400 chiller. A Biotronik HPLC pump BT 3020 pumped the two-phase solvent systems. The aqueous lower phase was used as mobile phase (elution mode, head to tail), and samples were loaded into the system by loop injection. The

HSCCC separation was monitored at λ = 280 nm with a Knauer UV–vis detector (Berlin, Germany). The chosen biphasic polar HSCCC solvent system consisted of *n*-hexane–methanol–ethyl acetate–water (3:5:3:5) (v/v/v/v), it was saturated in a separatory funnel at room temperature, and the two phases were divided before use and briefly (1 min) degassed by ultrasonication. The HSCCC-separation in the '*head-to-tail*' or reversed-phase mode defined the upper organic phase as the stationary phase, and the lower aqueous phase as the mobile phase. Reverse-phase elution-extrusion HSCCC conditions were applied as follows: the analytical HSCCC column was filled with the stationary phase (upper phase) at a flow rate of 5 mL/min. Then, the column was rotated clockwise at 1600 rpm to provide a 240×g centrifugal force field. The mobile phase (lower phase) was pumped in the direction from the center inlet of the column to the peripheral outlet at a flow rate of 1 mL/min when using the analytical column and at 5 mL/min when using the semi-preparative column. When the analytical column was used, 50 mg of the dried SPE extract were dissolved in 0.5 mL of each phase, while 250 mg were dissolved in 2.5 mL of each phase when the semi-preparative column was used. The sample solution was injected following equilibration of the column (the moment at which mobile phase elutes from the column outlet) and eluted with mobile phase for 25 min before extrusion with stationary phase at 2 mL/min when using the analytical column, and at 10 mL/min when using the semi-preparative column for 20 min. A column chamber temperature of 35°C was maintained. Finally, fractions were collected into test tubes with a fraction collector (LKB SuperRac 211, LKB, Bromma, Sweden) at 1-min intervals.

3. Results and discussion

3.1. Analytical characterization

Before the isolation of 3,4-DHPEA-EDA , the EVOO-PE was characterized at the analytical scale by means of HPLC-ESI-TOF/MS. Figure 36 shows the chromatogram of the EVOO-PE made at

analytical scale and Table 8 shows the main compounds found in the EVOO-PE. The compounds were assigned by taking into account their elution order and comparing MS with respect to EVOO phenolic compounds reported in the literature and in existing online public databases such as Metlin, Massbank or SciFinder Scholar. A total of 17 compounds belonging to different families (simple phenols, flavonoids, lignans, secoiridoids, and others) were identified, together with two unknown compounds. The first eluting compound was quinic acid (compound 1), a cyclic polyol characterized by a highly hydrophilic character. The hydroxytyrosol derivatives followed this molecule on the chromatographic run, giving the functional group a distinctive eluting behavior, i.e. the oxidized form (compound 2) eluted first, followed by the non-derivatized form (3), and followed by the acetate form (7). The EVOO-PE also showed elenolic acid isomers (8, and 13) as well as secoiridoids, which were formed from elenolic acid, a phenyl ethyl alcohol (hydroxytyrosol in the case of oleuropein derivatives (9, and 10), and tyrosol in the case of ligstroside derivatives (11, and 12), and a glucosidic residue except for the case of oleuropein aglycone (14)). Finally, flavones such as luteolin (15) and apigenin (17) eluted at the end of the chromatographic run due to their less hydrophilic character.

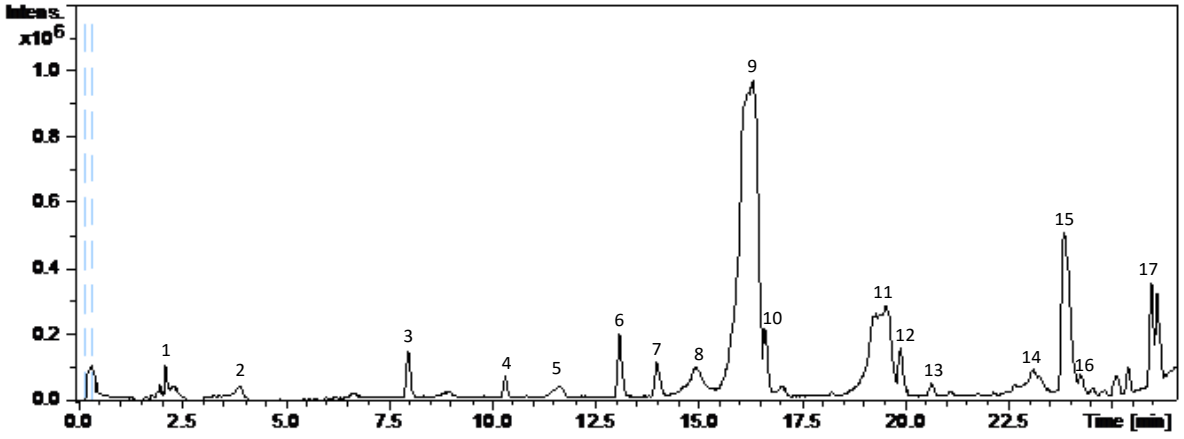

Figure 36. Base peak chromatogram (BPC) made by HPLC-ESI-QTOF/MS in negative ion mode of the EVOO-PE.

Peak	RT (min)	*m/z* experimental (M-H)	Molecular formula	*m/z* calculated (M-H)	Error (ppm)	mSigma value	Proposed compound
1	2.143	191.0558	$C_7H_{12}O_6$	191.0561	1.6	8.8	Quinic acid
2	3.949	151.0406	$C_8H_8O_3$	151.0406	-3.7	7.5	Oxidised hydroxytyrosol
3	7.996	153.0561	$C_7H_{10}O_4$	153.0557	-2.7	3.2	Hydroxytyrosol (3, 4-DHEPA)
4	10.321	172.0979	$C_8H_{15}NO_3$	172.0979	0.2	4.2	Unknown
5	11.609	151.0403	$C_8H_8O_3$	151.0401	1.5	9.4	Vanillin
6	13.064	186.1136	$C_9H_{17}NO_3$	186.1136	-0.0	5.2	Unknown
7	13.950	195.0663	$C_{10}H_{12}O_4$	195.0663	-0.3	10.8	Hydroxytyrosol acetate
8	14.920	241.0700	$C_{11}H_{14}O_6$	241.0718	7.1	9.6	Elenolic acid isomer 1
9	16.275	319.1220	$C_{17}H_{20}O_6$	319.1187	-10.6	28.4	Decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA)
10	16.575	335.1143	$C_{17}H_{20}O_7$	335.1136	-2.0	8.5	Hydroxy decarboxymethyl oleuropein aglycone (hydroxy-3,4-DHPEA-EDA)
11	19.501	303.1232	$C_{17}H_{20}O_5$	303.1238	1.9	3.7	Decarboxymethyl ligstroside aglycone (p-HPEA-EDA)
12	19.836	319.1185	$C_{17}H_{20}O_6$	319.1187	0.8	5.4	Hydroxy decarboxymethyl ligstroside aglycone (hydroxy-p-HPEA-EDA)
13	20.555	241.0710	$C_{11}H_{14}O_6$	241.0718	3.0	19.1	Elenolic acid isomer 2
14	20.030	377.1241	$C_{19}H_{22}O_8$	377.1242	0.4	2.8	Oleuropein aglycone (3,4-DHPEA-EA) isomer 1
15	23.782	285.0409	$C_{15}H_{10}O_6$	285.0405	1.5	7.5	Luteolin
16	24.184	377.1242	$C_{19}H_{22}O_8$	377.1242	-0.1	13.7	Oleuropein aglycone (3,4-DHPEA-EA) isomer 2
17	25.304	269.0452	$C_{15}H_{10}O_5$	269.0455	1.3	13.7	Apigenin

Table 8. HPLC-ESI-TOF-MS data of the identified compounds in the EVOO-PE.

3.2. Semi-preparative HPLC purification of 3,4-DHPEA-EDA

The establishment of the optimum analytical HPLC method for the separation of our target compound is essential for semi-preparative HPLC separation. We took as a reference the method developed by our research group at the analytical scale in which HPLC-ESI-TOF-MS was applied for qualitative and quantitative identification of the most important phenolic compounds of the same variety of EVOO (Lozano-Sánchez *et al.,* 2010). For the choice of the stationary phase, several factors had to be taken into account, such as particle size or length of the column. Smaller particle sizes had higher peak resolution, but allowed less capacity since these packed particles would increase the pressure in the column; therefore the intent was to reach a compromise value between particle size and resolution. Additionally, longer columns provided greater resolution (Huber & Majors, 2007). We chose a semi-preparative C18 250 x 10 mm, 5 µm particle-size column and performed a scaling-up process by trial-and-error measurements.

Lozano-Sánchez *et al.*, (2010) reported that the separation of secoiridoid derivates such as 3,4-DHPEA-EDA was more effective when methanol was used as opposed to acetonitrile or mixtures of acetonitrile/methanol as eluent B, and when using Milli-Q water with 0.25% acetic acid was used as opposed to a different percentage of acid as eluent A. These conditions and a column temperature of 25° C gave the best chromatographic resolution and therefore the aforementioned conditions were maintained. A preliminary gradient was developed taking into account the percentage of each mobile phase at which each compound eluted from the column. This was further optimized by flattening the gradient in the regions prior to our target compound in order to achieve the best separation results. Five different experimental gradients were tested, and among all of them, the best results were found with the multistep linear gradient detailed in the Materials and Methods section.

The flow rate used has an influence on peak resolution. At low flow rates an axial longitudinal diffusion results in band broadening (the analyte can diffuse against the flow), while at high flow

rates the meandering path followed by the eluent around the particles creates eddies, giving rise to poor longitudinal diffusion and inefficient mass transfer, which in turn results in band broadening (in effect, the analyte cannot keep up with the solvent front) (Wellings, 2006). In addition, by increasing the flow rate, the analysis time is reduced but pressure increases. It is necessary to achieve adequate separations, reaching a compromise between short times and good resolution between peaks. Flow rates of 2.5, 3, and 3.5 mL/min were tested, and the best chromatographic resolution was achieved with 3 mL/min, as shown in Figure 37. At this point, the column loading is maximized so that a high level of resolution is maintained, allowing peak skimming to achieve the required purity. The capacity of the column will determine both the concentration and volume injected and will lead to a lower resolution of the compounds. This implies a compromise between the column load, which favors the scan performance and decreases costs, and the resolution of the peaks. If the compounds are of low solubility in the mobile phase, increasing the volume of injection instead of the concentration will be favored (Huber & Majors, 2007). The values that provided the best resolution of target compounds were 200 µL of the EVOO-PE.

Once the semi-preparative conditions were established, we proceeded with the collection of fractions of 3,4-DHPEA-EDA. The fraction collector was synchronized with the chromatograph to collect the compound of interest, between 47 and 49 min. The use of a make-up pump and MRA splitter allowed us to couple the MS detector (TOF) to the chromatograph and thus monitor the target compounds and collect the MS-guided fraction. Twelve identical injections were performed with complete sample dissolution achieved in methanol, and a total amount of 15.3 mg of 3,4-DHPEA-EDA were isolated, achieving 99%target purity. The fraction was evaporated under vacuum, the residue was dissolved in an appropriate volume of methanol to give a concentration of 100 µg/mL, and was analyzed at the analytical scale. Analytical HPLC-ESI-TOF/MS base peak

chromatogram (BPC) of the purified fraction of the target compound 3,4-DHPEA-EDA is shown in Figure 38a, together with its corresponding mass spectrum (Figure 38b).

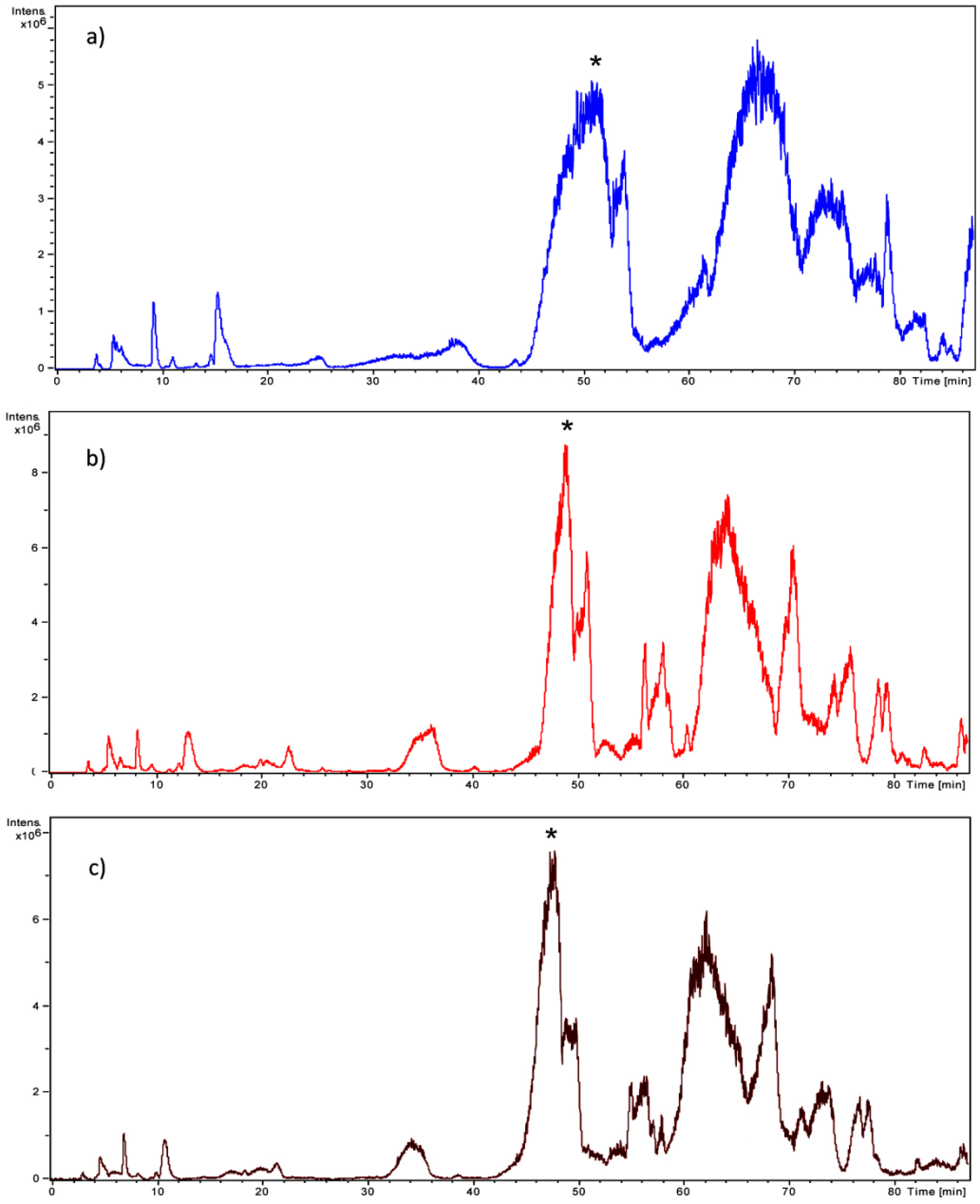

Figure 37. Semi-preparative HPLC-ESI-TOF/MS of the different runs at the flow rates of 2.5 (a), 3 (b), and 3.5 mL/min (c) for the optimization of the semi-peparative conditions.

Asterisk (*) indicates the target compound 3,4-DHPEA-EDA.

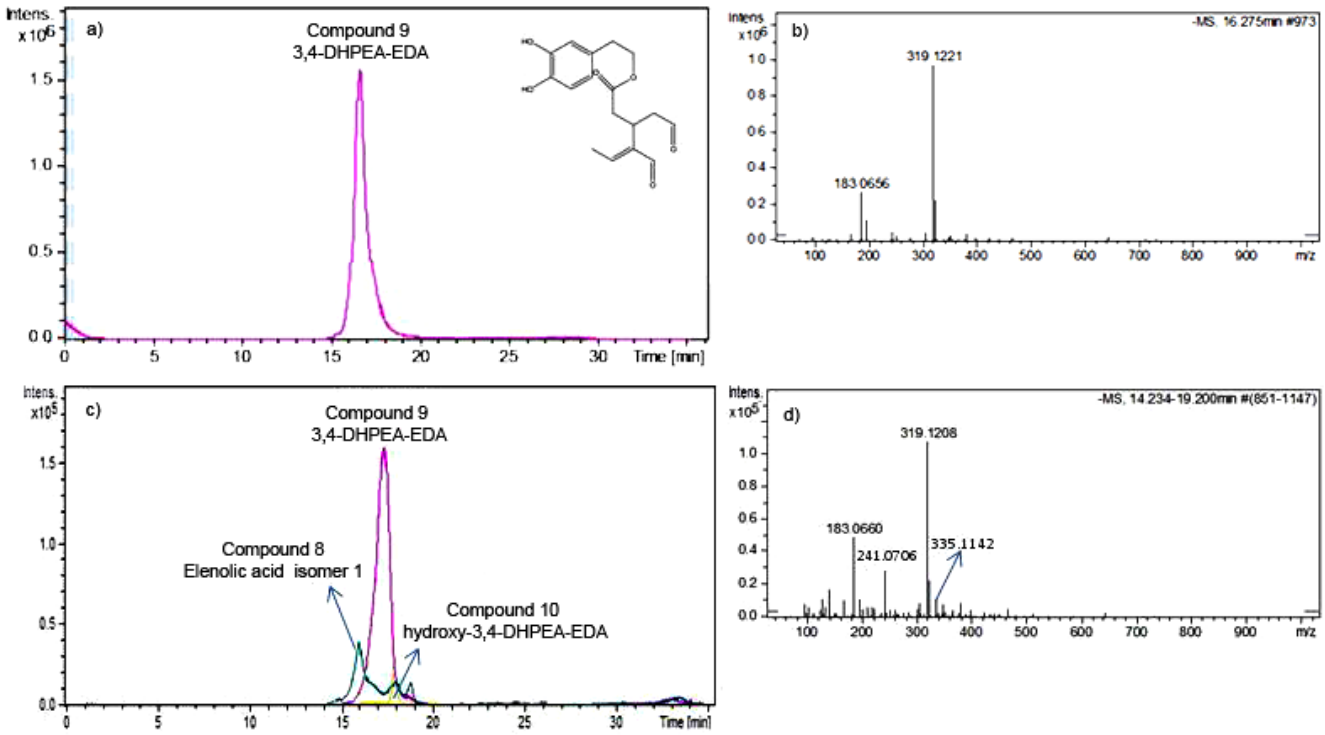

Figure 38. Analytical HPLC-ESI-TOF/MS base peak chromatogram (BPC) of the semi-preparative HPLC fraction (a) together with its corresponding mass spectrum (b); and of the HSCCC fraction (c), together with its corresponding mass spectrum (d).

3.3. Semi-preparative HSCCC purification of 3,4-DHPEA-EDA

In separation by HSCCC, the selection of the two-phase solvent system for the target compound(s) is the most important step, where this may be estimated as 90% of the entire work (Ito, 2005). To find the optimum solvent system for the isolation of individual 3,4-DHPEA-EDA by HSCCC, the HEMWat solvent system series was chosen for this work due to its excellent and broad range of selectivity and solubility characteristics for small-molecule chromatography (Garrard *et al.,* 2007). This solvent system series consists of 22 combinations of hexane, ethyl acetate, methanol, and water, which systematically change in polarity. To choose the most suitable MEMWat mix for semi-preparative HSCCC, we performed the trial described in the Materials and Methods section, starting by testing the combination of middle polarity (3:5:3:5) (v/v/v/v). We calculated the partition coefficient (*K*) of the target compound, which is defined as the ratio of the solute distributed between the two solvent phases. Usually it is expressed by the

amount of solute in the stationary phase divided by that of the mobile phase as in conventional liquid chromatography. The idea is to find systems with *K* values of the target compounds in an appropriate range: The suitable *K* values for HSCCC are $0.5 \leq K \leq 1.0$. A smaller *K* value elutes the solute closer to the solvent front with lower resolution while a larger *K* value tends to give better resolution but broader, more diluted peaks due to a longer elution time (Ito, 2005). The calculated *K* value of the target compound for the Hemwat system tested was 1.512 (see Table 9), signifying that the target compound was distributed mostly in the upper organic phase. Therefore, we continued the search by increasing order of hydrophobicity in the organic phase, and finally achieved an adequate *K* value of 0.776 with the HemWat system (4:5:4:5)(v/v/v/v).

HEMWat system (v/v/v/v)	*K* value
(5:5:5:5)	0.558
(4.5:5:4.5:5)	0.565
(4:5:4:5)	0.776
(3.5:5:3.5:5)	1.131
(3:5:3:5)	1.512

Table 9. *K* values of all the HemWat (hexane-ethyl acetate-methanol-water) systems tested.

The selected system was initially used on the analytical HSCCC scale, and satisfactory (65%) retention of the stationary phase and good resolution were achieved at a flow rate of 1 mL/min, sample capacity of 50 mg in 1 mL, and revolution speed of 1600 rpm. The calculated *K* value of 3,4-DHPEA-EDA will also predict its behavior in the HSCCC separation. Thus a solute eluting at a $K = 1$, equivalent to one column volume, is equally distributed between the upper and lower phases of the solvent system and will elute when one column volume of mobile-phase solvent has passed through the column. $K = 2$ is equivalent to two column volumes, and so on. In our case, a *K* value of 0.776 predicted an elution of nearly one column volume (25 mL at analytical scale), operating at a flow rate of 1 mL/min would mean that the target compound would start

eluting at 19.4 min, so that we fixed an elution time of 25 min, to allow enough time for the target compound to elute. Then, the rest of the chemical compounds were extruded by pumping stationary phase at 2 mL/min for 20 min, to allow enough time to exit the coil. The analytical method was then scaled-up to semi-preparative scale. Based on an approximate five-fold capacity increase between the analytical and semi-preparative HSCCC (130 mL column volume), a linear scale-up would result in a 5 mL sample volume, loading of 250 mg of the extract and a 5 mL/min mobile-phase flow rate with the same revolution speed. The representative HSCCC-UV chromatogram obtained for preparative separation is shown in Figure 39. Fractions were collected at 1-min intervals and analyzed by HPLC-UV-ESI-TOF/MS. The respective base peak chromatogram (BPC) and MS spectrum of the target fraction is shown in Figure 38b. As a result, 18.3 mg of 3,4-DHPEA-EDA were isolated from the EVOO-PE, achieving a target purity of 64%.

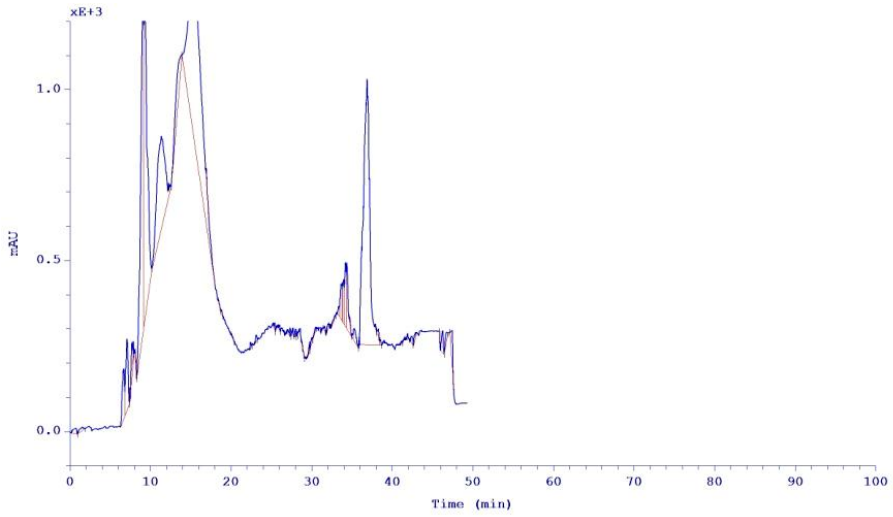

Figure 39. Semi-preparative HPCCC-UV chromatogram.

3.4. Comparison of semi-preparative HPLC and HSCCC

In this section, a comparison of semi-preparative HPLC and HSCCC used in semi-preparative isolation of 3,4-DHPEA-EDA is summarized. Table 10 shows the run parameters, together with purity and recovery of 3,4-DHPEA-EDA for the HPLC and HSCCC from the EVOO-PE. A higher

potential loading capacity of HSCCC will allow the samples to be processed in a single run whereas more injections would be necessary to process the same amount of sample by HPLC. This yielded significant increases of throughput and solvent usage using HSCCC rather than using HPLC. From the data provided, it can be calculated that a HSCCC run consumes nearly the same amount of solvent as does HPLC. However, the unit solvent consumption (mL/mg) of HSCCC was 28-fold lower than for HPLC. As for the stationary phase, a conventional stationary-phase column is necessary for HPLC separation while only solvent can be used as the stationary phase for HSCCC. Thus, generally higher cost for semi-preparative separation by HPLC was more necessary than that of HSCCC.

	HPLC	**HSCCC**
Amount sample/injection (mg)	10	250
Number of injections	3	3
Cycle time/injection (min)	85	45
Flow rate (mL/min)	3	5
Solvent consumption/injection (mL)	255	225
Solvent consumption/mg injected (mL/mg)	25	0.9
Target recovery mg 3,4-DHPEA-EDA /injection (mg/injection)	0.54±0.05	17.3±1.7
Target recovery mg 3,4-DHPEA-EDA /mg injected (mg/mg injected)	0.05±0.006	0.07±0.007
Target purity 3,4-DHPEA-EDA %	97±2	71±6

Table 10. Run parameters, purity and recovery for the HPLC and purification of 3,4-DHPEA-EDA from EVOO-PE.

It can be calculated that the recovery of target material is 36-fold greater by HSCCC than by HPLC. The greater recoveries by HSCCC are not surprising, as sample loss caused by irrecoverable adsorption to the solid support matrix is eliminated by this solvent-only technique (Edwards *et al.,* 2005). By contrast, the injected amount in semi-preparative HPLC could be improved by using columns of larger dimensions, with the subsequent increase in recovery. Therefore, due to the relatively small sample size and the large variability in HPLC recoveries, it

is not possible to draw firm conclusions as to whether the HSCCC recovery gains observed for this sample would be representative of a broader range of samples.

On the other hand, the purity of targets from HPLC was 35% superior to that of HSCCC, as the number of theoretical plates cannot match that of HPLC (Marston & Hostettmann, 2006). Although some authors have reported high purities (>90%) using HSCCC (Yu *et al.,* 2015; Yang *et al.,* 2015), this type of separations frequently require a pre-separation step employing macroporous resins when the crude extracts are complex (Wang *et al.,* 2015; Zhu *et al.,* 2015). In addition, HPLC is orthogonal and complementary to HSCCC, and can often provide straightforward resolution of otherwise difficult separations by HSCCC (Guo *et al.,* 2015; *Jiang et al.,* 2014). However, when considering these comparisons it should be noted that there is room for further optimization of both methodologies, in terms of both throughput and efficiency.

4. Conclusions

Both HSCCC and prep-HPLC purification methods can be effective tools to produce 3,4-DHPEA-EDA and can be a great alternative to total synthesis. For this sample set, the HSCCC method is a more efficient approach regarding loading capacity, solvent consumption, and throughput compared with the HPLC method. However, the purity of the target from HPLC was superior to that of HSCCC, as the number of theoretical plates could not match that of HPLC. Therefore, our results indicate that the optimized HSCCC is an economic process and hence a good candidate for further scaling-up to preparative and industrial scales, as long as the chromatographic profile of the sample itself is simple enough to achieve good resolution, or when a very high purity of the compound of interest is not required. For those cases, HPLC would provide superior target purity, but it would result in higher costs. In addition, HPLC is orthogonal and complementary to HSCCC, and can often provide straightforward resolution of otherwise difficult separations by HSCCC.

5. Acknowledgments

This work was supported by the projects BFU2014-52433-C3-2-R and AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as by P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are also grateful for the scholarship FPU AP2010-2793.

6. Conflict of interest

The authors of this manuscript declare no conflict of interest.

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Chapter 4

Bioguided semi-preparative isolation of active compounds through the modulation of AMPK activation in murine 3T3-L1 hypertrophic adipocyte model.

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Keywords:

Olea europaea, isolation, phenolic compounds, purification, RP-HPLC-ESI-TOF/MS, obesity, adipoRed, immunofluorescence, antibodies

Abbreviations:

ADP, adenosine diphosphate**; AICAR,** 5-Aminoimidazole-4-carboxamide ribonucleotide; **AMP,** adenosine monophosphate**; AMPK,** AMP-activated protein kinase; **BCS**, bovine calf serum; **BPC** Base peak chromatogram; **CaMKKβ**, Calcium/calmodulin-dependent protein kinase 2; **DEX,** Dexamethasone; **DMEM**, Dulbecco's modified Eagle's medium; **FA,** fatty acid**; FBS**, fetal bovine serum; **HPC**, high-precision calibration; **IBMX**, 3-isobutyl-1-methylxanthine; **LKB1**, liver kinase 1, **mTOR**, mammalian target of rapamycin; **pAMPK** phospho AMP-activated protein kinase; **PVD**, polyvinyldifluoride; **RP-HPLC-ESI-TOF/MS**, Reversed-phase High-Performance Liquid Chromatography coupled to Electrospray Time-of-Flight Mass Spectrometry; **Thr172**, Threonine172; **WHO,** World Health Organization.

Abstract

In this study, an *Olea europaea* leaf extract was analyzed following a RP-HPLC-ESI-TOF/MS methodology, and fractionated by means of semi-preparative HPLC to study of the capacity of its fraction to alleviate obesity-related disturbances in murine 3T3-L1 hypertrophic adipocyte model.

The capacity of the fractions to regulate intracellular lipids accumulation mediated through AMPK-dependent mechanisms was explored. The fractionation of the extract allowed us to delimit which compounds could possibly be responsible for such effect. We found out that the most active fractions in activating AMPK in 3T3-L1 hypertrophic adipocytes (p<0.001) contained compounds that belonged to the secoiridoids subclass (oleoside/secologanoside isomer 4, elenolic acid glucoside isomer 2, demethyloleuropein, oleuropein isomer 4, and hydroxyoleuropein isomer 1), cinnamic acids and derivatives (verbascoside and *p*-coumaric acid glucoside), flavonoids (luteolin rutinoside isomers 1 and 2, and glucosylrhamnosylquercetin isomer 2), and lignans (olivil). The olive leaf extract together with the mentioned fractions deserve further attention as a therapeutic aid in the management of obesity and/or associated disturbances.

1. Introduction

Obesity is a multifactorial complex disease of global significance. According to the World Health Organization (WHO), 39% of adults aged 18 years and over were overweight in 2014, and 13% were obese worldwide (WHO, January 2015). This disease is defined by excess adipose mass and adipose tissue expansion, which occurs through adipocyte hypertrophy and hyperplasia (Siriwardhana *et al.*, 2013), being adipocyte size a major determinant of obesity in adults (Ma *et al.*, 2015). Adipose tissue is an important energy storage organ with an important active metabolism (Kalupahana *et al.*, 2012). Indeed, adipose endocrine function is critical to overall energy balance and homeostasis with adipocyte-derived pro- and anti-inflammatory adipokines playing key roles. When the production and secretion of proinflammatory adipokines prevail, systemic inflammation, insulin resistance and obesity-related metabolic disorders arise (Wang *et al.*, 2014).

Recently, AMP-activated protein kinase (AMPK) has been revealed to be an important regulator of cellular energy homeostasis. AMPK is a sensor of cellular energy status that directs metabolic adaptation to support cellular growth and survival, restoring energy homeostasis. AMPK is

involved in the regulation of carbohydrate and lipid metabolism, resulting in inhibition of ATP-consuming anabolic pathways, including FA (fatty acid) synthesis, cholesterol and isoprenoid synthesis, hepatic gluconeogenesis and mTOR (mammalian target of rapamycin)-mediated protein translation. In parallel, AMPK activation stimulates ATP production by increasing FA oxidation, muscle glucose transport, mitochondrial biogenesis and caloric intake (Bijland *et al.*, 2013; Carling *et al.*, 2012). It also plays a major role in hormonal signaling, being a central node of signaling pathways. It can regulate the endocrine system, and at the same time, its activity is regulated by a number of hormones and cytokines (adipokines) such as leptin, interleukin-6, resistin, ghrelin, and adiponectin. In addition, it controls appetite through a neuroendocrine system that makes it a key regulator of energy metabolism at the whole body level *(Novikova et al.,* 2015).

AMPK is activated by phosphorylation at Thr172 that is modulated by the binding of AMP. Although allosteric activation is only caused by AMP, it has recently been found that the effects on phosphorylation and dephosphorilation can also be produced by ADP (Xiao *et al.*, 2011). Specifically, AMPK is phosphorilated by upstream kinases. The primary upstream AMPK kinase is the liver kinase B1 (LKB1), a product of a tumor suppressor gene, which provides anti-tumor functions through direct phosphorilation of AMPK Thr172 *in vitro* and *in vivo* (Shaw *et al.*, 2004; Shackelford & Shaw, 2009). Secondly, Calcium/calmodulin-dependent protein kinase 2 (CaMKKβ), triggers activation of AMPK in response to increases in cell Ca^{2+} (Woods *et al.*, 2005). Increase in Ca^{2+} influx usually accompanies such processes as activation of motor proteins and messages of increased energy consumption. Thus, this activation mechanism anticipates ATP deficiency before it has occurred (Sanders *et al.*, 2007). The classical pathways through which AMPK is activated by upstream kinases that respond to increases in AMP/ATP or ADP/ATP ratios, or by increases in Ca^{2+} are now becoming well understood, although the understanding of how phosphatases dephosphorylate the protein in Thr172 remains incomplete (Hardie *et al.,*

2012). Although changes in AMP, ATP, or Ca^{2+} are triggered by metabolic stresses, recent work suggest that AMPK can also be switched on by numerous plant-derived phenolic compounds (Ko, *et al.,* 2015; Kim & Lee, 2015; Herranz-López *et al.*, 2015). To date, sufficient evidence has been accumulated to bring up the fact that phenolic compounds of *Olea europaea* might be able to activate AMPK pathways in cancer cell lines though the AMPK/mTOR axis (Menendez *et al.*, 2013; Rigacci *et al.*, 2015; Zrelli *et al.*, 2011). These findings led us to postulate that these compounds could also have important implications in metabolic stress-related disorders such as obesity through AMPK-dependent mechanisms.

On the other hand, fractionation of phenolic extracts is of great interest in order to find putative candidates for the attributed biological activity. On this context, semi-preparative High Performance Liquid Chromatography (HPLC) plays a dominant role in separation of phenolic compounds due to its high resolving power, and to the fact that separation methods are already well established, and hyphenation with mass detectors is easy to achieve (Valls *et al.*, 2009). Consequently, the objective of this study was the analysis and fractionation of *Olea europaea* leaf extracts, a cheap and easily available natural source of these phytochemicals, for the study of their effect on obesity-related disturbances though AMPK modulation in murine 3T3-L1 adipocyte model.

2. Materials and methods

2.1. Materials

For the semi-preparative fractionation of the olive leaf extract, all chemicals were of analytical reagent grade and used as received. Methanol used for the extraction was purchased from Panreac (Barcelona, Spain). Acetic acid and acetonitrile for semi-preparative HPLC were purchased from Fluka and Sigma-Aldrich (Steinheim, Germany), respectively. Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA). 3T3-L1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dexamethasone (DEX), 3-isobutyl-1-

methylxanthine (IBMX), insulin, penicillin–streptomycin, calf serum (Hyclone®), fetal bovine serum (FBS) (Hyclone®), paraformaldehyde solution, and Triton X-100 were obtained from Sigma-Aldrich (Madrid, Spain). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco® (ThermoFisher Scientific, Waltham, MA, USA). Polyvinyldifluoride (PVD) filters, 0.22 µm, were obtained from Millipore (Bedford, MA, USA), and Dulbecco's phosphate buffered saline (PBS) from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of the olive leaf extract

Olive leaves (*Olea europaea*) from cultivar 'Arbequina' were used in this study, which were air-dried in the laboratory. Sample extraction was performed as described elsewhere *(Talhaoui, et al.*, 2014). Briefly, dry leaves (5 g) were crushed and extracted via Ultra-Turrax ® T18 basic (IKA, Staufen, Germany) using 300 mL of MeOH/H_2O (80/20). After solvent evaporation, the extracts were reconstituted with MeOH/H_2O (50/50) to achieve the desired concentration. Three extraction replicates were processed.

2.3. Analytical characterization of the olive leaf extract and isolated fractions

The olive leaf extract was analytically characterized by means of Reversed-Phase High-Performance Liquid Chromatography coupled to Electrospray Time-of-Flight Mass Spectrometry (RP-HPLC-ESI-TOF/MS), performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump, and a UV-vis detector. The chromatographic separation was carried out on a Zorbax Eclipse Plus C18 analytical column (4.6 mm x 150 mm, 1.8 µm particle size), at 25°C with a gradient elution programme at a flow rate of 0.5 mL/min. The mobile phases consisted of water plus 0.5% acetic acid (A) and acetonitrile (B). The following multistep linear gradient was applied: 0 min, 5% B; 5 min, 15% B; 25 min, 30% B; 35 min, 95% B; 40 min, 5% B. The initial conditions were maintained for 5 min, as reported elsewhere (Quirantes-Piné *et al.,*

2013). The injection volume in the HPLC system was 10 µL. The extract and the fractions were injected at a concentration of 1 mg/mL.

The compounds separated were monitored with a mass-spectrometry detector. MS was performed using the microTOF (Bruker Daltonik, Bremen, Germany), which was coupled to the HPLC system. At this stage, the use of a splitter was required for the coupling with the MS detector as the flow which arrived to the TOF detector had to be 0.2 mL/min in order to ensure reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative-ion mode. External mass spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of acetic) in quadratic high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to identification. The optimum values of source parameters were: capillary voltage of 4 kV; drying gas temperature, 190°C; drying gas flow, 9 L/min; nebulizing gas pressure, 2 bar, and end-plate offset, -0.5 kV. The values of transfer parameters were: capillary exit, -120 V; skimmer 1, -40 V; hexapole 1, -23 V, RF hexapole, 50 Vpp, and skimmer 2, -22.5 V. The source and transfer parameters were set for good sensitivity and a reasonable resolution of the mass range for compounds of interest (50-1000 *m/z*) in order to improve the ionization performance. The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor.

2.4. Semi-preparative fractionation of the olive leaf extract

Semi-preparative fractionation of the olive leaf extract was achieved using a Gilson preparative HPLC system (Gilson, 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). The extract was fractionated at room temperature. A 250 mm x 10 mm i.d., 5 µm Phenomenex

RP-C18 column was used for separating the compounds. The mobile phases consisted of acetic acid 0.5% (A) and acetonitrile (B). The following multi-step linear gradient was applied: 0 min, 5% B; 5 min, 15% B; 53 min, 27% B; 54 min, 28% B; 60 min, 100% B; 65 min, 100% B; 70 min, 5% B; 75 min, 5% B. The injection volume was 500 µL, at a concentration of 50 mg/mL. The flow rate used was set at 10 mL/min. The compounds separated were monitored with UV-Vis (240 and 280 nm) and MS using the time-of-flight mass spectrometer detector microTOF (Bruker Daltonik, Bremen, Germany) equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative-ion mode. At this stage, the use of a make-up pump and MRA splitter (model 307, Gilson, Middleton, WI, USA) was required to achieve a 0.2 mL/min flow rate for the coupling with the MS detector. Values of source and transfer parameters were as described in the previous section. Fractions were collected taking into account the UV and MS spectra.

2.5. Cell culture and treatment

3T3-L1 mouse embryo fibroblasts were obtained from American Type Culture Collection (Manassas, VA) and cultured as described elsewhere (Green H. & Kehinde O., 1975). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (1 mg/mL glucose) (GIBCO, Grand Island, NY) containing 10% bovine calf serum (BCS) until 80% confluence. At this point (day 0), the cells were stimulated to differentiate with high glucose (4.5 mg/mL) DMEM containing 10% fetal bovine serum (FBS), 1 µM insulin, 0.5 µM isobutylmethylxanthine (IBMX), and 1 µM dexamethasone for two days (day 2). Cells were then maintained in 10% FBS/high glucose (4.5 mg/mL) DMEM medium with 1 µM insulin for another sixteen days (day 18), at which time cells were hypertrophied, insulin-resistant adipocytes with accumulated intracellular lipids. The medium was freshly replaced every 48 h. All media contained 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 292 µg/mL pyruvate. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. At day 18, cells were treated with the respective extract or fractions, which were dissolved in 10% FBS/high glucose (4.5 mg/mL) DMEM medium with 1 µM insulin and

maintained for 48h. Cristal violeta assay was performed in order to dismiss possible cytotoxic effects of the extract/fractions at the working concentrations.

2.6. Quantification of intracellular lipid content

For the quantification of intracellular lipid droplets in high glucose-induced hypertrophic adipocytes the staining AdipoRed™ Assay Reagent (Lonza, Walkersville, MD USA) was used, which is a solution of the Hydrophilic Stain Nile Red. The reagent was diluted in PBS (1:40) and incubated for 30 min at ambient temperature in darkness. Florescence was measured with excitation at 528 nm and emission at 585 nm with Cytation 3 cell imaging multi-mode microplate reader (Biotek Instruments, Winooski, VT, USA).

2.7. Quantification of AMPK and pAMPK levels

For AMPK and pAMPK detection, an immunofluorescence assay was carried out. Cells were washed with PBS, fixed for 15 min in 4% paraformaldehyde, permeabilized in 0.25% Triton X-100 for 5 min, and washed with PBS. After blocking in 4% goat serum at room temperature for 1 h, the cells were washed and incubated overnight at 4°C with mouse monoclonal to AMPK alpha 1 + AMPK alpha 2 antibody (Abcam, Cambridge, UK) or rabbit monoclonal phospho-AMPKα (Thr172) (Cell Signalling Technology, Danvers, MA, USA). Cells were washed 3 times with PBS, and incubated at ambient temperature for 6 hours with Hoechst staining (2.5 µg/mL) together with each corresponding polyclonal secondary antibody, goat anti-rabbit IgG CF™ 594, or goat anti-mouse polyvalent immunoglobulins (G,A,M)-FITC, all three from Sigma-Aldrich (St. Louis, MO, USA). Cells were washed 3 times with PBS and read with Cytation 3 cell imaging multi-mode microplate reader (Biotek Instruments, Winooski, VT, USA). AMPK was detected by measuring the fluorescence with excitation at 490 nm and emission at 520 nm for AMPK, and with excitation at 590 nm and emission at 620 nm for pAMPK. To ensure that extracts/fractions were not cytotoxic, cell count was performed by taken microphotographs of the Hoechst-stained cells at 4x,

using the DAPI imaging filter cube. Microphotographs of pAMPK were taken at 20x, using the Texas Red imaging filter cube.

2.8. Statistical analysis

Values are represented as the mean ± standard deviation (S.D.) of the mean. The values were subjected to statistical analysis (one-way ANOVA, and Dunett's test for multiple comparisons/non parametric approaches). The differences were considered statistically significant at $p < 0.05$. All analyses were performed using Graph Pad Prism 6 (GraphPad Software, Inc. La Jolla, CA, USA). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ on bars indicate statistically significant differences versus control, unless otherwise stated. All cellular measurements derive from three independent experiments, wherein each performed in sextuplicates, unless specified.

3. Results and discussion

3.1. Characterization of the olive leaf extract by RP-HPLC-ESI-TOF

The olive leaf extract obtained as described in the materials and methods section was characterized using a RP-HPLC-ESI-TOF/MS methodology. The identification was performed by comparing retention times, together with the information provided with the MS data provided by the TOF analyzer, and literature (Figure 40).

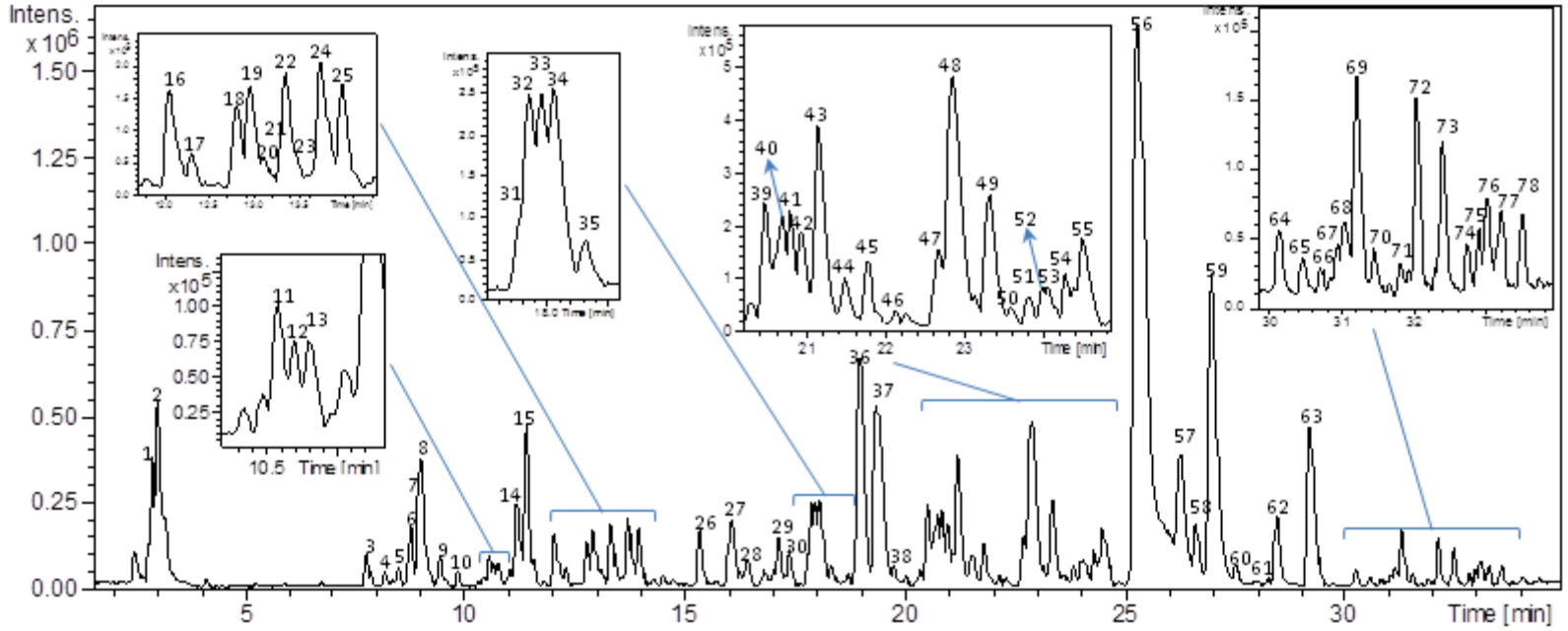

Figure 40. Base peak chromatogram (BPC) of the olive leaf extract in negative ion mode obtained by RP-HPLC-ESI-TOF/MS, in which the peaks are identified with numbers 1–78 according to the order of elution.

The chromatographic profile obtained by RP-HPLC-ESI-TOF/MS showed several peaks, of which a total of 78 are tentatively identified. Compound numbers have been assigned according to their elution order. These are listed in Table 11, which shows their retention time, experimental and calculated *m/z*, molecular formula, error, mσ, proposed compound, and matrix and reference in which they have been previously found. Mσ is a numerical value that indicates how similar the theoretical and measured isotopic patterns are, in such a way that a low mσ value indicates that the measured isotopic pattern of the peak is very similar to the theoretical isotopic pattern for the proposed molecular formula. The tolerance in the mσ value is usually established at 50, although it is influenced by co-eluting analytes or matrix compounds, so it may be higher in some cases (Peters *et al.*, 2009).

As can be seen from the data of Table 11, most of the compounds belonged to the phenolic compounds class, specifically to the iridoids, phenylethanol derivatives, cinnamic acids and derivatives, coumarins, flavonoids, and lignans subclass.

3.1.1. Iridoids

Structurally, they are bicyclic cis-fused cyclopentane-pyrans. This subclass included (epi)loganic acid isomers (3 and 9). This compound has been reported to be one of the key intermediates in the biosynthesis of most of the oleosides in *Olea europaea* (Obied *et al.,* 2008). Cleavage of a bond in the cyclopentane ring gives rise to a subclass known as secoiridoids, for which O*lea europaea* is known to be rich in, especially in oleosides. These are oleaceae-specific secoiridoids commonly esterified to a phenolic moiety. In fact one of the major compounds described previously as being the main component of olive leaves is oleuropein (Fu *et al.*, 2010; N. Talhaoui *et al.*, 2015a), a glucosilated hydroxytyrosol ester of elenolic acid. In our study, five isomers with the same molecular formula as oleuropein ($C_{25}H_{32}O_{13}$) and different eluting behavior were detected (compounds 53, 56, 57, 59, and 67). According to literature, two possible isomers with the same molecular formula have been described in olive leafs, oleuropein and oleuroside, which

differs from each other in the position of a double bound in the elenolic acid moiety (Michel *et al.*, 2015). Therefore, we were not able to distinguish between those two isomers. Other oleuropein/oleuroside derivatives previously described in olive-leaf extracts such as oleuropein/oleuroside aglycone (24), demethyloleuropein/demethoxyoleuroside (30), hydroxyoleuropein/oleuroside isomers (32 and 39), oleuropein/oleuroside glucoside isomers (31, 40, 42, 43, and 44), hydro-oleuropein/hydro-oleuroside (51), methoxyoleuropein /methoxyoleuroside (54), and oleuropein/oleuroside methyl ether (67) were also detected (Jerman *et al.*, 2015; Quirantes-Piné *et al.*, 2013; Talhaoui *et al.*, 2015a). Isomers detected at *m/z* 389 (4, 5, 8, and 15) were tentatively identified as oleoside or secologanoside, both of which have been reported in olive leaves before (Quirantes-Piné *et al.,* 2013), and show the same molecular formula. In addition, compounds 14, 16, 22, and 27 have been proposed as glucosylated forms of elenolic acid, a secoiridoid derivative, which has recently been identified in olive leaves (Michel *et al.*, 2015). Compounds 54, and 66 were proposed as ligstroside isomers, commonly described in *Olea europaea* leaves (Fu *et al.*, 2010). This compound is structurally related to oleuropein but contains a tyrosol moiety instead of a hydroxytyrosol one.

3.1.2. Phenylethanol derivatives

Compounds such as hydroxytyrosol (10), which has been widely described as one of the main components of olive leaves together with oleuropein (Fu et al., 2010), and its glycosylated form (6, and 7), together with the glycosylated form of tyrosol (11) were present in the olive leaf extract. Compounds at *m/z* 491 (50, and 52) were tentatively identified as calcelarioside isomers, a hydroxytyrosol-derived molecule which has been identified for the first time by Michel *et al.,* (2015) in *Olea europaea* leaves.

3.1.3. Cinnamic acids and derivatives

Compound 36 was identified as verbascoside according to the MS data and by comparison with the information reported previously. This molecule is a cinnamic acid derivative (also defined as a phenylpropanoid) commonly present in olive leafs (Fu *et al.,* 2010; Laguerre *et al.*, 2009; Quirantes-Piné *et al.*, 2013; Talhaoui *et al.*, 2014; Talhaoui *et al.*, 2015a). The hydromethanolic olive leaf extract also contained the derhamnosyl form of this molecule (38), the glucoside of caffeic acid (12) and *p*-coumaric acid (17).

3.1.4. Coumarins

Compound 13 was tentatively identified as esculin, a hydroxyl-coumarin which had been recently reported for the first time in *Olea europaea* organs (Michel *et al.*, 2015).

3.1.5. Flavonoids

The olive leaf extract contained several flavonoids. Among them, the aglycones of the flavonones luteolin (69) and apigenin (75), together with their glycosylated forms (34, 37, 48, and 55 for luteolin; and 41, and 47 for apigenin) and the glycosides of the methoxyflavone diosmetin (45, 46, and 49) were detected. In addition, flavonol quercetin (70) together with two respective glycosides (25, and 33), were also present. As it can be deduced from the assigned compound number, glycosylated forms eluted earlier from the chromatographic column than it did their respective aglycones, as it shoud be expected in reversed-phase chromatography. Our findings are in accordance with other authors, who have reported that flavonoids are one of the most common and widely distributed group of olive leaves phenolic compounds (Rahmanian *et al.*, 2015; Talhaoui, *et al.,* 2015b), and can be present in the aglycone or in the glycosylated form (Laguerre *et al.,* 2009; Talhaoui *et al.,* 2015b).

3.1.6. Lignans

Finally, compounds 23 and 35 and were tentatively identified as cycloolivil glucoside, and olivil. These are lignans that has previously been detected in *Olea europaea* organs (Michel *et al.,* 2015; Quirantes-Piné *et al.*, 2013).

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Pk	RT (min)	*m/z* exp.	*m/z* calc.	Mol. formula	Error (ppm)	mSigma	Proposed compound	Reference	Matrix
1	2.860	341.1086	341.1089	$C_{12}H_{22}O_{11}$	0.9	89.4	Sucrose	Guinda et al., 2015	Oive leaf
2	3.096	191.0570	191.0561	$C_7H_{12}O_6$	4.8	14.8	Quinic acid	Quirantes-Piné *et al.*, 2013	Oive leaf
3	7.811	375.1296	375.1297	$C_{16}H_{24}O_{10}$	0.3	9.2	(Epi)loganic acid isomer1	Peralbo-Molina, *et al.*, 2012	Olive pomace
4	8.229	389.1073	389.1089	$C_{16}H_{21}O_{11}$	4.1	9.9	Oleoside/ Secologanoside isomer 1	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
5	8.631	389.1074	389.1089	$C_{16}H_{22}O_{11}$	4	12.3	Oleoside/ Secologanoside isomer 2	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
6	8.797	315.1086	315.1085	$C_{14}H_{20}O_8$	0.1	16.5	Hydroxytyrosol-glucoside isomer 1	Fu *et al.*, 2010 Tóth *et al.*, 2015 Michel *et al.*, 2015	Oive leaf
7	8.849	315.1101	315.1085	$C_{14}H_{19}O_8$	5	33.3	Hydroxytyrosol-glucoside isomer 2	Fu *et al.*, 2010 Tóth *et al.*, 2015 Michel *et al.*, 2015	Oive leaf
8	9.048	389.1112	389.1089	$C_{16}H_{21}O_{11}$	5.7	13.7	Oleoside/ Secologanoside isomer 3	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
9	9.466	375.1299	375.1297	$C_{16}H_{24}O_{10}$	0.5	20.3	(Epi)loganic acid isomer 2	Peralbo-Molina *et al.*, 2012 Michel *et al.*, 2015	Olive pomace
10	9.868	153.0543	153.0557	$C_8H_{10}O_3$	9.3	16.0	Hydroxytyrosol	Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010 Michel *et al.*, 2015	Olive leaf
11	10.353	299.1136	299.1121	$C_{14}H_{20}O_7$	5	23.8	Tyrosol glucoside	Talhaoui *et al.*, 2015a	Olive leaf
12	10.486	341.0875	341.0878	$C_{15}H_{18}O_9$	1	5	Caffeoylglucoside	Borja et al., 1992	Olive mill wastewater
13	10.687	339.0702	339.0722	$C_{15}H_{16}O_9$	5.8	12.2	Esculin	Michel et al., 2015	Oive leaf
14	11.189	403.1246	403.1246	$C_{17}H_{24}O_{11}$	0.2	19.8	Elenolic acid glucoside isomer 1	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
15	11.423	389.1106	389.1089	$C_{16}H_{22}O_{11}$	4.2	14.6	Oleoside/ Secologanoside isomer 4	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
16	12.125	403.1241	403.1246	$C_{17}H_{24}O_{11}$	1.3	11.8	Elenolic acid glucoside isomer 2	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
17	12.309	325.0918	325.0918	$C_{15}H_{18}O_8$	3.5	5.7	*p*-coumaric acid glucoside	She et al., 2008	*Ligustrum purpurascens* leaves (Oleaceae)

18	12.794	519.1727	519.1872	$C_{22}H_{32}O_{14}$	2.7	50.6	Unknown	-	-
19	12.878	401.1476	401.1453	$C_{18}H_{26}O_{10}$	3.7	21.6	Unknown	Fu *et al.*, 2010	Oive leaf
20	12.944	593.1524	593.1512	$C_{27}H_{30}O_{15}$	2	9.2	Unknown	-	-
21	13.246	387.2001	387.2024	$C_{19}H_{32}O_{8}$	6.0	15.2	Unknown	-	-
22	13.329	403.1258	403.1246	$C_{17}H_{24}O_{11}$	2.9	20.5	Elenolic acid glucoside isomer 3	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
23	13.430	537.2002	537.1978	$C_{26}H_{34}O_{12}$	-4.5	3.3	Cycloolivil glucoside	Michel *et al.*, 2015	Olive stem
24	13.684	377.1461	377.1453	$C_{16}H_{25}O_{10}$	2.2	10.9	Oleuropein/oleuroside aglycone	Guinda *et al.*, 2015 Fu *et al.*, 2010	Oive leaf
25	14.099	609.1486	609.1461	$C_{27}H_{30}O_{16}$	4.1	10.4	Glucosyl rhamnosylquercetin (rutin) isomer 1	Talhaoui *et al.*, 2015a	Olive leaf
26	15.352	403.1222	403.1246	$C_{17}H_{24}O_{11}$	5.8	13.9	Elenolic acid glucoside isomer 4	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015	Olive leaf
27	16.055	415.1628	415.1610	$C_{19}H_{28}O_{10}$	4.4	21	Phenethyl primeveroside	Saimaru & Orihara, 2010	Isolated from olive cells
28	16.391	403.1958	403.1974	$C_{19}H_{32}O_{9}$	3.8	24.9	Ethyl-glucopyranosyloxy-oxopropyl-cyclohexaneacetic acid	Taamalli *et al.*, 2012)	Olive leaf
29	17.445	511.2374	511.2396	$C_{22}H_{40}O_{13}$	4.3	6.9	Unknown	-	-
30	17.476	525.1611	525.1614	$C_{24}H_{30}O_{13}$	0.4	7.6	Demethyloleuropein/demethyloleuroside	Michel *et al.*, 2015	Olive leaf
31	17.794	701.2279	701.2298	$C_{31}H_{42}O_{18}$	2.7	8.1	Oleuropein/oleuroside glucoside isomer 1	Fu *et al.*, 2010	Olive leaf
32	17.843	555.1744	555.1719	$C_{25}H_{32}O_{14}$	4.4	19.9	Hydroxyoleuropein/hydroxyoleuroside isomer 1	Quirantes-Piné *et al.*, 2013	Olive leaf
33	17.877	609.1476	609.1461	$C_{27}H_{30}O_{16}$	2.5	20.9	Glucosyl rhamnosylquercetin (rutin) isomer 2	Talhaoui *et al.*, 2015a	Olive leaf
34	17.795	593.1540	593.1512	$C_{27}H_{30}O_{15}$	4.7	29.2	Luteolin rutinoside isomer 2	Quirantes-Piné *et al.*, 2013 Michel *et al.*, 2015	Olive leaf
35	18.799	375.1426	375.1449	$C_{20}H_{24}O_{7}$	6.2	14.8	Olivil	Michel et al., 2015	Olive leaf
36	18.982	623.2004	623.1981	$C_{29}H_{36}O_{15}$	6.2	8.4	Verbascoside	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013	Oive leaf
37	19.601	447.0964	447.0933	$C_{21}H_{20}O_{11}$	6.9	61.7	Luteolin glucoside isomer 1	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015 Fu *et al.*, 2010	Olive leaf
38	19.735	477.1393	477.1402	$C_{23}H_{26}O_{11}$	2.0	26.7	Unknown	-	-
39	20.486	555.1722	555.1719	$C_{25}H_{32}O_{14}$	0.5	23.9	Hydroxyoleuropein/hydroxyoleuroside isomer 2	Quirantes-Piné *et al.*, 2013	Olive leaf

40	20.703	701.2329	701.2298	$C_{31}H_{42}O_{18}$	-4.4	16.5	Oleuropein/oleuroside glucoside isomer 2	Fu *et al.*, 2010	Olive leaf
41	20.806	577.1582	577.1563	$C_{27}H_{30}O_{14}$	-3.3	33.7	Apigenin rutinoside	Quirantes-Piné *et al.*, 2013 Michel *et al.*, 2015	Oive leaf
42	20.937	701.2318	701.2298	$C_{31}H_{42}O_{18}$	-2.8	15.9	Oleuropein/oleuroside glucoside isomer 3	Fu *et al.*, 2010	Oive leaf
43	20.772	701.2329	701.2298	$C_{31}H_{42}O_{18}$	-4.4	16.5	Oleuropein/oleuroside glucoside isomer 4	Fu *et al.*, 2010	Oive leaf
44	21.489	701.2374	701.2298	$C_{31}H_{42}O_{18}$	-10.7	28.2	Oleuropein/oleuroside glucoside isomer 5	Fu *et al.*, 2010	Oive leaf
45	21.756	607.1660	607.1668	$C_{28}H_{32}O_{15}$	1.4	17.3	Diosmetin rhamnoside glucoside (diosmin) isomer 1	Michel *et al.*, 2015	Oive leaf
46	22.275	607.1668	607.1668	$C_{28}H_{32}O_{15}$	0.0	26.8	Diosmetin rhamnoside glucoside (diosmin) isomer 2	Michel *et al.*, 2015	Oive leaf
47	22.642	431.0977	431.0984	$C_{21}H_{20}O_{10}$	1.5	22.8	Apigenin glucoside isomer 1	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013	Oive leaf
48	22.826	447.0982	447.0933	$C_{21}H_{20}O_{11}$	-11.1	38.6	Luteolin glucoside isomer 2	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015 Fu *et al.*, 2010	Oive leaf
49	23.311	461.1112	461.1089	$C_{22}H_{22}O_{11}$	-4.9	18.6	Diosmetin glucoside	Talhaoui *et al.*, 2015a	Olive leaf
50	23.463	491.1550	491.1559	$C_{24}H_{28}O_{11}$	1.8	5.6	Calceolarioside isomer 1	Michel *et al.*, 2015	Oive leaf
51	23.779	541.1925	541.1927	$C_{25}H_{34}O_{13}$	0.2	16.5	Hydro-oleuropein/hydro-oleuroside	Jerman *et al.*, 2015	Olives and olive oil-derived matrices
52	24.049	491.1535	491.1559	$C_{24}H_{28}O_{11}$	4.9	12.1	Calceolarioside isomer 2	Michel *et al.*, 2015	Oive leaf
53	23.948	539.1778	539.1770	$C_{25}H_{32}O_{13}$	-1.4	492.2	Oleuropein/oleuroside isomer 1	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010	Oive leaf
54	24.247	569.1906	569.1876	$C_{26}H_{34}O_{14}$	-5.4	7.6	Methoxyoleuropein/methoxyoleuroside	Tóth *et al.*, 2015	Oive leaf
55	24.532	447.0951	447.0933	$C_{21}H_{20}O_{11}$	-4.0	4.4	Luteolin glucoside isomer 3	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015 Fu *et al.*, 2010	Oive leaf
56	25.152	539.1808	539.1770	$C_{25}H_{32}O_{13}$	-7.0	25.3	Oleuropein/oleuroside isomer 2	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010	Oive leaf
57	26.221	539.1804	539.1770	$C_{25}H_{32}O_{13}$	-6.3	35.0	Oleuropein/oleuroside isomer 3	Guinda *et al.*, 2015	Oive leaf

								Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010	
58	26.756	537.1571	537.1614	$C_{25}H_{30}O_{13}$	8.0	223.0	Unknown	-	-
59	26.923	539.1793	539.1770	$C_{25}H_{32}O_{13}$	-4.3	7.1	Oleuropein/oleuroside isomer 4	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010	Oive leaf
60	27.441	557.2363	557.2240	$C_{26}H_{38}O_{13}$	-4.3	8.9	[dimetyl hydroxy octenoyloxi] secologanoside isomer 1	Quirantes-Piné *et al.*, 2013	Olive leaf
61	28.160	793.2821	793.2866	$C_{45}H_{46}O_{13}$	-1.9	59.1	Unknown	-	-
62	28.378	601.2156	601.2138	$C_{27}H_{38}O_{15}$	-3.1	6.0	Unknown	Fu *et al.*, 2010	Olive leaf
63	29.130	523.1802	523.1821	$C_{25}H_{32}O_{12}$	3.7	6.1	Ligstroside isomer 1	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010 Tóth *et al.*, 2015	Oive leaf
64	30.150	593.1285	593.1301	$C_{30}H_{26}O_{13}$	2.7	12.9	Unknown	-	-
65	30.485	557.2276	557.2240	$C_{26}H_{38}O_{13}$	6.6	18.7	[dimetyl hydroxy octenoyloxi] secologanoside isomer 2	Quirantes-Piné *et al.*, 2013	Olive leaf
66	30.719	523.1800	523.1821	$C_{25}H_{32}O_{12}$	3.9	31.6	Ligstroside isomer 2	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013 Fu *et al.*, 2010 Tóth *et al.*, 2015	Oive leaf
67	30.936	553.1948	553.1927	$C_{26}H_{34}O_{13}$	-3.9	19.0	Oleuropein/oleuroside methyl ether	Pérez-Bonilla *et al.*, 2011	Olive wood
68	31.020	539.1780	539.1770	$C_{25}H_{32}O_{13}$	1.7	4.4	Oleuropein/oleuroside isomer 5	Guinda *et al.*, 2015 Quirantes-Piné *et al.*, 2013	Oive leaf
69	31.204	285.0412	285.0405	$C_{15}H_{10}O_6$	-2.7	19.2	Luteolin isomer 1	Quirantes-Piné *et al.*, 2013 Tóth *et al.*, 2015 Michel *et al.*, 2015	Olive leaf
70	31.454	301.0360	301.0354	$C_{15}H_{10}O_7$	-2.1	5.9	Quercetin	Tóth *et al.*, 2015 Michel *et al.*, 2015	Olive leaf
71	31.789	613.1950	613.1927	$C_{31}H_{34}O_{13}$	-3.9	13.3	Resinoside	Goodger et al., 2009	Eucalyptus leaf
72	32.259	615.2125	615.2083	$C_{31}H_{36}O_{13}$	-6.8	6.5	Unknown	-	-
73	32.726	327.2178	327.2177	$C_{18}H_{32}O_5$	-0.3	14.0	Unknown	-	-
74	32.876	331.2502	331.2490	$C_{18}H_{36}O_5$	3.6	16.5	Trihydroxystearic acid	Meakins & Swindells, 1959	Olive leaf
75	32.960	269.0479	269.0455	$C_{15}H_{10}O_5$	-8.7	9.5	Apigenin	Fu *et al.*, 2010	Oive leaf
76	33.196	329.2339	329.2333	$C_{18}H_{34}O_5$	-1.8	28.9	Trihydroxy-octadecenoic acid	Zhao *et al.*, 2013 Melguizo-Melguizo *et al.*, 2014	*Arabidopsis thaliana* leaf

									Artemisia vulgaris leaf
77	33.380	285.0417	285.0405	$C_{15}H_{10}O_6$	-4.5	8.0	Unknown	-	-
78	33.715	287.2206	287.2228	$C_{16}H_{32}O_4$	7.6	4.6	Dihydroxyhexadecanoic acid	Meakins & Swindells, 1959	Olive leaf

Table 11. Proposed compounds detected in the olive leaf extract analyzed by RP-HPLC-ESI-TOF/MS. From left to right: peak number, retention time, calculated *m/z*, calculated *m/z*, molecular formula, error (ppm), milisigma value, proposed compound; and reference and matrix in which the proposed compound has been previously described.

3.2. Study of the effect of *Olea euroapea* leaf extract in alleviating obesity-linked disturbances in 3T3-L1 hypertrophic adipocytes

Cells maintained with high-glucose medium containing insulin 18 days after differentiation become hypertrophic adipocytes, a cell model characterized by high cytoplasmatic lipid accumulation, insulin-resitance, and exacerbated oxidative stress, a situation reasonably similar to the adipose tissue of an obese person. At this point, cells were treated for 48 hours with the crude extract at concentrations of 200, 400, 600, and 800 µg/mL. After 48h, the quantification of intracellular lipids was measured with the probe AdipoRed™. Cells treated decreased the lipid accumulation by 93, 93, 92, and 86 % ($p < 0.01$), respectively (Figure 41A), compared with the non-treated cells maintained in high glucose medium. Microscopic observation of the treated cells revealed a significant reduction in intracellular lipids accumulation at 800 µg/mL.

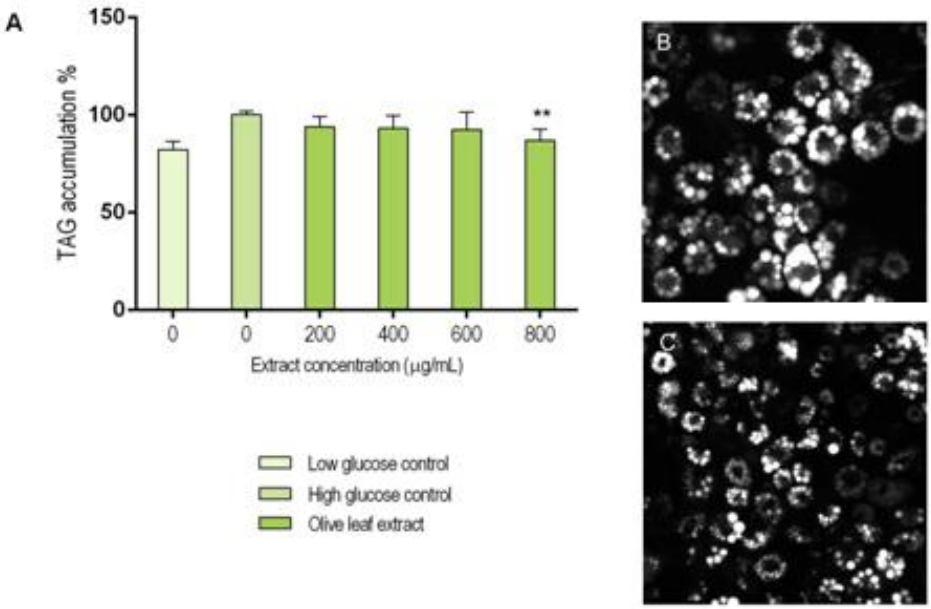

Figure 41. Intracellular lipid accumulation inhibitory effect of the complete olive leaf extract in 3T3-L1 hypertrophic adipocytes.A: Comparison of the olive leaf extract inhibitory effect at concentrations of 200, 400, 600, and 800 µg/mL incubated in high glucose medium. Values have been normalized with respect to the control incubated in high glucose medium only. With comparative aims, a control consisting on cells incubated in low glucose medium has also been included. ** indicates significant differences with respect to the control incubated in high glucose medium ($p<0.01$). B and C: Microphotographs taken with a fluorescence microscope at 20x using the GFP epifluorescence filter cube. Control cells incubated in high glucose medium (B) *vs*. cells incubated with 800 µg/mL of olive leaf extract (C).

Microphotographs of the stained intracellular lipids can be seen in Figure 41B and C, showing lipid intracellular droplets smaller in size in the treated cells at 800 µg/mL with respect to the control cells incubated in high glucose medium.

With the aim of unveiling the possible molecular mechanism of the reduction of intracelular lipids accumulation, we studied the effect of the olive leaf crude extract on the activation of AMPK through phosphorylation at Ser172. After 48 hours of treatment with the extract at increasing concentrations, the levels of AMPK and phospho AMPK at Ser 172 (pAMPK) were quantified by immunofluorescence. Minimal changes in total AMPK protein levels were detected, while phosphorylation of the protein increased at 800 µg/mL. Microscopic observation of the treated cells revealed a significant increase in the fluorescence intensity corresponding to pAMPK levels in cells treated with the complete extract at 800 µg/mL (Figure 42).

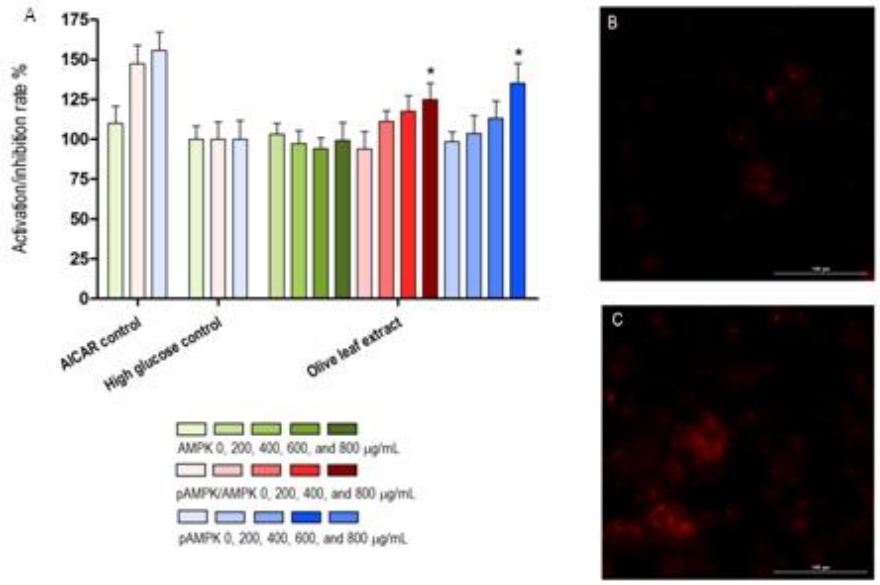

Figure 42. Activation/inhibition rate (%) of total AMPK and the ratio pAMPK/AMPK of the complete olive leaf extract in 3T3-L1 hypertrophic adipocytes. A: Comparison of the olive leaf extract activatory/inhibitory effect on AMPK levels and on the ratio pAMPK/AMPK quantified by immunofluorescence, at concentrations of 200, 400, 600, and 800 µg/mL incubated in high glucose medium. Values are normalized with respect to the high glucose control. With comparative aims, the positive control 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) has also been included. * indicates significant differences with respect to the control incubated in high glucose medium (p<0,05). B and C: Microphotographs taken with a fluorescence microscope at 20x using the Texas Red epifluorescence filter cube. Control cells incubated in high glucose medium (B) *vs*. cells incubated with 800 µg/mL of olive leaf extract (C).

3.3. Relationship between the bioactivity of the olive-leaf extract and its composition

To delimit the possible bioactive compounds responsible for the bioactivity of the olive-leaf extract, we developed a semi-preparative HPLC-ESI-TOF/MS methodology to fractionate the extract according to the UV and MS information.

For the scaling-up from analytical to semi-preparative scale, we used the methodology optimized by our laboratory for analytical scale as a starting point (Quirantes-Piné *et al.*, 2013), performing trial-and-error measurements monitored with the TOF mass spectrometer. While mobile phases and temperature were maintained the same as at analytical scale, for the choice of the stationary phase we kept the nature of the packing material (C18) but changed the dimensions and particle size (250 x 10 mm, 5 µm), with the aim of allowing higher loading capacity without excessively compromising resolution. The elution gradient was optimized in such a way to obtain the better resolution of the compounds of interest, while not compromising throughput, and the optimum flow rate was set at 10 mL/min. Finally, the column loading was maximized while a high level of resolution is maintained. The capacity of the column will determine both the concentration and volume injected and will lead to a lower resolution of the compounds. This implies a compromise between the column load, which favors the scan performance and decreases costs, and the resolution of the peaks. Therefore, the loading capacity was tested to its maximum level while maintaining resolution (500 µL/injection, at a concentration of 50 mg/mL) (See Figure 43).

Once the semi-preparative conditions were established, we proceeded with the collection of fractions. The fraction collector was synchronized with the chromatograph to collect fractions based on the UV and MS spectra.

To study which individual compounds are responsible for the mentioned AMPK activation, fractions were incubated 48 hours at a concentration of 400 µg/mL. The levels of AMPK and pAMPK were quantified by immunofluorescence (Figure 44).

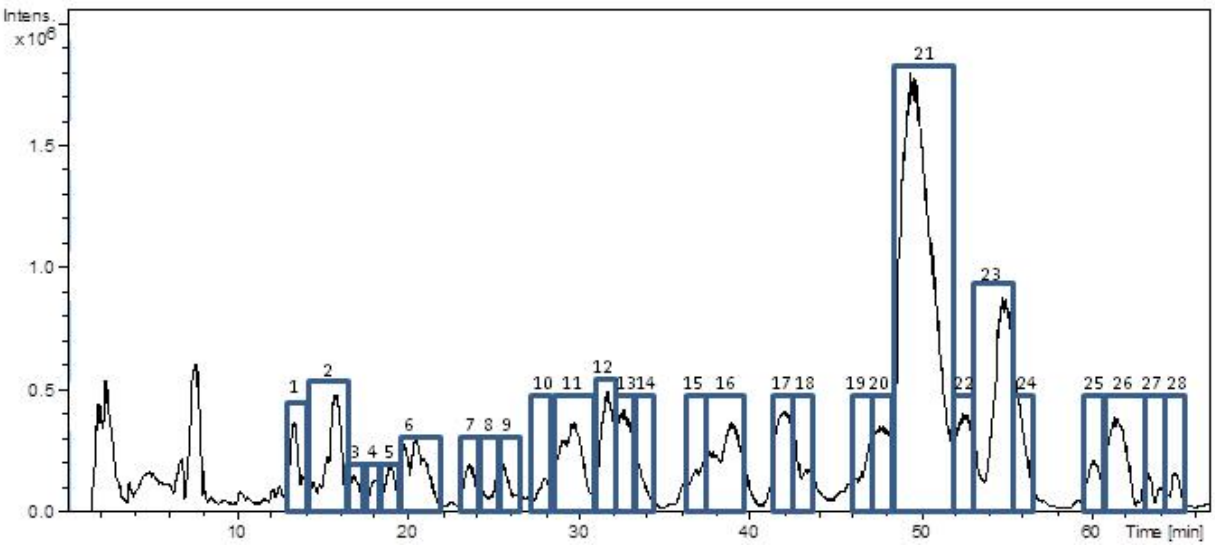

Figure 43. Base peak chromatogram (BPC) made by HPLC-ESI-TOF in negative ion mode of the olive leaf extract, obtained with the optimized semi-preparative conditions, and the collected fractions highlighted in blue.

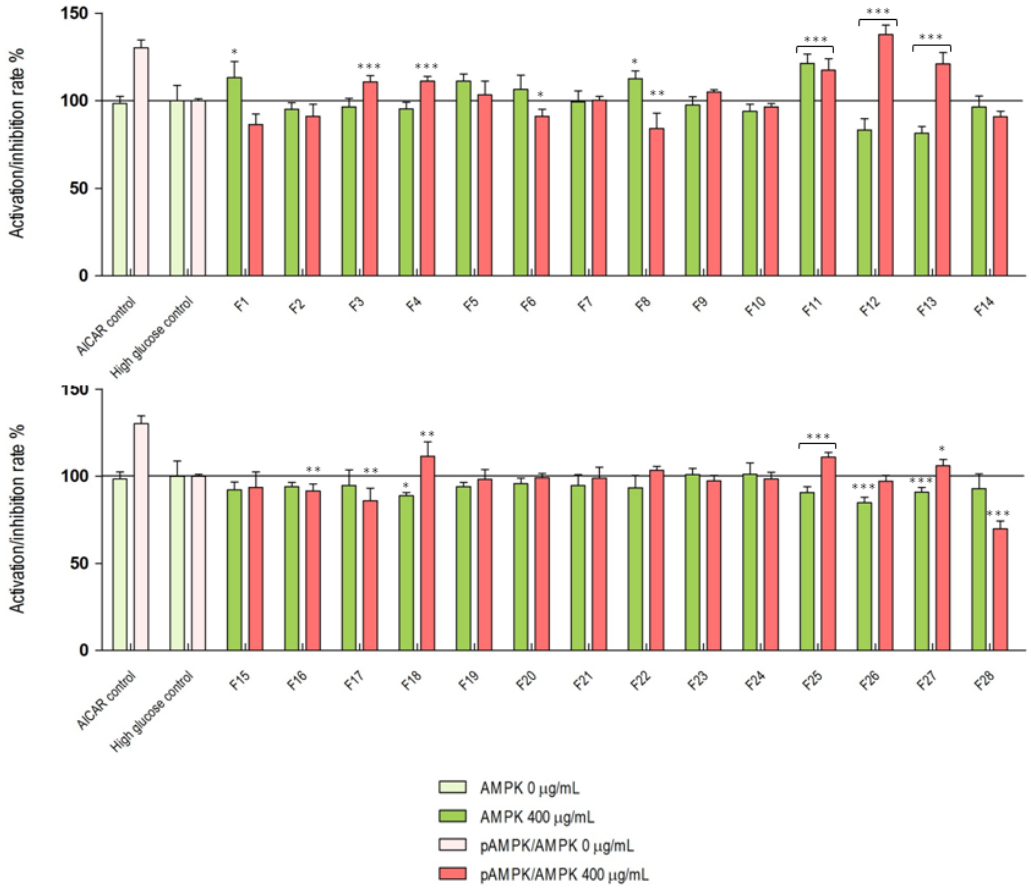

Figure 44. Activation/inhibition rate (%) of total AMPK and the ratio pAMPK/AMPK of the selected fractions in 3T3-L1 hypertrophic adipocytes. The fractions activatory/inhibitory effect on AMPK levels and on the ratio pAMPK/AMPK was quantified by immunofluorescence at a concentration of 400 µg/mL in cells incubated in high glucose medium. Values are normalized with respect to the high glucose control. With comparative aims, the positive control 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) has also been included. *,**, and *** indicate significant

differences with respect to the control incubated in high glucose medium (p<0,05, p<0,01, and p<0,001, respectively).

The fractions were analyzed at analytical scale using the HPLC-ESI-TOF methodology described in the materials and methods section, and fully characterized (See Figure 45). Results suggest that fractions 3, 4, 11, 12, 13, 18, and 25 significantly activated AMPK in hypertrophic and insulin-resistant adipocytes through phosphorylation (p<0.001). Fraction 3 contained compounds 15 (oleoside/secologanoside isomer 4), 16 (elenolic acid glucoside isomer 2), and 17 (p-coumaric acid glucoside); fraction 4 presented compounds 16, 17, 18 (unknown), and 20 (unknown), fraction 11 was composed of compounds 29 (unknown), 30 (demethyloleuropein/demethyloleuroside), 32 (hydroxyoleuropein/hydroxyoleuroside isomer 1), 33 (glucosyl rhamnosylquercetin isomer 2), and 34 (luteolin rutinoside isomer 2); fraction 12 presented compounds 32 (hydroxyoleuropein7hydroxyoleuroside isomer), 33 (glucosyl rhamnosylquercetin isomer 2), 34 (luteolin rutinoside isomer 2), 35 (olivil), 36 (verbascoside), 37 (luteolin glucoside isomer 1); fraction 13 contained compounds 36, and 37; fraction 18 presented compounds 44 (Oleuropein/oleuroside glucoside isomer 5), 48 (Luteolin glucoside isomer 2), diosmetin glucoside (49), and calceolarioside isomer 1 (50); and fraction 25, compounds 59 (oleuropein/oleuroside isomer 4), unknown 6 (60), and unknown (61). Although fractions share a potent AMPK activation capacity through phosphorylation, a distinct behavior is observed when concerning the total AMPK levels. In this regard, total AMPK levels were not significantly altered by fractions 3, and 4, significantly increased (p<0.001) by fraction 11, and significantly reduced (p<0.001) by fractions 12, 13, 18, and 25. In contrast, fraction 28, which contained compounds 73 (9-(epi)F1t-Phytoprostane/trihydroxy octadecadienoic acid/seimatopolide A), 74 (trihydroxystearic acid), 75 (apigenin), 76 (trihydroxy-octadecenoic acid), 77 (unknown), and 78 (dihydroxyhexadecanoic acid), decreased AMPK activation through phosphorylation by 30% (p<0.001).

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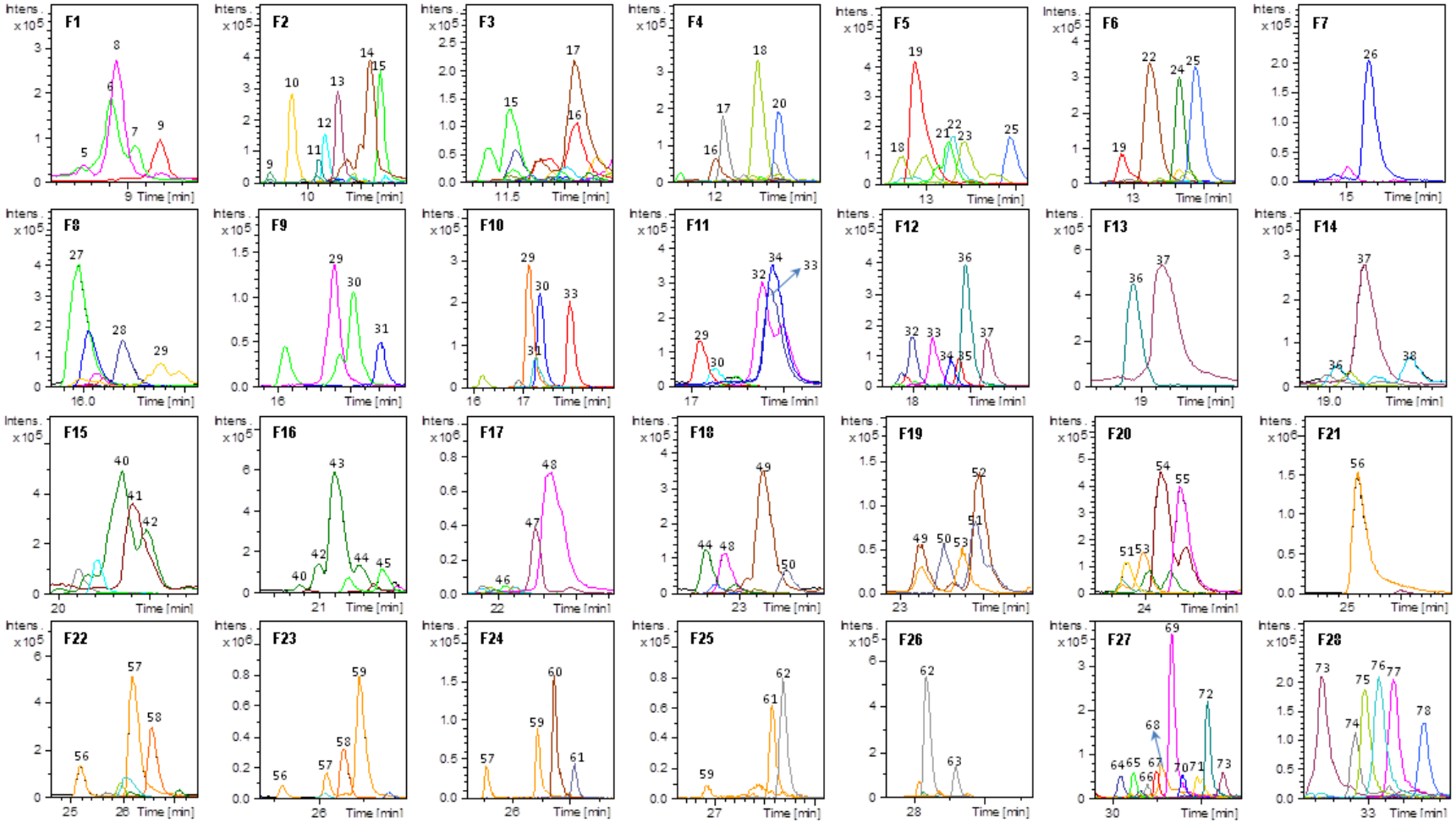

Figure 45. Base peak chromatogram (BPC) of the selected fractions (F1-F28) in negative ion mode obtained by RP-HPLC-ESI-TOF/MS, in which the peaks are identified with numbers 1–78, according to the order of elution.

4. Discussion

Phenolic compounds are the most intensively studied natural products as a recognized source of pharmacologic compounds (Herranz-López *et al.,* 2015). Several lines of evidence suggest a significant impact of phenolic compounds in obesity, which is an increasingly prevalent condition. Although plant phenolic extracts such as *Oiltea Camelia* (Chen *et al.,* 2014), *Lippia Citriodora* (Herranz-López *et al.,* 2015), or *Hibiscus sabdariffa* (Herranz-López *et al.*, 2012) have demonstrated an attenuation of intracellular lipids accumulation in 3T3-L1 adipocytes, a well characterized and widely accepted model of *in vitro* adipogenesis and lipid accumulation that becomes hypertrophic and insulin resistant when induced with high-glucose conditions (Green & Kehinde, 1975; Herranz-López *et al.*, 2012; Herranz-López *et al.,* 2015), to date there are no other reports on this ameliorative effect for olive leaf extracts. Our results suggest that olive leaf extract might have a potential in reducing lipid accumulation in hypertrophic and insulin resistant adipocytes. Researchers have suggested that one of the mechanisms by which phenolic extracts reduce lipids accumulation in the cytoplasm of the adipocyte might be due to AMPK activation (Sung & Lee, 2016; Herranz-López *et al.*, 2012; Herranz-López *et al.,* 2015; Qin *et al.*, 2016). This is in accordance to our results, which have confirmed AMPK activation in 3T3-L1 hypertrohic and insulin resistant adipocytes by phosphorylation at Thr-172.

In this regard, several lines of evidence have indicated a direct link between AMPK and lipid metabolism. It has been reported that AMPK exerts a multiple effects that lead to normalization of lipid metabolism in adipose tissue; directly phosphorylates acetylCoA carboxylase (ACC), which leads to inhibition of fatty acid synthesis and stimulation of fatty acid uptake into mitochondria (González-Barroso *et al.*, 2012; Kim & Lee, 2015; Rao *et al.*, 2015); inhibits lipolysis by phosphorylation of hormone sensitive lipase (Kang *et al.,* 2012), inhibits synthesis of triglycerides and phospholipids via inactivation of glycerol phosphate acyl transferase (GPAT), and

downregulates the transcription of lipogenic genes, including those encoding ACC, fatty acid synthase (FAS), and glycerol phosphate acyl transferase (GPAT) (Yuan & Piao, 2011; Li *et al.*, 2011). Although the olive leaf extract is able to reduce the intracellular lipid accumulation in 3T3-L1 hypertrohic adipocytes though AMPK-dependent mechanisms, more studies need to be performed in order to decipher which downstream targets are involved.

Additionally, the fractionation of the olive leaf extract through semi-preparative RP-HPLC-ESI-TOF/MS allowed us to delimit the number of possible candidate compounds responsible for the observed activation of AMPK. Specifically, the components that might contribute to this effect in a higher extent belonged to the secoiridoids (oleoside/secologanoside isomer 4, elenolic acid glucoside isomer 2, demethyloleuropein/demethyloleuroside, and hydroxyoleuropein/ hydroxyoleuroside isomer 1), cinnamic acids and derivatives (verbascoside and *p*-coumaric acid glucoside), flavonoids (luteolin rutinoside isomers 1 and 2, glucosylrhamnosylquercetin isomer 2, and diosmetin glucoside), and lignans (olivil) subclass.

The facts that fractions 12 and 13 share compounds 36 (verbascoside) and 37 (luteolin glucoside isomer 1) and that the adjacent fraction (14) contained almost exclusively compound 37 and didn't exhibit AMPK activation, suggest that verbascoside could be the main responsible for AMPK activation in these fractions. However, confirmation with the pure standard should be carried out in order to verify this hypothesis. Moreover, elenolic acid glucoside and p-coumaric glucoside, which are both present in fractions 3 and 4, diosmetin glucoside in fraction 18 and unknown compounds 61 an 62 in fractions 25 and 26, might contribute to the observed AMPK activation in these particular fractions.

It has been reported that *p*-coumaric, luteolin, and quercetin, in their aglycone form, are able to increase phosphorylation of AMPK in 3T3-L1 cells (Kang *et al.,* 2012; Herranz-López *et al.*, 2015; Xiao *et al.*, 2014; Strobel *et al.*, 2005), Unlike previous findings, our results point out that some of

the phenolic compounds exhibit AMPK activation as glycosides when added at the extracellular media. In agreement to our results, some authors have previously proved that compounds such as luteolin glucoside have an antiadipogenic effect on 3T3-L1 adipocytes, which might indicate that such phenolic glucosides could be able to go through the plasmatic membrane (Kim *et al.*, 2010) or is metabolized to luteolin aglycone at intracellular level. However, we should express caution when extrapolating *in vitro* data to actual actions of phenolic compounds in the body. First, the intestinal flora is likely to metabolize some of these compounds. Once the glucoside is cleaved, the released aglycone is subjected to the action of specific enzymes in the wall of the small intestine leading to glucuronide, sulphated, and methylated metabolites, which may reach their target tissues and organs (Herranz-López *et al.*, 2012). Obviously, our *in vitro* assays do not take into account the *in vivo* bioavailability issue and can lead to false positive interpretations.

In addition, *Olea europaea* secoiridoids have been proposed to activate AMPK leading to the inhibition of the mammalian target of rapamycin (mTOR) in breast cancer cells (Menendez *et al.*, 2013), which is overactivated not only in cancer, but also in other human chronic diseases such as obesity (Barrajón-Catalán *et al.*, 2014). These findings might support the hypothesis that some *Olea europaea* secoiridoids are responsible for the activation of AMPK in 3T3-L1 adipocytes, although this should be confirmed using the same cell line.

Due to the diverse nature of all the natural AMPK activators, one important question arises: how do they manage to activate AMPK despite the fact that their structures are so different? Hawley *et al.,* (2010) used a cell line that expressed an AMP- and ADP- insensitive AMPK mutant, and proposed that phenolic compounds such as quercetin activate AMPK indirectly by increasing cellular AMP, and ADP, usually by inhibiting mitochondrial ATP synthesis. It has also been reported that oeluropein aglycone indirectly actives AMPK through calcium concentration and subsequent activation of CaMKKβ in SH-SY5Y neuroblastoma cells (Rigacci *et al.*, 2015).

Nevertheless, the direct activation of kinases, such as AMPK, by phenolic compounds at the nucleotide binding site has also been proposed (Corominas-Faja *et al.*, 2014; Taha & Khanfar, 2015). Whether phenolic compounds exert their AMPK modulation through direct or indirect mechanism in our cell model should be further confirmed.

It is also interesting to point out the potent inhibitory effect of fraction 28 (p<0.001). It is known that the phosphorylation of AMPK is reversed by the phosphatases, although the exact mechanisms that modulate their action remain poorly understood (Russo *et al.,* 2013). Yet it is out of the scope of the present dissertation, it has been found that overactivation of AMPK has been observed in several neurodegenerative diseases such as Alzheimer's disease, progressive supranuclear palsy, corticobasal degeneration, Pick's disease, Parkinson and others (Novikova *et al.,* 2015). Therefore, AMPK inhibitors could also be relevant as therapeutic therapies against these types of diseases.

5. Conclusion

In conclusion, we propose that *Olea europaea* olive leaf extract could regulate intracellular lipids accumulation mediated through AMPK-dependent mechanisms. The fractionation of the extract allowed us to delimit which compounds could possibly be responsible for that effect. We found out that most active fractions in activating AMPK in 3T3-L1 hypertrophic adipocytes (p<0.001 y p<0.01) contained compounds that belonged to the secoiridoids subclass (oleoside/secologanoside isomer 4, elenolic acid glucoside isomer 2, demethyloleuropein/demethyloleuroside, oleuropein/oleuroside isomer 4, and hydroxyoleuropein/hydroxyoleuroside isomer 1), cinnamic acids and derivatives (verbascoside and *p*-coumaric acid glucoside), flavonoids (luteolin rutinoside isomers 1 and 2, glucosylrhamnosylquercetin isomer 2, and diosmetin glucoside), and lignans (olivil). Yet the value of the complete extract cannot be discarded, these compounds deserve further attention as a

therapeutic aid in the management of obesity and/or associated disturbances. It should be mention that synergistic or antagonistic interactions may occur among these components, and therefore, studies with pure standards when available should be carried out in order to find the most efficient theraphy. In addition, although *Olea europaea* phenolic compounds show potential for clinical applications in obesity, it needs to be verified *in vivo* to be able to extrapolate the results to actual actions of phenolic compounds in the human body.

6. Acknowledgements

This work was supported by the projects BFU2014-52433-C3-2-R and AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as by P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science). The authors are also grateful for the scholarship FPU AP2010-2793.

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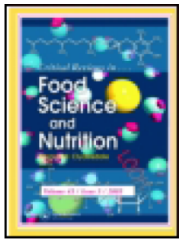

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Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

To cite this article: Cecilia Jiménez-Sánchez, Jesús Lozano-Sánchez, Antonio Segura-Carretero & Alberto Fernández-Gutiérrez (2015): Review: Alternatives to Conventional Thermal Treatments in Fruit-juice Processing. Part 1: Techniques and Applications, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2013.867828

To link to this article: http://dx.doi.org/10.1080/10408398.2013.867828

Review: Alternatives to Conventional Thermal Treatments in Fruit-Juice Processing. Part 1: Techniques and Applications

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Abstract

This article provides an overview of alternatives to conventional thermal treatments and a review of the literature on fruit-juice processing for three key operations in fruit-juice production such as microbial inactivation, enzyme inactivation, and juice yield enhancement, these being radiation treatments (UV light, high-intensity light pulses, γ-irradiation), electrical treatments (pulsed electric fields, radiofrequency electric fields, ohmic heating), microwave heating, ultrasound, high hydrostatic pressure, inert gas treatments (supercritical carbon dioxide, ozonation), and flash-vacuum expansion. The non-thermal technologies discussed in this review have the potential to meet industry and consumer expectations. However, the lack of standardization in

operating conditions hampers comparisons among different studies, and consequently ambiguity arises within the literature. For the juice industry to advance, more detailed studies are needed on the scaling-up, process design, and optimization, as well as on the effect of such technologies on juice quality of juices in order to maximize their potential as alternative non-thermal technologies in fruit-juice processing.

Keywords

Microorganisms, enzyme, quality, technological, innovation

2 ACCEPTED MANUSCRIPT

1. Introduction

Beverages, concentrated juices, and purees are vital food products due to the massive demand of the global market (Tumpanuvatr and Jittanit. 2012). Over the last few years, the consumption of fruit juices has been rapidly increasing (Duthie *et al.,* 2000, Netzel *et al.,* 2007, Tiwari *et al.,* 2009c), making the fruit-juice industry among the largest agro-based industries worldwide (Ribeiro *et al.,* 2010).

Fruit juices are finished products that have been subjected to a transformation process. According to the final product desired and the fruit used, the transformation has numerous variations (Jeantet *et al.,* 2007). The initial steps in the juice extraction include washing, sorting, and crushing of fruits in a mill. All fruits designated for juice processing must be healthy, free of contamination and major bruises, especially free of mold or rot that would lead to defective juices (McLellan and Padilla-Zakour, 2004). In general, juice extraction should be done as rapidly as possible so as to minimize its oxidation by naturally present enzymes.

A variety of methods is used to open the fruit to release the juice, where pressure is applied to the mash the fruit or force it through a press. Several styles of separators are available for both batch and continuous production, such as the pack press (Taylor, 2005), the Bucher-Guyer horizontal rotary press (Downes, 1999), the belt press (Downes, 1999; Shaw, 1994), and the filter press, among others. Heating and addition of enzymes might also be included before the mash is transferred to the extraction stage.

The immediate turbidity in freshly pressed fruit juices is generally considered to be a result of suspended pectin particles from the plant-cell walls, but other disrupted cell-wall and cell materials may also contribute to juice cloudiness (Weiss, 1987; Binning & Possmann 1993). A common problem arising mostly in cloudy fruit juices is the spontaneous clarification during storage. This, usually referred to as haze formation, is assumed to be caused by interactions

between haze-active proteins and phenolic compounds that form insoluble multi-molecular structures (Siebert *et al.,* 1996, Siebert, 2006, Pinelo *et al.,* 2010). Industrial juice clarification typically involves enzyme-catalyzed depectinization and fining by the addition of pectinases, gelatin, silica sol, and/or bentonite, respectively, to encourage pectin degradation and subsequent physico-chemical precipitation of sediments and haze-active components (Konja & Lovric, 1993; Grassin & Fauquembergue, 1996). These treatments are followed by filtration and/or centrifugation (Weiss, 1987; Grassin & Fauquembergue, 1996; Pinelo *et al.,* 2010). Juices are pasteurized immediately after pressing so as to denature any residual enzymes. Centrifugation then removes large pieces of debris, leaving most of the small particles in suspension (Kashyap *et al.,* 2001). The final step is usually a heat treatment or equivalent nonthermal process to achieve a safe and stable juice. For a concentrate, the juice is transferred to an evaporator to remove water to the desired concentration level. Other processes used for water removal include reverse osmosis and freeze concentration, which are best suited for heatsensitive juices. The concentrate is then ready for final processing, packaging, and storage (McLellan & Padilla-Zakour, 2004; Downing, 1996).

Thermal processing is the most commonly used processing technique (Suh *et al.,* 2003). However, it may degrade organoleptic, physical, and physicochemical properties, and the nutritional quality of juices (Kubo *et al.,* 2013; Qin *et al.,* 1995; Charles-Rodríguez *et al.,* 2007; Ibarz *et al.,* 1999; Suh *et al.,* 2003; Chen *et al.,* 2013).

Therefore, recent consumer demands for safe and minimally processed foods with high-quality attributes have encouraged the food industry and scientific researchers to find innovative foodprocessing techniques to produce foods with a minimum of changes induced by the technologies themselves (Enomoto *et al.,* 1997; Hong & Pyun, 2001; Liao *et al.,* 2007; Esteve & Frígola, 2007). Intense investigation has evaluated alternative and complementary processes to

thermal treatments (Heinz *et al.,* 2003; Stewart *et al.,* 2002). Innovations in fruit-juice processing have focused in recent years on microbial and enzymatic inactivation and on increasing juice yield. Therefore, the present paper provides an overview of alternatives to conventional thermal treatments and the published literature in fruit-juice processing for these three key operations in fruit-juice production, such as UV light, high-intensity light pulses, γ-irradiation, pulsed electric fields, radiofrequency electric fields, ohmic heating, microwave heating, ultrasound, high hydrostatic pressure, supercritical carbon dioxide, ozonation, and flash-vacuum expansion.

2. Alternative technologies to conventional thermal treatments in fruit-juice processing for microbial inactivation

Fruit-juice producers have traditionally relied on the acidity of their products to ensure microbiological safety. Nevertheless, several incidents of food-borne disease have been associated with juices. Recent regulations by the Food and Drug Administration (FDA) have required processors to achieve a 5-log reduction in the numbers of the most resistant pathogens in their finished products. The ruling has accelerated the search for novel non-thermal processes that can ensure product safety yet maintain the desired nutritional and sensory characteristics (Tiwari *et al.,* 2009c). In this review, radiation, electrical, ultrasound, high-hydrostatic-pressure, and inert-gas treatments are examined as alternatives to conventional thermal methods for the preservation of fruit juices. Table 12 presents the results achieved during the last decade in terms of microbial inactivation in fruit juices.

Technique	Scale	Microorganisms inactivated	Parameters assayed	Combinations with enhanced efficiency	References
UV light	Laboratory	Aerobic plate count Total yeasts Total moulds *Saccharomyces cerevisiae* *Escherichia coli*	UV dose: 0.087-6 J/cm^2	Ultrasound: 20 kHz, 95 µm	Oteiza *et al.,* 2005, Tran & Farid. 2004, Koutchma *et al.,* 2004, Koutchma *et al.,* 2006, Char *et al.,* 2010, Oteiza *et al.,* 2010
HILP	Laboratory	*Escherichia coli* *Listeria innocua*	Light dose: 1.15-1.2 J/cm^2/pulse Pulse width: 360 µs Treatment length: 1-8 s	Thermosonication: 24 kHz, 100 µm, 2.8-5 min, 40-53°C PEF 24-34kV/cm, 93µs, 92 s	Palgan *et al.,* 2011b, Pataro *et al.,* 2011, Muñoz *et al.,* 2011, Caminiti *et al.,* 2011, Caminiti *et al.,* 2009b
γ-irradiation	Laboratory	Total aerobic counts Coliforms Total moulds *Escherichia coli*	Irradiation dose: 1-5 kGy	Preservative addition: Citric acid (0.3%), sodium benzoate (0.015%), potassium sorbate (0.025%), and sucrose (10%)	Jo & Lee. 2012, Jo *et al.,* 2012, Mishra *et al.,* 2011, Alighourchi *et al.,* 2008, Kim et al 2007, Wang.*et al.,* 2006, Song *et al.,* 2006
PEF	Laboratory, pilot plant and industrial	Total aerobic mesophilic bacteria Total moulds Total yeasts Total enterobacteriaceae *Escherichia coli* *Salmonella enteritidis* *Listeria monocytogenes* *Staphylococcus aureus*	Electric field: 15-35 kV/cm Pulse rise time: 200 ns-4 µs Treatment length: 2 ms-250 s Bipolar mode	Heating: 60-72°C, 15-30 s Preservative addition: citric acid (1.5-2%); bark oil (0.2 %)	Mosqueda-Melgar *et al.,*2008, Morales-de la Peña *et al.,* 2010, Chen *et al.,* 2010, Evrendilek *et al.,* 2000, Akin *et al.,* 2009, Elez-Martínez *et al.,* 2006, Yeom *et al.,* 2000, Zhang *et al.,* 2010, Walkling-Ribeiro *et al.,* 2008, Nguyen 2007
RFEF	Laboratory	*Escherichia coli*	Electric field: 15-18 kV/cm Frequency: 20-40 Hz Treatment length: 190-270 µs	-	Geveke *et al.,* 2004, Geveke *et al.,* 2007, Ukuku *et al.,* 2008, Ukuku & Geveke 2010
OH	Laboratory	*Escherichia coli* *Salmonella typhimuriums* *Listeria monocytogenes* *Clostridium perfringens* *Alicyclobacillus acidoterrestris*	Electric field: 10-40 kV/cm Treatment length. 300 s-30 min Temperature. 70-150°C	-	Leizerson *et al.,* 2005b, Baysal *et al.,* 2010, Onwnka *et al.,* 2008, Sagong *et al.,* 2011, Lee *et al.,* 2012
Ultrasound	Laboratory	Total lactic acid bacterium Total yeasts *Escherichia coli* *Fusarium oxysporum* *Saccharomyces cerevisiae*	Wave amplitude: 23-120 µm Frequency: 20-24 kHz Treatment length: 26 s-20.4 min	Heating: 40-72°C Pressure: 400 kPa Osmotic pressure: 12.6 MPa Additive addition: benzoate (100 ppm) and citrus extract (1800	Adekunte et al 2010a, Adekunte *et al.,* 2010, Bermúdez-Aguirre & Barbosa-Cánovas. 2012, Palgan *et al.,* 2011, Bevilacqua *et al.,* 2012, Wong *et al.,* 2010

		Salmonella spp. *Shigella sp.*		ppm)	
HHP	Laboratory	Total aerobic count Total mesophilic bacteria count Total psicotrophs Total lactic acid bacteria Total enterobacteriaceae Total moulds and yeasts Total coliforms *Staphylococcus aureus* *Cryptosporidium parvum* oocysts *Bacillus coagulans* *Lactobacillus plantarum* *Alicyclobacillus sp.* *Escherichia coli* *Salmonella enteritidis* *Listeria innocua* *Listeria monocytogenes* *Yersinia pseudotuberculosis* *Fracisella tubularensis* *Saccharomyces cerevisiae*	Pressure: 207-700 MPa Treatment length: 2-60 min	Heating: 45-71°C High pressure CO_2: 4.9 MPa Additive addition: sucrose laurate (1%), chitosan (0.01-0.1%), nisin (100IU/mL)	Alpas *et al.,* 2000, Slifko *et al.,* 2000, Shearer *et al.,* 2000, Park *et al.,* 2002, Lee *et al.,* 2002, Alpas *et al.,* 2003, Doğan & Erkmen. 2003, Briñez *et al.,* 2006, Bayındırlı *et al.,* 2006, Lee *et al.,* 2006, Bari *et al.,* 2007, Dede *et al.,* 2007, Hsu *et al.,* 2008, Buzrul *et al.,* 2008, Lavinas *et al.,* 2008, Schlesser *et al.,* 2009, Kumar,S. *et al.,* 2009, Xu *et al.,* 2009, Suárez-Jacobo *et al.,* 2010, Ferrari *et al.,* 2010, Carreño *et al.,* 2011, Varela-Santos *et al.,* 2012, Patterson *et al.,* 2012, Zhao *et al.,* 2013, Mert *et al.,* 2013, Sokolowska *et al.,* 2013a, Sokolowska *et al.,* 2013b
SC-CO_2	Laboratory	Total yeasts *Escherichia coli*	Pressure: 6.9-48.3 MPa CO_2 concentration: 70-170 g/kg Temperature: 25-45°C	-	Gunes *et al.,* 2005, Gunes *et al.,* 2006
Ozonation	Laboratory	Total aerobic count *Listeria innocua* *Listeria monocytogenes* *Escherichia coli* *Salmonella spp.*	Flow rate: 0.12-2.4 L/min Ozone concentration:0.048-0.098 mg/mL/min Treatment length: 5-240 min	Additive addition: dimethyl dicarbonate (250-500 ppm), hydrogen peroxide (300-600 ppm)	Patil,S. *et al.,* 2010b, 534 Patil,S. 2009b Williams *et al.,* 2005, Williams *et al.,* 2004

Table 12. Alternative technologies to conventional thermal treatments in fruit-juice processing for microbial inactivation: summary.

Abbrevations: UV, Ultraviolet; HILP, High Intensity Light Pulses, PEF, Pulsed Electric Fields, RFEF, Radiofrequency Electric Fields, OH, Ohmic Heating, HHP, High Hydrostatic Pressure, SC-CO_2, supercritical CO_2

2.1. Radiation treatment

2.1.1. Ultraviolet light

Fresh food products may be processed using ultraviolet (UV) light as a germicidal medium to reduce the food-borne microbial load. The radiation absorbed by DNA may stop cell growth and lead to cell death (Liltved and Landfald. 2000), the most lethal effect taking place at 254 nm (Murakami *et al.,* 2006, Oteiza *et al.,* 2005, Ibarz *et al.,* 2005). Compared to thermal pasteurization, UV-treated juice may have the added benefit of having a more fresh-like quality in addition to a simpler process with lower operating costs. In addition, photoreactivation may occur when the UV-C injured cells are exposed to wavelengths higher than 330nm (Liltved & Landfald, 2000). In this ca se, the damage occurring at the DNA level could be repaired by protein factors (DNA repair genes) (Yajima *et al.,* 1995). However, a dark environment might avoid photoreactivation of irradiated products (Stevens *et al.,* 1998, Guerrero-Beltrán & Barbosa-Cánovas, 2004).

UV treatment of juices is difficult due to their low UV transmittance through the juice containing dense suspended solids, where microorganisms continue to survive despite continued exposure to high amounts of UV-light energy. Juices with high amounts of suspended matter need a stronger UV dose than do clear juices, i.e. apple juice needs a lower UV dose to achieve effective reduction as expected, whereas orange juice and tropical juices require higher UV dosages to achieve the reductions needed (Keyser *et al.,* 2008). In recent years, to treat juices having high amounts of suspended solids and low UV transmittance, different approaches have been employed, such as extremely thin film UV reactors (Oteiza *et al.,* 2005, Tran & Farid, 2004) to decrease the path length of UV light and thus avoid problems associated with lack of penetration and reactors used to increase the turbulence within a UV reactor to bring all material into close exposure to the UV light (Koutchma *et al.,* 2006). Flow rates and mixing in the turbulent flow also

affected microbial inactivation, i.e. the higher the flow rates the higher inactivation rates in a turbulent flow UV reactor (Koutchma *et al.,* 2004).

UV radiation has been reported to be effective in inactivating *Escherichia coli* ATCC 35218 in apple juice, *Escherichia coli* ATCC 35218 and its cocktail were more sensitive than *Saccharomyces cerevisiae* KE162 and the cocktail of yeasts (Char *et al.,* 2010). In addition, Oteiza *et al.,* (2010) reported that the presence of native yeast cells in orange juice weakens the UV inactivation of *Escherichia coli*. UV-absorption coefficients in the juice increase at greater yeast concentrations, and higher UV doses are necessary to inactivate bacteria.

UV radiation has also been combined with sonication, enhancing the inactivation effect in orange juice, and being more effective simultaneously rather than in a series of ultrasound-UV-C approach (Char *et al.,* 2010). The disadvantages are the possible flavor and color changes in some juice products (Murakami *et al.,* 2006). Furan has also been reported to be formed during UV treatments (Bule *et al.,* 2010).

2.1.2. High-intensity light pulses

High-Intensity Light Pulses (HILP), also known as pulsed light, is an emerging non-thermal technology which uses light pulses of short duration (100-400 μs) ranging from ultraviolet to infrared wavelengths (200-1100 nm), for microbial inactivation. The lethal effect of HILP on microorganisms is attributed mostly to the photochemical action of the UV part of the spectrum emitted by the flash lamp.

Several critical parameters should be considered when designing experiments to assess the suitability of HIPL, such as transparency of the medium, type of microorganism, energy dose supplied, the number of pulses, and the depth of the samples (Palgan *et al.,* 2011b). As expected, and as in UV treatments, microbial inactivation declines with decreasing transparency

of the medium (Pataro *et al.,* 2011). Indeed, treatment at total energy doses of 7-28 J/cm2 during 2-8 s has been demonstrated to be efficient to inactivate *Escherichia coli* in apple juice, but not in orange juice, due to its lower transparency (Palgan *et al.,* 2011b).

Results also depend on the type of microorganism examined. *Escherichia coli* cells showed greater susceptibility to the HILP treatment than did *Listeria innocua* cells in both apple and orange juices. Furthermore, it should be noted that the HILP sensitivity of the various groups of microorganisms may be diverse since each organism has a different requirement in terms of lethal dose (Pataro *et al.,* 2011). Although no clear pattern can be established regarding the differences in HILP sensitivity of the different microorganisms investigated (Gómez-López *et al.,* 2005), it has been generally observed that Gram-positive bacteria are more resistant than Gram-negative ones (Anderson *et al.,* 2000; MacGregor *et al.,* 1998; Rowan *et al.,* 1999; Sharifi-Yazdi and Darghahi. 2006; Pataro *et al.,* 2011).

Concerning lethal doses, results have highlighted that the lethal effect of HILP depended on the energy dose supplied, i.e. the higher the quantity of the energy delivered to the juice stream, the greater the inactivation level in a range of energy dosages from 1.8 to 5.5 J/cm2 (Pataro *et al.,* 2011). By contrast, other findings suggest that the application of a higher energy dose (5.1 J/cm^2) did not significantly raise levels of *Escherichia coli* inactivation in comparison to a lower dose (4.03 J/cm^2) (Muñoz *et al.,* 2011). Similarly, energy doses of 5.1 J/cm^2 or 4.0 J/cm^2 gave no significant differences regarding microbial reduction (Caminiti *et al.,* 2009).

However, the combination of HILP as an initial hurdle followed by thermosonication, led to significant inactivation of 3.37 log CFU/mL and 3.46 log CFU/mL when applied at either the lower and higher energy settings for each hurdle, respectively, giving significantly greater reductions than any of the treatments applied individually (Muñoz *et al.,* 2011); and also followed by manothermosonication, performed to reduce *Escherichia coli* and *Pichia fermentans* in a blend of

fresh apple and cranberry juice, inactivation levels of approximately 6 log have been achieved (Palgan *et al.,* 2011a).

2.1.3. γ-Irradiation

For the γ-Irradiation of food, the product is exposed to a source of gamma rays (Cobalt-60) (Mahapatra *et al.,* 2005). X-rays, or electrons may also be used, although few fruit-juice applications are available in the literature. Microorganisms are inactivated by γ-irradiation primarily due to DNA damage, which destroys the reproductive capabilities and other functions of the cell (Tiwari *et al.,* 2009c; De Ruiter & Dwyer, 2002).

Several factors such as composition of the medium, the moisture content, presence or absence of oxygen, influence radiation resistance, particularly in case of vegetative cells reportedly influence the process (Farkas, 2006). However, some evidence calls into question the link between the microbial-inactivation efficiency and the composition of the medium (Niemira, 2001); as the dose required to achieve 90% destruction of salmonella Enteritidis in orange versus orange-tangerine blend varied only slightly (0.35 to 0.37 kGy), with no significant differences among the juices. Other authors reported that *Salmonella Enteritidis* sensitivity to γ-irradiation is not strongly affected by the composition of formulated commercial orange juices, and in further studies they asserted that neither the resistance of each *Salmonella* isolate inoculated in juice preparations of varying turbidity nor the pattern of relative resistance among isolates was altered in juice preparations of reduced turbidity, therefore proposing the variable resistance of *Salmonella* isolates to irradiation as a more significant factor than turbidity in designing antimicrobial juice-irradiation protocols (Niemira *et al.,* 2001).

Improvement in microbiological quality by radiation processing was evidenced by the dosedependent reduction in total viable counts, yeasts, and molds (Chervin & Boisseau, 1994; Niemira *et al.,* 2003; Kim *et al.,* 2007; Lee *et al.,* 2009; Alighourchi *et al.,* 2008). Irradiation doses

of more than 2 kGy were sufficient to completely inactivate the total bacteria and fungi counts and to retard microbial growth during storage in pomegranate juices (Alighourchi *et al.,* 2008). Similarly, γ- irradiation of 5 kGy at 15°C for carrot juice (Jo & Lee, 2012) and ashitaba and kale juices (Jo *et al.,* 2012) showed 99% or higher sanitation compared to control. They reported that γ-irradiation was superior to UV treatment in terms of energy efficiency and post-treatment effect on microorganism growth (Jo & Lee, 2012).

On the other hand, resistance to γ-irradiation depends on the microbial type examined. A study of microorganism survival indicated that *Escherichia coli* was sensitive to irradiation and can be reduced by 7 log at 1 kGy, whereas the total colony and *Bacillus subtilis* spore bacteria endured stronger irradiation (Wang *et al.,* 2006). Irradiation at 3 kGy showed no viable cell growth of *Salmonella typhimurium* and *Escherichia coli* in carrot or kale juices, *Escherichia coli* being more sensitive than *Salmonella typhimurium* to radiation (Song *et al.,* 2006). However, other studies have reported the presence of two typical radiation-resistant bacteria, *Bacillus megaterium* and *Exiguobacterium acetylicum* in 5 kGy-irradiated kale juices (Kim *et al.,* 2007). Differences in radiation sensitivities among the microorganisms are related to differences in their chemical and physical structure, and in their ability to recover from radiation injury. The amount of radiation energy required to control microorganisms in food, therefore, varies according to the resistance of the particular species and according to the number of organisms present.

Nonetheless, evidence suggests off-odor formation in γ-irradiated juices. Strong off-odor at 2 kGy and higher was found in melon juice (Wang *et al.,* 2006), which agrees with findings of other authors (Fan *et al.,* 2002; Foley *et al.,* 2002; Yoo *et al.,* 2003), who found that total volatile off-odor compounds increased in a dose-dependent manner.

Combinations of γ-irradiation with heat sterilization at 85-95°C have been reported to lower the doses required for elimination of the *Alicyclobacillus acidoterrestris* spores (Nakauma *et al.,*

2004). Similarly, the combination of frozen storage (-20°C) plus irradiation at doses from 0.5-2.0 kGy has resulted in greater overall reductions than either process alone (Niemira *et al.,* 2003). Other approaches such as the addition of preservatives, including citric acid (0.3%), sodium benzoate (0.015%), potassium sorbate (0.025%), and sucrose (10%), have been used to extend the shelf life of sugarcane juice (Mishra *et al.,* 2011).

2.2. Electrical treatment

2.2.1. Pulsed electric fields

Pulsed electric field (PEF) technology involves the application of high-voltage pulses to liquid or semi-solid foods placed between two electrodes. Although a temperature might rise due to the electric current flowing through the liquid food (ohmic heating) (Vega-Mercado *et al.,* 1997; Lindgren *et al.,* 2002; Barsotti *et al.,* 1999), PEF is intended to be a non-thermal technique.

The pulse caused by the discharge of electrical energy is extremely short (with nanoseconds to microseconds), while the interval between discharges is comparatively long (1 millisecond to seconds). The strength of the electric field that passes through the food is directly proportional to the voltage supplied across the electrodes and inversely proportional to the gap or distance between the electrodes. PEF technology utilizes electric field strengths of 10-80 kV/cm (Deeth *et al.,* 2008).

To establish a typical PEF system, a pulse generator is needed. A typical system comprises a high-voltage power supply, one or more (energy storage) capacitors, a high-voltage switch and a treatment chamber. The chamber design exhibits a significant influence on the effectiveness of the process by affecting treatment uniformity, peak electrical field strength, and product throughput (Buckow *et al.,* 2011; Qiu *et al.,* 1998; Buckow *et al.,* 2013). One challenge is to design a treatment chamber capable of operating at high and uniform electric field intensities and which prevents dielectrical breakdowns (Qiu *et al.,* 1998), which may occur when the applied

electrical field strength exceeds the dielectric strength of the treated food product in the chamber (Zhang et al. 1995; Buckow *et al.,* 2013). Dielectrical breakdowns can also be caused by local field enhancement and impurities (gas bubbles or solids) in liquid foods. Batch chambers with parallel plate electrode configurations provide relatively low throughputs but high-treatment uniformity. Treatment chambers with colinear configurations of electrodes allow continuous operation at high throughputs, but often exhibit poor treatment uniformity. Nonuniform electric field and flow velocity distributions can result in under- (often in central regions or dead spaces) or overtreated (often in boundary regions) volume elements, which lead to an increased chance of electrical sparks and system breakdown, as well as degradation of the quality of the treated product. The majority of pilot and industrial-scale PEF systems comprise treatment chambers (flow cells) with cofield and colinear configurations of electrodes. This configuration provides a very nonuniform electrical field which may result in insufficient treatment, dead spaces and, thus, possibly recontamination of the treated medium with microorganisms. To reduce the risk of under- or overprocessing, multiple PEF treatment chambers can be placed in series. This numbering up of treatment zones also means that the required processing time can be broken up into smaller fractions allowing intermediate cooling of the product. This can slightly reduce the effectiveness of the treatment but will preserve the quality of the product (Buckow *et al.,* 2013).

The application of PEF pulses leads to the permeabilization of biological membranes. The plasma membranes of cells become permeable to small molecules after being exposed to an electric field; permeation then causes swelling and the eventual rupture of the cell membrane (Vega-Mercado *et al.,* 1997), reducing or eliminating the microbial load. Indeed, several authors have also reported total inactivation (<1 log CFU/mL) of mesophilic bacteria, mold, and yeast in different fruit juices, after applying different PEF procedures (Li and Zhang. 2004; Mosqueda-Melgar *et al.,* 2008; Morales-de la Peña *et al.,* 2010). This phenomenon depends on parameters

such as fruit maturity, the degree of material fragmentation (particle size), and oxidationpreventing methods, among others (Turk *et al.,* 2010). Intrinsic characteristics of the juice such as its conductivity can influence the microbial inactivation rate. Chen *et al.,* (2010), studying *Staphylococcus aureus* inactivation in apple juice of different conductivities (1.5, 2.0 and 2.5 mS/cm), achieved inactivation with a 75 μs treatment at electric-field strengths of 25, 30, and 35 kV/cm, respectively.

Optimization of electrical parameters (including pulse profile, pulse polarity, pulse duration, number of pulses, pulse frequency, and electric-field strength) and expression parameters (pressure, type of equipment, length of treatment) is crucial to achieve the desired output (Bi *et al*., 2013). It has been reported that as the field strength was increased during stand-alone PEF treatment from 24 to 34 kV/cm, a greater number of *Escherichia coli* cells were inactivated (2.8 compared with 4.2 log CFU/mL), in a tropical fruit smoothie (Walkling-Ribeiro *et al.,* 2008a), and the same effect was found in carrot juice (Akin & Evrendilek. 2009).

Increasing the treatment time also had an impact on microbial reduction, although according to Akin & Evrendilek (2009), not as pronounced as offered by field strength. Morales-de la Peña *et al.,* (2010) reported that by prolonging PEF treatment from 800 to 1400 μs, microbial shelf-life of juice and soymilk juices could be extended from 31 to 56 days.

Other authors have reported that the pulse with shorter rise time (200ns) had a better effect of inactivation on *Staphylococcus aureus* incubated into apple juice compared to a longer one (2 μs) (Chen *et al.,* 2010).

Some researchers even recommend that PEF should be used together with moderate temperatures, i.e. approximately 45-55 °C for increasing the microbicidal effect (Lindgren *et al.,* 2002; Walkling-Ribeiro *et al.,* 2008a; Dunn & Pearlman. 1985). Walkling-Ribeiro *et al.,* (2008a) investigated the effect of PEF-treatment combined with moderate heat from 25°C to either 45 or

55°C over 60 s in a tropical fruit smoothie inoculated with *Escherichia coli* K12. The higher temperature during the PEF treatment induced a greater inactivation, these results being comparable (6.9 log CFU/mL) with those for thermal pasteurization (6.3 log CFU/mL; 72°C, 15 s). These results are in agreement with other authors (Evrendilek *et al.,* 2000; Yeom *et al.,* 2000). However, when a PEF treatment at 32 kV/cm for 90 s was compared to heat treatment at 100°C for 1 min, PEF treatment proved to be efficient in yeast inactivation and moderate in *Escherichia coli* inactivation, although thermal pasteurization was effective for *Escherichia coli* and yeast inactivation (Zhang *et al.,* 2010).

Other studies have demonstrated the efficiency of the addition of antimicrobials to the PEF treatment. When tomato juice was treated alone at mild heat and PEF at field strength of 80 kV/cm, 20 pulses and 50°C, microbial reduction proved minor. However, with the addition of small amount of nisin (100 U/mL) the microbial reduction became significantly greater, about 4.4-log reductions in microbial counts (Nguyen and Mittal. 2007). Positive results have also been achieved by combining PEF with citric acid (0.5-2.0%, w/v) or cinnamon-bark oil (0.05-0.30%, w/v) against populations of *Escherichia coli* O157:H7, *Salmonella Enteritidis* and *Listeria monocytogenes* in melon and watermelon juices. However, the taste and odor in those PEFtreated melon and watermelon juices containing antimicrobials were significantly affected (Mosqueda-Melgar *et al.,* 2008).

Synergistic effects have been reported for combinations of PEF treatment with UV light (Noci *et al.,* 2008; Walkling-Ribeiro *et al.,* 2008b; Caminiti *et al.,* 2011a), and HILP (Caminiti *et al.,* 2009a; Caminiti *et al.,* 2011b).

2.2.2. Radiofrequency electric fields

Radiofrequency electric fields (RFEF) or electric currents that oscillate at radio frequencies in the range of about 3 Hz to 300 GHz could be used as a non-thermal pasteurization for the

inactivation of bacteria in foods. The RFEF process is similar to the PEF process. The difference is that in PEF processing, the high voltage is applied in pulses using a pulse generator, whereas in RFEF processing, the voltage is applied continuously using an alternating current generator (Geveke *et al.,* 2007). Figure 46 shows a schematic diagram of a continuous radio frequency electric fields process including two treatment chambers in series. It has been reported that inactivation of bacteria is by disruption of the bacterial surface structure, leading to the damage and leakage of intracellular biological active compounds (Ukuku *et al.,* 2012, Ukuku *et al.,* 2008).

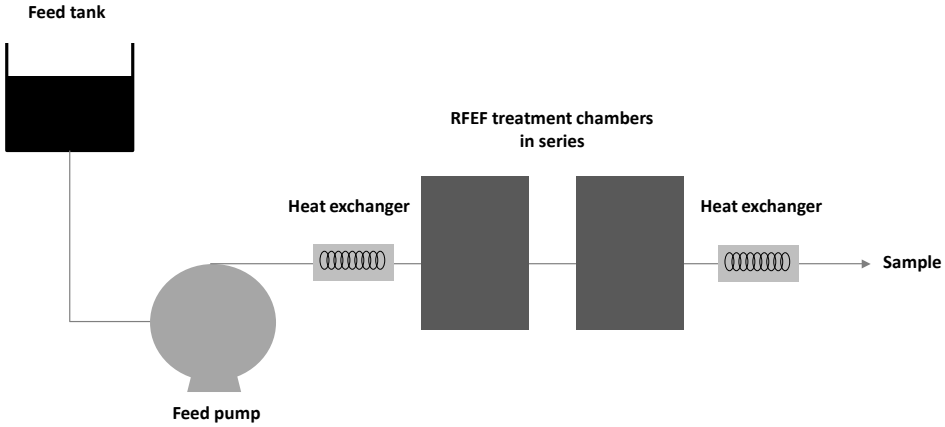

Figure 46. Schematic diagram of continuous radio frequency electric fields process including two treatment chambers in series (Geveke *et al.,* 2007).

It seems clear that increasing the number of treatments and temperature improved inactivation (Geveke *et al.,* 2007, Geveke *et al.,* 2002, Geveke and Brunkhorst. 2003, Geveke and Brunkhorst. 2004). However, the electric-field strength and frequency involves certain complications. It has been stated that increasing the field strength strengthened inactivation within a range of 20-60 kHz (Geveke & Brunkhorst, 2003). However, when experiments were made at lower field strengths (15-20 kV/cm), varying the electric field strength had no effect on the inactivation (Geveke *et al.,* 2007). Frequencies in the range of 20-60 kHz were at first reported to have no effect on microbial inactivation (Geveke and Brunkhorst. 2003). However, it

was subsequently shown that lower frequencies of 15 and 20 kHz inactivated *Escherichia coli* better than did frequencies of 30 to 70 kHz (Geveke & Brunkhorst, 2004).

It has been stated that the RFEF application of moderate heat provides a much higher inactivation than when used alone. Increasing the temperature to 55°C enhanced the inactivation of the bacteria, leading to 99.99% reduction (Ukuku *et al.,* 2008).

RFEF has been studied alone and in combination to UV light for inactivating *Escherichia coli* K-12 in apple juice. At 40°C, UV-light treatment alone caused a 5.8-log reduction of *Escherichia coli* in apple juice while RFEF caused only a 2.8-log reduction. A combination of the two processing treatments did not increase cell injury or leakage of intracellular bacterial UV substances more than that from the UV-light treatment (Ukuku and Geveke. 2010). In further studies, RFEF at 25 kV/cm, 75°C and 3.4 ms was used in combination to mild heat at 75°C and the viability loss for *Escherichia coli* averaged 7 log CFU/mL (Ukuku *et al.,* 2012).

2.2.3. Ohmic heating

Ohmic heating (OH) of food products involves the passage of alternating current through them, thus generating internal heat as a result of electrical resistance of the food components (Reznick. 1996; Valero *et al.,* 2010). Figure 47 shows a schematic diagram of an ohmic heating circuit. This technique differs from PEF, as the latter involves the application of a short burst of high voltage to a food placed between two electrodes, destroying the cell membrane by mechanical effect with no intended heating (Barbosa-Cánovas *et al.,* 1999).

This may be considered a thermal treatment although it has some advantages over the conventional heating system because the heat is instantly generated within the food with the passing of the electrical current (Icier *et al.,* 2006; Icier & Tavman, 2006) whereas the conventional method must rely on the heat-transfer mechanisms, especially heat conduction and

convection, which are usually limited due to the thermo-physical properties of food and the fouling on the heat contact surface (Tumpanuvatr & Jittanit, 2012; Lalande, 1985; Bansal & Chen, 2006). Therefore, ohmic heating has been attributed to a high heating rate, high energyconversion efficiency, uniform volumetric heating (Assawarachan, 2010; Castro *et al.,* 2004; de Alwis & Fryer, 1990) and therefore inflicts less thermal damage on the product (Leizerson & Shimoni. 2005a; Leizerson & Shimoni, 2005b).

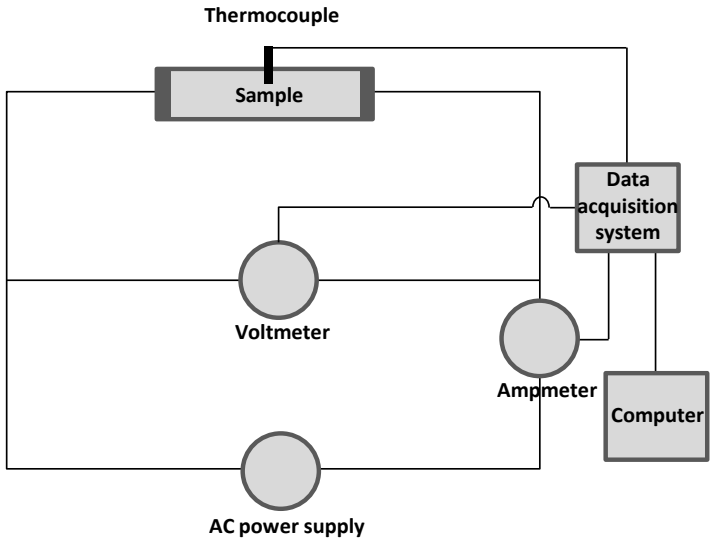

Figure 47. Schematic diagram of an ohmic heating circuit (Tumpanuvatr & Jittanit, 2012).

The heating rate is directly proportional to the square of the electric field strength, and the electrical conductivity of the product. Thus, foods having lower electrical conductivities heat more slowly than do those of higher electrical conductivities if the same electrical-field strength is applied. The electrical conductivities of food products normally depend on their temperatures and constituents, especially salt, acid, and fat (Tumpanuvatr & Jittanit, 2012; Shiksat *et al.,* 2004; Sarang *et al.,* 2008). Many published works have demonstrated that the electrical conductivities of foods would increase if their temperatures were raised (Shiksat *et al.,* 2004; Sarang *et al.,* 2008; Icier & Ilicali. 2005b; Icier, 2009, Assawarachan, 2010; Lee *et al.,* 2012; Darvishi *et al.,*

2011; Singh *et al.,* 2008; Kong *et al.,* 2008). If the product has more than one phase, such as in juices containing a mixture of liquid and particulates, the electrical conductivity of all the phases should be considered (Ruan *et al.,* 2001; Zhou *et al.,* 2011) concluded that the conductivity of apple juice with granules increases linearly as temperature increases and particle size and mass fraction decreases. However, other authors found that the electrical conductivities of liquid food matrices might instantaneously drop after the occurrence of bubbling (Icier & Ilicali, 2005a; Icier & Ilicali, 2005b). The bubbles are characterized as electrical insulators; therefore, they will interfere the flow of electrical current and lessen the electrical conductivity of food matrices as a whole (Tumpanuvatr & Jittanit, 2012).

Increases in electric-field strength will enhance OH treatment efficiency. Lee *et al.,* (2012) reported that as the electric-field strength increased from 25 to 40 V/cm, surviving populations of foodborne pathogens decreased more effectively for lower electric field strength, longer treatments were needed to achieve the same microorganism reduction (Sagong *et al.,* 2011; Baysal & Icier, 2010).

Not all microbial species are equally resistant to the OH treatment, and thus Onwnka *et al.,* (2008) found significant differences in bacterial survival according to the pathogen type, with *Salmonella* spp. being the most resistant one to a treatment at 100°C and *Clostridium perfringens* at 70°C, while *Escherichia coli* proved to be the least resistant, the total destruction of the pathogens being achieved at 20 min of treatment. Sagong *et al.,* reported in 2011 that *Escherichia coli* O157:H7 was more resistant than other species such as *Salmonella Typhimurium* or *Listeria monocytogenes* to a treatment at 10-20 V/cm for up to 540 s. On the other hand, significantly higher lethality for of *Alicyclobacillus acidoterrestris* spores treated with OH was achieved than for spores treated with conventional heating in orange juice (Baysal & Icier, 2010). However, other authors have reported that the type of thermal treatment applied did

not significantly affect the shelf life in terms of microbial counts (Leizerson & Shimoni, 2005b). Also it has been found that the significant parameter in the inactivation of microorganisms is the thermal effect, regardless of the kind of thermal treatment (ohmic or conventional) (Leizerson & Shimoni, 2005a).

Moreover, conditions such as pressure at 121°C in combination with OH have been used to raise the boiling point and thus reduce the viable *Bacillus subtilis* spores in orange juice four logarithmic orders in less than 1 s of treatment (Uemura & Isobe, 2003).

The type of electrode is also a factor to be considered. Onwuka & Ejikeme (2005) suggested that heavy electrolysis could occur with the use of both Cu/Cu and Cu/Al electrodes, resulting in reactions on the metals, which could enter the juice solution, thereby making the product unsafe to consume. They advise that the enhancement of juice extraction by OH should therefore use electrodes devoid of electrolysis, such as platinum coated with titanium.

2.3. Ultrasound treatment

Power ultrasound (US) may also be employed as an alternative processing option to conventional thermal approaches for pasteurization and sterilization of food products.

The propagation of high power US in a liquid induces bubble cavitation due to pressure changes. These resulting micro-bubbles collapse violently in the succeeding compression cycles of propagated ultrasonic waves. These results were found with the use of localized high temperatures and pressures, and high shearing effects. Consequently, the intense local energy and high pressure bring about a localized pasteurization effect without causing a significant rise in macro-temperature (Tiwari *et al.,* 2009c). Figure 48 shows a schematic diagram of an ultrasound exposure system for fruit juice.

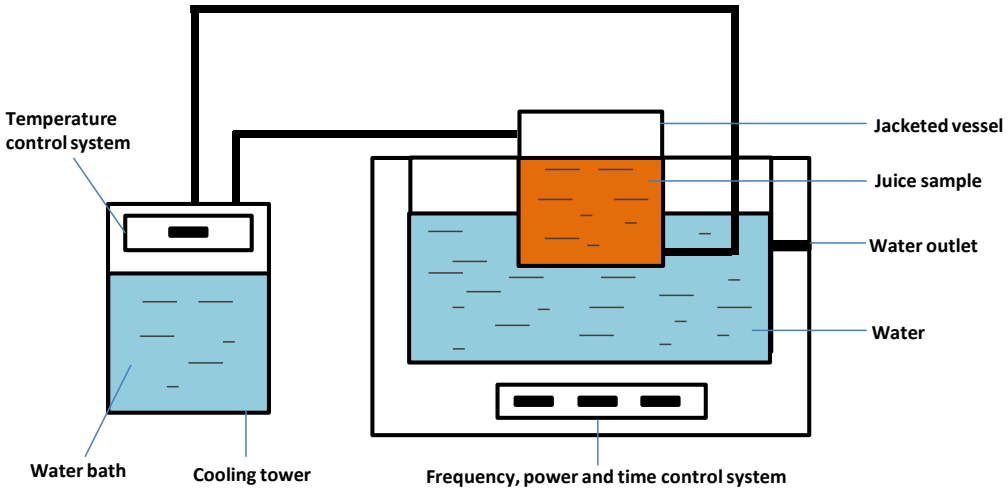

Figure 48. Schematic diagram of an ultrasound exposure system (Abid *et al.,* 2013).

It has been reported that viscosity of the juice influence the degree of cavitation (Patil *et al.,* 2009b), as the composition of each juice could provide some protective effect on the cells against the effect of cavitation. It has also been reported that grape juice showed the highest inactivation of *Saccharomyces cerevisiae*, followed by pineapple and cranberry juice (Bermúdez-Aguirre & Barbosa-Cánovas, 2012).

Other authors, modeling the inactivation kinetics of yeasts in tomato juice, found that parameters such as amplitude level and processing time had a significant effect on increasing the inactivation of yeasts, but the effect was relatively weak at lower amplitude levels and processing times (Adekunte *et al.,* 2010a). These authors also observed that yeast inactivation followed the Weibull model, as in the work of (Bermúdez-Aguirre & Barbosa-Cánovas, 2012), the yeast survival curves being concave downward. Further studies found an increase in temperature with a higher amplitude level (Adekunte *et al.,* 2010b). Other authors, increasing temperature to between 40°C and 60°C, achieved higher *Saccharomyces cerevisiae* inactivation rates during ultrasonic treatment at 24 kHz and 120 μm for 10 min. Moreover, a continuous mode was found to be more

effective in inactivating *Saccharomyces cerevisiae* than the pulsed mode (Bermúdez- Aguirre & Barbosa-Cánovas, 2012).

It has been stated that US treatment is more effective than the treatment alone for microbial inactivation when combined with moderate heat, as previously mentioned, and other conditions such as pressure (manosonication) (Arroyo *et al.,* 2011), mild heat and pressure (manothermosonication) (Palgan *et al.,* 2011a; Caminiti *et al.,* 2011a), osmosonication which combines US with non-thermal concentration (Wong *et al.,* 2010; Wong *et al.,* 2012), or antimicrobials (Ferrante *et al.,* 2007; Bevilacqua *et al.,* 2012). Nevertheless, other combinations such as sonication coupled with carbonation were found to be of little value in inactivating microorganism at room temperature (Cheng *et al.,* 2007).

The US-assisted process reportedly required a higher total energy input than conventional heating due to the high amount of electrical energy required for US generation (Zenker *et al.,* 2003). The choice of implementation of this technology should be based on the determination of whether the product quality improvements resulting from US-assisted thermal treatment may justify its increased energy requirement (Valero *et al.,* 2007). Although the possibility of deactivating micro-organisms by ultrasonic processing has been demonstrated under laboratory conditions, industrial adoption of this technology is limited, due to the significant challenges encountered in industrial scaling-up (O'Donnell *et al.,* 2010).

2.4. High-hydrostatic-pressure treatment

In the high-hydrostatic-pressure (HHP) process, the juice is subjected to pressures from 100 MPa to 900 MPa. The pressurization is applied for the duration of the treatment and then released. Pressure is applied isostatically, i.e. equally applied in all directions. The pressure applied and the holding time depends on the type of product treated and the expected final result. HHP has also been used for enzyme inactivation, but microbial inactivation generally requires lower pressures

than enzyme inactivation (San Martín *et al.,* 2002). Although HHP in intended to be a non thermal technique, an inherent mild increase in temperature may occur. The combined pressure and mild heat might improve the application of HPH treatment for liquid food pasteurization (Belloch *et al.,* 2012).

The efficacy for inactivating microorganisms in fruit juices is strongly affected by the juice matrix, with the concentration and pH being determinant factors (Wang *et al.,* 2012). The effectiveness of the treatment may change with the juice concentration (Basak *et al.,* 2002).

Some authors hold that differences in water availability explain the greater resistance of *Alicyclobacillus acidoterrestris* spores to high-pressure inactivation in the juice with high soluble-solid content (Sokolowska *et al.,* 2013a, Sokolowska *et al.,* 2013b). Other authors reported that during the 45 min pressurization (200 MPa, 50°C) of *Alicyclobacillus acidoterrestris* spores in concentrated apple juice (71.1°Brix) no significant changes were detected in their number. However, in the juices with a lower soluble-solid content of 35.7, 23.6, and 11.2°Brix, the reduction in spores was 1.3-2.4 log, 2.6-3.3 log, and 2.8-4.0 log, respectively (Lee *et al.,* 2006). Aerobic bacteria are more pressure resistant in a neutral system than in an acid system (Patterson. 2005, Zhao *et al.,* 2013).

In general, microorganisms showed pressure sensitivity that was more pronounced at higher pressure levels (Basak *et al.,* 2002). Research has revealed that the microbial-inactivation curve, at pressures of 120-400 MPa and temperatures ranging from -5 to 45°C showed a biphasic trend of pressure destruction, i.e. an initial rapid drop in the survival curves described by first-order kinetics followed by a long tailing (Erkmen. 2009, Reyns *et al.,* 2000). Erkmen attributes the tailing phenomena to a smaller portion of the microorganism population being more pressureresistant or adapted to the pressure stress that made the remaining cells more resistant.

Other authors, investigating the use of pulsed HHP in apple juice using pressure of 150 to 300 MPa, 25 to 50°C, 1 to 10 pulses of 60 s, concluded that the efficiency of pulsed HHP processes depends on the combination of the pulse holding time and number of pulses (Donsì *et al.,* 2010). They asserted that the efficacy of the single pulses diminishes with the greater the pulse number and pressure level, and therefore the first pulse cycle is more effective than the following ones. Other authors (Buzrul *et al.,* 2008) suggest that multiple pulses for a total holding time of 5 min at 300 MPa instead of continuous (single pulse) treatment had no significant effect on the microbial inactivation in kiwifruit juice; however, it did in pineapple juice for *Escherichia coli* and *Listeria innocua*. Similarly, other findings suggest that repeating the high-pressure treatment at least 4 times at 300 MPa for a total of 40 min would inactivate more *Escherichia coli* strains in tomato juice than using continuous pressure for the same amount of time (Bari *et al.,* 2007).

The effect of decompression has also been tested. The results found in the literature agree that faster compression rates have a greater impact on *Escherichia coli* inactivation in orange and apple juices treated at 0.1-600 MPa at 4 and 25°C than at slower compression rates (Abbas Syed *et al.,* 2013, Noma *et al.,* 2004). However, when it comes to *Bacillus subtilis* spores, slow compression combined with slow decompression had a greater impact on their inactivation than did any combination of fast compression and fast decompression at 600 MPa at 60°C and 70°C (Syed *et al.,* 2012).

The choice of an optimal temperature is crucial in the HHP process. Normally, a synergic effect between pressure and temperature is reported in literature (Lee *et al.,* 2006, Donsì *et al.,* 2010, Briñez *et al.,* 2006, Suárez-Jacobo *et al.,* 2010, Mert *et al.,* 2013). It bears noting that when moderate heating is coupled with high pressure, thermal degradation of the products may be detected, and therefore the optimization of the process conditions results in a compromise between the reduction of the pressure value, due to the synergetic temperature action, and the

achievement of quality of the final production (Donsì *et al.,* 2010). Conversely, Buzrul *et al.,* 2008) reported that using low (0°C) or sub-zero (−10°C) temperatures instead of an ambient temperature (20°C) during pressurization did not change the effectiveness of HHP treatment on *Escherichia coli* or *Listeria innocua* in kiwifruit and pineapple juices treated at 300 MPa for 5 min. Other authors, instead, reported higher *Escherichia coli* inactivation at 4°C than at 25°C in orange and apple juices treated at 0.1-250 MPa for 20 min (Noma *et al.,* 2004). Different processing conditions, matrix, as well as differences in the equipment may explain these differences (Suárez-Jacobo *et al.,* 2010, Vachon *et al.,* 2002). It is also worth noting that the temperature might rise due to the HHP treatment. Carreño *et al.,* (2011) worked at 200 MPa at 30°C for 10 s, achieving a final temperature of 45°C and at 400 MPa at 45°C for 1 min, achieving a final temperature of 60 °C. Likewise, it has been reported a surge in temperature at 600 MPa, from 25 to 43°C, which is calculated as 3°C/100 MPa (Cao *et al.,* 2012).

The type of microorganism to be inactivated strongly affects the efficacy of the process (Briñez *et al.,* 2006), determining the optimum combination of pressure, time, and temperature. For example, all *Salmonella* serovars tested in orange juice treated at 550 MPa for 2 min at 6°C and held for 24 h showed a >5-log decrease, while *Escherichia coli* O157:H7 strains required greater pressure, a higher temperature, longer pressurization, or a chemical additive to achieve a 5-log decrease (Whitney *et al.,* 2007). Similarly, *Saccharomyces cerevisiae* was more resistant than *Escherichia coli* and *Listeria innocua* when orange juice was processed in the high-pressure range of 103 to 241 MPa. However, at pressures higher than 241 Mpa, a rapid inactivation was observed for the three types of microorganisms (Guerrero-Beltran *et al.,* 2011).

There is abundant research assessing the performance of HHP regarding microbial inactivation. For example, Sokolowska *et al.,* (2012, 2013a) achieved a 3.5-log reduction of *Saccharomyces cerevisiae* NCFB 3191 using high hydrostatic pressure of 300 MPa at 20°C with a holding time of

0, 1, 5, and 10 min in beet juice, the inoculum being 5.4 log CFU/mL. Total inactivation (5 and more than 7 log for red and white grape juices, respectively) were observed at 250 MPa, 40°C for 10 and 15 min and 200 MPa, 40°C for 5, 10, and 15 min for red and white grape juices, respectively (Mert *et al.,* 2013). Moreover, reduced microbial counts have been maintained under storage at 4°C up for 22 days (Patterson *et al.,* 2012), for more than 35 days (Varela-Santos *et al.,* 2012), and at room temperature up to 90 days (Mert *et al.,* 2013).

Other combinations such as added dissolved CO_2 (Wang *et al.,* 2012) have been reported to inactivate bacteria effectively in high-acid fruit juice. Other authors have added chitosan, (Kumar *et al.,* 2009), nisin (Zhao *et al.,* 2013) or essential oils (Espina *et al.,* 2013) which act synergistically with the pressure to give higher microbial inactivation.

2.5. Inert-gas treatment

2.5.1. Supercritical carbon dioxide

Supercritical carbon dioxide (SC-CO_2) inactivation technology represents a non-thermal processing method for the inactivation of microorganisms. The mechanism of microbial inactivation by SC-CO_2 has not yet been fully elucidated. SC-CO_2 combines the solvent capacity of liquids with the mobility of gases. This is because, in supercritical state (31.1°C; 7.38 MPa), the density and viscosity of CO_2 lie midway between a gas and a liquid (Berna *et al.,* 2000), so it may quickly penetrate complex structures and porous materials. Several theories explaining the inactivation mechanism of SC-CO_2 involve the diffusion of CO_2 into the cells (Ortuño *et al.,* 2012, Ortuño *et al.,* 2013). SC-CO_2 is reported to have significant lethal effects on microorganisms in liquid foods (Park *et al.,* 2002).

The most important parameter in SC-CO_2 is the adequate combination of pressure and temperature. Liao *et al.,* (2008) concluded that when using SC-CO_2 at 32°C and 20 MPa, 75 min were required to reduce the population of *Escherichia coli* by a 5-log reduction, whereas 42°C

and 30 MPa were needed to achieve a reduction of 7 log in the same time. Other authors achieved 4.7 log reduction of *Saccharomyces cerevisiae* at 10 MPa at 36°C in apple juice (Spilimbergo *et al.,* 2007), and also in peach and kiwi juices (Spilimbergo & Ciola, 2010). Researchers have suggested that as the CO_2-to-juice concentration (0, 85, and 170 g/kg), temperature (25 and 35°C), and pressure (6.9, 27.6, and 48.3 MPa) increase, the yeastinactivation rate increases, and they have also reported that CO_2 was more effective in the supercritical state than in the subcritical state for inactivating yeast (Gunes *et al.,* 2005). In subsequent studies, the same authors suggested that *Escherichia coli* was very sensitive to dense CO_2 treatment, with a more than 6-log reduction in treatments containing 70 and 140 g/kg CO_2, irrespective of temperature and pressure. The CO_2:product ratio was the most important factor affecting the inactivation rate of *Escherichia coli*. No effect of temperature or pressure was detected, given the high sensitivity of the cells to dense CO_2 (Gunes *et al.,* 2006).

Other combinations such as PEF (Spilimbergo *et al.,* 2003) or USs (Ortuño *et al.,* 2012, Ortuño *et al.* 2013) have been shown to have additive or synergistic effects on SC-CO_2 microbial inactivation, reducing the SC-CO_2 processing requirements (time, temperature, and pressure).

2.5.2. Ozonation

The FDA approval of ozone as a direct food additive for the treatment, storage, and processing of foods in 2001 (Khadre *et al.,* 2001) has resulted in food scientists and the juice-processing industries employing ozone for pasteurization of fruit juices (Cullen *et al.,* 2010).

Ozone is a powerful broad-spectrum agent active against bacteria and fungi, and their spores, as well as viruses and protozoa. The wide antimicrobial spectrum, combined with a high oxidation potential make it an attractive processing option for the food industry. Ozone processing within the food industry has been used for fresh fruits and vegetables either by gaseous treatment or washing with ozonated water (Tiwari *et al.,* 2009b, Tiwari *et al.,* 2009c, Tiwari *et al.,* 2009d, Tiwari

et al., 2009e). However, liquid phases are most frequently ozonated by injecting ozone gas (mixtures of air/ozone or oxygen/ozone) through a sparger into a liquid. Figure 49 shows the schematics of ozone processing equipment for fruit juice. Residual ozone is the concentration of ozone that may be detected in the medium after application to the target surface. Both the instability of ozone under certain conditions and the presence of ozone-consuming materials affect the level of residual ozone available in the medium. Therefore, it is important to distinguish between the concentration of applied ozone and residual ozone necessary for effective disinfection (Cullen *et al.,* 2010).

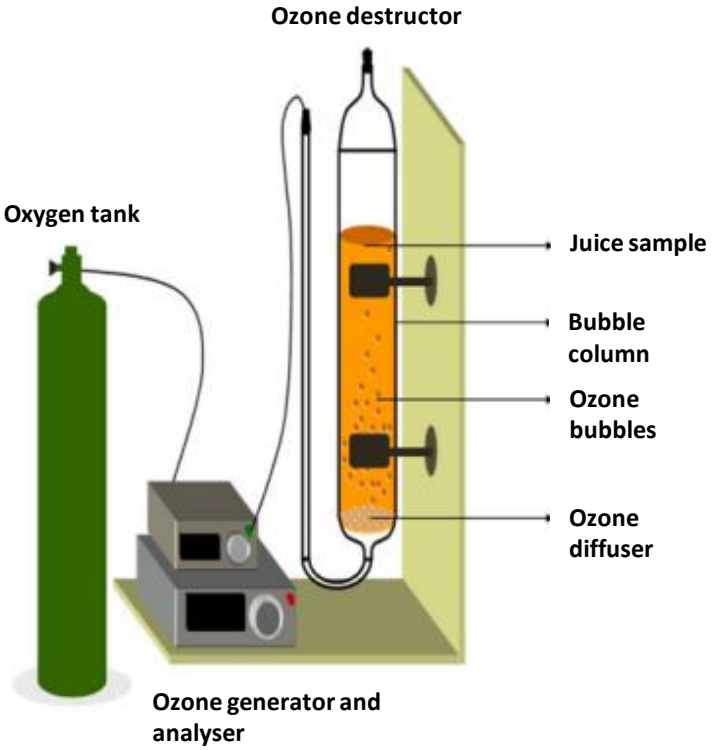

Figure 49. Schematics of ozone processing equipment (Patil *et al.,* 2011).

Reviewed modeling approaches employed for describing the kinetics of microbial inactivation include non-linearities. These non-linearities could include shoulder and tailing effects of the microbial kinetics, the Weibull model frequently being adopted (Choi *et al.,* 2012, Patil *et al.,*

2010). The formation of a shoulder or a tailing effect on the microbial kinetics during ozone treatment could also be due to rapid reactivity between ozone and microorganisms, and environmental factors, such as the presence of organic material (Restaino *et al.,* 1995, Hunt and Mariñas. 1997). Patil *et al.,* (2011) developed a new model that described the growth of a *Saccharomyces cerevisiae* population in unprocessed and ozone-processed apple juice under dynamic conditions that simulated a storage-temperature abuse. At the lower temperatures (4 and 8°C), the longer lag phase indicates that the yeast population needed a longer time to adapt to the environment. However, at higher storage temperatures (12 and 16°C), this effect was not evident, indicating the ability of yeasts to grow at these temperatures with a reduced or seemingly absent lag time.

Process parameters that highly influence the efficacy of ozone treatment include flow rate, ozone concentration, and temperature. At high flow rates a small number of large bubbles are produced, which rise to the liquid surface quickly and result in poor gas dissolution. These large bubbles escape the medium quickly, thereby reducing the contact time, leading to a lower inactivation rate. At low flow rates, small bubbles are produced, but, as the amount of ozone applied is low, inactivation is slow (Cullen *et al.,* 2010). Patil *et al.,* (2009c) explored the effect of the ozone flow rate on inactivation of *Escherichia coli* in liquid food, regardless of the lag time; after 25 min, complete inactivation was achieved using flow rates ranging from 0.03-0.5 L/min. However, complete inactivation was not achieved with flow rates of 0.3 and 0.75 L/min after a 30-min treatment. The optimum flow rate was 0.12 L/min, being the time required to achieve a 5-log reduction of 20 min.

Effect of ozone concentration on *Escherichia coli* ATCC 25922 has also been explored. Concentrations ranging from 17 to 75 µg/mL for a flow rate of 0.12 L/min, and from 28 to 120 µg/mL in the case of 0.6 L/min were assessed. They reported that for both flow rates, the highest

concentration was the most effective to inactivate *Escherichia coli* (Patil *et al.,* 2009a, Patil *et al.,* 2009b, Patil *et al.,* 2009c).

Same authors have assayed the effect of temperature on the efficacy of ozone inactivation *Escherichia coli* ATCC 25922 at an optimum inactivation flow rate of 0.12 L/min. Four different temperatures have been investigated: ambient temperature (12–15°C), 20, 25, and 30°C, ambient temperature giving the best inactivation levels (Patil *et al.,* 2009c). Williams *et al.,* reported that in orange-juice samples, *Salmonella* and *Escherichia coli* O157:H7 populations were undetectable after 15 and 75 min of ozonation process at 50°C, respectively. Ozonation at 4°C reduced *Salmonella* and *Escherichia coli* O157:H7 by4.2 log CFU/mL and 4.8 log CFU/mL in orange juice, respectively. Treatment at ambient temperature resulted in population reductions of less than 5.0 log CFU/ml (Williams *et al.,* 2004). There is no consensus on the effect that temperature exerts on the biocidal efficacy of ozone. For example, a drop in the temperature of the aqueous medium increases ozone solubility and stability, augmenting its availability in the medium and consequently boosting its efficacy. The simultaneous contribution of these two factors (solubility/stability and reactivity) to ozone efficacy may vary with experimental conditions, making it difficult to predict the influence of temperature on a particular application (Cullen *et al.,* 2010, Pascual *et al.,* 2007).

The effect of ozone in combination with dimethyl dicarbonate and hydrogen peroxide for orangejuice preservation has been explored by (Williams *et al.,* 2005). These researchers reported that a 5-log reduction of *Escherichia coli* O157:H7 was achieved using ozone in combination with mentioned antimicrobials followed by refrigerated storage.

Ozone has also been used routinely for washing and storing fruits and vegetables (Karaca & Velioglu, 2007, Wu *et al.,* 2013, Bataller *et al.,* 2012). Technologies that use ozonated water could result in an improvement of the quality characteristics of intact fruits by aiming for example

at reducing fruit damage, excessive softening as well as the decontamination of product surfaces, resulting in higher quality juices (Rodoni *et al.,* 2010).

3. Enzymatic inactivation

The use of enzymes in the food industry has expanded significantly, generating more added value to the final product and production rise while lowering costs. The juice industry has been using enzymes, especially in extraction for yield increase, juice clarification, filtration and stability (Ribeiro *et al.,* 2010).

Pectinases were among the first enzymes used for processing juices. Studies suggest that the use of these enzymes, whether isolated or associated with other enzymes may enhance yield and help in the clarification of a wide range of juices, as they are able to degrade pectin. Although there are several types of pectinases, the most widely studied are polygalacturonases and pectin methyl esterases. Polygalacturonases catalyze the hydrolysis of glycosidic linkages in polygalacturonic acid, producing D-galacturonate, while pectin methyl esterases catalyze deesterification of the methoxyl group of pectin, forming pectic acid and methanol (Pedrolli *et al.,* 2009). These enzymes are normally present in plant tissues. However, the technological effect produced by these endogenous enzymes, is reportedly unsubstantial, and exogenous pectinases are added to produce the desired technological effect (Mahfuzur Rahman & Rakshit, 2004).

Other enzymes of interest in fruit-juice processing naturally present in fruits are polyphenoloxidases, peroxidases, lipooxygenases and ascorbate peroxidases. Polyphenoloxidase oxidizes *o*-diphenols into *o*-quinones, which condense, via a non-enzymatic pathway, with amino acids, proteins or other compounds to form brown pigments (Coseteng & Lee, 1987). These browning products decrease both the acceptability and nutritional quality of the fresh juice (de la Rosa *et al.,* 2011). Peroxidase may oxidize not only various substrates in the presence of hydrogen peroxide, but can also produce reactive oxygen species (Mohamed *et al.,*

2011, Falguera *et al.,* 2012), which are found in most raw and unblanched fruit and vegetables, and are associated with the development of off-flavors and browning pigments (O'Donnell *et al.,* 2010). Lipoxygenase activity in fruit and fruit products is reported to be related to oxidation of fatty acids and pigments. Lipoxygenase catalyzes the oxidation of polyunsaturated fatty acids containing a cis, cis-1, 4-pentadiene system, which produces 9- or 13-cis, trans-hydroperoxides. Lipooxigenase has been associated with quality deterioration because of its negative effects on pigments such as carotenes during storage, and its role in off-flavor and odor production (Aguiló-Aguayo *et al.,* 2008, O'Donnell *et al.,* 2010, King and Klein. 1987). Ascorbate peroxidase detoxifies peroxides such as hydrogen peroxide using ascorbate as a substrate (Raven, 2000). This would lead to a detriment of ascorbate in fruit juices, lowering their nutritional quality.

Therefore, inactivation of these and other enzymes is required during fruit-juice processing to produce high-quality products. Heat treatment of fruits can be critical in decreasing the activity of these enzymes. However, high-temperature heating may also degrade other important components (Threlfall *et al.,* 2005) and trigger chemical reactions that lower the quality of juices (Garde-Cerdán *et al.,* 2007). For this reason, numerous alternatives to heat treatments have been developed over the years, including UV light, γ-irradiation, pulsed electric fields, ohmic heating, ultrasound, and high hydrostatic pressure, which are reviewed in this section. Table 13 gathers the results achieved during the last decade in terms of enzymatic inactivation in fruit juices.

Technique	Scale	Enzymes inactivated	Parameters assayed	Combinations with enhanced efficiency	References
UV light	Laboratory	Pectin methyl esterase Polyphenoloxidase Peroxidase	UV dose: $3.88 \cdot 10^{-7}$ E/min Treatment length: 15-100 min	-	Manzocco *et al,.* 2009; Oteiza *et al.,* 2005; Tran & Farid, 2004; Koutchma *et al.,* 2004; Koutchma *et al.,* 2006; Char *et al.,* 2010; Oteiza *et al.,* 2010;
γ-irradiation	Laboratory	Polyphenoloxidase Peroxidase Lipooxigenase	Irradiation dose: 1-5 kGy Irradiation rate. 0.5 kGy/h	-	Kim *et al.,* 2007; Wang *et al.,* 2006
PEF	Laboratory	Pectin methyl esterase Polygalacturonase Polyphenoloxidase	Electric field: 35-80 kV/cm Pulse rise time: 1-7 µs Treatment length: 59-100 µs Bipolar mode	-	Aguiló-Aguayo *et al.,* 2010; Elez-Martínez *et al.,* 2006; Nguyen & Mittal, 2007; Irwe & Olsson, 1994; Yeom *et al.,* 2000
OH	Laboratory	Pectin methyl esterase Polyphenoloxidase Peroxidase	Electric field: 20-40 kV/cm Treatment length. 0.63 s-120 min Temperature: 54-150°C	-	Jakób *et al.,* 2010; Demirdöven & Baysal, 2012; Icier *et al.,* 2006; Içier *et al.,* 2008; Leizerson & Shimoni, 2005a; Leizerson & Shimoni, 2005b; Castro *et al.,* 2004; Yildiz *et al.,* 2009
US	Laboratory	Pectin methyl esterase Polygalacturonase Polyphenoloxidase Peroxidase Ascorbate peroxidase	Wave amplitude: 65-75 µm Frequency: 19-20 kHz Acoustic energy density: 0.42-376 W/cm^2 Treatment length: 2-10 min	-	Tiwari *et al.,* 2009g; Terefe *et al.,* 2009, Fonteles *et al.,* 2012; Costa *et al.,* 2013
HHP	Laboratory	Pectin methyl esterase Polygalacturonase Polyphenoloxidase	Pressure: 300-800 MPa Treatment length: 5-10 min	High pressure carbon dioxide	Jolie *et al.,* 2009; Stoforos *et al.,* 2002; Hsu, 2008; McKay *et al.,* 2011

Table 13. Alternative technologies to conventional thermal treatments in fruit-juice processing for enzymatic inactivation: summary.

3.1. Radiation treatment

In this section, UV light and γ-irradiation are reviewed. It is generally accepted that the enzymes in raw food materials and non-thermally treated foods are stable against low-dose radiation. Nevertheless, there few works examine UV influence on some enzymatic activities. Falguera *et al.,* (2011) reported that pectin methyl esterase was inactivated after 40 min of irradiation at an incident energy of 3.88·10−7 E/min, while polyphenoloxidase was completely inactivated after 100 min of treatment, and peroxidase after only 15 min, regardless its initial activities. High intensity treatments, being either in the visible or UV-C spectral ranges, have been associated with non-reversible structural changes which result in enzyme inactivation (Manzocco *et al.,* 2009).

Regarding γ-irradiation, it has been reported that in kale juice a dose of 5 kGy did not affect polyphenoloxidase (Kim *et al.,* 2007). By contrast, other authors achieved 53.34%, 34.31%, and 45.54% decreases in peroxidase, polyphenoloxidase, and lipooxygenase activities, respectively, at 1 kGy, and 56, 32, and 85%, respectively, at 5 kGy in cantaloupe juice. Enzymeactivity determination indicated that lipooxygenase was the easiest one to be inactivated, followed by polyphenoloxidase and peroxidase, but the 3 enzymes still remained active even at 5 kGy. These authors observed that at doses higher than 1 kGy a slight irradiation off-odor was produced, and a strong off-odor at 2 kGy and higher. Therefore, they indicate that γ-irradiation may not completely inactivate the enzymes on the premise of acceptable off-odor (Wang *et al.,* 2006).

3.2. Electric treatment

3.2.1. Pulsed electric fields (PEF)

PEF has been also used to inactivate enzymes. Some authors have achieved residual activity of pectolitic enzymes involved in viscosity changes of watermelon juices processed at pulse frequencies from 50 to 250 Hz and pulse widths ranging from 1.0 to 7.0 μs in monopolar or

bipolar mode (Aguiló-Aguayo *et al.,* 2010). When PEF treatment was compared to conventional heat treatments, a decrease in pectin methyl esterase activity of 88% was achieved in orange juice with a PEF treatment at 35 kV/cm for 59 µs while heat pasteurization at 94.6 °C for 30 s inactivated 98% of pectin methyl esterase activity, which was not restored during storage at 4 and 22°C for 112 days, in both cases (Yeom *et al.,* 2000). According to Elez-Martínez *et al.,* (2006), 100% inactivation of the pectin methyl esterase when processing orange juice at 90°C for 1 min was reached while PEF treatment inactivated 81.6% activity in the juice without exceeding 40°C. These researchers suggest that enzyme inactivation was probably due entirely to the PEF treatment itself, as temperature was not high enough to inactivate pectin methyl esterase. Similarly, PEF treatment at 80 kV/cm 2 µs reduced pectin methyl esterase activity up to 55% in tomato juice (Nguyen & Mittal, 2007). Given that a 90-100% reduction of the pectin methyl esterase activity is normal in commercial heat-pasteurized orange juice (Yeom *et al.,* 2000, Irwe & Olsson, 1994), a better optimization of the variables or a complementary heat treatment would be necessary in cases that did not achieve this reduction. However, PEF treatment at 80 kV/cm 2 µs did not affect polygalacturonase activity, while conventional heating to 50°C its activity was reduced significantly (Nguyen & Mittal, 2007).

Bi *et al.,* (2013) analyzed the effectiveness of PEF as a method of inactivating enzymes to prevent enzymatic discoloration. It was concluded that with increasing the electric-field strength and pulse rise time, the residual activity of polyphenoloxidase and peroxidase decreased, almost completely inactivating both enzymes at 35 kV/cm-electric-field strength and 2 µ-pulse rise time. Other authors found the greatest decrease in enzymatic activity by using a combination of preheating to 50°C, and a PEF treatment time of 100 µs at 40 kV/cm, this being significantly higher than that recorded in juice processed by conventional mild pasteurization (Riener *et al.,* 2008).

3.2.2. Ohmic heating (OH)

OH proved to be promising in enzyme-inactivation experiments. Some experiments have achieved additional inactivation of food enzymes with OH when compared to conventional heating in juices and other foods (Icier *et al.,* 2006, Demirdöven and Baysal. 2009, Jakób *et al.,* 2010). Leizerson and Shimoni (2005a, 2005b) found no significant effect for the type of thermal treatment on pectin methyl esterase inactivation in orange juice. According to other authors, pectinase in lemon juice was inactivated with a very short time length, 0.63 sec at 75°C (Inoue *et al.,* 2007). The effects of voltage gradient, temperature, and holding time on the polyphenoloxidase activity were investigated for grape juice, and it was found that the critical deactivation temperatures were 60°C or lower for 40 V/cm, and 70°C for 20 and 30 V/cm (Içier *et al.,* 2008). In other experiments, OH heating of polyphenoloxidase caused their inactivation to be faster than during conventional heating (Castro *et al.,* 2004). Moreover, OH has been attributed to cause less browning than conventional heating (Leizerson & Shimoni, 2005a; Leizerson & Shimoni, 2005b; Jakób *et al.,* 2010; Yildiz *et al.,* 2009).

3.3. Ultrasound (US) treatment

Studies on US treatment for enzyme inactivation in fruit juices include Tiwari *et al.,* (2009a, 2009b), who assayed different ultrasonic acoustic energy-density levels of 0.42, 0.47, 0.61, 0.79, and 1.05 W/mL with treatment times of 0, 2, 4, 6, 8, and 10 min, concluding that ultrasonic acoustic energy density decreased pectin methyl esterase activity in orange juice by 62% at the highest level. Results indicate that sonication alone cannot completely inactivate pectin methyl esterase under the experimental conditions employed. However, it has been reported that the particle suspension stability of juice depends not only on pectin methyl esterase activity but also on alterations of the pectin. Changes in chord-length distributions as a function of US intensity have been reported, this size reduction contributing to improved cloudiness stability (Tiwari *et al.,*

2009a). The sonication in combination with heat (thermosonication) has been reported to inactivate pectin methyl esterase following first-order kinetics (Terefe *et al.,* 2009). The results of these authors showed that thermosonication (20 kHz, 75 μm) processing of tomato juice at 75°C caused almost complete inactivation of pectin methyl esterase and about 72% inactivation of polygalaturonase after 4 min. These researchers asserted that under these conditions, the residual polygalacturonase activity is unlikely to cause substantial pectin degradation as the preferred substrate for polygalacturonase is demethylated pectin, which would be less available because pectin methyl esterase is almost completely inactivated.

The minimal residual activity (23.29%) resulted from the most drastic treatment (376 W/cm2 for 10 min), achieving 23% residual activity for peroxidase, 70% for polyphenoloxidase and 2% for ascorbate peroxidase, being a function of US power intensity and processing time (Fonteles *et al.,* 2012), which is consistent with the results of Costa *et al.,* (2013).

3.4. High-hydrostatic-pressure treatment

HHP has also been used for enzymatic inactivation; however, less information is available than for microbial inactivation. Concerning pectin methyl esterase inactivation, the literature reports that it is rather heat-labile but pressure-stable (Jolie *et al.,* 2009). More concretely, Stoforos *et al.,* explored the inactivation of endogenous pectin methyl esterase in tomato juice during combined HHP (ambient to 800 MPa) and moderate-temperature (60 to 75°C) treatments under isobaric and isothermal processing conditions. Their findings suggest that pressure and temperature did not act synergistically with respect to tomato pectin methyl esterase inactivation, in the whole range of pressure and temperature investigated, but they had mainly counteracting effects. Consequently, in the cases where a certain level of residual pectin methyl esterase inactivation activity is desired, high pressure could be used to inactivate other, undesired, enzymes while reserving this one, leading to a desired viscosity/texture, in some products (Stoforos *et al.,* 2002).

This agrees with Hsu *et al.,* 2008), who reported pectin methyl esterase inactivation reduction of 27.8% using the treatment of 200 MPa at 25°C, 10 min, this being the most efficient when compared to the cases of higher-pressure treatments (beyond 300 MPa) at all temperatures in this study (4, 25 and 50°C).Therefore the highest efficiency was found with low-pressure/mild-temperature treatments. This fact has been attributed to reversible configuration and/or conformation changes of the enzyme and/or substrate molecules (Hsu *et al.,* 2008, Ogawa *et al.,* 1999).

Concerning polygalacturonase activity, pressures beyond 400 MPa at ambient and low temperatures (25 and 4°C) strongly reduce its activity up to 90% while pressure from 100 to 300 MPa has slight or insignificant effects on the inactivation of polygalacturonase (up to 14%), demonstrating a certain pressure resistance of polygalacturonase (Hsu *et al.,* 2008).

Regarding polyphenoloxidase, studies in cloudy apple juice performed by Buckow *et al.,* (2009) showed synergistic effects of pressure and temperature on the inactivation of apple polyphenoloxidase at pressures above 300 MPa and antagonistic effects at lower pressures. Compared to ambient pressure conditions, temperatures required to inactivate polyphenoloxidase in apple juice were increased 10-15°C at 100-300 MPa. Other authors did not inactivate polyphenoloxidase with treatment for 1 min at 500 and 600 MPa at 20°C in apple juices, which turned brown during storage (McKay *et al.,* 2011).

4. Yield improvement

Since juice extraction is a slow, laborious and highly energy-consuming step in fruit-juice production, various methods have been tested to improve efficiency and augment yield (Wang & Sastry, 2002). Although enzymatic inactivation would also boost juice yield, there are some other methods that directly offer improvements such as pulsed electric fields, ohmic heating, microwave heating, ultrasound, and flash-vacuum expansion, which are reviewed in this section.

4.1. Electric treatment

4.1.1. Pulsed electric fields

Efficiency of hydraulic pressing can be increased by raw material plasmolysis, cellular damage or permeabilization prior to expression. This can be achieved by combined pulsed-electric-field application and pressure, found to be useful as a pretreatment to enhance juice expression and solute extraction (Flaumenbaum, 1968; Jemai & Vorobiev, 2002). Pressure damages cells exposed to PEF, which have developed pores in the cell membrane, allowing diffusion migration of moisture and depressing the cell-resealing processes, thereby increasing juice yield (Grimi *et al.,* 2009, Schilling *et al.,* 2007). The effectiveness of the PEF treatment depends on the uniform and tight packing of raw material between electrodes. The excessive quantity of extraparticle high-conductive liquid (from cells destroyed by cutting) increases electrical energy losses because of great current flow through the system. Moreover, the low values of external moisture content also may restrict the effect of the PEF because of the absence of contact between solid particles. Thus, we would expect greater PEF treatment efficiency after pre-compression of the raw material and removal of excess liquid from the extracellular volume at the initial steps of compression. PEF application at the moment when the press-cake's specific electrical conductivity reaches a minimum and the pressure achieves its constant value seems to be the most optimal (Bazhal *et al.,* 2001).

This technology has been previously used on a laboratory scale, with improvements in juice yields in apple juice production after adding ascorbic acid to prevent oxidation (Schilling *et al.,* 2007). Similarly, other authors augmented juice yield from 49-54% to 76-78% at 45 min of pressing in grapes, the energy input on the order of 20 kJ/kg at electric-field strength 750 V/cm being sufficient for optimum PEF-assisted expression. These authors also found that PEF pretreatment application before pressurization exerted the most pronounced effects on the

expression kinetics and on the juice yield and quality, though it was accompanied by the highest electric-energy consumption (Praporscic *et al.,* 2007).

When PEF are combined with heating at moderate temperatures (~40° C), juice extraction from apples is notably enhanced (Schilling *et al.,* 2008, Lebovka *et al.,* 2004), resulting in noticeable softening of the tissue, resulting in more damage of apple tissue than with the PEF treatment alone.

In further studies, same authors (Praporscic *et al.,* 2007) tested the effect of the size of slices of apples and carrots on juice yield. They reached the conclusion that PEF treatment at moderate electric field strength (250–400 V/cm) resulted in more pronounced additional expression of juice for the larger slices. However, at the end of the treatment at 1000s, little difference was detected between small and large slices. Similarly, (Turk *et al.,* 2010) found scant difference between small and large mash using 10 pulses delivered continuously at the electric field strengths of 450 V/cm. Other authors (Grimi *et al.,* 2011) compared the effect of PEF treatment on whole and sliced apples, sliced apples resulting in higher juice yield and faster extraction kinetics compared to whole ones.

PEF experiments have also been extrapolated to a continuous pilot-plant scale using a single-belt press, increasing cider and apple-juice yield by 4.1% (Turk *et al.,* 2012a) and by 5.2% on an industrial scale (Turk *et al.,* 2012b). In further studies, Jaeger *et al.,* made a systematic study on an industrial scale with various de-juicing systems, achieving substantial improvements in juice yield for apple, 77.5% (belt press) and 86.1% (pack press), and for carrots, up to 76% (belt press and filter press) (Jaeger *et al.,* 2012).

4.1.2. Ohmic heating (OH)

OH is believed to have an additional electopermeabilization effect leading to extraction improvement even at moderate temperatures that do not exceed 50°C (Wang and Sastry. 2002, Praporscic *et al.,* 2005). A 13.76% increase in juice recovery over control from carrot was demonstrated using 2-stage pressing with OH heating, achieving maximum juice recovery of 98.9% with a first pressing of 2.72 min., OH up to a final temperature of 65.6°C under a voltage gradient of 15 V/cm followed by a second pressing of 10 min (Ranmode & Kulshreshtha, 2011). Similarly, other authors achieved greater juice yield in ohmically treated samples than in non-treated, the former showing less mechanical resistance than the raw material. These researchers also studied the effect of frequency and concluded that lower frequency caused a greater effect on increasing moisture diffusion, which resulted in high juice yield (Wang & Sastry, 2002). This agrees with other authors (Lima & Sastry, 1999, Lima *et al.,* 1999), who also had greater yields at lower frequencies.

Moreover, satisfactory results were achieved when OH and PEF at electric field strength less than 100 V/cm were combined, promoting a high level of membrane destruction and mechanical softening of tissues even at a moderate temperature under 50°C of apples, potatoes, and sugar beets. Both treatments might have led to some synergetic effect, related to electroporation of cell membranes and thermal softening of tissue (Praporscic *et al.,* 2005, Praporscic *et al.,* 2006).

4.2. Microwave heating treatment

Microwave heating (MH) is caused by the ability of the materials to absorb microwave energy and convert it into heat. The presence of moisture or water causes dielectric heating due to the dipolar nature of water (Datta and Davidson. 2000). There are many factors which affect MH and its heat distribution and the most important of them are the dielectric properties and penetration depth (Chandrasekaran *et al.,* 2013).

Cendres *et al.,* (2011) implemented a system for extraction of fruit juice using a microwave heater with a multimode 2450 MHz microwave oven with a maximum delivered power of 1000 W at 10 W increments. These authors concluded that the process of microwave extraction of fruit juice could thus be divided in four stages. Stage 1 corresponds to the time necessary for heating the matrix from room temperature to the boiling point of water; it is also the time needed for the first fruit juice droplets to get outside the microwave cavity. Stage 2 corresponds to an intensive extraction of fruit juice at an increasing flow rate; it is also the phase of extraction of easily exchangeable water in the fruits. Stage 3 corresponds to a decrease in the flow rate of extraction, when the remaining water is more linked to plant structure and becomes increasingly difficult to extract. Finally, stage 4 is the end of extraction process, when the fruit is almost dry and the temperature increases rapidly due to the absence of water which regulated the temperature at its boiling point (100°C) in the preceding stages. They also observed different diffusion behavior during extraction according to the type of fruit used. The best yields were consistently obtained for grapes, followed by plums and apricots. These authors attribute this fact to the different availability of water in fruit as well as the thickness and texture of the layer of pulp, the viscosity of the juice, the porosity of the wall, etc. For all three fruits, increasing power densities from 0.5 W/g to 1.5 W/g markedly shortened the duration of extraction but also of the yields. Higher yields were obtained with initially frozen fruits, in which ice might interfere in the process by acting as a buffer, a power sink, as some heat is consumed for melting.

Other authors reported that the thermal effect of MH heating on increasing juice yield decreased with the pretreatment temperature from 40 to 50°C, which might be due to moisture loss at a higher temperature. Pretreated samples also showed less mechanical resistance than did the raw material, suggesting that less input work was required for expression, possibly due to a breakdown of cell membrane and wall constituents, not only increasing juice yield but also saving

energy. However, when it was compared to ohmic heating, less effect in increasing juice yield and increasing input work was found (Wang & Sastry, 2002).

Other researches developed a tunable microwave applicator for extracting date juice and similar products consisting of a microwave unit and a press/filter unit, showing good performance characteristics with power conversion efficiency of more than 70% (Ali, 2000).

4.3. Ultrasound treatment (US)

US treatment may also been used to enhance juice yield. However, there is little evidence to support this statement. Lieu & Le (2010) have assayed the effect of ultrasound together with temperature at 74 °C. They reached the conclusion that US treatment increased extraction yield 3.4% and shortened treatment time by more than three-fold; meanwhile, a combined US and enzyme treatment increased extraction yield only 2%, but shortened treatment time by more than four-fold. After US treatment, enzymatic treatment (0.05% pectinase) boosted extraction yield 7.3% and the total treatment time of this method was still shorter than that of the traditional enzymatic treatment method.

4.4. Flash-vacuum expansion treatment (FVE)

FVE is another technology developed to improve yield and also the nutritional quality of juice. In this technique, plant material at 60-90°C is rapidly placed in a vacuum chamber at 1-10 kPa absolute pressure. Figure 50 shows a FVE squeme. At this reduced pressure (high vacuum), the boiling point of water in the tissues is much lower than the temperature of the plant material (60-90°C). The plant material expands or disintegrates due to instantaneous evaporation of constituent water and forms micro-channels inside the tissues (Paranjpe *et al.,* 2012). Steam heating induces a thermal denaturation of endogenous oxidases, while the whole process,

performed with the absence of oxygen, prevents oxidation and subsequent browning of the products (Brat *et al.,* 2001b).

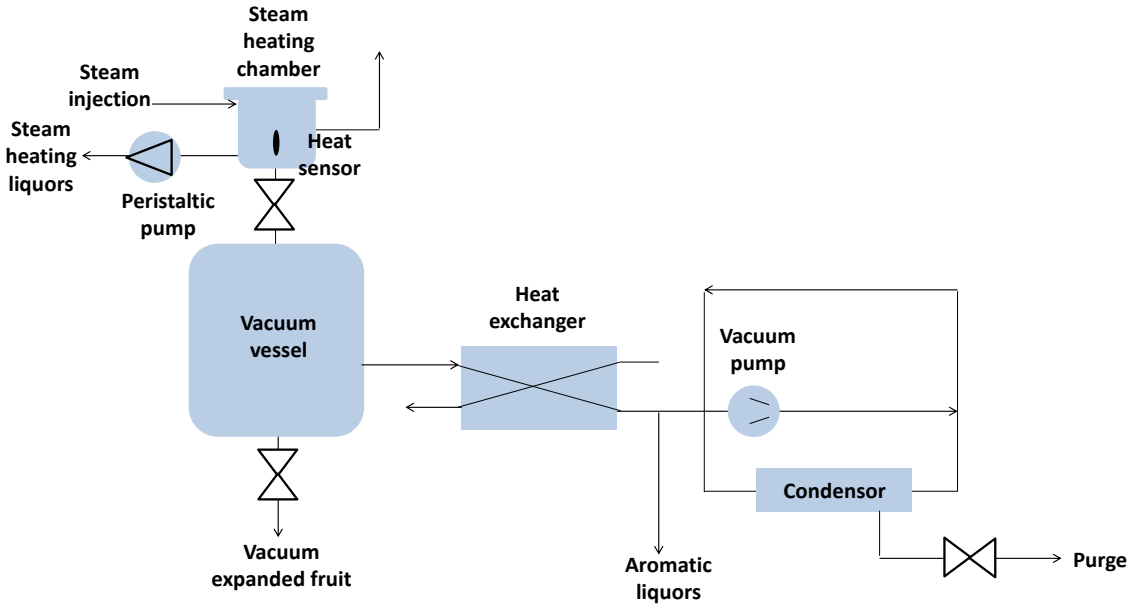

Figure 50. Flash-vacuum expansion scheme. Adapted from Brat *et al.,* 2002.

Pressure and temperature are significant factors in this procedure. Some researchers have assayed temperatures of 60, 75, and 90°C and pressure of the vacuum chamber of 1, 5 and 10 kPa absolute in extracting grapes. It is concluded that higher temperatures and lower pressures of flash-vacuum expansion improve juice yield with pressure being a more significant factor (Paranjpe *et al.,* 2012, Anonymous 2007).

Brat *et al.,* compared passion fruit puree obtained by flash-vacuum expansion with steamheated puree and cold-pressed juice, all passed through the same screen. It is stated that FVE gave about two-fold higher yield compared to the steam treatment and produced a significantly thicker puree, which is related to its content in de-starched alcohol-insoluble residue (Brat *et al.,* 2001b). Other studies have improved grape-juice yield by 5-20% as compared to heat treatment (Anonymous, 2007).

After vacuum-expansion, the puree is impoverished in volatile components because of instant evaporation of some water when steam-heated fruits are placed in the vacuum vessel. However, most of these volatiles can be recovered in aromatic liquors generated by the vacuum-expansion step, and they could be added back to the vacuum-expanded puree (Brat *et al.,* 2001a, 2001b).

FVE has also been used to recover essential oils from citrus peels, allowing yields comparable to the Food Machinery Corporation Process (Brat *et al.,* 2001a).

5. Conclusion

In this paper, non-thermal alternatives to conventional heating in fruit-juice processing are reviewed. The literature reveals that research is still underway, and many aspects remain to be fully studied and understood.

Ensuring food safety and at the same time meeting the demand for nutritious foods, has intensified interest in non-thermal preservation techniques. The non-thermal technologies discussed in this review have the potential to meet industry and consumer expectations. However, the lack of standardization in operating conditions makes comparisons between different studies difficult. Consequently ambiguity arises within the literature, as these control conditions may not be reported in detail or are reported differently. More complex considerations arise for combinations of technologies, particularly with respect to optimization of practical applications. In many cases, combination with mild heat has been used, which does not reach usual heating temperatures, and achieve a synergistic effect. A fundamental understanding of these phenomena is essential for optimum process design.

Thus, in order to advance in the juice industry, more studies need to be carried out in detail on the scaling-up, process design, and optimization, as well as the effect of such technologies in the

overall quality of fruit juices in order to maximize their potential as alternative non-thermal technologies in fruit juice processing.

6. Acknowledgements

This work was supported by the project AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as by P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science) projects. The authors are also grateful for the scholarship FPU AP2010-2793.

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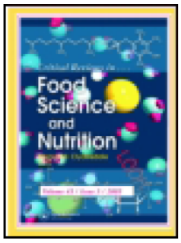
Chapter 6



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Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

To cite this article: Cecilia Jiménez-Sánchez, Jesús Lozano-Sánchez, Antonio Segura-Carretero & Alberto Fernández-Gutiérrez (2015): Alternatives to Conventional Thermal Treatments in Fruit-juice Processing. Part 2: Effect on Composition, Phytochemical Content, and Physicochemical, Rheological, and Organoleptic Properties of Fruit Juices, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2014.914019

To link to this article: http://dx.doi.org/10.1080/10408398.2014.914019

Review: Alternatives to conventional thermal treatments in fruit-juice processing. Part 2: Effect on composition, phytochemical content, and physicochemical, rheological, and organoleptic properties of fruit juices

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Abstract

Traditional thermal techniques may cause losses in nutritional quality and phytochemical contents, and also in physicochemical, rheological, and organoleptic properties of processed fruit juices. This article provides an overview of the effect on these quality by the use of alternatives to traditional thermal treatments in fruit-juice processing, for three key operations in fruit-juice production such as microbial inactivation, enzyme inactivation, and juice-yield improvement. These alternatives are UV light, high-intensity light pulses, γ-irradiation, pulsed electric fields, radiofrequency electric fields, ohmic heating, microwave heating, ultrasound, high hydrostatic

pressure, supercritical carbon dioxide, ozonation, and flash-vacuum expansion. Although alternatives to heat treatments seem to be less detrimental than the thermal treatment, there are many parameters and conditions that influence the output, as well as the nature of the juice itself, hampering comparisons between different studies. Additionally, future research should focus on understanding the mechanisms underlying the changes in the overall quality of fruit juices, and also on scaled-up processes, process design, and optimization that need to be deal with in detail to maximize their potential as alternative non-thermal technologies in fruit-juice processing while maintaining fruit-juice attributes to the maximum.

Keywords

Polyphenols, carotenoids, vitamin C, color, flavor, viscosity

2 ACCEPTED MANUSCRIPT

1. Introduction

Today, consumer habits are changing with increasing demands for fresh-like products. Thus, fruit-juice industries have directed their studies to the search for alternative processing technologies in order to produce foods with a minimum of changes induced by the technologies themselves (Velázquez-Estrada *et al.,* 2012).

It is well established that traditional thermal techniques can extend the shelf life of juices, ensure their safety, and also maximize performance in fruit-juice processing. However, they may cause losses in nutritional, physicochemical, rheological, and organoleptic parameters (Gómez *et al.,* 2011). Growing interest in a healthy diet and a better quality of life, together with a general knowledge linking phytochemical-containing fresh food and a protective role against chronic diseases such as coronary heart disease, cancer, and others (Mullen *et al.,* 2007) point out the importance of preserving nutritional and phytochemical quality of fruit juices. Similarly, physicochemical attributes of fruit juices such as pH, Brix grade, or cloudiness determine their stability and storage ability, highlighting the importance of producing a stable product able to meet the requirements of an increasingly demanding market. It is generally believed that food texture and rheology are major determinants of consumer acceptance and preference, and the rheological characterization of food is also important for the design of unit operations, process optimization, and high-quality product assurance (Ibarz, 2003; Rao. 1999). Finally, the preservation of organoleptic attributes is crucial, as products are designed to please a more and more discerning consumer palate.

To overcome these difficulties, the fruit-juice-processing industry over the years has introduced novel technologies to extend shelf life, ensure safety, improve quality, and appeal to consumer perception without any adverse effect or damage on quality parameters of the juice (Abid *et al.,* 2013). These technologies are “non-thermal” processing technologies intended to achieve similar

microbial and enzymatic inactivation, and yield enhancement with reduced or no application of heat (Noci *et al.,* 2008), and they have been under investigation to evaluate their potential as an alternative or as a complementary process to conventional thermal treatments (Heinz *et al.,* 2003; Stewart *et al.,* 2002). Consequently, an understanding of the effects that these alternative technologies, meant to replace heat treatments, exert on the quality attributes of juices becomes vital in order to design and optimize technological parameters in an effort to produce high-standard products.

In this context, the present work provides an overview of the impact of three key processes in fruit-juice production in fruit-juice preparation: microbial inactivation, enzyme inactivation, and juice-yield enhancement. A study is made these processes in relation to composition, phytochemical content and physicochemical, rheological and organoleptic properties of fruit juices, these being considered essential quality parameters. The technologies examined include UV light, high-intensity light pulses (HILP), γ-irradiation, pulsed electric fields (PEF), radiofrequency electric fields (RFEF), ohmic heating (OH), microwave heating (MH), ultrasound (US), high hydrostatic pressure (HHP), supercritical carbon dioxide (SC-CO_2), ozonation, and flash-vacuum expansion (FVE).

2. Effect of technological processing on the composition of fruit juices

In this section, carbohydrates, amino acids, proteins, lipids, and vitamins are reviewed.

2.1. Carbohydrates

Regarding radiation treatments, sugars have been reported to keep their original value constant during the time the UV experiment lasted when treating apple juices of different varieties at an incident energy of $3.88 \cdot 10^{-7}$ E/min (Falguera *et al.,* 2011).

Electrical treatments such as PEF have not been reported to affect the concentrations of glucose, fructose, or sucrose at different field strengths (Schilling *et al.,* 2007; Vervoort *et al.,* 2011). Similarly, the pectin contents of the juices were not diminished, amounting to 1.1 g galacturonic acid/L in apple juice extraction on the laboratory scale (Schilling *et al.,* 2007; Schilling *et al.,* 2008).

On the other hand, microwave treatments have led to an effective extraction of pectins (Cendres *et al.,* 2012) and also to an increased pectin solubilization (Igual *et al.,* 2010a).

Other types of treatment such as US have resulted in a significant increase in sucrose (53.60%) and glucose (4.24%) concentrations in cantaloupe juices, when compared to non-treated samples (Fernandes *et al.,* 2009). These results agree with previous researchers, who have reported that US promotes high extractability for sugars (Lieu & Le, 2010).

HHP processing has been reported to exert no effect on sugar composition (Vervoort *et al.,* 2011). Similarly, no significant differences were detected for glucose, fructose, or sucrose after ozone treatment of apple cider (Choi & Nielsen, 2005).

And finally, FVE has shown an effect on carbohydrates of fruit juices. Two carbohydrates fractions have been studied in FVE passion fruit puree: the destarched alcohol-insoluble residue and water-soluble polysaccharides. The former from the vacuum-expanded fruit puree was richer in cellulose and xylose-containing polysaccharides and poorer in uronic-acid-containing polysaccharides than that of reference and steam-heated puree. Other neutral sugars were present in similar proportions. The latter were mainly pectic substances with dominant proportions of galacturonic acid highly esterified with methanol, while the reference-fruit puree had a different composition with a lower level of galacturonic acid and much higher proportions of arabinose and galactose (Brat *et al.,* 2002).

2.2. Amino acids and proteins

Regarding electrical treatments, it has been reported that PEF treatment halved the protein content in grape juice (Marsellés-Fontanet *et al.,* 2013). Similarly, evidence suggests that microwave treatment could reduce the content of total amino-nitrogen in apple juices (Zhang *et al.,* 2010a). During US treatment, protein concentration has been found to be significantly affected by processing time and US intensity; however, higher protein content in treated juices than in non-treated ones might be attributable to protein release due to cell disruption by sonication. Conversely, upon increasing the US intensity and exposure time (above 7 min at intensities higher than 226 W/cm^2), a decrease in protein content was observed. This reaction may occur when the rate of protein loss surpasses the extraction rate (Costa *et al.,* 2013).

2.3. Vitamins

Fruits and vegetables are good sources of vitamin C (Zen *et al.,* 2002), which is the main vitamin found and therefore the vitamin reviewed in this section.

2.3.1. Radiation treatment

Vitamin C is a light-sensitive vitamin in fruit juices and can be degraded by UV treatment. Authors have reported that ascorbic acid degradation occurred more rapidly at higher UV dosages and the reaction accelerated with increasing exposure times (Tikekar *et al.,* 2011). Falguera *et al.,* 2011 reported a slight decrease during UV experiments with treatments up to 100 min applying an incident energy of $3.88 \cdot 10^{-7}$ E/min. UV pulsed light has been reported to cause a minor reduction in vitamin C, between 0.85-12.31% at 31-644 J/pulse, and 0.5 Hz (Orlowska *et al.,* 2012).

It has been stated that 5 kGy of γ-irradiation decreased the vitamin C percentage gradually to 62.5% in carrot-juice samples (Jo & Lee, 2012, Jo *et al.,* 2012). A dose-dependent decrease in vitamin C content has also been reported in previous studies (Song *et al.,* 2007; Jo *et al.,* 2012).

2.3.2. Electric treatment

The effects of PEF processing on content of vitamin C have been reported extensively in literature. Scientific evidence indicates high vitamin C retention after PEF processing in orange juice and gazpacho (Elez-Martínez & Martín-Belloso, 2007), tomato juice (Nguyen & Mittal, 2007), and orange-carrot (Torregrosa *et al.,* 2006), carrot (Akin & Evrendilek, 2009), and strawberry (Odriozola-Serrano *et al.,* 2009b), apple juice (Evrendilek *et al.,* 2000), and beverages containing fruit juice and milk (Salvia-Trujillo *et al.,* 2011). However, other findings suggest that vitamin C retention is reduced after the PEF treatment (Bi *et al.,* 2013; Oms-Oliu *et al.,* 2009), although there is no consensus regarding the effect of electric-field strength on this reduction. Under similar PEF conditions, differences in vitamin C retention among PEF-treated juices could be due to their different pH, since more acidic conditions are known to stabilize vitamin C (Tannenbaum *et al.,* 1985; Bi *et al.,* 2013; Oms-Oliu *et al.,* 2009). Several authors have compared the impact of PEF with the impact of thermal processing on vitamin C stability in different juices. Thus, thermal pasteurization is often found to be more detrimental than PEF pasteurization to vitamin C (Vervoort *et al.,* 2011; Elez-Martínez *et al.,* 2006; Elez-Martínez & Martín-Belloso, 2007; Yeom *et al.,* 2000; Min *et al.,* 2003; Zhang *et al.,* 2010b; Odriozola-Serrano *et al.,* 2008a; Odriozola-Serrano *et al.,* 2008b; Cortés *et al.,* 2008).

Regarding RFEF treatments, Geveke *et al.,* (2007) processed orange juice on a pilot-plant scale at 15 and 20 kV/cm at frequencies of 21, 30, and 40 kHz, and at an outlet temperature of 65°C with a holding time of 2 s, finding no loss in ascorbic acid due to RFEF processing, probably due to the short treatment time and low temperature.

Several authors have asserted that the type of heating (ohmic or conventional) had no significant effect on vitamin C degradation (Leizerson & Shimoni, 2005a; Leizerson & Shimoni, 2005b; Lima *et al.,* 1999; Tumpanuvatr & Jittanit, 2012). However, the vitamin C contents of ohmically heated juices were significantly lower than those of unheated juices. Similarly, Demirdöven & Baysal (2012) found minor differences between vitamin C contents of OH treated samples compared to conventional treatments. On the other hand, other authors have reported that the concentration of vitamin C in orange juice continuously ohmic-heated at 25-45 V/cm was significantly higher than in the conventionally heated juice (Lee *et al.,* 2012; Vikram *et al.,* 2005).

2.3.3. Microwave heating treatment

According to Vikram *et al.,* (2005), the degradation of vitamin C was highest during MH at 455W 180s due to uncontrolled temperature generated during processing compared to OH, infrared heating, and conventional heating. However, other authors reported that treatment at 900 W for 30 s preserved vitamin C (Igual *et al.,* 2010b).

2.3.4. Ultrasound (US) treatment

US processing of juices is reported to have minimal effects on the degradation of key quality parameters such as vitamin C in orange juice during storage at 10°C (Tiwari *et al.,* 2009a; Tiwari *et al.,* 2009b; Tiwari *et al.,* 2009d). Positive effect of US on vitamin C retention was also found by (Cheng *et al.,* 2007; Zenker *et al.,* 2003; Bhat *et al.,* 2001b; Zafra-Rojas *et al.,* 2013; Abid *et al.,* 2013). Some authors attribute this fact to the elimination of dissolved oxygen, which is essential for ascorbic acid degradation during cavitation, produced during sonication treatments (Bhat *et al.,* 2001b; Adekunte *et al.,* 2010a; Adekunte *et al.,* 2010b; Knorr *et al.,* 2004; Zafra-Rojas *et al.,* 2013). However, the ascorbic acid content significantly decreased in juice processed by sonication when compared to control (Santhirasegaram *et al.,* 2013), in agreement with other authors (Gómez-López *et al.,* 2010; Gómez-López *et al.,* 2005) and also with Adekunte *et al.,*

(2010b), who also reported that the decrease in ascorbic acid content is influenced by amplitude level and treatment time. The degradation of ascorbic acid could be explained by the formation of free radicals by sonochemical reaction, associated with oxidative processes (Hart and Henglein, 1985).

2.3.5. High hydrostatic pressure

Queiroz *et al.,* (2010) reported that vitamin C content in cashew juice did not change significantly in all samples treated at 250 MPa. However, contents significantly fell in samples pressurized at 400 MPa for 7 min compared to control. Similarly, other authors reported that HHP did not cause any significant changes in vitamin C in cloudy apple juice at 500 MPa, 3 min, and 25°C (Kim *et al.,* 2012).

In terms of vitamin C stability after processing, after 6 months of storage at 4°C ascorbic acid decreased by 39.41% in cloudy juices, and by 48.91% in clear juices (Cao *et al.,* 2012) in strawberry juices treated at 600 MPa for 4 min, at 25°C. A high-pressure treatment of 500 MPa at 35°C for 5 min led to a better ascorbic acid retention during post-processing storage of orange juice at 0-15°C compared to conventional thermal pasteurization (80°C, 30 s) (Polydera *et al.,* 2003).

2.3.6. Inert-gas treatment

Vitamin C content remained unchanged after supercritical CO_2 treatment of juices under mild conditions. However, when higher pressure and higher flow rates were employed (23 MPa, 5 L/h, 36°C), this treatment proved detrimental for this compound (Fabroni *et al.,* 2010).

Significant reductions in ascorbic vitamin C content (85.8%-96%) were observed at an ozone concentration of 7.8% w/w and a treatment time of 10 min (Tiwari *et al.,* 2009a; Tiwari *et al.,* 2009f) in tomato and strawberry juices. Similarly, orange juice decreases from 41.59 to 12.70

mg/100 mL were registered after 10 min of treatment time at a gas-flow rate of 0.0625 L/min, the ascorbic acid degradation-rate constant increasing exponentially with respect to ozone concentration (Tiwari *et al.,* 2008b).

3. Effect of technological processing on phytochemical content of fruit juices

In this section, the effect of technological fruit-juice processing on phytochemical content of juices is examined. The phytochemicals considered include terpenoids, alkaloids, and polyphenols. Carotenoids, being a particular class of terpenoids together with polyphenols have received growing attention in recent years due to the numerous publications that reveal their benefits for human health (Fernández-García *et al.,* 2012). Tables 14 and 15 present the results achieved during the last decade in terms of the effect of selected alternatives to conventional thermal treatments in fruit-juice processing on their polyphenol and carotenoids content respectively.

3.1. Phenolic compounds

3.1.1. Radiation treatment

Total phenols, flavonols, and flavonoids have shown enhancement on exposure to UV, significant at 60 min (Bhat, 2011a). Other authors found no variations in total phenolics with treatments of up to 100 min applying an incident energy of $3.88 \cdot 10^{-7}$ E/min (Falguera *et al.,* 2011). UV radiation also reportedly exerts a negative influence on anthocyanins. Bakowska *et al.,* (2003) found a strong negative influence of UV irradiation on the complex of cyanidin-3-glucoside with copigment compared to thermal treatment at 80°C.

Technique	Scale	Effect on phenolic compounds	Optimun parameters	References
UV light	Laboratory	Enhancement	UV dose: 2.158 J/m^2 Treatment length: 30-60 min	Bhat *et al.,* 2011a
		No significant differences	UV dose: 3.88·10^{-7} E/min Treatment length: 20-120 min	Falguera *et al.,* 2011
		Depletion	UV dose: 2.1 mW/cm2	Bakowska *et al.,* 2003
HILP	Laboratory	No significant differences	Light dose:1.17-5.1 J/cm^2/pulse Pulse width: 360 µs Treatment length: 2-8 s	Palgan *et al.,* 2011; Pataro *et al.,* 2011; Caminiti *et al.,* 2009
		Depletion	Light dose: 1.313 J/cm^2/pulse Energy dosage: 3.3 J/cm2 Pulse width: 360 µs	Caminiti *et al.,* 2012
γ-irradiation	Laboratory	No significant differences	Irradiation dose: 5 kGy	Mishra *et al.,* 2011
PEF	Laboratory, Pilot plant and industrial scale	Enhancement	Electric field: 0.4-3 kV/cm Pulse rise time: 100-1000 µs Treatment length: 10-100 ms Bipolar mode	Grimi *et al.,* 2009; Grimi *et al.,* 2011; Schilling *et al.,* 2008
		Depletion	Electric field: 0.45-1 kV/cm Pulse rise time: 10-32 ms Treatment length: 100 µs Bipolar mode	Turk *et al.,* 2010; Turk *et al.,* 2012a
		No significant differences	Electric field: 0.6-35 kV/cm Pulse rise time: 0.2 - 100 µs Treatment length: 75 µs - 23 ms Bipolar mode	Bi *et al.,* 2013; Odriozola-Serrano *et al.,* 2009; Morales-de la Peña *et al.,* 2010; Odriozola-Serrano *et al.,* 2008[a]; Odriozola-Serrano *et al.,* 2008b; Turk *et al.,* 2012b
OH	Laboratory	Enhancement	Electric field:10-40 kV/cm Treatment length: 0-12 min Temperature. 90°C	Yildiz *et al.,* 2009
MW	Laboratory	No significant differences	Microwave power: 435-850 W Frequency: 2450 MHz Treatment length: 15-60 s Temperature: 70-80°C	Picouet *et al.,* 2009; De Ancos *et al.,* 1999

Ultrasound	Laboratory	Enhancement	Wave amplitude: 24-60 µm/40-100% Power: 1500 W Frequency: 20-40 kHz Treatment length: 2-90 min Mode: pulsed and continuous	Santhirasegaram *et al.,* 2013; Tiwari *et al.,* 2010; Zafra-Rojas *et al.,* 2013; Abid *et al.,* 2013; Bhat *et al.,* 2011b
		Depletion	Power: 100-500 W Treatment length: 2-10 min Continuous mode	Fonteles *et al.,* 2012
HHP	Laboratory	Enhancement	Pressure: 200-500 MPa Treatment length: 2.5-25 min Temperature: 20-25°C	Chen *et al.,* 2013; Queiroz *et al.,* 2010; Kim *et al.,* 2012
		Depletion	Pressure: 600 MPa Treatment length: 30 min Temperature: 70°C	Corrales *et al.,* 2008
		No significant differences	Pressure: 400-600 MPa Treatment length: 5-30 min Temperature: 20°C	Corrales *et al.,* 2008; Ferrari *et al.,* 2010
SC-CO_2	Laboratory	Depletion	Pressure: 23 MPa CO_2 concentration: 5 L/h Temperature: 36°C	Fabroni *et al.,* 2010
		No significant differences	Pressure: 35.4 MPa CO_2 concentration: 8-16% Temperature: 30°C	Del Pozo-Insfran *et al.,* 2006
Ozonation	Laboratory	Depletion	Flow rate: 0.0625-0.125 L/min Ozone concentration:0.016-0.078 mg/kg Treatment length: 0-18 min	Torres *et al.,* 2011; Patil *et al.,* 2010b, Patil *et al.,* 2010[a]; Tiwari *et al.,* 2009[a]; Tiwari *et al.,* 2009b; Tiwari *et al.,* 2009c; Tiwari *et al.,* 2009f
FVE	Laboratory	Enhancement	Heating conditions: 60-95°C, 6 min Vacuum conditions: 1-10 kPa, 0.5 s	Morel-Salmi *et al.,* 2006; Paranjpe *et al.,* 2012; Anonymous 2007; Brat *et al.,* 2002

Table 14. Effect of selected alternatives to conventional thermal treatments in fruit-juice processing on their phenolic content.

Abbrevations: **UV**, Ultraviolet; **HILP**, High Intensity Light Pulses; **PEF**, Pulsed Electric Fields; **OH**, Ohmic Heating; **MW**, Microwave; **HHP**, High Hydrostatic Pressure; **SC-CO_2**, Supercritical CO_2, **FVE**, Flash Vacuum Expansion.

Technique	Scale	Effect on polyphenols	Optimun parameters	References
PEF	Laboratory, Pilot plant and industrial scale	No significant differences	Electric field: 25-40 kV/cm Pulse rise time:1-7 µs Treatment length: 30-1500 µs Bipolar mode	Odriozola-Serrano *et al.,* 2009[a]; Odriozola-Serrano *et al.,* 2009b; Torregrosa *et al.,* 2006; Cortés *et al.,* 2006; Vervoort *et al.,* 2011
		Conversion	Electric field: 35 kV/cm Pulse rise time:1-7 µs Treatment length: 100-2500 µs Bipolar mode	Odriozola-Serrano *et al.,* 2007; Odriozola-Serrano *et al.,* 2008[a]; Odriozola-Serrano *et al.,* 2008b; Odriozola-Serrano *et al.,* 2009a; Oms-Oliu *et al.,* 2009
MW	Laboratory	Depletion	Microwave power: 435-850 W Frequency: 2450 MHz Treatment length: 30s-10 min Temperature: 60-70°C	Fratianni *et al.,* 2010; De Ancos *et al.,* 1999; Kim *et al.,* 2001
Ultrasound	Laboratory	Enhancement	Power: 130 W Frequency: 40 kHz Treatment length: 15-60 min	Santhirasegaram *et al.,* 2013; Demirdöven & Baysal, 2009
HHP	Laboratory	Enhancement	Pressure: 300 MPa Treatment length: 10 min Temperature: 4-25°C	Hsu *et al.,* 2008
		No significant differences	Pressure: 395-445 MPa Treatment length: 8-11 min Temperature: 70°C	Kim *et al.,* 2001

Table 15. Effect of selected alternatives to conventional thermal treatments in fruit-juice processing on their carotenoid content.

Abbrevations**: PEF**, Pulsed Electric Fields; **MW**, Microwave; **HHP**, High Hydrostatic Pressure

The phenolic compounds content has been found not to be affected by HILP treatment at dosages of 7-28 J/cm^2 (Palgan *et al.,* 2011a; Palgan *et al.,* 2011b) nor by the combination of HILP at dosages of 5.1-4.0 J/cm^2 and PEF at two field strengths (24-34 kV/cm) (Caminiti *et al.,* 2009). Conversely, Caminiti *et al.,* (2012) reported a significant decrease in total phenolics when applying a combination of HILP at 3.3 J/cm^2 with manothermosonication at 400 kPa and 35°C.

It has been stated that γ-irradiation induces negligible or subtle losses of nutrients and sensory qualities in food compared to thermal processing as it does not substantially raise the temperature of food during processing (Wood & Bruhn, 2000). Accordingly, the total phenolics in processed sugarcane juice did not change significantly upon addition of preservatives plus gamma-radiation processing at 5 kGy (Mishra *et al.,* 2011).

However, other authors have reported an increase in the total phenol contents at 3-5 kGy in carrot and kale juices (Song *et al.,* 2006) as well as tamarind juice, in a dose-dependent manner (Lee *et al.,* 2009; Lee *et al.,* 2006). Similarly, other authors (Bhat *et al.,* 2011b; Ayed, N. 1999) reported that the anthocyanin content in grape pomace increased with the irradiation dose, at an optimum at 6 kGy. This increase in measured content may be due to the extraction of bound pigments by the degradation of the cell wall. Conversely, Alighourchi *et al.,* (2008) found a significant reduction in the total and individual anthocyanin content in pomegranate juice after irradiation at higher doses (3.5-10 kGy) and that irradiation effects on anthocyanin pigments depend upon the nature of anthocyanin; for example, diglycosides are relatively stable towards the irradiation dose compared to monoglycosides.

3.1.2. Electric treatment

Regarding the effect of PEF on the phenolic compounds content of fruit juices, Odriozola-Serrano *et al.,* (2009b) reported that the maximum values of anthocyanins were reached by combining high frequencies and low pulse widths irrespective of the pulse polarity. The effect of electric-field

strength was assessed by Bi *et al.,* (2013), who concluded that the retention of total phenols increased with greater electric-field strength regardless pulse rise times, so that total phenols treated at higher electric-field strengths did not significantly differ from control. There is no consensus in the literature regarding the retention of phenolic compoundss. Numerous results show no significant differences in total phenolic content between untreated and PEF-treated tomato juices (Odriozola-Serrano *et al.,* 2009a; Odriozola-Serrano *et al.,* 2009b), juice-soymilk beverage (Morales-de la Peña *et al.,* 2010), apple juice (Schilling *et al.,* 2007), and tomato juice (Odriozola-Serrano *et al.,* 2009a; Odriozola-Serrano *et al.,* 2008a) with pulses of 4-400 μs, at 1-35 kV/cm field strength. Similarly, Odriozola-Serrano *et al.,* (2008b) found no significant differences in flavonol content between treated and non-treated strawberry juice but detected minor differences in the anthocyanin content, which proved higher in untreated samples. Likewise, after the scaling-up on an industrial scale of apple-juice production, PEF treatment at 650 V/cm, 23.2 ms did not significantly affect the concentration of native phenolic compounds in the mash. Furthermore, the concentration of total native phenolic compoundss in the juices increased by 8.8% due to PEF treatment of the mash when the samples were preserved from enzymatic oxidation (Turk *et al.,* 2012b). In contrast to the above findings, evidence in grape-juice extraction also suggests that PEF application might have increased the phenolic compounds content in a regime of progressive pressure increase (from 0 to 1 bar in 1 h) at 400 V/cm as compared with juice produced in the regime of constant pressure (Grimi *et al.,* 2009). A positive impact of PEF on the extraction of phenolic compounds was also observed in juices after PEF treatment of apple juice (Grimi *et al.,* 2011; Bhat *et al.,* 2011b; Schilling *et al.,* 2008) and grape juice (Puértolas *et al.,* 2010; Puértolas *et al.,* 2009). Likewise, same authors found that the concentration of anthocyanins and phenolic compounds during fermentation of red grapes was higher in the PEF-treated samples than in controls, this effect increasing with the electric-field

strength until 5 kV/cm (Puértolas *et al.,* 2010). Similarly, fewer phenolic compounds losses (18%) have been noted during PEF treatments at 32-35 kV/cm, 3-4 µs when compared to conventional heat treatments at 90-100°C for a holding time of 30 s to 1 min (32-42%) (Aguilar-Rosas *et al.,* 2007; Zhang *et al.,* 2010b). Additionally, Zhang *et al.,* (2008) reported that the degradation rate constant of cyanidin-3-glucoside, which compares favorably to that for thermal treatment. By contrast, Turk *et al.,* (2010) found a decline in native phenolic compounds yield due to PEF treatment at 450 V/cm and 10 ms, representing 54% of the phenolic compounds for a large mash and 17% for a small mash compared to untreated samples. These authors attributed this decrease to greater oxidation of phenolic compounds in cells due to the electroporation of the inner-cell membrane and the adsorption of the oxidized products onto the mash. When apple mash was treated on a pilot-plant scale (1000 V/cm, 100 µs), a 17.8% loss was recorded (Turk *et al.,* 2012a).

Regarding OH treatments, Yildiz *et al.,* (2009) found that the heating process increased the amount of phenolics, with no difference between ohmic and conventional heating. Both treatments reached 90°C and remained at this temperature for 0-12 min.

3.1.3. Microwave heating treatment

Only scant research is available concerning the effect of MH on phenolic compounds during fruit-juice processing. Research on apple puree showed that heating to 70-80°C maintained the phenolic compounds content after the treatment (Picouet *et al.,* 2009). Similarly, De Ancos *et al.,* (1999) found no significant change in total anthocyanin concentration.

3.1.4. Ultrasound treatment

The application of US-assisted extraction has been reported to improve the extraction yield of bioactive compounds by between 30 and 35% compared to freshly squeezed juice

(Santhirasegaram *et al.,* 2013). This result agrees with the findings of other authors (Tiwari *et al.,* 2010; Zafra-Rojas *et al.,* 2013; Abid *et al.,* 2013; Bhat *et al.,* 2011b), who found a significant increase in the total phenolic content in sonicated juice samples when compared to a control, this being due possibly to the greater disruption of cell walls, facilitating the release of bound phenolic contents.

The effect of US on anthocyanins was studied in strawberry juice by Tiwari *et al.,* (2008a). These researchers detected a slight increase (1–2%) in the pelargonidin-3-glucoside content of the juice at lower amplitude levels and treatment times, possibly due to the extraction of bound anthocyanins from the suspended pulp.

Conversely, according to other authors (Fonteles *et al.,* 2012), the phenolic compounds content of juice degraded by 30%. This degradation of anthocyanins might have been due to cavitation, which involves the formation, growth, and collapse of microscopic bubbles (Tiwari *et al.,* 2009b).

3.1.5. High-hydrostatic-pressure treatment

HHP treatments have been found to augment the total phenolic compounds content at 200-500 MPa, 2.5-25 min at room temperature (Chen *et al.,* 2013; Queiroz *et al.,* 2010; Kim *et al.,* 2012). This could have resulted from plant-cell disruption caused by pressure, leading to higher extractability of these compounds.

HPP treatment at ambient temperature is reported to have minimal effects on the anthocyanin content of various fruits and vegetables (Tiwari *et al.,* 2009b). Many researchers examine the retention of anthocyanins, including Corrales *et al.,* (2008), who reported an insignificant reduction in cyanidin-3-glucoside in a model solution at processing conditions of 600 MPa, 20°C and 30 min. However, these authors reported a 25% loss at 600 MPa, 70°C for 30 min compared

to a 5% loss at 70°C for 30 min, indicating that HHP accelerates anthocyanin reduction at elevated temperatures.

Nevertheless, other authors reported a very specific trend regarding anthocyanin retention after the HHP treatment. At room temperature, their concentration decreased with the pressure level and processing time. However, at temperatures higher than ambient, the treatment time is not a relevant parameter, and the application of the high pressure slightly depresses the anthocyanin content. If the operating temperature exceeds 45°C and the pressure level is higher than 400 MPa, anthocyanin content is similar or higher than the value estimated for the fresh juice. These researchers attribute this result to the fact that in this particular range of processing conditions the high-pressure treatment alters mainly the mechanism of anthocyanin degradation by affecting the molecules involved in the reaction kinetics, such as enzymes (Ferrari *et al.,* 2010).

The stability of anthocyanins under storage after HHP treatment was evaluated by Zabetakis *et al.,* (2000), the lowest losses being recorded after treatment at 800 Mpa under refrigerated storage when compared to treatments at 200-600 Mpa and ambient storage. Other studies have demonstrated that after 6 months of storage at4°C, anthocyanins diminished by 7-30% and total phenolic compounds by 14-16 % in strawberry juices (Cao *et al.,* 2012).

3.1.6. Inert-gas treatment

The retention of total phenolics, flavonoids and anthocyanins was observed for supercritical CO_2 juices under mild conditions. However, when higher pressure and higher flow rates were employed (23 MPa, 5 L/h, 36°C), this treatment proved detrimental for these compounds (Fabroni *et al.,* 2010). In another study conducted by Del Pozo-Insfran *et al.,* (2006), no significant changes in total anthocyanin content were reported for SC-CO_2 processed muscadine grape juice compared to a 16% loss in thermally processed juice. Enhanced anthocyanin stability was also

found in the processed juice during storage for 10 weeks at 4°C, its fortification being due perhaps to the prevention of oxidation by the removal of dissolved oxygen.

Ozone treatment is generally expected to cause the loss of antioxidant constituents, because of its strong oxidizing activity. During ozonation, at higher processing conditions (4.8% w/w ozone concentration for 10 min processing time) a fall of 99.1%, 96.6%, 99.8%, and 49.7% was reported for chlorogenic acid, caffeic acid, cinnamic acid, and the total phenol content, respectively (Torres *et al.,* 2011). Similarly, according to Patil *et al.,* (2010b), a processing time required to achieve 5-log reductions for *E. coli* under similar experimental conditions showed a 66.5%, 73.5% and 65.0% drop for chlorogenic acid, caffeic acid, and cinnamic acid, respectively. This is in accordance to further research by same authors, who also found a significant reduction in phenolic compounds for longer ozonation times (Patil *et al.,* 2010a). Moreover, the ozonation of anthocyanin-rich fruit juices such as strawberry and blackberry has been reported to cause significant decline in these bioactive compounds. A reduction of 98.2% in the pelargonidin-3-glucoside content of strawberry juice was reported at an ozone concentration of 7.8% w/w processed for 10 min (Tiwari *et al.,* 2009a; Tiwari *et al.,* 2009b; Tiwari *et al.,* 2009c; Tiwari *et al.,* 2009f). Significant losses in the anthocyanin content were recorded during ozonation. During ozonation, cyanidin-3-O-glucoside was found to be more stable compared to delphinidin-3-O-glucoside and malvidin-3-O-glucoside. Similarly, a decrease of 99.1%, 96.6%, 99.8%, and 49.7% was observed for chlorogenic acid, caffeic acid, cinnamic acid, and total phenol content, respectively (Tiwari *et al.,* 2009e).

3.1.7. Flash-vacuum expansion (FVE) treatment

The FVE process allows the fast extraction of all phenolic compounds (hydroxycinnamic acids, flavonols, anthocyanins, catechins, and proanthocyanidins) and can be used to produce phenolic compounds-enriched grape juices (Morel-Salmi *et al.,* 2006; Paranjpe *et al.,* 2012; Anonymous

2007; Brat *et al.,* 2002). Paranjpe *et al.,* (2012) reported that lower pressures (in the flash chamber) and higher initial fruit temperatures promote the extraction of phenolics into the juice.

3.2. Carotenoids

3.2.1. Electrical treatment

While Vervoort *et al.,* (2011) found that PEF at 23 kV/cm, 2 μs had no significant effect on the carotenoid profile of orange juice, the authors agree on an increase in total carotenoids on applying 25-35 kV/cm (Odriozola-Serrano *et al.,* 2009a; Odriozola-Serrano *et al.,* 2009b; Torregrosa *et al.,* 2006; Cortés *et al.,* 2006; Vervoort *et al.,* 2011). Regarding individual carotenoids, Odriozola-Serrano *et al.,* (2007, 2008a, 2008b) found higher lycopene concentrations in PEF-processed tomato juice than in the untreated. The increase ranged from 1.0% to 46.2% after applying different PEF treatments, the maximum lycopene relative content being when PEF treatment was applied at 35 kV/cm for 1000 μs with bipolar pulses of 7 μs at 250 Hz. This increase was higher in the PEF-treated samples than in pasteurized samples at 90°C for 30 and 60 s. The increase in lycopene just after processing coincided with a depletion of phytoene and neurosporene contents compared to the untreated juice. Thus, PEF might stimulate the transformation of some carotenoids into lycopene (Odriozola-Serrano *et al.,* 2007; Odriozola-Serrano *et al.,* 2009a). Similarly, Oms-Oliu *et al.,* (2009) found 14% more lycopene in watermelon juice with 7-μs bipolar pulses for 1050 μs at 35 kV/cm and frequencies ranging from 200 to 250 Hz. Odriozola-Serrano *et al.,* (2007) also found that β-carotene in treated tomato juice significantly augmented (31-38%), whereas γ-carotene content was depleted (3-6%) after PEF treatment. A plausible explanation for this is that γ-carotene may undergo cyclization to form six-membered rings at one end of the molecule, giving β-carotene as a product.

3.2.2. Microwave heating treatment

Carotenoid degradation in orange juice was monitored during MH at different time/temperature conditions. Various carotenoids were identified and quantified by HPLC. The degradation rate of carotenoids was influenced by MH temperatures: at 60°C and 70°C for 10 min, violaxanthin and antheraxanthin were the most unstable compounds, while lutein and provitamin A carotenoids were more stable. At 85°C a decrease of about 50% was observed for almost all carotenoids after 1 min of MH (Fratianni *et al.,* 2010). Microwave treatment has been demonstrated to induce the degradative loss of total carotenoid content, 475W for 45s provoking the greatest loss of the total carotenoid (57%) in papaya puree (De Ancos *et al.,* 1999).

3.2.3. Ultrasound treatment

Juice samples subjected to sonication showed significant rises in carotenoid content, registering great improvement in extractability of carotenoids (9%) when compared to the control (Santhirasegaram *et al.,* 2013). Thus, carotenoids are still stable after 15 and 30 min of sonication. This may be explained by the inactivation of enzymes responsible for carotenoid degradation due to cavitation-induced shock waves and sonochemical reaction (Demirdöven & Baysal, 2009).

3.2.4. High hydrostatic pressure

Kim *et al.,* (2001) concluded that α- and β-carotene were relatively stable at 395 to 445 MPa, 70°C, for 8 to 11 min. It has also been reported that high-pressure treatment at 300 MPa at 4 and 25°C, 10 min, total carotenoid and total lycopene contents significantly increased up to 62 and 56% respectively as compared with control in tomato juices (Hsu *et al.,* 2008).

4. Effect of technological processing on physicochemical properties of fruit juices

In this section, pH and total soluble solids, and cloudiness and turbidity are reviewed.

4.1. Total soluble solids and pH

Radiation treatment has been reported to have no effect on the total soluble solids or pH, regardless of the type of treatment (Vanamala *et al.,* 2007; Azhuvalappil *et al.,* 2010; Falguera *et al.,* 2011; Noci *et al.,* 2008; Ibarz *et al.,* 2005), and the same was found with the HILP treatment (Caminiti *et al.,* 2009; Caminiti *et al.,* 2011; Palgan *et al.,* 2011b; Vanamala *et al.,* 2007).

Similarly, electrical treatments exerted no effect with respect to pH or total soluble solids (Azhuvalappil *et al.,* 2010; Zárate-Rodríguez *et al.,* 2000). However, when the effect of OH treatments on the pH of the samples was studied, a drop in pH was found, attributed by Tolstoguzov (1990) to the ionization that took place inside the pulp during the migration of ions from the positive anode to the negative cathode, which produced more hydrogen ion than hydroxyl ion. In contrast to this finding, other authors have reported that OH exerts no significant changes in pH or total soluble solids of juices (Yildiz *et al.,* 2009).

The MH treatment has been reported to slightly increase the acidity of apple juice (Zhang *et al.,* 2010a). This agrees with Igual *et al.,* (2010a), who reported that jams processed with 900 W of power microwave exhibited significantly lower pH values.

Moreover, the literature shows that the sonication treatment and time had no effect on pH or total soluble solids in juices (Zafra-Rojas *et al.,* 2013; Abid *et al.,* 2013; Santhirasegaram *et al.,* 2013, Bhat *et al.,* 2011; Adekunte *et al.,* 2010b). However, other authors found significant changes in pH after processing fruit-juice samples. With the processing of pineapple juice, pH fell as temperature rose to 40, 50, and 60°C and when the US treatment was pulsed instead of continuous. In contrast, when processing cranberry juice, this trend was reversed and pH rose when temperature was raised and pulsed treatments were applied (Bermúdez-Aguirre & Barbosa-Cánovas, 2012). The change of pH in the thermo-sonicated juices could be due to the formation of some chemical products in the treatment medium, such as nitrite, hydrogen

peroxide, and nitrate, as shown by Supeno and Kruus (2000) when sonication was applied in aqueous media.

Other treatments such as HHP or inert-gas treatments (SC-CO_2 and ozonation) were found not to prompt any change in pH of fruit juices nor total soluble solids (Hartyáni *et al.,* 2013; Mert *et al.,* 2013; Hartyáni *et al.,* 2011; Fabroni *et al.,* 2010; Choi & Nielsen, 2005; Chen *et al.,* 2013).

Similarly, FVE treatment had no significant effect on grape juice pH, or total soluble solids (Paranjpe *et al.,* 2012; Tiwari *et al.,* 2008b). However, Brat *et al.,* (2002) reported lower total soluble solids and higher pH in flash-vacuum-expanded passion fruit purees than those of reference juice. This is explained by a dilution of the inner juice from the aril by the rind.

4.2. Cloudiness and turbidity

After electrical treatments such as PEF, juices mechanically expressed from treated samples usually have less turbidity (Grimi *et al.,* 2011; Grimi *et al.,* 2007; Praporscic *et al.,* 2007).

By contrast, it has been demonstrated that sonication treatment significantly increased the degree of cloudiness of apple juice (Abid *et al.,* 2013). The higher cloudiness value might be due to the high-pressure gradient by cavitation during the sonication treatment, causing colloidal disintegration plus the dispersion and breakdown of macromolecules to smaller ones, thereby making the juice properly homogenized and more consistent. Some studies indicate that US may reduce the molecular weight of pectin by breaking its linear molecule and thus producing a weaker network (Seshadri *et al.,* 2003).

Other treatments such as HHP have significantly reduced the transmittance of orange juices, leading to a more opaque juice, which may be due to greater stabilization of the juice cloud (Carreño *et al.,* 2011).

With regards to CO_2 treatment, an influence on cloudiness has been noted, density being not only preserved but even augmented (Fabroni *et al.,* 2010). This phenomenon it attributable, as other authors have previously proposed, to the depressurization of the system, which homogenizes orange juice, rupturing particles in the juice colloid, thereby increasing cloudiness (Kincal *et al.,* 2006). On the contrary, other authors appreciated no significant differences between ozone-treated samples and their respective controls. However, ozone treatment preserved turbidity to a far higher degree than in thermally pasteurized juices (Choi & Nielsen. 2005).

5. Effect of technological processing on rheological properties of fruit juices

Radiation treatments such as UV have been reported to exert no changes in juice viscosity as a function of exposure time after UV treatment (Manzocco *et al.,* 2009).

Similarly, no variations in PEF-treated apple mash were observed, having no effect on the flow properties of the apple juices relative to their controls (Schilling *et al.,* 2007). However, Bi *et al.,* (2013) found less apparent viscosity and a lower consistency index of apple juice as well as a higher flow-behavior index on increasing the electric-field strength. This author attributes these findings to the breakdown of a colloidal suspension, which may be caused by the depolymerization of macromolecules present in the juice suspension (Tiwari *et al.,* 2008d). By contrast, Aguiló-Aguayo *et al.,* (2010) reported that PEF treatment led watermelon juices to have a higher viscosity than in the unprocessed products. It was suggested that the strong reduction of pectin methylesterase activity achieved by PEF treatments may disrupt the chain reaction of pectin methylesterase and polygalacturonase to avoid losses in the viscosity of the processed juices, and more soluble pectin might be leached from cell walls, resulting in a product with higher viscosity. The difference between these results might be caused by different juice preparations and juice varieties, resulting in varying pectin contents and components. During OH treatments, it has been reported that consistency coefficients significantly decreased while temperatures

increased (Icier & Tavman. 2006; Yildiz *et al.,* 2009). Through OH treatments, during the heating-up period of juices to 90°C, the consistency coefficient fell compared to that of the fresh juice, whereas no significant change was detected in this value during a holding period at 90°C. A similar trend was observed for flow-behavior indexes. Same trend was found during conventional heating, and therefore it was concluded that, in addition to thermal effects, ohmic heating had no electrical effect on rheological characteristics (Yildiz *et al.,* 2009).

Although no information is available regarding the effect of MH on fruit juices, the microwave reportedly increases the consistency of jam samples (Igual *et al.,* 2010a).

US processing reportedly diminishes juice viscosity by 75% of the initial value for non-sonicated juice (Costa *et al.,* 2013), so that the higher the US intensity and processing time, the lower the viscosity. However, viscosity increased in tomato juice after thermosonication, which was attributed to the reduction of the average particle size compared to the untreated juice (Wu *et al.,* 2008). Seshadri *et al.,* (2003) explain that, depending on the intensity of US, the viscosity might thicken or thin.

High-hydrostatic-pressure treatments have shown some effects on viscosity of fruit juices. Higher apparent viscosity values were determined for highly pressurized orange juices compared to thermally treated ones immediately after processing. Suspended pulp decreased significantly after the application of pressure and was more pronounced after HPH treatment. HPH treatment is known to reduce particle size, converting part of the sedimentable pulp into colloidal pulp (Polydera *et al.,* 2003).

Similarly, significant changes were observed in consistency and flow-behavior indices of apple juice after ozonation. The consistency index decreased as a function of ozone concentration (Torres *et al.,* 2011). A decline in the apparent viscosity value during ozonation result from the

breakdown of a colloidal suspension which may be caused by the depolymerization of macromolecules present in the juice suspension (Tiwari *et al.,* 2008d).

Regarding FVE treatments, the consistency and viscosity of the puree from treated samples was far higher than that of the reference puree and also the steam-heated puree, even with the reincorporation of aromatic liquors recovered during the treatment. The high viscosity is attributable mainly to its insoluble destarched alcohol content (Brat *et al.,* 2002).

6. Effect of technological processing on organoleptic properties of fruit juices

In this section color, and aroma and flavor parameters are reviewed.

6.1. Color

6.1.1. Radiation treatment

Color values are usually monitored by *L, a, b* color space scales. Most popular color scales are Hunter *L, a, b* and CIE *L*, a*, b**; while similar in organization, a color will have different numerical values. In both of them the parameter *L* expresses whiteness or brightness/darkness, *a* representing the variation between red and green and *b* representing the variation between yellow and blue (Tiwari *et al.,* 2009b). The total color difference (TCD or Δ*E*) indicates the magnitude of color change after treatment. Differences in perceivable color can be analytically classified as not noticeable (0-0.5), slightly noticeable (0.5-1.5), noticeable (1.5-3.0), well visible (3.0-6.0) and great (6.0–12.0) (Clydesdale, 1978; Cserhalmi *et al.,* 2006). In UV-radiated apple juices, L has been reported to increase in samples of Golden, Starking, and Fuji apple varieties (Ibarz *et al.,* 2005; Falguera *et al.,* 2011; Manzocco *et al.,* 2009). However, this measurement is influenced by the fruit variety, normally fruit juices with high initial brightness, such as juice from King David apples, have kept its *L* value almost invariable. The parameter *a* has been reported to decrease during irradiation in the samples from Golden, Starking, and Fuji, signifying that the

juices became less red (Falguera *et al.,* 2011; Ibarz *et al.,* 2005). Similarly, parameter *b* reportedly fluctuates greatly according to the variety employed, its value decreasing in the samples from Golden (which had the highest initial one), slightly increasing in the samples from Starking and Fuji, and remaining almost constant in the juice from King David, which had the lowest initial value (Falguera *et al.,* 2011). Other authors have found a decline in this parameter in apple and lemon juices (Ibarz *et al.,* 2005), in agreement with Guerrero-Beltrán & Barbosa-Cánovas (2005) and Falguera *et al.,* (2011), who reported photodestruction of pigments in apple juices.

HIPL treatment has revealed a slightly noticeable TCD (Caminiti *et al.,* 2011) difference apple juice, regardless of the exposure time. To the contrary, other authors found no significant differences among HIPL-treated samples and controls (Caminiti *et al.,* 2011).

6.1.2. Electrical treatment

Juice color has not been found to be adversely affected by PEF (Schilling *et al.,* 2008). Higher ΔE, *L,* and *b* values have been reported in apple juice after PEF treatments, revealed as brighter color and more yellowness juice visible to the naked eye. It has been reported that color change was closely related to the enzymatic activity, such as polyphenoloxidase and peroxidase, which catalyzes the oxidation of phenolic compounds and causes enzymatic browning. Thus, the maintenance of apple-juice color might be due to the inactivation of these enzymes by PEF (Bi *et al.,* 2013). Azhuvalappil *et al.,* (2010) attributed the greater luminosity in treated cloudy apple juice to partial precipitation of insoluble suspended particles.

In terms of treatment combinations, PEF/HILP combinations affected the *L* and *b* color attributes with respect to the untreated control, although all combinations caused “slightly noticeable” color changes (Caminiti *et al.,* 2011).

However, Walkling-Ribeiro *et al.,* (2008a, 2008b) found no significant difference in color between an untreated reconstituted apple juice and the product processed by UV/PEF combined treatments, while thermal processing (94°C for 26 s) caused a significant change in all color attributes. In a recent study on fresh apple juice, Noci *et al.,* (2008) found that batch UV and PEF combinations also had less adverse effects on juice color than did heat pasteurization.

The *L* value of orange juice exposed to ohmic and conventionally heated juices did not significantly differ from the control. Minor changes in *a* and *b* values were appreciated after both heating treatments. However, *a* and *b* color values of continuous ohmic-heating-treated samples were much closer to that of control than were the conventionally heated counterparts (Lee *et al.,* 2012). In other studies, the color of ohmically heated juices proved insignificantly different from those of conventionally heated specimens; however differences with respect to control were found by a sensory panel (Tumpanuvatr & Jittanit, 2012). Ohmic heating also reportedly caused less browning during holding time than did conventional heating (Yildiz *et al.,* 2009).

6.1.3. Microwave heating (MH) treatment

Research has indicated that microwave processing provokes lower degradation of color compared to infrared and ohmic heating (Vikram *et al.,* 2005). Similarly, Yousefi *et al.,* (2012) reported that all color parameters decreased with time; however, color degradation was more notable in conventional heating compared to the MH method. Nevertheless, microwave processing affected the color parameters *b* and *L* compared to non-treated samples (Stinco *et al.,* 2013). By contrast, Cendres *et al.,* (2011) produced brightly colored juices with MH, as this method may inactivate endogenous enzymes such as polyphenol oxidase prior to fruit grinding (Walkling-Ribeiro *et al.,* 2008b).

6.1.4. Ultrasound treatment (US)

High-intensity US might release intracellular contents, which may affect the product color. The results have shown that sonication treatment significantly changes color between treated and non-treated juice samples. The sample treated for longer times registered the lowest *L* value, probably due to the release of intracellular compounds that slightly darkened the juice. On the other hand, *a* and *b* values rose, this being indicative of the release of red and yellow pigments, respectively (Abid *et al.,* 2013; Costa *et al.,* 2013). The results agree with the observations of sonicated kasturi lime juice (Bhat *et al.,* 2011b); and Bermúdez-Aguirre & Barbosa-Cánovas (2012), who also found significant color differences between treated and non-treated samples. The color changes in fruit juice might be attributed to the cavitation during sonication (Tiwari *et al.,* 2008a). However, shifts in apple-juice color after sonication could not be easily judged with the naked eye (Abid *et al.,* 2013). In contrast, it has also been stated that US processing has no effect on color (Fonteles *et al.,* 2012).

Manothermosonication applied as a second hurdle following UV and HILP treatment darkened the product. This was reflected in significantly lower *L* value and *a* values, indicating that the juice processed by the selected combinations was less bright and less red than was control. Also, a lowering of the *b* value suggested a trend towards a bluish color. Color measurements performed on the juice after processing by the first hurdle showed only that the product did not significantly differ from the untreated sample, regardless of the nature of the hurdle (UV or HILP). This result suggests that the MTS treatment was responsible for the color changes observed in the product (Caminiti *et al.,* 2011).

6.1.5. High hydrostatic pressure (HHP)

Effects of HHP in pomegranate juices have been assessed. Compared with the freshly squeezed juice, the *L* value of the HHP-treated juice slightly declined, while the high-temperature short-time

pasteurized juice exhibited a significant rise in value, indicating greater brightness. The *a* values did not change after treatment, indicating no significant influence in terms of redness. The *b* value rose significantly, indicating that the blueness had faded and the yellowness had intensified. The Δ*E* value was less than 1.5, indicative of slightly noticeable changes, while changes after high-temperature short-time pasteurization were noticeable. This implies that the effect of the HHP treatment on the pigments, such as chlorophylls, lycopenes, and anthocyanins, was weaker than that of high-temperature short-time pasteurization (Chen *et al.,* 2013). By contrast, when the HPP treatment was applied to orange juice, the L value increased while the *a* value decreased and the *b* value was higher compared to the untreated sample (Hartyáni *et al.,* 2011). This might be due to the different nature of the color pigments present in different fruit juices.

On examining the Δ*E* values, (Hartyáni *et al.,* 2013) found only slightly noticeable differences in comparison to the control samples. The *L* value followed a rising trend with increasing pressure.

6.1.6. Inert-gas treatment

With regard to color parameters, the *L*, *a*, and *b* values decreased after CO_2 treatment. However, these changes were not pronounced enough to significantly alter the characteristic color of the juice (Fabroni *et al.,* 2010).

During ozonation, apple-juice samples were observed to be lighter in color, i.e. higher *L* and *b* values, whereas *a* values of juice samples were found to decline with longer processing times and higher ozone concentrations (Torres *et al.,* 2011; Patil *et al.,* 2010a). However, ozonated strawberry juice samples were observed to be lighter in color, i.e. increased *L* value, whereas *a* and b^* values were found to fall with rising ozone concentrations and longer treatment times (Tiwari *et al.,* 2009f); the same trend was followed by blackberry juice (Tiwari *et al.,* 2009c). It has been reported that the chromophore of conjugated double bonds for anthocyanins or carotenoids,

which are responsible for berry-juice color, may be degraded (Tiwari *et al.,* 2009c). In orange juice a clear increase in lightness value (*L*) was discerned with an increased gas flow rate, ozone concentration, and treatment time. Conversely, *a* and *b* values decreased (Tiwari *et al.,* 2008c). In further studies in orange juice, *L, a* and *b* values rose with respect to the ozone concentration (% w/w) at each gas-flow rate (Tiwari *et al.,* 2008b).

6.1.7. Fash-vacuum expansion treatment

Vacuum-expanded passion-fruit purees exhibited higher *a* values and lower *L* and *b* values than did the reference juice, which had a red-purple tone. By contrast, the reference juice was orangish-yellow due to carotenoids. This difference might be owed to the fact that the mesocarp containing most of anthocyanins is not disintegrated by the pulping step, while the FVE treatment may have loosen the tissue cohesion by creating microchannels, thus separating cells at their cell-wall level and facilitating extraction of color (Brat *et al.,* 2002).

6.2. Aroma and flavor

6.2.1. Radiation treatment

Sensory evaluation has shown that samples treated with radiation dosages up to 10.62 J/cm^2 were comparable to control in terms of acceptability, though higher dosages adversely affected flavor (Caminiti *et al.,* 2012). UV+PEF and HILP PEF combinations did not alter either the odor or the flavor of the juice (Caminiti *et al.,* 2011).

6.2.2. Electric treatment

After PEF treatment, volatile aroma components of orange juice, namely ethyl butyrate, linalool, decanal and valencene are not significantly lost. In the case of lemon juice, no marked decrease was detected in the amount of neral and geranial, character impact compounds. Meanwhile, the concentration of known off-flavor compounds (α-terpineol and terpinen-4-ol) remained constant

(Cserhalmi *et al.,* 2006). Similarly, nootkatone, a key aroma compound in grapefruit, did not significantly change (Cserhalmi *et al.,* 2006). These observations agree with those of Aguilar-Rosas *et al.,* (2007) and Yeom *et al.,* (2000), who reported that volatile chemical compounds responsible for fruit-juice flavor are retained at a higher ratio in comparison to high-temperature short-time pasteurization with PEF-treated samples.

Ohmic-heated orange juice maintained higher amounts of the five representative flavor compounds than did heat-pasteurized juice. Moreover, sensory-evaluation tests revealed no difference between fresh and ohmic-heated orange juice (Leizerson & Shimoni, 2005a).

6.2.3. Ultrasound treatment

Results for the aromatic profile of juices has shown that, compared to untreated samples of juices and nectars, ultrasonic treatment prompts the formation of new compounds or the disappearance of compounds that were present in the untreated samples, this being influenced by US amplitude level, sonication time, and temperature (Šimunek *et al.,* 2013).

Combinations of manothermosonication, UV, and HIPL have been shown to adversely affect aroma and flavor attributes (Caminiti *et al.,* 2011). By contrast, in terms of odor, flavor, and overall acceptability, no significant differences were detected by panelists between high-temperature short-time pasteurization (94°C for 26 s) and batch thermosonication at 55°C for 10 min followed by continuous PEF at a field strength of 40 kV/cm for 150 μs (Walkling-Ribeiro *et al.,* 2009).

6.2.4. High hydrostatic pressure (HHP)

The results of aroma analysis indicate that the quantity of ethyl-esters did not decrease in orange juice, except for beta-mircene, which slightly diminished after HHP treatment. A similar trend was observed for grapefruit juice, in which only ethyl octanoat decreased, while tangerine juice preserved all its aroma compounds (Hartyáni *et al.,* 2011). In another study, concentrations of

limonene, α-terpineol, and carvone were measured after HHP treatment. Pressure, temperature, and treatment time were found to be critical factors influencing changes in concentrations. Both pressure and temperature could cause limonene degradation, resulting in significant increases of α-terpineol and carvone concentrations (Pan *et al.,* 2011).

Electronic noses have been used to predict aroma changes in treated apple and orange juices. Neither the pressure application nor the different pressure levels appeared to be relevant, as no significant differences were found in the separation of treated samples and controls applying a statistic supervised LDA method. By contrast, the electronic nose was able to differentiate clearly other parameters such as temperature and storage time regarding aroma changes (Hartyáni *et al.,* 2013). Similarly, Laboissière *et al.,* (2007) reported no significant changes in compounds responsible for the yellow passion fruit juice aroma and flavor, when the data were analyzed through QDA and PCA.

7. Conclusion

The effects of alternatives to conventional heat treatments on nutritional, physicochemical, rheological, and organoleptic parameters in fruit-juice processing, are reviewed in this paper. These effects should be considered by processors as consumer preferences currently point towards products that, in addition to having longer shelf-life than fresh ones, need to have similar characteristics to the original product and preserve their overall quality. The literature reveals that research is still underway, and many aspects remain to be fully studied and understood. In general, temperature during processing and storage is an important factor affecting the quality of the processed product. However, not only temperature but other factors are involved in quality losses. Although alternatives to heat treatments seem to be less detrimental than the thermal treatment, there are many parameters and conditions that influence the yield as well as the nature of the juice itself, making it difficult to compare different studies. There is also a lack of

standardization in operating conditions, and consequently ambiguity arises within the literature, as these control conditions may not be reported in detail or are reported differently. Fundamental understanding of these phenomena is essential for optimum process design to ensure high-standard products. Additionally, future research should focus on understanding the mechanisms underlying the changes in the overall quality of fruit juices and should also delve into scaled-up processes, process design, and optimization. This investigation is needed in detail to maximize the potential of alternative non-thermal technologies in fruit-juice processing while maintaining maximum-quality fruit-juice attributes.

8. Acknowledgments

This work was supported by the project AGL2011-29857-C03-02 (Spanish Ministry of Science and Innovation), as well as by P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 (Andalusian Regional Government Council of Innovation and Science) projects. The authors are also grateful for the scholarship FPU AP2010-2793.

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Chapter 7

Food Chemistry 182 (2015) 282–291

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Characterization of polyphenols, sugars, and other polar compounds in persimmon juices produced under different technologies and their assessment in terms of compositional variations

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ARTICLE INFO

Article history:
Received 8 March 2013
Received in revised form 2 March 2015
Accepted 3 March 2015
Available online 11 March 2015

Keywords:
High performance liquid chromatography
Mass spectrometry
Polyphenols
Persimmon juice
Fruit juice processing

ABSTRACT

Persimmon juice is emerging in the global juice market as a new wholesome commercial juice that could effectively complement a healthy diet, given the epidemiological evidence linking a diet rich in fruits and vegetables with reduced incidences of chronic diseases. However, little data are available on the persimmon-juice composition or on the effect of the technological treatment employed for its production. The present work performs a complete qualitative analytical characterization through high-performance liquid chromatography coupled to electrospray time-of-flight mass spectrometry (HPLC–DAD–ESI-TOF/MS) of the diverse persimmon juices produced under different technologies in a pilot plant (clarification, astringency removal, flash vacuum expansion, centrifugation and pasteurization) in order to evaluate the effect of the different production procedures on the polar chemical profile of persimmon juice. Persimmon-juice extracts have been found to be a source of sugars, protein derivatives, organic acids, vitamins, and polyphenols, including simple polyphenols (phenolic acids and flavonoids) and polymerized flavan-3-ols. A marked influence of processing on the composition of the juices has been noticed. Extracts 3 and 7 (undergoing the combinations of clarification and centrifugation, and astringency removal, centrifugation and pasteurization, respectively) contained more polyphenols, which may help reduce risk of chronic diseases.

1. Introduction

The persimmon (*Diospyros kaki* Thunb.) is a commercially important fruit crop, particularly widespread in Asian countries and spreading to other regions of the world, more concretely Europe, because of greater consumer acceptability and the characteristics of the Mediterranean climate, which is suitable for growing most persimmon cultivars (Del Bubba *et al.,* 2009). The persimmon is a nutritionally beneficial fruit, the main compounds in the fresh fruits being sugars, vitamin C, carotenoids, and phenolic compounds; the sugars in mature fruit are fructose, glucose, and sucrose (Candir *et al.,* 2009; Del Bubba *et al.,* 2009; Veberic *et al.,* 2010; Ittah, 1993). Vitamin C exists as two vitamers in persimmon (L-ascorbic acid and dehydro-L-ascorbic acid). Carotenoids give the fruit its characteristic reddish-orange colour, consisting of β-β carotene, β-ε

carotene, and β-cryptoxanthin (Veberic *et al.,* 2010; Kondo, *et al.,* 2004). Phenolic compounds comprise simple phenolic compounds together with highly polymerized tannins. The former group includes benzoic acid derivatives, monomeric flavanols, flavonols, and flavanones, while the latter includes polymerized flavan-3-ols procyanidins). Persimon tannins may be either of low molecular weight and hence water soluble, and astringent, making the fruit not edible at harvest time; or of high molecular weight and thus water insoluble, and non astringent, making the fruit edible at harvest time (Giordani *et al.,* 2011).

The persimmon fruit may be used for juice production. Traditional uses for persimmon juice include raw material for the production of a fermented products used for dying in Japan called *kaki-shibu* (Imai *et al.,* 2001), production of persimmon vinegar (Gao *et al.,* 2010; Lee & Kim, 2009), and persimmon juice as a dye for noodles (Han *et al.,* 2012). However, persimmon juice is emerging now into the global juice market as a new wholesome, commercial juice that promotes health, but little data are available on persimmon juice composition or the effect(s) of the technological processing.

There is epidemiological evidence linking a diet rich in fruits and vegetables with reduced incidences of coronary heart disease, cancer, and various chronic diseases (Margetts & Buttriss, 2008). Indeed, there is scientific evidence for the recommendation of several fruits and vegetables due to health benefits associated with their consumption. Cranberries have been recommended for the treatment of urinary tract infection (Howell *et al.,* 1998; Pinzón-Arango *et al.,* 2009; Takahashi *et al.,* 2013). Both observational and intervention studies have provided evidence in support of a protective role of green tea intake in the development of oral–digestive tract cancer or an inhibitory role of oral supplementation of green tea extract on a precancerous lesion of oral cavity (Li *et al.,* 1999; Tsao *et al.,* 2009; Yuan, 2013). Moderate consumption of red wine is widely believed to reduce the incidence of heart disease, an effect known as the French

paradox (Renaud & De Lorgeril, 1992). Fruits and vegetables contain numerous health-promoting compounds such as fibre and high concentrations of phenolic compounds, vitamins, and minerals. Among these, phenolic compounds although not essential for survival, may over the long term protect against these chronic diseases (Mullen *et al.,* 2007). In this regard, persimmon juice could be an effective complement to nutrition linked to a healthy diet (Endrizzi *et al.,* 2009).

The purpose of this work was the chemical characterization of persimmon juice produced using technologies applied by the fruit-juice industry. For this goal, nine persimmon juices were produced using different combinations of the following technologies: clarification, astringency removal, centrifugation, and pasteurization. Qualitative variations were evaluated by high-performance liquid chromatography coupled to electrospray time-of-flight mass spectrometry (HPLC-DAD-ESI-TOF/MS), to examine the impact of production technologies on chemical composition.

2. Materials and methods

2.1. Chemicals and apparatus

All chemicals were of analytical reagent grade and used as received. Methanol used for the extraction of the phenolic compounds from the persimmon juice samples was purchased from Panreac (Barcelona, Spain) and acetic acid from Fluka and Sigma-Aldrich (Steinheim, Germany). Double-deionized water with conductivity lower than 18.2MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Extracts were dried using a rotary vacuum evaporator model R-200 coupled to a heating bath model B-490, both from Büchi Labortechnik (9230 Flawil, Switzerland.). Authentic standards were used for comparison of the retention time, MS, and UV-Vis characteristics with the compounds identified. D-(-)-fructose, citric acid anhydrous, gallic acid, (+)-catechin, vanillic acid, and L-tyrosine were purchased from Sigma-Aldrich (San Luis, USA);

Hesperidin, quercetin-3-O-rhamnoside (quercitrin), and kaempferol-3-O-glucoside from Extrasynthese (Lyon, France); and quercetin-3-O-glucoside from Fluka (San Luis, USA).

2.2. Samples

Persimmon fruits of the variety 'Rojo Brillante' were harvested at the yellow-orange ripening state. Once in the Pilot Plant, fruits of a uniform size were selected and randomly grouped into batches of 50 kg each. The fruits were washed in cold tap water and drained. For juice extraction, fruits were cut in halves and pedicles were removed. Fruit pieces were ground into slurry and squeezed with a laboratory pilot press (Zumonat C-40; Somatic AMD, Valencia, Spain). Then the slurry was treated with 200 mg/kg macerating enzyme pectinase (Novozymes, Bagsvaerd, Denmark) for 24 h at room temperature to facilitate juice extraction, following the method reported elsewhere (Gorinstein *et al.,* 1993). Enzymes were inactivated at 95°C for 5 min and the juice, after being cooled, was centrifuged at 8000xg for 15 min. The supernatant was then collected, bottled and stored at 4°C for further use.

The supernatant was submitted to different technological procedures used in the fruit-juice-processing industry: centrifugation, pasteurization, astringency removal, clarification, and flash-vacuum expansion. Samples were taken in triplicate. Juice samples were stored in screw-cap 20-mL polypropylene containers, which were stored frozen at -20°C until analysis.

The experimental conditions for each stage were the following:

Astringency was removed by storing the fruits in a chamber with 95% carbon dioxide for 24 h at 20°C and 90% relative humidity prior to juice extraction. Centrifugation was performed at 8000xg for 15 min in a Lemitec MD 80 decanter centrifuge (Lemitec GMBH, Berlin, Germany). Pasteurization consisted of heat treatment at 95°C for 30s in a semi-tubular pasteurizer 25 L/h (Mipaser Prototype). Clarification was performed by maceration of the slurry with 800 mg/kg

enzyme pectinase (Novozymes, Bagsvaerd, Denmark) for 24 h at room temperature, according to the method reported elsewhere (Gorinstein *et al.,* 1993). Flash-vacuum expansion was performed in a cylindrical stainless-steel, steam-heating chamber at 85°C for 10 min. The juice was then placed in a cylindrical quartz vacuum vessel at 3 KPa coupled to the steam-heating chamber through a manual pneumatic valve (Brat, Olle, Reynes, Cogat, & Brillouet, 2001).

Combination of different fruits and treatments resulted in nine juices: sample 1 (centrifuged and pasteurized persimmon juice), sample 2 (non-astringent and centrifuged juice), sample 3 (clarified and centrifuged juice), sample 4 (non-astringent, flash-vacuum expansion-treated, and centrifuged juice), sample 5 (clarified, non-astringent, and pasteurized juice), sample 6 (clarified, centrifuged, and pasteurized juice), sample 7 (non-astringent, centrifuged, and pasteurised), sample 8 (clarified, non astringent, centrifuged, and pasteurized juice), sample 9 (clarified, non-astringent, and centrifuged juice).

2.3. Extract preparation

Extract preparation aimed to extract polar fractions of the nine juices. For the preparation of the extracts, 10 ml of each juice were mixed with 40 ml of methanol:water in a proportion of 80:20, as previously reported by other authors for a high recovery (Giordani *et al.,* 2011), stirred for 1 h at ambient temperature and then passed through filter paper. This extraction procedure was performed in triplicate. After this, the solvent was evaporated under vacuum using a rotary vacuum-evaporator at 40°C. The residue was dissolved in 10 ml of methanol:water (80:20), filtered through a cellulose syringe filter (0.45 µm) and conserved at -20°C prior to analysis.

2.4. Analytical characterization

The nine juice extracts were analytically characterized by HPLC coupled to electro-spray time-of-flight mass spectrometry (HPLC-ESI-TOF/MS). The HPLC-ESI-TOF/MS method was performed

using an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) of the Series Rapid Resolution equipped with a vacuum degasser, autosampler, a binary pump, and diode-array detector (DAD). The chromatographic separation was performed in a Zorbax Eclipse Plus RP-C18 analytical column (Agilent Technologies, Palo Alto, CA, USA) 150 x 4.6 mm i.d., 1.8 µm particle size). The flow rate was 0.80 ml/min, and the temperature of the column was maintained at 25°C. The mobile phase used was water with 0.25% acetic acid as eluent A, and methanol as eluent B. The total run time was 27 min using the following multistep linear gradient: 0min, 5%B; 7 min, 35%B; 12 min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5%B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis (Lozano-Sánchez *et al.,* 2010). The injection volume in the HPLC was 20 µl. The compounds separated were monitored in sequence first with DAD (240, 280 and 350 nm) and then with a mass-spectrometry detector. The MS analysis was performed using the microTOF (Bruker Daltonik, Bremen, Germany), which was coupled to the HPLC system. A splitter was used to couple the MS detector, as the flow arriving to the TOF detector had to be 0.2mL/min to ensure reproducible results and stable spray. The TOF mass spectrometer was equipped with an ESI interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) operating in negative ion mode. External mass-spectrometer calibration was performed with sodium acetate clusters (5 mmol/l sodium hydroxide in water/2-propanol 1/1 (v/v), with 0.2% of acetic) in quadratic high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to identification. The optimum values of source and transfer parameters were established according to Lozano-Sánchez et al.(2010). The accurate mass data for the molecular ions were processed using the software Data Analysis 3.4 (Bruker Daltonik), which provided a list of possible elemental formulas using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as

minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalent, as well as a sophisticated comparison of the theoretical with the measured isotopic pattern (Sigma-Value) for increased confidence in the suggested molecular formula. The accuracy threshold for confirmation of elemental compositions was established at 10 ppm for most of the compounds.

3. Results and discussion

3.1. Composition of the methanolic persimmon juice extracts

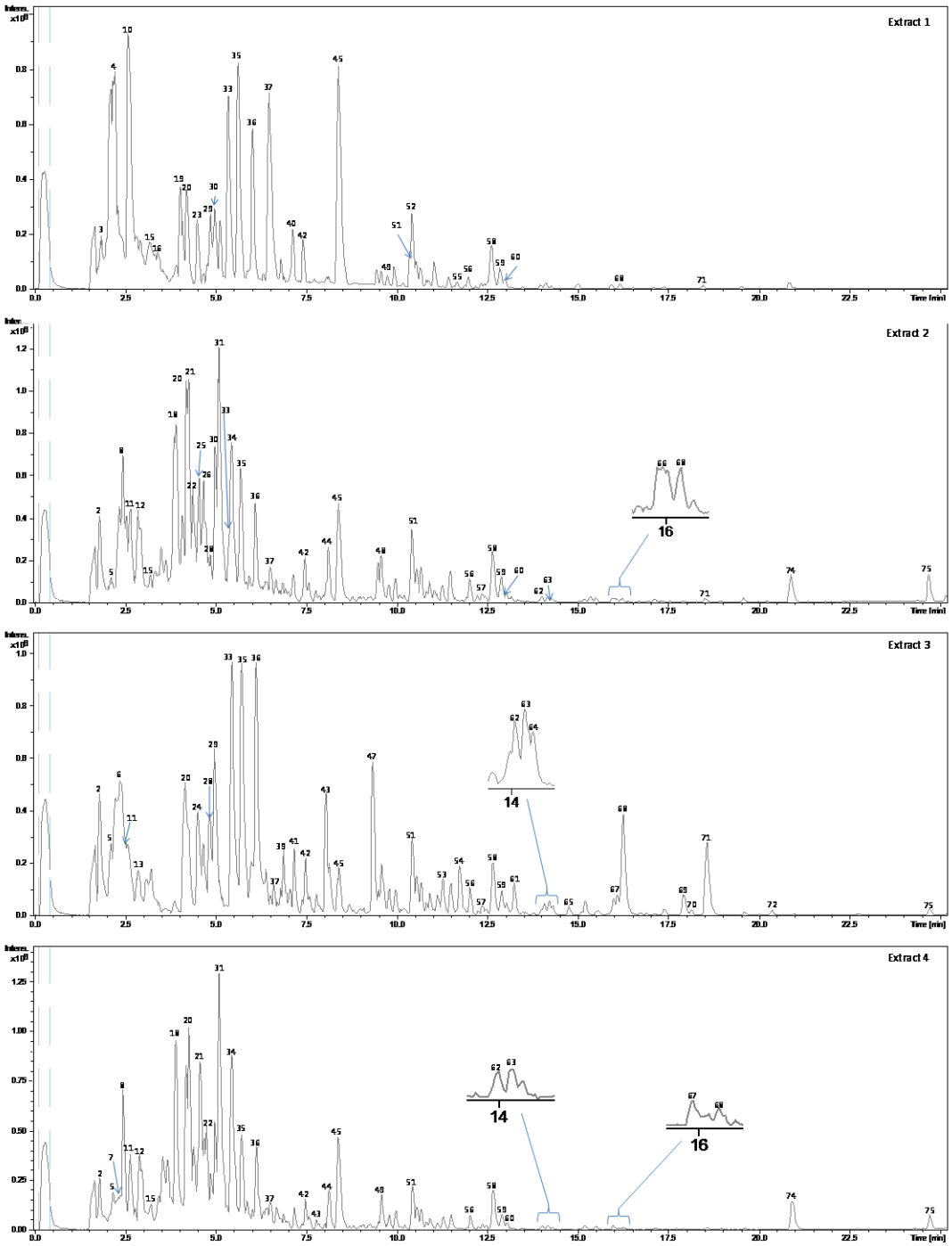
This is the first report to characterize persimmon juices using HPLC-DAD-ESI-TOF/MS. The extracts were qualitatively characterized by taking into consideration the retention time, UV-absorbance maxima, MS data provided by the TOF analyzer, and pure standards confirmation of some compounds. Figure 51 shows chromatograms for the nine juice extracts. A total of 74 compounds were detected, among these, 44 compounds belonging to different chemical classes (sugars, organic acids, protein derivatives, vitamins, phenolic compounds, and their derivatives) were tentatively characterised in the nine juice extracts. Table 16 shows the proposed compounds.

Peak	Proposed compound	Molecular formula	RT	λ	*m/z* calc.	*m/z* exp.	Error (ppm)	mσ	Extracts present
1	Digalacturonic acid	$C_{12}H_{18}O_{13}$	1.76	234	369.0675	369.0695	-5.5	6.1	5*
2	Galacturonic acid	$C_6H_{10}O_7$	1.81	230	193.0354	193.0364	-5.3	13.2	2, 3, 5, 7*
3	Xylonic acid	$C_5H_{10}O_6$	1.83	236	165.0405	165.0407	-1.3	2.3	1, 2, 3, 4, 5, 6, 7*, 8, 9
4	Sucrose	$C_{12}H_{22}O_{11}$	2.12	266	341.1089	341.1088	0.3	3.8	1*, 4, 5
5	Fructose	$C_6H_{12}O_6$	2.14	238	179.0571	179.0564	1.7	6.9	1, 2, 3, 4, 5, 6, 7*, 8, 9
7	Malic acid	$C_4H_6O_5$	2.41	230, 245	133.0142	133.0157	-10.8	5.9	1, 2, 3, 4, 5, 6, 7*, 8, 9
10	Hexosyl hexosyl hexoside	$C_{18}H_{32}O_{16}$	2.59	255	503.1618	503.1625	-1.6	3.2	1*, 4, 5, 6, 7, 8, 9
11	Ascorbic acid hexoside	$C_{12}H_{18}O_{11}$	2.64	235, 287	337.0779	337.0776	0.7	6.9	1, 2, 3, 4, 5, 6, 7*, 8, 9
13	Methyl hexosyl hexoside	$C_{13}H_{24}O_{11}$	2.88	238	355.1246	355.1246	-0.1	1.3	1, 2, 3*, 4, 5, 6, 7, 8, 9
15	Citric acid	$C_6H_8O_7$	3.18	280	191.0197	191.0206	-4.8	11.5	1*, 3, 4, 5, 6, 8, 9
16	Pyroglutamic acid	$C_5H_7NO_3$	3.41	212	128.0353	128.0363	-8.1	12.6	1*, 2, 3, 4, 5, 6, 7, 8, 9
17	L-leucine	$C6H_{13}NO_2$	3.88	219, 276	130.0874	180.0892	-14.2	5.2	1, 2, 4, 5, 6*, 7, 8, 9
19	L-tyrosine	$C_9H_{11}NO_3$	4.03	214, 275	180.0666	180.0680	-7.5	0.7	1*, 2, 3, 4, 5, 6, 7, 8, 9
28	Galloyl glucoside	$C_{13}H_{16}O_{10}$	4.80	272	331.0671	331.0673	0.7	6.8	1, 2, 3, 4, 5, 6, 7*, 8, 9
33	Galloyl glucoside II	$C_{13}H_{16}O_{10}$	5.35	272	331.0671	331.0660	3.3	6.6	1, 2, 3, 4, 5, 6, 7*, 8, 9
35	Gallic acid	$C_7H_6O_5$	5.62	270	169.0142	169.0152	-5.4	7.3	1, 2, 3, 4, 5, 6, 7*, 8, 9
36	Galloyl glucoside III	$C_{13}H_{16}O_{10}$	6.02	272	331.0671	331.0676	-1.7	5.2	1, 2, 3, 4, 5, 6, 7*, 8, 9
37	L-phenylalanine	$C_9H_{11}NO_2$	6.47	208, 236	164.0717	164.0721	-2.6	5.3	1, 2, 3, 4, 5, 6, 7*, 8, 9
38	L-phenylalanine hexoside	$C_{15}H_{21}NO_7$	6.84	213, 275	326.1249	326.1245	1.9	1.6	1, 6*
39	10-Hydroxyoleoside 7-methyl ester	$C_{18}H_{26}O_{12}$	6.88	220, 283	433.1351	433.1355	-0.8	3.2	1, 2, 3*, 4, 5, 6, 7, 8, 9
40	Galloyl glucoside IV	$C_{13}H_{16}O_{10}$	7.13	272	331.0671	331.0684	-3.9	9.8	1, 2, 3, 4, 5, 6, 7*, 8, 9
42	Pantothenic acid	$C_9H_{17}NO_5$	7.46	223, 242	218.1034	218.1042	-3.7	1.4	1, 2, 3, 4, 5, 6, 7*, 8, 9
43	Procyanidin B2	$C_{30}H_{26}O_{12}$	8.05	228, 280	577.1351	577.1363	-1.9	2.8	3*, 6, 8
45	L-tryptophan	$C_{11}H_{12}N_2O_2$	8.38	220, 270	203.0826	203.0832	-3.1	5.7	1*, 2, 3, 4, 5, 6, 7, 8, 9
46	L-tryptophan hexoside	$C_{17}H_{22}N_2O_7$	8.86	221, 239, 275	365.1354	365.1339	4.3	5.4	1, 6*
47	Catechin	$C_{15}H_{14}O_6$	9.33	228, 280	289.0718	289.0732	-5.0	11.6	1, 2, 3*, 5, 6, 7, 8, 9
49	Dihydroxyphaseic acid hexoside	$C_{21}H_{32}O_{10}$	9.57	230, 275	443.1933	443.1936	-3	12.6	1, 2, 3*, 4, 5, 6, 7, 8, 9
51	Eriocitrin/neoeriocitrin	$C_{27}H_{32}O_{15}$	10.35	280	595.1668	595.1660	1.4	2.4	1, 2, 3*, 4, 5, 6, 7, 8, 9
52	Tetrahydro-β-carboline-3-carboxylic acid	$C_{13}H_{13}N_2O_2$	11.02	245, 278	229.0983	229.0983	-0.2	1.2	1*
54	Vanillic acid hexoside	$C_{14}H_{18}O_9$	11.66	237, 278	329.0878	329.0878	-0.0	9.6	1, 3*, 5, 6, 7, 8, 9
55	Asperuloside	$C_{18}H_{22}O_{11}$	11.88	269	413.1089	413.1091	-0.4	10.5	1*

56	Dihydrophaseic acid	$C_{15}H_{22}O_5$	11.96	267	281.1394	281.1399	-1.7	11.9	1, 2, 3*, 4, 5, 6, 7, 8, 9
57	Naringenin hexoside	$C_{21}H_{22}O_{10}$	12.49	285	433.1140	433.1164	-5.5	12.6	3*, 4, 5, 6, 7, 8, 9
60	Phenethyl-β-primeveroside	$C_{19}H_{28}O_{10}$	12.96	223, 275	415.1610	415.1614	-1.0	9.5	1, 2, 3, 4, 5, 6*, 7, 8, 9
62	Peptide: L-lysine, L-serine, L-tyrosine/ L-serine, L-proline, L-proline, L-proline	$C_{18}H_{28}N_4O_6$	13.93	240, 280	395.1936	395.1950	-3.4	10.7	1, 2, 3, 4, 5, 6*, 7, 8, 9
63	Peptide: L-lysine, L-threonine, L-histidine	$C_{16}H_{28}N_6O_5$	14.10	240, 276	383.2048	383.2106	-15.1	14.2	1, 2, 3, 4, 5, 6*, 7, 8, 9
64	Myricetin-hexoside	$C_{21}H_{20}O_{13}$	14.18	260, 358	479.0831	479.0819	2.6	57.7	1, 3*, 6
65	Vanillic acid	$C_8H_8O_4$	14.72	290	167.0350	167.0362	-7.2	14.2	3*, 5, 7
66	Hesperidin/neohesperidin	$C_{28}H_{34}O_{15}$	15.94	277	609.1825	609.1824	0.2	17.8	2, 4, 5*, 6, 7, 8, 9
67	Quercetin-O-hexoside-gallate	$C_{28}H_{24}O_{16}$	15.96	265, 358	615.0992	615.0992	-0.1	8.4	3*, 5, 6, 7
68	Quercetin-3-O-glucoside	$C_{21}H_{20}O_{12}$	16.12	256, 355	463.0882	463.0904	-4.8	6.3	1, 2, 3*, 4, 5, 6, 7, 8, 9
69	Quercetin acetyl hexoside	$C_{23}H_{22}O_{13}$	17.26	259, 358	505.0998	505.104	-3.4	15.0	2, 3*, 4, 5, 7, 8, 9
70	Kaempferol -O-hexoside-gallate	$C_{28}H_{24}O_{15}$	18.10	265, 352	599.1042	599.1037	0.9	11.0	3*, 5, 6
71	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{11}$	18.43	265, 351	447.0933	447.0947	-3.2	4.8	1, 2, 3*, 4, 5, 6, 7, 8
72	Kaempferol acetyl hexoside	$C_{23}H_{22}O_{12}$	20.19	263, 355	489.1038	489.1031	1.6	20.3	2, 3*, 4, 5, 7, 8, 9

Table 16. Identified compounds in the persimmon juice extracts by HPLC-DAD-ESI-TOF/MS.

*Error, mσ and *m/z* exp. values are referred to this extract.


Extract 1
Extract 2
16
Extract 3
14
Extract 4
14
16

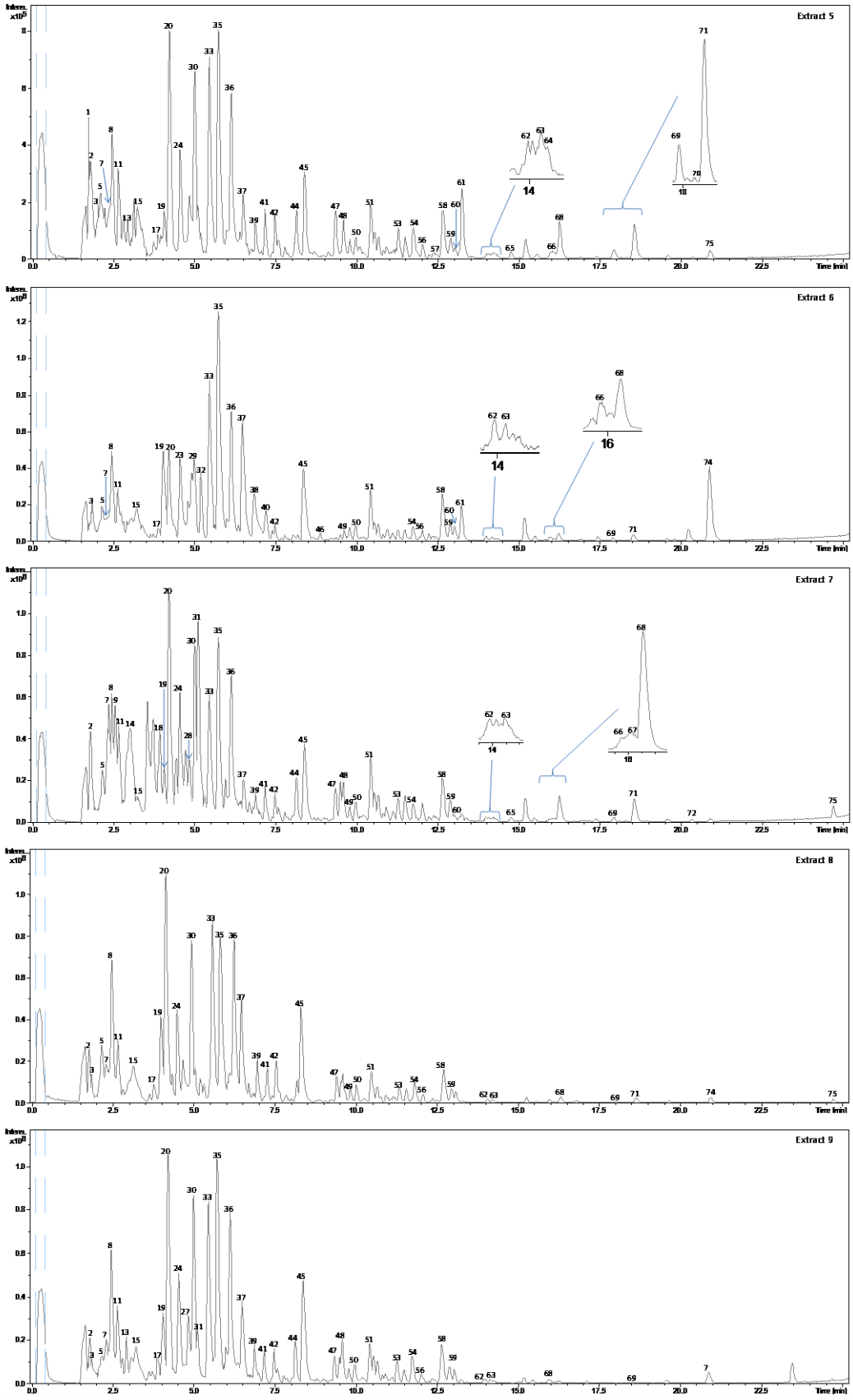


Figure 51. Base peak chromatograms of 1-9 persimmon juice extracts obtained by HPLC-DAD-ESI-TOF/MS.

3.1.1. Sugars

Peaks 1, 2, 4, 5, 10, and 13 were characterized as sugars. These compounds eluted earlier in the chromatographic process due to their highly hydrophilic character. Compounds 1, 2, and 3 had a deprotonated molecule at *m/z* 369, 193, and 165, respectively, and were identified as sugar acids (digalacturonic, galacturonic, and xylonic acid). These compounds showed low absorption in the UV/VIS region near to 230 nm, which is consistent with the data reported in the literature (Moreira *et al.,* 2010). Compound 4, found to be a disaccharide at *m/z* 341, was tentatively identified as sucrose, while peak 5 was designated as fructose by comparison to the respective standard. These compounds present in persimmon juices come from the flesh of the fruit, which has been shown to contain predominantly sucrose, fructose, and glucose (Giordani *et al.,* 2011). Compound 10 at *m/z* 503 was characterized as a trisaccharide, any combination of glucose or fructose being possible. Peak 13 at *m/z* 355 was identified as a methoxylated disaccharide, eluting later than its corresponding non-methoxylated form (2.12 and 2.88 min, respectively).

3.1.2. Protein derivatives

With regard to proteins, some amino acids were identified (peaks 19, 37, and 45) eluting from the chromatographic column at 4.03, 6.47, and 8.38 min, at *m/z* 180, 164, and 203, respectively. These compounds were identified as L-tyrosine, L-phenlyalanine and L-tryptophan. This identification was based on their MS data and elution order, and L-tyrosine was confirmed by the relevant standard. Peaks 62 and 63 at *m/z* 395 and 383 were characterized as peptides, considering the possibilities of L-lysine, L-serine, and L-tyrosine or L-serine, L-proline, L-proline, L-proline for the former and L-lysine, L-threonine, and L-histidine for the latter. Degradation compounds from amino acids were also detected in some samples corresponding to peaks 16, 38, and 46 at *m/z* 128, 326, and 365 and characterized as pyroglutamic acid, L-phenylalanine hexoside, and L-tryptophan hexoside.

3.1.3. Organic acids

Various organic acids and derivatives were found, more specifically malic acid, citric acid, dihydrophaseic acid hexoside, and dyhydrophaseic acid, corresponding to peaks 7, 15, 49, and 56, ranked by elution order. They yielded deprotonated molecules at *m/z* 133, 191, 443, and 281, respectively. Malic and citric acids presented absorbance maxima at 230 and 245, and 280 nm, respectively. This is consistent with the metabolite analysis performed in persimmon fruits by Veberic *et al.* (2010). Moreover, the presence of citric acid was confirmed by injection of the authentic standard. Dihydroxyphaseic acid hexoside presented a higher polarity due to the greater number of hydroxyl groups provided by the hexose and, therefore, eluted earlier (9.57 min) than the non-glycosylated form dihydrophaseic acid (11.96 min). These compounds are involved in the abscisic acid metabolism and hence related to environmental stress tolerance in plants (Setha *et al.,* 2004). Other authors have found these compounds in other fruits (Hurtado-Fernández *et al.,* 2011).

3.1.4. Vitamins

It is well known that the predominant vitamin in persimmon fruit is vitamin C, existing as two vitamers: L-ascorbic acid and dehydro-L-ascorbic acid (Giordani *et al.,* 2011). However, in the persimmon juices studied here, neither of these forms was found, except for the glycosylated form of ascorbic acid at *m/z* 337 (peak 11), in this case the 6-atom-monosaccharide linked to the vitamer provides the molecule a more hydrophilic character, eluting at the beginning of the chromatographic run, soon after the sugars. This form might come from the fruit, as it has previously been reported in other fruits and vegetables (Toyada-Ono *et al.,* 2005; Hancock, Chudek, Walker, Pont & Viola, 2008). However, as the glucosylated form of ascorbic acid is more stable than its aglycone form (Kanawati, Von Saint Paul, Herrmann, Schäffner & Schmitt-Kopplin, 2011), the latter might have been degraded and only the former was detected. Another water-

soluble vitamin designated as pantothenic acid was found at *m/z* 218, eluting at 7.46 min. This is the first report of the presence of this vitamin in persimmon fruit.

3.1.5. Phenolic compounds

Among phenolic compounds in fully ripe persimmon fruit, simple phenolic compounds as well as polymerized flavan-3-ols (tannins or procyanidins) are reported in the literature (Veberic *et al.,* 2010; Akagi *et al.,* 2011; Del Bubba *et al.,* 2009; Gu *et al.,* 2008).

In the former group, phenolic compounds belonging to the group of flavonoids and phenolic acids are present. All phenolic acids detected in persimmon juice extracts belonged to the hydroxybenzoic acid subclass, i.e. gallic acid and vanillic acid. Compound 35 was considered gallic acid, as it eluted at 5.62 min and presented absorbance maxima at 218 and 270 nm, in accordance with the literature (Engels *et al.,* 2012). Furthermore, retention time, MS, and UV-Vis characteristics were similar to those of the authentic standard. Four compounds (peaks 28, 33, 36 and 40) with same molecular formula at *m/z* 331 in the MS spectra were detected at 4.80, 5.35, 6.02, and 7.13 min, indicating the occurrence of isomeric structures that significantly differ in their elution behaviour. These isomeric compounds were tentatively identified as galloyl esters of glucose, with highly correlated UV spectra, which are also similar to gallic acid. These molecules may come from the hydrolysis of gallotannins composed of a glucose core esterified with gallic acid residues, which have previously been reported in other fruits such as pomegranate or mango (Fischer *et al.,* 2011; Barreto *et al.,* 2008) but never in persimmon. Compound 65 was characterized as vanillic acid, since it eluted later and had a methyl group in its structure, giving it a less soluble character. In addition, its retention time was comparable to that of the relevant standard. It presented an absorbance maximum at 290 nm, in accordance with existing data (Fischer *et al.,* 2011). Its glycosylated form (vanillic acid hexoside) eluted from the column earlier than the non-glycosylated form (11.66 min) as a result of the contribution of the hydroxyl groups

provided by the hexose. Only one compound belonging to the subclass flavanol was present, and this was designated as catechin (peak 47), eluting at 9.33 min, by comparison to the corresponding standard. Within the flavonol subclass, eight compounds were detected (peaks 64, 67, 68, 69, 70, 71, 72, and 73), i.e. myricetin hexoside, quercetin hexoside gallate, quercetin hexoside, quercetin acetyl hexoside, kaempferol hexoside gallate, kaempferol hexoside, and kaempferol acetyl glucoside, respectively, which share the common skeleton of the flavonol nucleus but vary in the number and structure of their hydroxyl and methoxyl moieties. As expected in reverse-phase liquid chromatography, hexoside gallate derivatives eluted earlier because of the larger numbers of hydroxyl groups, followed by hexoside forms, and finally by the less polar acetyl hexoside forms. In addition, the elution behaviour agreed with the polarity of the flavonol moieties, the more hydroxyl groups they possessed, the earlier they eluted from the columns and, therefore, myricetin derivatives eluted first, followed by quercetin derivatives and finally by kaempferol derivatives. The presence of quercetin-3-O-glucoside, and kaempferol-3-O-glucoside was confirmed with the corresponding standards. Furthermore, they all presented absorbance maxima peaks typical of flavonols between 253-265 and 352-360. Aglycones of the flavonols were not detected but three flavanones were found (peaks 51, 57, and 66), these being considered eriocitrin/neoeriocitrin, naringenin hexoside, and hesperidin, respectively. These compounds showed a $[M-H]^-$ ion at *m/z* 595, 433, and 609, respectively, and all presented a maximum absorbance peak between 275 and 289 nm, typical of flavanones. The assignment of hesperidin was confirmed with the respective standard.

Among the second group, polymerized flavan-3-ols were present only with a low degree of polymerization (up to the dimer). Compound 43 had an *m/z* value at 577, indicative of a B-type procyanindin dimer. This compound showed absorbance maxima at 280 nm, consistent with data from other authors (Liu *et al.,* 2010).

3.1.6. Other compounds

Other compounds, not belonging to any of the previous groups, were identified on the basis of their mass spectrometry spectra and their elution time. Compound 39 at *m/z* 433 was found to be 10-hydroxyoleoside-7-methyl ester, a compound that exists as a metabolite in fruits (Guo *et al.,* 2011). Compound 52 at *m/z* 229 was identified as tetrahydro-β-carboline-3-carboxylic acid, a biologically active alkaloid found as a naturally occurring substance in some fruit and fruit juices that might act as an antioxidant when absorbed and accumulated in the body (Herraiz & Galisteo, 2003; Herraiz, 2000). Compound 55 had a deprotonated molecule at *m/z* 413 and was characterized as asperuloside, a terpenoid previously found in fruits (Su *et al.,* 2005). Compound 60 was found to be phenetyl-β-primeveroside, previously found in plants (Fernández-Arroyo *et al.,* 2010).

3.1.7. Unknown compounds

Although ions may undergo a modest degree of in-source decomposition in the electrospray ionization source, additional information on post-source fragmentations in tandem MS experiments is required to elucidate the identity of unknown compounds. A number of compounds (30) remained unknown, as indicated in Table 17. The information provided by retention, time, UV absorption, and mass spectra was not sufficient to determine their identity. Among these, compounds with very different retention times but similar *m/z* were observed for compounds at *m/z* 643 (three peaks), 555 (four), 645 (four), 647 (two), 745 (two), 373 (two), and 381 (two). This indicates the presence of isomeric forms.

Peak	Proposed compound	Molecular formula	RT	λ	*m/z* calc.	*m/z* exp.	Error (ppm)	mσ	Extracts present
6	Unknown 643	$C_{24}H_{36}O_{20}$	2.37	212, 331	643.1727	643.1749	-3.3	2.7	3*, 4, 5, 7, 8, 9
8	Unknown 605	$C_{22}H_{38}O_{19}$	2.44	211	605.1935	605.1942	-1.2	1.8	1, 2, 3, 4*, 5, 6, 7, 8, 9
9	Unknown 643 II	$C_{24}H_{36}O_{20}$	2.54	232, 328	643.1727	605.1731	-0.7	6.1	4, 5, 7*, 8, 9
12	Unknown 541	$C_{19}H_{26}O_{18}$	2.86	213, 240	541.1046	541.1043	0.7	7.7	2, 4*, 7
14	Unknown 534	$C_{22}H_{21}N_3O_{13}$	3.03	255	534.1002	534.0992	1.8	43.8	7*
18	Unknown 555	$C_{20}H_{28}O_{18}$	3.92	209, 236	555.1203	555.1211	-1.4	3.8	2, 4*, 7, 8, 9
20	Unknown 645	$C_{24}H_{38}O_{20}$	4.20	211, 273	645.1884	645.1891	-1.2	2.9	1, 2, 3*, 4, 5, 6, 7, 8, 9
21	Unknown 555 II	$C_{20}H_{28}O_{18}$	4.27	209, 236	555.1203	555.1208	-0.9	4.6	2, 4*, 7, 8, 9
22	Unknown 731	$C_{26}H_{36}O_{24}$	4.37	211, 252	731.1524	731.1540	-2.2	3.1	2*, 4, 7, 8, 9
23	Unknown 645 II	$C_{24}H_{38}O_{20}$	4.50	211, 273	645.1884	645.1871	1.9	3.4	1, 2, 3*, 5, 6, 8, 9
24	Unknown 647	$C_{24}H_{40}O_{20}$	4.52	213, 281	647.2040	647.2024	2.5	3.8	1, 2, 3, 4, 5, 6, 7, 8*, 9
25	Unknown 555 III	$C_{20}H_{28}O_{18}$	4.55	209, 236	555.1203	555.1200	0.5	1.3	2, 4*, 7, 8, 9
26	Unknown 731 II	$C_{26}H_{36}O_{24}$	4.64	211, 252	731.1524	731.1545	-2.9	1.4	2*, 4, 7, 8, 9
27	Unknown 555 IV	$C_{20}H_{28}O_{18}$	4.73	209, 236	555.1203	555.1202	0.1	9.5	2, 4*, 7, 8, 9
29	Unknown 645 III	$C_{24}H_{38}O_{20}$	4.87	211, 273	645.1884	645.1872	1.9	17.4	1, 3*
30	Unknown 645 IV	$C_{24}H_{38}O_{20}$	4.99	211, 273	645.1884	645.1872	1.7	14.4	1, 3*, 4, 5, 6, 7, 8, 9
31	Unknown 745	$C_{27}H_{38}O_{24}$	5.10	213, 235, 283	745.1680	745.1683	-0.3	1.5	2, 4*, 7, 8, 9
32	Unknown 647 II	$C_{24}H_{40}O_{20}$	5.14	213, 281	647.2040	647.2049	-1.3	3.3	1, 5, 6*, 8, 9
34	Unknown 745 II	$C_{27}H_{38}O_{24}$	5.44	213, 275	745.1680	745.1678	0.3	4.7	4*, 7, 8, 9
41	Unknown 405	$C_{16}H_{22}O_{12}$	7.14	216, 267	405.1038	405.1050	-2.9	2.1	1, 2, 3*, 4, 5, 6, 7, 8, 9
44	Unknown 373	$C_{15}H_{18}O_{11}$	8.11	225, 272	373.0776	373.0787	-3.0	10.2	2, 3*, 4, 5, 6, 7, 8, 9
48	Unknown 373 II	$C_{15}H_{18}O_{11}$	9.57	225, 272	373.0776	373.0787	-2.8	7.2	2, 3*, 4, 5, 7, 8, 9
50	Unknown 367	$C_{15}H_{28}O_{10}$	9.97	239, 280	367.1610	367.1613	-0.9	2.2	3*
53	Unknown 461	$C_{19}H_{26}O_{13}$	11.26	280	461.1301	461.1319	-4.0	3.6	2, 3*, 4, 5, 6, 7, 8, 9
58	Unknown 381	$C_{16}H_{30}O_{10}$	12.60	247, 288	381.1766	381.1779	-3.4	8.5	1, 3*, 4, 5, 6, 7, 8, 9
59	Unknown 381 II	$C_{16}H_{30}O_{10}$	12.83	247, 288	381.1766	381.1766	-0.1	2.2	1, 2, 3*, 4, 5, 6, 7, 8, 9
61	Unknown 613	$C_{27}H_{34}O_{16}$	13.23	239, 287	613.1774	613.1796	-3.5	2.4	5*, 6, 7, 8, 9
73	Unknown 737	$C_{42}H_{74}O_{10}$	20.81	240, 278	737.5209	737.5162	6.5	18.5	2, 4, 5, 6*, 7, 8, 9
74	Unknown 301	$C_{16}H_{30}O_5$	24.65	254	301.2020	301.2033	-4.2	2.4	2, 4, 5, 6*, 7, 8, 9

Table 17. Unknown compounds found in the persimmon juice extracts by HPLC-DAD-ESI-TOF/MS. *Error, mσ and *m/z* exp. values are referred to this extract.

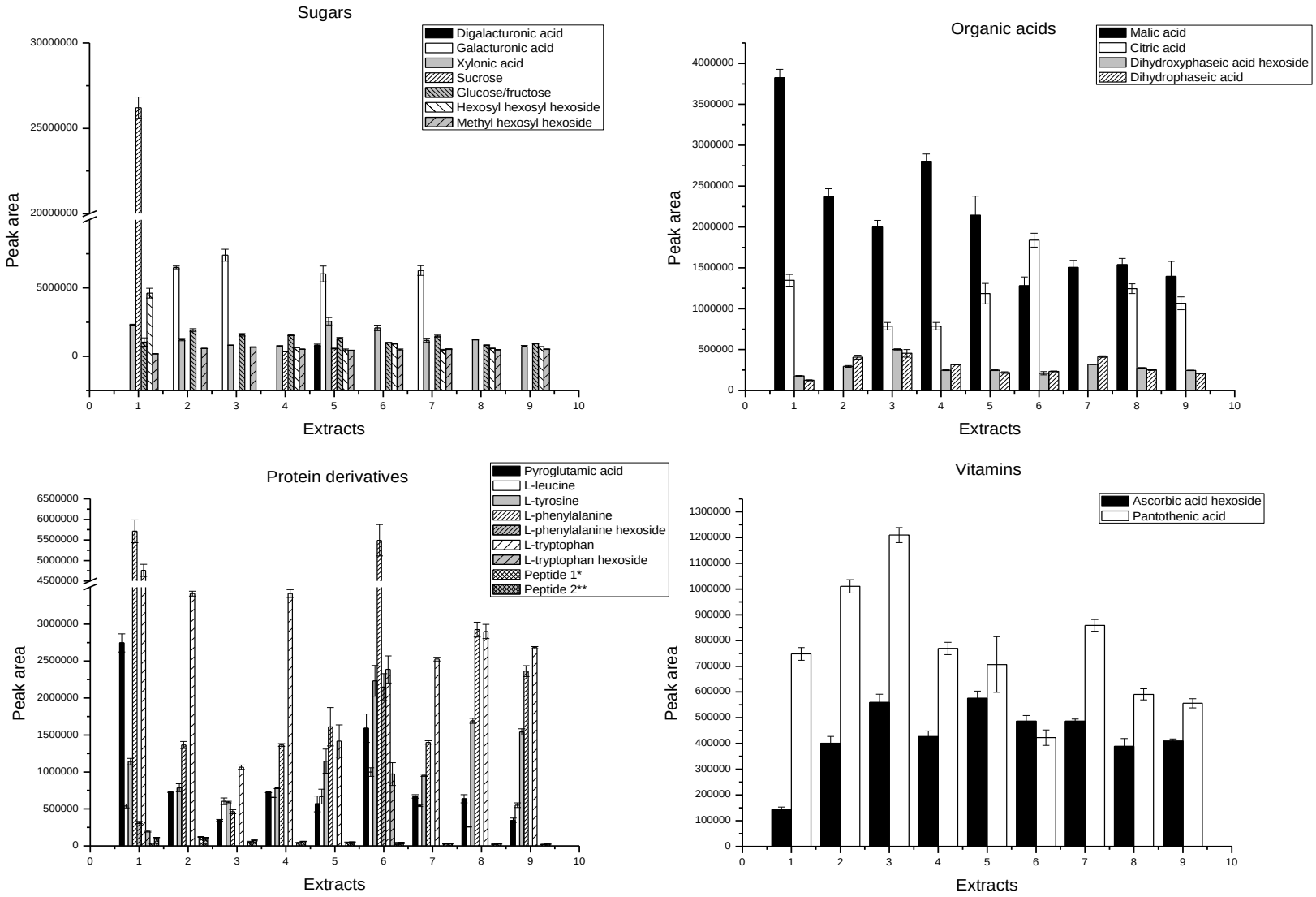


Figure 52. Variations of sugars, organic acids, protein derivatives and vitamins in terms of peak area for each compound in all persimmon juice extracts (1-9).

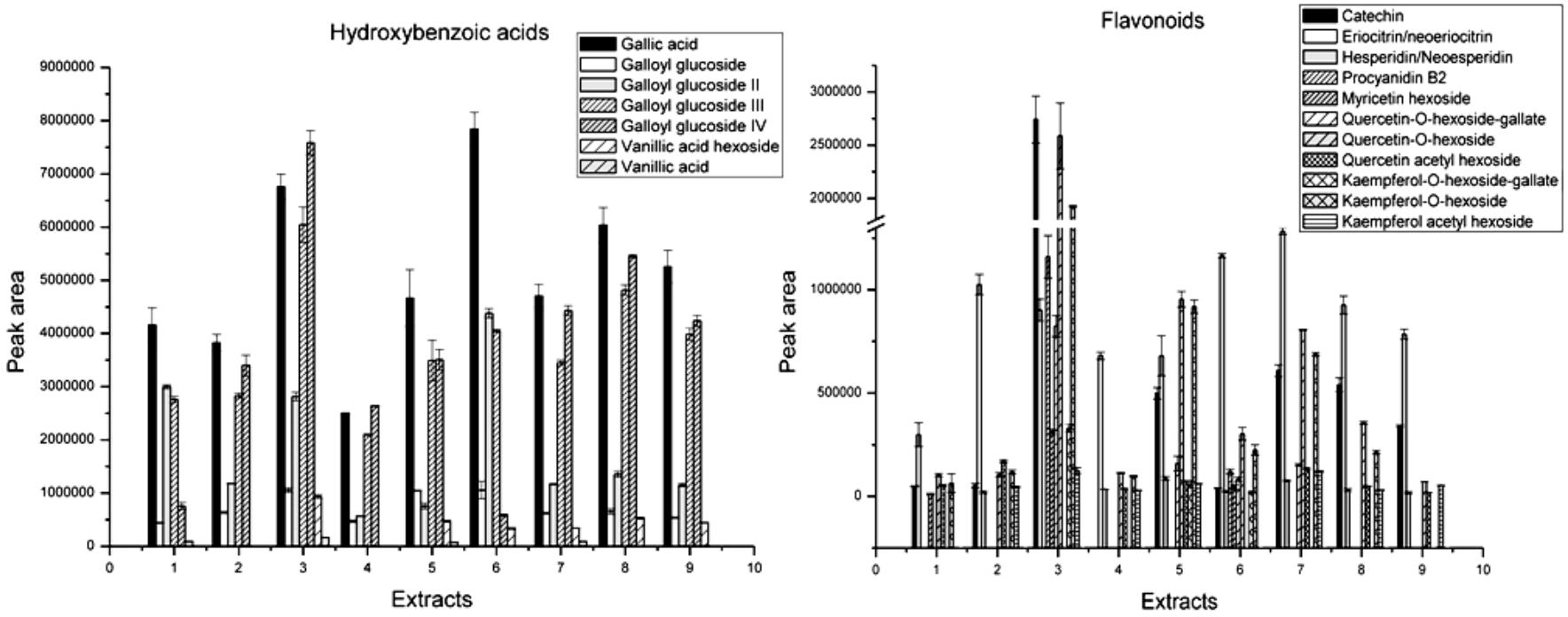


Figure 53. Variations of phenolic compounds (hydroxybenzoic acids and flavonoids) in terms of peak area for each compound in all persimmon juice extracts (1-9).

3.2. Relationship between the phenolic and other polar compounds and the technology production applied

Once the chemical composition of the different extracts was established, the technological processing applied to the different juices was evaluated in terms of compositional variations. These differences are illustrated in Figures 52 and 53 by means of comparisons of the area of the different compounds in all the extracts.

3.2.1. Changes in sugars

Sugar acids (digalacturonic acid and galacturonic acid) were not present in all the samples. Galacturonic acid was present in samples 2, 3, 5, and 7, and digalacturonic acid was present in sample 5. These compounds are naturally found in persimmon juices as they are constituents of pectins, composed of repeat units of galacturonic acid in which carboxyl groups of galacturonic acid may either remain as free acids, be esterified with methanol, or be neutralized with cations (Moreira *et al.,* 2010). The presence of these compounds is due to the natural persimmon ripening, in which the activity of the hydrolytic enzymes of the cell wall like pectin methylesterase causes fruit softening, characterized by the solubilization and depolymerization of pectic polysaccharides (Souza *et al.,* 2011). The different pectinases used for clarification, may contribute as well to the presence of pectin monomers and dimers in the juice. Xylonic acid was present in all samples, previously found as a metabolite in plants (García-Villalba *et al.,* 2008; Qin *et al.,* 2009). Regarding disaccharides and trisaccharides, a considerably higher amount was found in sample 1, corresponding to the less-treated sample, and this had not undergone clarification or the astringency-removal processes, which may have led to their hydrolysis. In addition, invertase activity might result from the release of the enzyme from specific vacuoles during these processes, facilitating the conversion of sucrose into glucose and fructose and

therefore being higher in the rest of the samples. No difference was observed for the methylated sugar.

3.2.2. Changes in protein derivatives

Amino acids and peptides were present in all samples. Although persimmons do not usually represent a major dietary source for amino acids and proteins, these are present in the fruit and hence in the juice. Amino acids were present in higher amounts in samples 1 and 6; again the juice underwent less intense processing than other extracts. Our results show only a slight variation for peptides. Some degradation products were detected. Pyroglutamic acid as glutamine and glutamic acid degradation product (Montevecchi *et al.,* 2011) was present in all samples, being predominant in sample 1. Phenylalanine-hexoside and tryptophan-hexoside were present in samples 1 and 6, both underwent pasteurization, a process that may have led to their formation. These products are formed by a condensation between the free amino group of an amino acid, peptide, or protein and the carbonyl group of a reducing sugar, leading to the formation of Amadori compounds in the early stages of the Maillard reaction (Del Castillo *et al.,* 1999). These generally arise before sensory changes become noticeable and it should be noted that the extent of the early Maillard reaction is low for heat-treated products with high water contents such as juices. For such samples the Maillard reaction does not have a significant impact on the nutritional quality of the final product (Wellner *et al.,* 2011).

3.2.3. Changes in organic acids

No significant differences were found in dihydroxyphaseic acid and dihydroxyphaseic acid hexose contents among samples. Citric acid was found in all samples except for number 2 and 7, while malic was found in all samples. As reflected in Figure 2 (a), extract 1 yielded higher amounts of organic acids.

3.2.4. Changes in vitamins

The values for the amounts of vitamins showed little fluctuation in response to the processing and were present in all samples. There was a slight tendency to higher pantothenic acid concentrations in sample 3 and higher ascorbic acid hexoside in samples 3 and 5.

3.2.5. Changes in phenolic compounds

Due to the assumed association with health-related properties, many industrial juices aim at high yields of phenolics. In this case the phenolic content varied among the different samples according to the technological approach applied, as illustrated in Figure 3. Generally, sample 1 presented lower amounts of phenolic compounds (hydroxybenzoic acids as well as flavonoids). This can be attributed to the fact that phenolic compounds are located within the vacuole of the plant cell, enclosed by tonoplast and cytoplasmic lipid membranes, which are in turn encapsulated by the plant cell wall (cellulose and pectin) (Rodríguez *et al.,* 2004; Padayachee *et al.,* 2012). Sample 1 was pasteurizated after pressing and received no other treatment, and although processing may release a certain amount of phenolic compounds from the cell wall, many may remain attached and, thus, were not detected. Sample 1 also contained more amino acids. This may have resulted in lower amounts of phenolic compounds, as it is well known that phenolic compounds interact with proteins turning juices and other type of beverages turbid (Siebert, 1999).

On the other hand, sample 3 presented the most phenolic compounds. This juice was submitted to clarification in addition to the processes undertaken for sample 1. This clarification step by means of enzyme addition may have led to the release of the phenolic compounds attached to the cell wall.

Compounds such as procyanidin B2 appeared only in samples 3 and 6, neither of which was treated to remove astringency. This process implies the condensation or polymerization of soluble tannins or procyanidins (which cause the astringent taste) into insoluble, non-astringent forms, by acetaldehyde produced in the flesh during treatment with CO_2.-During this process complex formation between pectins and tannins is possible (Taira *et al.,* 1997), though not shown in the samples.

4. Conclusions

A complete analytical characterization of extracts of nine persimmon juices produced using different technologies was performed with HPLC-DAD-ESI-TOF/MS for the first time. Persimmon juice extracts were found to be a source of sugars (sugar acids, disaccharides, and trisaccharides), protein derivatives (amino acids and derivatives), organic acids (malic, citric, and others), vitamins (ascorbic acid derivatives and pantothenic acid) and phenolic compounds including simple phenolic compounds and polymerized flavan-3-ols. Simple phenolic compounds consisted of phenolic acids i.e. gallic acid and galloyl esters of glucose and flavonoids (flavanols such as (epi)catechin, flavonols such as myricetin, quercetin, and kaempferol derivatives and flavanones such as eriocitrin/neoeriocitrin). Polymerized flavan-3-ols included a B-type procyanidin dimer. Some of these compounds have been described for the first time in persimmon products. A preliminary approach assessing compositional variations arising from different technological processing was performed. A marked influence was observed on the composition of the juices. Extracts 3 and 7 (undergoing the combinations of clarification and centrifugation, and astringency removal, centrifugation, and pasteurization, respectively) contained more phenolic compounds, which are thought in part to be responsible of protective effect against chronic diseases. These results pose the necessity of evaluating quantitative

variations for a better understanding of the impact of technology on fruit-juice production as well as further studies on the scaling-up of such technology.

5. Acknowledgements

Authors are grateful to the Spanish Ministry of Education and Science for the scholarship FPU AP2010-2793 and for the Project AGL2011-29857-C03-02, as well as to the Andalusian Regional Government Council of Innovation and science for the projects P09-CTS-4564, P10-FQM-6563 and P11-CTS-7625 as well as for the project GREIB.PT.2011.18.

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Pk.	RT	m/z calc.	Proposed compound	Peak area								
				1	2	3	4	5	6	7	8	9
1	1.76	369.0675	Digalacturonic acid	ND	ND	ND	ND	811245±82397	ND	ND	ND	ND
2	1.81	193.0354	Galacturonic acid	ND	6499601±102291	7401532±435484	ND	6028729±576722	ND	6277614±357891	ND	ND
3	1.83	165.0405	Xylonic acid	2310856±35884	1225540±83867	813002±9568	748921±39513	2562273±268157	2083799±200259	1173953±147718	1218024±20714	736224±54415
4	2.12	341.1089	Sucrose	26209584±626329	ND	ND	341122±20790	563499±28457	ND	ND	ND	ND
5	2.14	179.0571	Fructose	861303±26771	1378092±1037355	1573479±99156	1558676±42897	1327723±60558	999346±19772	1480262±73681	814649±26236	945581±9964
6	2.37	643.1727	Unknown 643	ND	ND	1959437±53071	119655±31284	137608±20952	ND	809825±58304	ND	248605±2696
7	2.41	133.0142	Malic acid	3826410±102270	2370071±98359	1998710±82061	2804021±89057	2142872±234475	1281167±107121	1505106±86188	1538505±77357	1395436±183911
8	2.44	605.1935	Unknown 605	71332±19537	872990±105493	941680±113091	1663085±99239	1657077±164102	1722543±87086	1840619±99288	1287603±91119	1506673±72187
9	2.54	643.1727	Unknown 643 II	ND	ND	ND	167461±21852	139423±24732	ND	1671015±88474	496132±38340	356717±19542
10	2.59	503.1618	Hexosyl hexosyl hexoside	4622415±361540	ND	ND	656903±13315	462571±81474	927856±36381	460703±48833	581838±12302	692153±16429
11	2.64	337.0779	Ascorbic acid hexoside	143415±9539	400733±26437	559373±31569	427001±21871	575608±27959	486272±22052	486332±8555	388652±30436	409207±8662
12	2.86	541.1046	Unknown 541	ND	3073623±34209	ND	3532504±739023	ND	ND	2039407±19800	ND	ND
13	2.88	355.1246	Methyl hexosyl hexoside	186520±13682	577180±11608	676944±23514	522700±9202	432000±27638	476058±69873	528223±31971	475561±1462	521744±20253
14	3.03	534.1002	Unknown 534	ND	ND	ND	ND	ND	ND	3057379±200246	ND	ND
15	3.18	191.0197	Citric acid	3245347±190683	ND	1347761±71474	787878±45228	1184975±124765	1838771±84776	ND	1244815±59895	1066025±78398
16	3.41	128.0353	Pyroglutamic acid	2745965±122509	729624±7930	346903±10381	591270±10593	568726±107017	1591113±192714	669208±22769	637455±54719	345386±30244
17	3.88	130.0874	L-leucine	540274±25200	603360±45703	ND	655115±1383	666423±100387	998086±58504	546406±12933	258854±4111	550040±31606
18	3.92	555.1203	Unknown 555	ND	4539569±464097	ND	4714908±71906	ND	ND	1607878±48916	102388±1856	137940±4038
19	4.03	180.0666	L-tyrosine	1140589±43482	785966±53664	593174±10124	619011±9952	1145790±163043	2232037±208973	954521±14304	1691402±34355	1542114±41640
20	4.20	645.1884	Unknown 645	554758±6413	3206050±222936	1054836±9133	2742482±493970	2564086±277845	1555408±202005	4863341±102825	4897344±122834	4190619±52640
21	4.27	555.1240	Unknown 555 II	ND	4163121±202414	ND	5111958±113397	ND	ND	1408549±40840	119908±4309	168766±5523
22	4.37	731.1524	Unknown 731	ND	1953319±116158	ND	1674854±60982	ND	ND	1318065±30416	58675±10829	70799±12627
23	4.50	645.1884	Unknown 645 II	870332±21704	1910538±111788	860748±110993	ND	941509±94136	2004980±104304	ND	1708917±48532	1389055±78089
24	4.52	647.2040	Unknown 647	252035±41750	1250785±55347	1304360±308052	1116959±53018	1153481±94136	854409±115409	2137722±8250	2389052±40886	1978814±75166
25	4.55	555.1203	Unknown 555 III	ND	2217126±72865	ND	3497809±51784	ND	ND	1324470±37541	172155±5360	210386±4364
26	4.64	731.1524	Unknown 731 II	ND	2130779±105268	ND	1563907±11063	ND	ND	1288238±82488	141651±6356	142394±17746
27	4.73	555.1203	Unknown 555 IV	ND	1687617±238903	ND	2822396±59301	ND	ND	962629±64324	182551±1848	198827±19678
28	4.80	331.0671	Galloyl glucoside	441814±5643	635036±17900	1055616±44126	469825±23889	1043647±12790	1058305±164455	623983±18973	651674±49037	541212±15841
29	4.87	645.1884	Unknown 645 III	634005±53353	ND	453881±30967	ND	ND	ND	ND	ND	ND
30	4.99	645.1884	Unknown 645 IV	711637±51805	ND	1055656±46976	1623415±8772	2413585±395861	3007500±68683	3169890±67310	4810980±122289	3967939±56026
31	5.10	745.1680	Unknown 745	ND	3888689±131599	ND	4771642±87773	ND	ND	3846381±21315	476994±26746	801812±30431
32	5.14	647.2040	Unknown 647 II	464316±15683	ND	ND	ND	273063±60140	1080476±111332	ND	369218±36938	294135±14422
33	5.35	331.0671	Galloyl glucoside II	3000924±37462	1179798±9423	2816487±77423	568048±7208	758836±54622	4382802±86807	1164698±13297	1354656±52965	1153848±33006
34	5.44	745.1680	Unknown 745 II	ND	ND	ND	3435872±85774	ND	ND	2612608±32707	341978±23010	601265±4253
35	5.62	169.0142	Gallic acid	4157587±327145	3827600±153057	6755182±238250	2469156±19900	4665432±532769	7844918±310920	4706310±213800	6038232±327626	5257891±305555
36	6.02	331.0671	Galloyl glucoside III	2757343±57654	2832246±46619	6044637±333807	2092855±17887	3491252±382548	4047872±23556	3450493±52358	4816705±93872	3982816±113970
37	6.47	164.0717	L-phenylalanine	5711385±277431	1365146±47095	463131±27466	1160006±19469	1611623±259774	5491656±380444	1398573±23566	2924656±100521	2363935±73854
38	6.84	326.1249	L-phenylalanine hexoside	317635±14211	ND	ND	ND	ND	2149128±181789	ND	ND	ND
39	6.88	433.1351	10-Hydroxyoleoside 7-methyl ester	445930±9208	408793±2503	1138548±12265	385658±8567	487290±54013	600777±9832	568995±2078	796619±19960	613474±14507
40	7.13	331.0671	Galloyl glucoside IV	757489±66816	3399473±195000	7583654±226458	2635531±7366	3505753±192546	581156±22572	4432631±90321	5450846±28538	4237832±103740
41	7.14	405.1038	Unknown 405	256830±10544	424888±27770	827722±11096	390119±14603	520295±5304	389874±46781	548331±6237	429108±9033	387381±6296
42	7.46	218.1042	Pantothenic acid	747835±24610	1010452±26103	1209419±29189	769051±23564	706811±107745	422827±29917	858655±22638	590772±21539	556265±17835
43	8.05	577.1363	Procyanidin B2	ND	ND	1159087±103020	ND	ND	121315±12154	ND	67883±692	ND

44	8.11	373.0776	Unknown 373	ND	859740±41451	692402±17555	618888±15736	546573±14970	70251±5003	794993±4927	688692±10377	616930±7493
45	8.38	203.0832	L-tryptophan	4760478±146044	3412320±32530	1065115±29133	2781598±52315	1417936±217723	2386579±182474	2527194±25058	2900189±95561	2682247±13979
46	8.86	365.1339	L-tryptophan hexoside	198976±10775	ND	ND	ND	ND	972222±154115	ND	ND	ND
47	9.33	289.0732	Catechin	48941±2833	52714±10612	2742244±220883	ND	498964±27470	39733±1180	608789±28175	539328±33892	339853±5668
48	9.57	373.0776	Unknown 373 II	ND	1015520±55722	1069949±42956	949908±22808	699780±32346	ND	1045417±3160	1199354±23155	728188±540880
49	9.57	443.1936	Dihydroxyphaseic acid hexoside	179993±4629	294868±9798	501025±10650	248663±4531	248485±4892	211093±19285	320159±3140	277614±2687	245349±2530
50	9.97	367.1610	Unknown 367	ND	ND	459770±28826	ND	ND	ND	ND	ND	ND
51	10.36	595.1660	Eriocitrin/neoeriocitrin	282124±31900	1025173±49091	901759±52930	680495±18024	680381±4892	1165364±10436	1283499±16445	926077±42871	785217±24617
52	11.02	229.0983	Tetrahydro-β-carboline-3-carboxylic acid	1033136±93470	ND	ND	ND	ND	ND	ND	ND	ND
53	11.26	461.1301	Unknown 461	ND	252417±19682	563495±38635	234693±10964	445467±21609	244908±11525	438317±7044	408985±18802	348727±7160
54	11.66	329.0878	Vanillic acid hexoside	88344±6842	ND	936696±34974	ND	472393±21049	337284±17544	344096±4804	529466±14226	446045±2201
55	11.88	413.1091	Asperuloside	44814±2724	ND	ND	ND	ND	ND	ND	ND	ND
56	11.96	281.1399	Dihydrophaseic acid	125554±5391	408056±24597	455102±46330	317562±1902	219698±8928	233677±4170	415590±9105	252721±6803	209408±1157
57	12.49	433.1164	Naringenin hexoside	ND	ND	86149±2549	24428±1041	60512±646	56764±2405	68208±1994	44874±1963	31262±3207
58	12.60	381.1766	Unknown 381	707155±28918	ND	1013285±99460	1031227±7246	972574±79144	1250309±147688	1036503±37315	1048216±4027	829946±29386
59	12.83	381.1766	Unknown 381 II	375311±23585	680650±29055	623761±27434	536285±8289	485004±37424	534948±58399	631584±30071	540790±30303	440359±34401
60	12.96	415.1614	Phenethyl-β-primeveroside	161138±21445	261307±14884	263237±51480	210372±3189	230900±11874	472227±32180	233172±7195	203878±6676	159719±2059
61	13.23	613.1774	Unknown 613	ND	ND	ND	ND	1220038±160218	1017628±81503	103902±3355	30748±3199	27217±2721
62	13.93	395.1950	Peptide: L-lysine, L-serine, L-tyrosine/L-serine, L-proline, L-proline, L-proline	37000±1603	121437±4794	117046±9451	114828±1917	98939±5291	120096±16017	101510±2466	108633±6064	82634±2269
63	14.10	383.2106	Peptide: L-lysine, L-threonine, L-histidine	108251±7277	109597±6061	110690±6809	116832±2876	90139±4434	110395±11525	93787±5239	106683±4811	86213±2563
64	14.18	479.0819	Myricetin-hexoside	13304±1218	ND	312188±11777	ND	ND	44624±10481	ND	ND	ND
65	14.72	167.0362	Vanillic acid	ND	ND	165635±3434	ND	72882±8423	ND	89430±402	ND	ND
66	15.94	609.1825	Hesperidin/neohesperidin	ND	20363±5598	ND	34045±273	86582±9042	24708±3939	75875±5091	30151±6406	18422±4742
67	15.96	615.0992	Quercetin-O-hexoside-gallate	ND	ND	823923±51545	ND	158744±35260	83381±10150	152173±5185	ND	ND
68	16.12	463.0882	Quercetin-3-O-glucoside	103349±7337	105221±9858	2586923±309631	112730±2180	954105±37907	302524±31125	806644±3496	356084±6350	71013±384
69	17.26	505.0998	Quercetin acetyl hexoside	ND	53893±4668	170054±5844	36770±3050	75016±907	ND	134069±3653	49885±2479	20741±536
70	18.10	599.1042	Kaempferol -O-hexoside-gallate	ND	ND	329639±18808	ND	58763±16913	20267±4648	ND	ND	ND
71	18.4	447.0933	Kaempferol-3-O-	63706±45299	118870±9412	1924874±7780	97089±2563	918149±31894	224729±24670	688729±8273	214816±8299	ND

	3		glucoside									
72	20.19	489.1038	Kaempferol acetyl hexoside	ND	45413±4171	125065±14990	27759±2565	60553±1099	ND	120902±3778	30407±1329	52302±951
73	20.81	737.5209	Unknown 737	ND	193087±27436	ND	678202±28095	157932±50697	2346131±461651	90889±1319	99054±7342	202112±3855
74	24.65	301.2020	Unknown 301	ND	1062408±93691	15017±2235	711906±9388	166563±11496	79551±12657	801298±9169	231065±13904	181370±22821

Table S1. Peak area of the identified compounds in all persimmon juice samples by HPLC-DAD-ESI-TOF/MS.

Conclusions

The following general and specific conclusions can be drawn from the work conducted in this dissertation.

General conclusions:

1. Green asparagus (*Asparagus officinalis*), marula bark (*Sclerocarya birrea*), extra-virgin olive oil, olive leaf (*Olea europaea*), and persimmon juice (*Diospyros kaki*) plant matrixes represent valuable sources of bioactive compounds for future applications in developing functional foods and nutraceuticals.

2. The application analytical methodologies based on based on liquid-liquid extraction , solid-liquid extraction and solid phase extraction (SPE), followed by analytical characterization by reverse phase high-resolution liquid chromatography coupled to detection systems of visible ultraviolet spectroscopy and mass spectrometry (HPLC -DAD - RP - ESI -TOF / MS and RP-HPLC-ESI-QTOF/MS^2) enabled the characterization of bioactive compounds profile of extracts from the aforementioned plant sources, thus contributing to the analytical knowledge of their composition.

3. The development of chromatographic techniques based on semi-preparative HPLCand HSCCC allowed the purification of fractions from complex *Olea europaea* extracts. It showed the potential of both techniques and their complementarity, and the choice of one or the other technique will depend on the required purity and yield based on the specific application that is to be developed.

4. The semi-preparative HPLC separation technique allowed a bioassay-guided fractionation of bioactive compounds from olive leaf based on their potential for modulation of AMPK in a murine hypertrophic adipocyte model of obesity.

5. There are new technologies in food-industry alternatives to conventional heat treatment that cause minor alterations in the quality of fruit juices. It is therefore necessary to evaluate the effect of the technological processing on fruit juice, so that the final product is safe and meets the quality expectations of consumers.

Specific conclusions:

1. A solid-liquid hydro-alcoholic extraction from green asparagus was performed. The RP-HPLC-ESI-QTOF/MS^2 methodology allowed the identification of a total of 94 compounds in the extract, belonging to different chemical classes such as organic acids; amino acids, peptides and derivatives; phenolic compounds (hydroxycinnamic acids, flavonols, lignans and norlignans); oxylipins; and glyceroglycolipids, among others. Of these, 74 are referred to for the first time in this matrix. MS detection in tandem elucidated some structural features as a starting point before using nuclear magnetic resonance (NMR) spectroscopic studies.

2. Several hydroalcoholic extracts were made from marula bark by solid-liquid extraction. The RP-HPLC-ESI-QTOF/MS^2 methodology helped identify a total of 95 compounds in the extracts, these proving to be very rich in phenolic compounds, and more specifically in proanthocyanidins. This methodology successfully detected from monomers up to dimers of (epi) catechin, (epi) gallocatechin and (epi) afzelechin, with one or two units of gallic acid, for which some structures have been proposed.

3. A methanolic extract of extra-virgin olive oil was made by solid-phase extraction, which was characterized by RP-HPLC-ESI-TOF/MS. Semi-preparative purification methodologies for the secoiridoid decarboxymethyl oleuropein aglycone, a compound with demonstrated anti-cancer activity, were developed by HSCCC and HPLC, for later comparison in terms of purity, throughput, and yield. The HSCCC method is more efficient in terms of loading capacity, solvent

consumption, and performance compared with the HPLC method. However, the purity of the purified compound was greater by HPLC than by HSCCC.

4. A solid-liquid hydroalcoholic extract was made from olive leaf, which was further characterized by ESI-RP-HPLC-TOF/MS. Its anti-obesity potential was assessed, demonstrating a reduction of intracellular lipid accumulation through AMPK-dependent mechanisms in an *in vitro* murine hypertrophic adipocyte model. The fractionation of the extract by semi-preparative HPLC identified the compounds that could be responsible for that effect. The most active fractions in AMPK activation (p<0,001 and p<0.01) contained compounds belonging to the subclass secoiridoids (oleoside/secologanoside isomer 4, elenolic acid glucoside isomer 2, demethyl oleuropein/demethyl oleuroside, oleuropein/oleuroside isomer 4, and hydroxyoleuropein/hydroxyoleuroside isomer 1), cinnamic acids and derivatives (verbascoside and *p*-coumaric glucoside), flavonoids (luteolin rutinoside isomers 1 and 2, glucosylrhamnosylquercetin isomer 2, and diosmetin glucoside), and lignans (olivil).

5. The non-thermal alternatives to conventional heating in fruit juice processing were reviewed. These alternatives included radiation treatments (ultraviolet light, high-intensity light pulses, and γ radiation), electrical treatments (pulsed electric fields, radiofrequency electric fields, and ohmic heating), microwave heating, ultrasound, high hydrostatic pressure, inert gas treatments (ozonation and supercritical carbon dioxide), and flash-vacuum expansion. Research continues and many aspects have not yet been fully studied or understood. These technologies have great potential for the development of products that meet the expectations of the industry and consumers. However, the lack of standardization in operating conditions hampers comparisons between different studies.

6. The literature was reviewed on the effects that the above technologies could have on the content of bioactive compounds as well as on physicochemical, rheological, and organoleptic

properties. Generally, the temperature during processing and storage is an important factor affecting the quality of the finished product. However, not only the temperature but also other factors are involved in quality losses. Although alternatives to heat treatments appear to be less harmful in these terms, it is difficult to compare different studies for the lack of standardization in the operating conditions, and therefore there is great ambiguity in literature.

7. The RP-HPLC-ESI-TOF/MS methodology allowed the complete analytical characterization of nine extracts of persimmon juice produced in pilot plant using different technologies. These extracts proved to be a good source of sugars (sugar acids such as galacturonic, digalacturonic, and xylonic acids; disaccharides, and trisaccharides); amino acid derivatives and peptides; organic acids (malic, citric, and others); vitamins and derivatives; and phenolic compounds including simple phenolics and polymerized flavan-3-ols. Simple phenolics were phenolic acids (i.e. gallic acid, and galloyl glucose esters), and flavonoids (flavanols such as [epi] catechin; flavonols such as myricetin, quercetin, and kaempferol derivatives; and flavanones such as eriocitrin/neoeriocitrin). Flavan-3-ols included polymerized procyanidins dimers type B. In addition, an initial attempt was made to assess the qualitative composition variations resulting from different technological processing. Extracts juices undergoing combinations of clarification, and centrifugation; or astringency removal, centrifugation, and pasteurization a showed higher content in phenolic compounds.

Como fruto de la realización del presente trabajo se pueden obtener las siguientes conclusiones generales y específicas:

Conclusiones generales:

1. Las matrices vegetales de espárrago verde (*Asparagus officinalis*), corteza de marula (*Sclerocarya birrea*), aceite de oliva virgen extra, hoja de olivo (*Olea europaea*), y zumo de caqui (*Diospyros kaki*) representan fuentes interesantes de compuestos bioactivos que pueden ser utilizadas en futuras investigaciones dirigidas al desarrollo de alimentos funcionales y nutracéuticos.

2. La aplicación de metodologías analíticas basadas en extracción líquido-líquido, extracción sólido-líquido y extracción en fase sólida (SPE), seguidas de la caracterización analítica mediante cromatografía líquida de alta resolución en fase reversa acoplada a sistemas de detección de espectroscopia ultravioleta visible y espectrometría de masas (RP–HPLC–DAD–ESI–TOF/MS y RP–HPLC–ESI–QTOF/MS^2) hizo posible la caracterización del perfil de compuestos bioactivos de extractos procedentes de las mencionadas fuentes vegetales, contribuyendo así a profundizar en el conocimiento de la composición de las fracciones analizadas.

3. El desarrollo de metodologías basadas en las técnicas cromatográficas de purificación HPLC y HSCCC a escala semi-preparativa permitió la obtención de fracciones purificadas a partir de extractos complejos de *Olea europaea*. Debido a que se puso de manifiesto el potencial de ambas técnicas así como su complementariedad, la elección de una u otra metodología de fraccionamiento y/o purificación dependerá de la pureza y rendimiento requeridos en base a la aplicación concreta que se pretenda desarrollar.

4. Se demostró el potencial de un extracto de hoja de olivo así como fracciones obtenidas a partir del mismo en un modelo celular 3T3-L1 de obesidad (modelo hipertrófico adipocitario murino).

5. Se evaluó el efecto que, nuevas combinaciones de etapas tecnológicas del proceso de elaboración de los zumos, ejercen sobre la composición de compuestos con potenciales propiedades funcionales.

Conclusiones específicas:

1. Se llevó a cabo una caracterización de la fracción polar del espárrago verde. Para ello, se realizó una extracción sólido-líquido de los polifenoles presentes en la porción comestible de este vegetal seguido de su análisis mediante RP–HPLC–ESI–QTOF/MS^2. En base a la información proporcionada por el analizador de masas (masa exacta, distribución isotópica y patrón de fragmentación) se identificaron un total de 94 compuestos en el extracto perteneces a diferentes clases químicas. De ellos, se propusieron por primera vez en esta matriz 74 compuestos como posibles candidatos.

2. Se caracterizó mediante RP–HPLC–ESI–QTOF/MS^2 el perfil de compuestos presentes en cuatro extractos de corteza de marula (*Sclerocarya birrea*) que de acuerdo con la bibliografía científica consultada presentaban bioactividad frente a diferentes patologías de la sociedad actual. Los extractos fueron obtenidos mediante maceración convencional empleando los siguientes disolventes: agua, metanol y diclorometano. Se identificaron un total de 95 compuestos, siendo los componentes más numerosos los compuestos fenólicos (proantocianidinas). La metodología analítica seguida posibilitó la caracterización de monómeros y dímeros de (epi) catequina, (epi) galocatequina y (epi) afzelequina, con una o dos unidades de ácido gálico.

3. Se realizó un estudio comparativo de técnicas cromatográficas a escala semi-preparativa de HPLC acoplada a espectrometría de masas de tiempo de vuelo (TOF-MS) y HSCCC acoplada a espectroscopía ultravioleta-visible (UV-Vis) para purificar el compuesto decarboximetil oleuropeina aglicona, con bioactividad anti-cáncer demostrada. Para tal finalidad

se partió de un extracto de polifenoles de aceite de oliva virgen extra obtenido mediante extracción en fase sólida (SPE) y caracterizado mediante RP–HPLC–ESI–TOF/MS. Una vez caracterizado el extracto y confirmada la presencia del compuesto de interés se procedió a su purificación por ambas técnicas. La colección de fracciones fue monitorizada en base a la información proporcionada por los detectores TOF-MS y UV-Vis para la técnica separativa HPLC y HSCCC, respectivamente. De los resultados obtenidos se confirmó que la pureza de compuesto purificado mediante HPLC fue superior a la de la fracción obtenida mediante HSCCC. Sin embargo, es necesario tener en cuenta que el método de HSCCC presenta una mayor capacidad de carga, menor consumo de disolvente, y por tanto mayor rendimiento en comparación con el método de HPLC.

4. Se evaluó la bioactividad de un extracto de hoja de olivo en la línea celular 3T3-L1 de obesidad (modelo hipertrófico adipocitario murino). Se llevó a cabo la extracción y caracterización de la fracción fenólica la hoja de olivo mediante maceración asistida con Ultra-Turrax seguida de su análisis mediante RP–HPLC–ESI–TOF/MS. Se demostró el potencial anti-obesidad *in vitro* del extracto completo mediante una reducción de la acumulación de lípidos intracelulares a través de mecanismos dependientes de AMPK. El fraccionamiento del extracto de hoja de olivo mediante HPLC a escala semi-preparativa y el análisis de la bioactividad de cada una de las fracciones aisladas permitió delimitar qué compuestos podrían ser responsables de la activación de AMPK en 3T3-L1 adipocitos hipertróficos, siendo las fracciones caracterizadas por la presencia de secoiridoides, áciods cináminos, flavonoides y lignanos las que presentaron una mayor bioactividad.

5. Se realizó una revisión de la literatura científica acerca de los tratamientos no térmicos aplicados en la industria de los zumos de fruta como alternativa para la inactivación microbiológica, enzimática, y al aumento en el rendimiento de la producción. Estas alternativas

incluyeron: tratamientos de radiación (luz ultravioleta, pulsos de luz de alta intensidad, y radiación γ), tratamientos eléctricos (campos eléctricos pulsados, campos eléctricos de radiofrecuencia, y calentamiento óhmico), calentamiento por microondas, ultrasonidos, alta presión hidrostática, tratamientos con gas inerte (dióxido de carbono supercrítico y ozonización), y expansión súbita. Los resultados derivados del análisis del estado del arte pusieron de manifiesto la necesidad de profundizar en el conocimiento científico de aspectos relevantes para este sector.

6. El análisis del efecto de las etapas anteriormente citadas en las propiedades fisicoquímicas, reológicas, y organolépticas de los zumos de fruta puso de manifiesto que a pesar de que algunas de estas tecnologías pueden tener un gran potencial para desarrollar productos capaces de satisfacer las expectativas de la industria y de los consumidores, la falta de estandarización en las condiciones experimentales desarrolladas por los diferentes grupos de investigación dificulta la extrapolación de los resultados al sector.

7. Se evaluó el efecto de diferentes tecnológicas aplicadas en la elaboración del zumo de caqui sobre la composición fenólica y otros compuestos polares en el producto final. Para llevar a cabo el estudio se elaboraron nueve zumos en planta piloto obtenidos con diferentes combinaciones de los siguientes procesos: clarificación, reducción de la astringencia, expansión súbita, centrifugación, y pasteurización. Para establecer el estudio comparativo entre los diferentes diagramas de flujo se llevó a cabo una extracción líquido-líquido de cada uno de los nueve zumos obtenidos al final de cada lineal para su posterior análisis mediante RP-HPLC–DAD–ESI-TOF/MS. La caracterización analítica de los extractos puso de manifiesto que todos los zumos obtenidos constituían una buena fuente de azúcares y derivados, derivados de aminoácidos y péptidos, ácidos orgánicos, vitaminas y derivados, y compuestos fenólicos. Dentro de este último grupo se identificaron compuestos pertenecientes a fenoles simples tales como

ácidos fenólicos y flavonoides (flavanoles como (epi) catequina, flavonoles como miricetina, quercetina, y derivados de kaempferol, y flavanonas como eriocitrina/neoeriocitrina). Los flavan-3-oles polimerizados incluyeron dímeros de procianidinas de tipo B. Además, se realizó una primera aproximación para evaluar variaciones de composición semi-cuantitativa resultantes de las diferentes combinaciones de los procesos citados anteriormente. Estos resultados mostraron que los extractos obtenidos de zumos producidos a través de las combinaciones de procesos basados en (1) clarificación y centrifugación y (2) eliminación de la astringencia, centrifugación, y pasteurización, presentaron mayor contenido en compuestos fenólicos.