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ANALYTICAL, AGRONOMIC, AND BIOLOGICAL EVALUATION OF PHENOLIC COMPOUNDS IN *Olea europaea* PRODUCTS AND BY-PRODUCTS

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Submitted for a Ph.D. Degree in Chemistry

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ANALYTICAL, AGRONOMIC AND BIOLOGICAL EVALUATION OF PHENOLIC COMPOUNDS IN *Olea europaea* PRODUCTS AND BY-PRODUCTS.

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That the work presented in this doctoral thesis entitled "**ANALYTICAL, AGRONOMIC AND BIOLOGICAL EVALUATION OF PHENOLIC COMPOUNDS IN *Olea europaea* PRODUCTS AND BY-PRODUCTS**" has been carried out under our direction in the laboratories of the Department of Analytical Chemistry and Functional Food Research and Development Center (CIDAF), and shows all requirements of eligibility to obtain the Doctor of Philosophy Degree in Chemistry from the University of Granada.

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La doctoranda Nassima Talhaoui y los directores de la tesis Dr. Antonio Segura Carretero y Dra. Ana Maria Gómez Caravaca garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

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LIST OF ABBREVIATIONS

ANOVA: Analysis of variance

B.C: Before Christ

BPC: Base peak chromatogram

CAT: Catalase

CC: Column chromatography

CD: Crohn's disease

CID: Collision induced-dissociation

CLC: Capillary liquid chromatography

DAD: Diode array detector

DHPEA-EA: Dihydroxyphenylethanol-elenolic acid mono-aldehyde (Oleuropein-aglycone)

DHPEA-EDA: Dihydroxyphenylethanol-elenolic acid dialdehyde (Decarboxymethyl oleuropein aglycone)

DIECA: Sodium diethyldithiocarbamate

DNA: Deoxyribonucleic acid

DOA: Decarboxymethyl oleuropein-aglycone

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EC50: Half maximal effective concentration

EIC: Extract ion chromatogram

eNOS: Endothelial nitric oxide synthase

ESI: Electrospray ionization

EVOO: Extra virgin olive oil

FAO: Food and agriculture organization

FrDW: Fruit dry weight

FrFW: Fruit fresh weight

FrM: Fruit moisture.

GC: Gas chromatography

GIT: Gastrointestinal tract

HPEA-EA: Hydroxyphenylethanol-elenolic acid (Ligstroside-aglycone)

HPLC: High-performance liquid chromatography

HPTLC: High performance thin-layer chromatography

IBD: Inflammatory bowel diseases

IELC: Ion exchange liquid chromatography

iNOS: Isoform nitric oxide synthase

IOC: International olive council

IT: Ion trap

LC: Liquid chromatography

LDL: Low-density lipoprotein

LLE: Liquid liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

LPS: Lipopolysaccharide

m/z: Mass ratio to charge

MAE: Microwave-assisted extraction

MD: Mediterranean diet

MeOH: Methanol

MS: Mass spectrometry

MUFAs: Monounsaturated fatty acids

NE: North east

NMR: Nuclear magnetic resonance

nNOS: Neuronal nitric oxide synthase

NO: Nitric oxide

NOS: Nitric oxide synthase

NPLC: Normal phase liquid chromatography

OCFrDW: Oil content in fruit dry weight

OCFrFW: Oil content in fruit fresh weight

OxLDL: Oxidized low-density lipoprotein

PAL: Phenylalanine ammonia lyase

PCA: Principal component analysis

PEP: Pentafluorophenyl

PET: Polyethylene terephthalate

PLE: Pressurized phase extraction

PPO: Polyphenol protein oxidase

QTOF: Quadrupole time-of-flight

RI: Ripening index

RNA: Ribonucleic acid

ROS: Reactive nitrogen species

RPHPLC: Reversed-phase high performance thin-layer chromatography

RPLC: Reversed phase liquid chromatography

RSD: Relative standard deviation

SD: Standard deviation

SELC: Size-exclusion chromatography

SFE: Supercritical fluid extraction

SLE: Solid liquid extraction

SOD: Superoxide dismutase

SPE: Solid phase extraction

TBARS: Thiobarbituric acid

TC: Cholesterol

TG: Triglyceride

TLC: Thin-layer chromatography

TOF: Time of flight

TP: Total Phenol

UAE: Ultrasound-assisted extraction

UC: Ulcerative colitis

USLE: Ultrasound-assisted solid-liquid extraction

UV: Ultraviolet

UV-Vis: Ultraviolet-Visible

VOO: Virgin olive oil

SUMMARY

The olive tree (*Olea europaea*) is a common feature of the Mediterranean landscape, olive fruit and olive oil having been a basic element in the nutrition of civilizations around the Mediterranean basin for millennia. Even olive leaves have been used in folk medicine. In recent decades, extensive research has examined the olive's beneficial properties for human health, highlighting the prominence of phenolic compounds in these benefits.

The present doctoral thesis offers a new perspective on olive products and by-products: olive leaves, fruit, and oil. In particular, this study identifies, quantifies, and evaluates the patterns of phenolic compounds in the main products and by-products of six of the leading cultivars currently cultivated in Spain. Moreover, a preliminary bioactive study concerns the phenolic compounds in olive leaves. The thesis is organized into six chapters.

Chapter 1 provides the introduction, giving an overview of the importance of olive leaves as a by-product from the olive-oil industry. It also provides a general view of the types phenolic compounds in the olive leaves, explaining the extraction procedures and the analytical tools used for determining these compounds. The abiotic and biotic factors affecting the content in quantitative and qualitative phenolic compounds in leaves are emphasized throughout the study and, finally, the health potential of phenolic compounds in olive leaves is stressed by reviewing the main *in vitro* and *in vivo* studies on these compounds during the last decade.

Chapter 2 deals with the identification and quantification of the phenolic fraction of olive leaves in the cultivars 'Arbequina' and 'Picual' and, for the first time, in 'Sikitita'. This latter cultivar has recently been developed in a collaborative olive-breeding program involving the IFAPA of Cordoba and University of Cordoba (Spain). This chapter also demonstrates the efficiency of high-performance liquid chromatography coupled to diode array detector and electrospray time-of-flight mass spectrometry (HPLC-DAD-ESI-TOF-MS) for

determining phenolic compounds in olive leaves. This technique has provided the phenolic profiles of the three cultivars and has enabled the heredity phenolic profiles to be compared between 'Sikitita' and its parent cultivars 'Arbequina' and 'Picual'. The three cultivars were grown under the same environmental and agronomic conditions.

Chapter 3 focuses on the effect that the cultivar and sampling time exert on the phenolic content variation in olive leaves. For this, olive leaves from 'Arbequina', 'Picual', 'Sikitita', 'Arbosana', 'Changlot Real', and 'Koroneiki' cultivars grown under the same environmental and agronomic conditions, at the IFAPA of Cordoba, were collected at four different times over fruit ripening (June, August, October and December) and were analyzed by HPLC-DAD-ESI-TOF-MS. The general trend in variation of all the cultivars was a decline of phenolic contents in summer and a surge in winter. The phenolic compound contents were chemometrically analyzed to discriminate between cultivars as well as between sampling times, and the phenolic contents in leaves were clearly defined for different sampling times and cultivars.

Chapter 4 analytically follows the same study scheme as in Chapter 2 but introducing a preliminary study of the bioactivity of olive leaf extracts. In consideration of the results reported in Chapter 3, 'Arbequina', 'Picual', and 'Sikitita' olive leaves were sampled again in December. The phenolic compounds were accurately determined by HPLC-DAD-ESI-TOF-MS. Finally, olive leaf phenolic extracts were used to determine the *in vitro* immunomodulatory properties of the leaves, presenting promising results. The study was conducted with the collaboration of the "Pharmacology of Natural Products" group from Pharmacology Department at the University of Granada (Spain).

Chapter 5 concerns the variation patterns of the phenolic compounds during the ripening of olive fruits from the same six cultivars 'Arbequina', 'Picual',

‘Sikitita’, ‘Arbosana’, ‘Changlot Real’ and ‘Koroneiki’. This study reports, for the first time, the determination of phenolic compounds and their evolution during ripening in the cultivars ‘Sikitita’, ‘Arbosana’, ‘Changlot Real’, and ‘Koroneiki’. The olive fruits were harvested at the same time as the olive leaves sampling mentioned above in reference to Chapter 3. The phenolic fraction in the fruits was also determined using HPLC-DAD-ESI-TOF-MS. In addition, some agronomic traits such as ripening index, fruit size, and moisture and oil contents in the fruits were determined in order to identify a relationship between fruit traits and phenolic profiles. As in the leaves, the fruits were chemometrically studied to discriminate cultivars and sampling times using phenolic compounds as the discrimination parameters.

Chapter 6, the last study of the thesis, analyzes the phenolic fraction of virgin olive oil (VOO) in comparison to the phenolic fraction of the corresponding olive fruits. In this sense, with the VOO available from the cultivars studied in this PhD thesis, and given that all cultivars were grown in the same orchards under the same agronomic and environmental conditions, the phenolic composition was determined both in fruits and in oils using HPLC-DAD-ESI-TOF-MS. A qualitative and quantitative comparison was made of the phenolic compounds in all the cultivars studied. In addition, the transfer rates of phenolic compounds from fruits to oils were calculated in order to highlight the effect of the cultivar on the presence of phenolic compounds and their transfer between olive fruits and VOOs. Finally, the principal component analysis again confirmed a strong genetic effect of the phenolic profile both in olive fruits and oils.

RESUMEN

El olivo (*Olea europaea*) es uno de los elementos más característicos del paisaje mediterráneo. Tanto por sus aceitunas como por el aceite obtenido de sus frutos, el olivo ha sido siempre una de las bases alimenticias más importantes que ha acompañado a las civilizaciones de la cuenca mediterránea. Asimismo, desde la antigüedad sus hojas han sido apreciadas por sus propiedades medicinales. En las últimas décadas, se han realizado extensas investigaciones sobre los efectos beneficiosos de los productos del olivo sobre la salud humana. Dichas investigaciones han puesto de manifiesto el papel que juegan los compuestos fenólicos en las propiedades beneficiosas del olivo.

La presente tesis doctoral supone un nuevo aporte científico acerca de productos del olivo como las aceitunas y el aceite de oliva virgen, y de subproductos como las hojas de olivo. Concretamente, esta tesis aborda la identificación y la cuantificación de los compuestos fenólicos, así como la evaluación de dichos compuestos a lo largo del tiempo, en los principales productos y subproductos de seis variedades de olivo entre las más expandidas actualmente en España. Por otra parte, se ha introducido también un estudio preliminar de la bioactividad de los compuestos fenólicos de la hoja de olivo. Por lo tanto, esta tesis doctoral se estructura en seis capítulos.

El capítulo 1 se considera una introducción donde se ofrece una visión general de la importancia de la hoja de olivo como importante subproducto de la industria del aceite de oliva. También se lleva a cabo una revisión de las distintas clases de compuestos fenólicos presentes en la hoja de olivo, y se exponen los procedimientos de extracción y las herramientas de análisis empleados para la determinación de dichos compuestos. Asimismo, se resumen en una gran parte del capítulo, los factores abióticos y bióticos que afectan cualitativamente y cuantitativamente al contenido de los compuestos fenólicos en la hoja. Y por último, se subraya el potencial saludable de estos compuestos fenólicos exponiendo los diferentes estudios *in vitro* e *in vivo* llevados a cabo en la última década.

El capítulo 2 versa sobre la identificación y la cuantificación de la fracción fenólica de hoja de olivo de las variedades ‘Arbequina’ y ‘Picual’ y, por primera vez, de la variedad ‘Sikitita’. ‘Sikitita’ es una nueva variedad recién obtenida dentro de un programa de mejora genética llevado a cabo entre el IFAPA de Córdoba y la Universidad de Córdoba. En este capítulo se demuestra también la eficacia de la cromatografía líquida de alta resolución acoplada a un detector de diodos y a un espectrómetro de masas de tiempo de vuelo con ionización por electrospray (HPLC-DAD-ESI-TOF-MS) para la determinación de los compuestos fenólicos de la hoja de olivo. Esta herramienta ha permitido destacar los perfiles fenólicos de las tres variedades, así como la comparación entre los perfiles fenólicos de la variedad ‘Sikitita’ y sus progenitores ‘Picual’ y ‘Arbequina’. Cabe destacar que los olivos de las tres variedades fueron cultivados bajo las mismas condiciones agronómicas y ambientales.

El capítulo 3 se centra principalmente en el efecto de la variedad y la época de muestreo sobre la variación en el contenido fenólico de la hoja de olivo. Para ello, se han usado muestras de hojas de olivo de las variedades ‘Arbequina’, ‘Picual’, ‘Sikitita’, ‘Arbosana’, ‘Changlot Real’ y ‘Koroneiki’ cultivadas en las mismas condiciones ambientales y agronómicos, en el IFAPA de Córdoba. La toma de muestras se realizó en cuatro épocas diferentes a lo largo de la maduración de las aceitunas (junio, agosto, octubre y diciembre), y posteriormente se llevó a cabo el análisis de compuestos fenólicos mediante HPLC-DAD-ESI-TOF-MS. Además, los contenidos de los compuestos fenólicos se analizaron utilizando herramientas quimiométricas con el fin de clasificar las muestras en función de su variedad y el momento del muestreo.

El capítulo 4 vuelve a emplear el mismo esquema de trabajo desarrollado en el capítulo 2, y además incluye un estudio preliminar acerca de la bioactividad de los extractos de hoja de olivo. Teniendo en cuenta los resultados obtenidos en el capítulo 3, se llevó a cabo un nuevo muestreo de las variedades ‘Arbequina’, ‘Picual’ y ‘Sikitita’ en diciembre. A continuación, se determinaron los

compuestos fenólicos en las diferentes hojas de olivo mediante HPLC-DAD-ESI-TOF-MS. Por último, los extractos fenólicos de las hojas de olivo se utilizaron para la evaluación *in vitro* de sus propiedades inmunomoduladoras. Este estudio se llevó a cabo gracias a la colaboración con el grupo de "Farmacología de Productos Naturales" del Departamento de Farmacología de la Universidad de Granada.

El capítulo 5 describe el estudio de los patrones de variación de los compuestos fenólicos de seis variedades de aceituna ('Arbequina', 'Picual', 'Sikitita', 'Arbosana', 'Changlot Real' y 'Koroneiki') a lo largo de su proceso de maduración. Cabe destacar que en este capítulo se estudia, por primera vez, el perfil fenólico y su evolución durante la maduración de cuatro de estas variedades ('Sikitita', 'Arbosana', 'Changlot Real' y 'Koroneiki'). Las muestras de aceituna se tomaron en los mismos cuatro tiempos previamente mencionados en el capítulo 3. El análisis de la fracción fenólica se realizó mediante HPLC-DAD-ESI-TOF-MS. Además, se determinaron otros parámetros tales como el índice de madurez, el tamaño de los frutos, así como su humedad y contenido en aceite con el fin de encontrar una posible relación entre estos parámetros y los perfiles de compuestos fenólicos. Del mismo modo que para las hojas de olivo, se llevó a cabo un estudio quimiométrico usando los contenidos en compuestos fenólicos como parámetros de discriminación para poder diferenciar entre las variedades y el momento del muestreo.

El capítulo 6 presenta el último trabajo llevado a cabo en la presente tesis doctoral y describe el análisis de la fracción fenólica de aceite de oliva virgen comparándolo con el contenido en compuestos fenólicos de sus aceitunas de proveniencia. De esta manera, teniendo en cuenta la disponibilidad de aceite de oliva virgen procedente de las aceitunas de las seis variedades estudiadas durante la tesis doctoral, y que todas las variedades se cultivaron en el mismo campo experimental y bajo las mismas condiciones agronómicas y ambientales, se determinaron los compuestos fenólicos de aceitunas y aceites mediante HPLC-

DAD-ESI-TOF-MS. A continuación se realizó una comparación tanto cualitativa como cuantitativa de los compuestos fenólicos entre aceitunas y aceites de cada una de las seis variedades estudiadas. Además, se calcularon los grados de transferencia de los compuestos fenólicos de las aceitunas al aceite con el fin de determinar el efecto de la variedad en esa transferencia y en la presencia o no de determinados compuestos fenólicos. Por último, el análisis de componentes principales se empleó para evaluar el efecto genético en los perfiles fenólicos de aceitunas y aceites.

RESUME

L'olivier (*Olea europaea*) est l'un des éléments caractéristiques du paysage méditerranéen. Le fruit ou l'huile issus de l'olivier ont toujours été des éléments fondamentaux dans la nutrition des civilisations du bassin méditerranéen. De plus, le rôle de la feuille d'olivier dans la médecine traditionnelle a aussi eu une importance conséquente. Depuis les dernières décennies, des recherches approfondies ont été faites sur les bienfaits de l'olivier sur la santé de l'être humain. Ces recherches ont mené à souligner la grande importance que jouent les composés phénoliques de l'*O. europaea* dans les vertus bénéfiques de l'olivier.

Cette présente thèse de doctorat est une nouvelle contribution scientifique aux produits et sous-produits de l'olivier: les feuilles, les fruits de l'olivier, mais aussi l'huile d'olive vierge. Cela concerne particulièrement l'identification et la quantification des composés phénoliques, ainsi que l'étude de l'évolution de ces dits composés au cours du temps dans les principaux produits et sous-produits de l'olivier. Six variétés parmi les variétés les plus étendues actuellement en Espagne ont été étudiées dans cet objectif. De plus, une étude préliminaire sur la bio-activité des composés phénoliques des feuilles d'olivier a été aussi présentée. Cette thèse de doctorat est ainsi organisée en six chapitres.

Le chapitre 1 fait office d'introduction. Il donne un aperçu général sur l'importance des feuilles d'olivier comme sous-produit issu de l'industrie de l'huile d'olive. Ce chapitre procure aussi une vue d'ensemble sur les types de composés phénoliques des feuilles d'olivier, tout en exposant les procédures d'extraction et les outils analytiques utilisés pour la détermination de ces composés. De plus, il résume dans la majeure partie de cette révision bibliographique, les facteurs biotiques et abiotiques qui affectent le contenu quantitatif et qualitatif des composés phénoliques dans les feuilles d'olivier. Finalement, ce chapitre met l'accent sur le potentiel bénéfique des composés phénoliques des feuilles d'olivier sur la santé en rassemblant les différentes études scientifiques *in vitro* et *in vivo* menées au cours de la dernière décennie.

Le chapitre 2, lui, traite de l'identification et la quantification de la fraction phénolique des feuilles d'olivier dans les variétés 'Arbequina' et 'Picual' et pour la première fois dans la variété 'Sikitita'. Cette dernière est une nouvelle variété récemment obtenue suite à un programme d'amélioration génétique mené au sein du centre de recherche IFAPA de Cordoue et de l'Université de Cordoue. Ce chapitre démontre l'efficacité de la chromatographie en phase liquide à haute performance couplée au détecteur à barrettes de diodes et à la spectrométrie de masse electrospray à temps de vol (HPLC-DAD-ESI-TOF-MS), pour la détermination des composés phénoliques dans les feuilles d'olivier. Cet outil a permis de mettre en évidence les profils phénoliques des trois variétés mais aussi de comparer les profils phénoliques de 'Sikitita' avec ses progéniteurs 'Arbequina' et 'Picual', d'autant plus que ces trois variétés ont été cultivées dans les mêmes conditions agronomiques et environnementales.

Le chapitre 3 se concentre principalement sur l'effet de la variété et de l'époque d'échantillonnage sur la variation du contenu phénolique dans les feuilles d'olivier. A cette fin, des feuilles d'olivier issues de variétés 'Arbequina', 'Picual', 'Sikitita', 'Arbosana', 'Changlot Real' et 'Koroneiki', ont été utilisées. Ces variétés ont été cultivées dans les mêmes conditions agronomiques et environnementales dans le centre de l'IFAPA de Cordoue. L'échantillonnage a été effectué en quatre périodes différentes au cours de la maturation du fruit (juin, août, octobre et décembre). Par la suite l'analyse chromatographique des composés phénoliques a été réalisée par l'outil HLPC-DAD-ESI-TOF-MS. En outre, une analyse chimiométrique a été appliquée aux contenus phénoliques des six variétés afin de classifier les échantillons en fonction des variétés et du moment de la réalisation de l'échantillonnage.

Le chapitre 4 reprend le même plan de travail développé dans le chapitre 2. En outre, une étude préliminaire de l'activité biologique des extraits de feuilles d'olivier a été introduite. Compte tenu des résultats obtenus dans le chapitre 3, un nouvel échantillonnage des feuilles d'olivier des trois variétés 'Arbequina',

'Picual' et 'Sikitita' a été réalisé au mois de décembre. Une nouvelle détermination des fractions phénoliques des feuilles de ces variétés a été effectuée ensuite par le biais de l'HPLC-DAD-ESI-TOF-MS. Les extraits phénoliques issus des feuilles sujettes de ce travail ont été finalement utilisés dans une étude conduite en *in vitro* afin d'évaluer leurs propriétés immuno-modulatrices. L'étude a été réalisée avec la collaboration du groupe de recherche «Pharmacologie des Produits Naturels» qui est un groupe issu du Département de Pharmacologie de l'Université de Grenade.

Le chapitre 5 concerne l'étude des variations des composés phénoliques au niveau des olives durant la maturation des six variétés 'Arbequina', 'Picual', 'Sikitita', 'Arbosana', 'Chaglot Real' et 'Koroneiki'. Cette étude démontre pour la première fois la détermination des composés phénoliques et leurs évolutions au cours de la maturation au niveau des variétés ('Sikitita', 'Arbosana', 'Chaglot Real' et 'Koroneiki'). Les échantillons des olives ont été prélevés dans les mêmes quatre périodes précédemment mentionnées au chapitre 3 et la fraction phénolique dans les olives a aussi été déterminée en utilisant l'HPLC-DAD-ESI-TOF-MS. De plus, certaines caractéristiques agronomiques comme l'indice de maturité, la taille du fruit, l'humidité et le contenu en huile ont été déterminés dans le but de découvrir une éventuelle relation entre les caractéristiques agronomiques des olives et leurs profils phénoliques. Il en est de même pour les feuilles, en prenant les composés phénoliques comme paramètres de discrimination, l'étude chimiométrique a donné lieu à une séparation entre les différentes variétés d'une part et entre des différentes périodes d'échantillonnage des olives d'autre part.

Chapitre 6 représente les derniers travaux entrepris dans cette thèse et décrit l'analyse de la fraction phénolique de l'huile d'olive vierge par rapport aux composés phénoliques des olives d'où ils proviennent. En ce sens, étant donné la disponibilité de l'huile d'olive vierge issue des variétés étudiés dans cette thèse de doctorat et en tirant profit du fait que toutes les variétés ont été cultivées dans

le même verger et dans les mêmes conditions agronomiques et environnementales, la composition phénolique a été déterminée dans les deux produits olives et huiles d'olive vierge en utilisant l'HPLC-DAD-ESI-TOF-MS. Une comparaison qualitative et quantitative des composés phénoliques dans toutes les variétés a été effectuée par la suite. Par ailleurs, des taux de transfert des composés phénoliques des fruits vers l'huile ont été calculés dans le but de souligner mettre en exergue l'effet de la variété sur le transfert et l'apparition ou la disparition des composés phénoliques entre les olives et l'huile d'olive vierge. Finalement, l'analyse du principal composant a été utilisée pour évaluer l'effet génétique de la variété sur les profils phénoliques des olives ainsi que de l'huile d'olive.

OBJECTIVES

The use of *Olea europaea* products (fruits and oil), particularly in the context of the Mediterranean diet, has been advocated for many years for their health-promoting properties. Besides, throughout history, Mediterranean olive leaves have been used in traditional medicine. The olive tree has benefited from general improvement in the current agriculture so that the surface areas of cultivation as well as the volume of production have greatly increased. Currently, olive tree is cultivated not only in the traditional Mediterranean countries but is also grown worldwide (the so-called new olive world). Furthermore, ample evidence is available concerning the health benefits resulting from olive-product consumption. These benefits are associated with the presence of bioactive compounds in the olive matrices, among which phenolic compounds have been demonstrated to prevent several human diseases.

Thus, the main objective of this present thesis is to determine through agronomic, analytical, and biological evaluation, the phenolic compounds from different products of *O. europaea*: olive leaves, olive fruits and virgin olive oil from six olive cultivars (‘Arbequina’, ‘Picual’, ‘Sikitita’, ‘Arbosana’, ‘Changlot Real’ and ‘Koroneiki’) grown at the same orchard under the same agronomic and environmental conditions. This objective can be itemized into the following categories:

✓To identify and quantify the phenolic compounds in olive leaves, fruits and virgin olive oils using high performance liquid chromatography coupled to diode array detector and electrospray time-of-flight mass spectrometry.

✓To evaluate qualitatively as well as quantitatively the time course of the main phenolic compounds in leaves and fruits, and the evolution of the agronomical traits changes of fruits over the ripening period.

✓To qualitatively and quantitatively evaluate the transfer of single and total phenolic compounds from fruits to virgin olive oil at the laboratory scale.

OBJETIVOS

El uso de los productos derivados de *Olea europaea* (aceite y aceitunas), en particular en el contexto de la dieta mediterránea, ha sido defendido a lo largo de los años debido a sus propiedades promotoras de la salud. Además, a lo largo de la historia, diferentes civilizaciones también han ensalzado las virtudes de uno de sus subproductos como son las hojas de olivo en la medicina tradicional. El árbol del olivo se ha beneficiado de las mejoras generales de la agricultura actual. Por ello, la superficie de cultivo así como la producción han aumentado enormemente. Hoy en día, el olivo no solo se cultiva en los países mediterráneos sino también en otros muchos países en todo el mundo (el llamado nuevo mundo del olivo). Asimismo, existen numerosas evidencias de los beneficios que lleva asociado el consumo de productos derivados del olivo para la salud. Estos beneficios para la salud se relacionan con la presencia de compuestos bioactivos. Entre ellos se encuentran los compuestos fenólicos, compuestos que han demostrado que contribuyen en la prevención de diversas enfermedades.

Por todo ello, el principal objetivo que se planteó en esta Tesis Doctoral fue la determinación y la evaluación agronómica, analítica y biológica de los compuestos fenólicos de diferentes productos de *O. europaea*: hoja de olivo, aceituna y aceite de oliva virgen de seis variedades (‘Arbequina’, ‘Picual’, ‘Sikitita’, ‘Arbosana’, ‘Changlot Real’ y ‘Koroneiki’) cultivadas en el mismo campo experimental bajo las mismas condiciones agronómicas y ambientales. Este objetivo principal se puede desglosar en los siguientes puntos:

✓Identificar y cuantificar los compuestos fenólicos de las hojas de olivo, las aceitunas y los aceites de oliva virgen de las seis variedades mediante cromatografía líquida de alta resolución acoplada a un detector de diodos en fila y a un espectrómetro de masas por tiempo de vuelo (HPLC-DAD-ESI-TOF-MS).

✓Evaluar el patrón de evolución del contenido en compuestos fenólicos de las hojas de olivo y las aceitunas, así como el cambio de los parámetros agronómicos

de las aceitunas, durante el periodo de maduración de las seis diferentes variedades.

✓Evaluar cualitativamente y cuantitativamente la transferencia de los compuestos fenólicos individuales y totales desde la aceituna al aceite de oliva virgen en las seis variedades estudiadas a escala del laboratorio.

OBJETIFS

Depuis de nombreuses années, la consommation des produits de l'*Olea europaea* (olives et huile d'olive), notamment dans le contexte du régime méditerranéen été préconisée en vertu des propriétés bénéfiques de ces produits sur la santé humaine. En outre, les feuilles d'olivier ont toujours été encensées par de nombreuses civilisations à travers l'histoire grâce à leurs vertus dans la médecine traditionnelle.

La culture de l'olivier a profité du développement général de l'agriculture contemporaine qui a engendré une forte croissance tant en surface comme en production. Actuellement, la culture de l'olivier n'est pas seulement pratiquée dans les zones dites traditionnelles au niveau du bassin méditerranéen mais elle s'est aussi développée dans de nouvelles zones autour du monde. D'autre part, de nombreuses recherches scientifiques ont mis en évidence les bienfaits que procure la consommation des produits de l'olivier sur la santé grâce à leur contenu en composé bioactifs. Les composés phénoliques de l'*O. europaea* sont parmi les composés bioactifs responsables de la prévention contre plusieurs maladies.

Par conséquent, l'objectif principal de cette thèse de doctorat est de déterminer et d'évaluer les aspects agronomique, analytique et biologique des composés phénoliques de différents produits et sous-produits de l'*O. europaea*: feuilles d'olivier, olives et huile d'olive vierge provenant de six variétés ('Arbequina', 'Picual', 'Sikitita', 'Arbosana', 'Changlot Real' et 'Koroneiki') cultivées dans le même verger et dans les mêmes conditions agronomiques et environnementales. Cet objectif principal peut être détaillé en les points suivants:

✓ Identifier et quantifier les composés phénoliques des feuilles d'olivier, olives et huiles d'olive vierge de six variétés d'olivier en utilisant la chromatographie en phase liquide à haute performance couplée au détecteur à barrettes de diodes et à la spectrométrie de masse electrospray à temps de vol.

Objetifs

✓Evaluer l'évolution des composés phénoliques contenus dans les feuilles d'olivier et dans les olives, ainsi que les changements survenus au niveau des caractéristiques agronomiques des olives pendant la maturation des six variétés.

✓Evaluer qualitativement et quantitativement le transfert des composés phénoliques simples et totaux à partir des fruits à l'huile d'olive vierge au niveau des six variétés étudiées, à l'échelle du laboratoire.

INTRODUCTION

1. Olive crops: History and importance

1.1. Origin and domestication

The olive tree (*Olea europaea* L.), probably the first domesticated fruit tree in the Mediterranean, remains one of the most important crops of this region, both economically and culturally. Archaeological evidence and clues provided by living plants and particularly by wild relatives of the olive tree indicate that olive was already under cultivation in proto-historic times (3500-4000 years B.C.) in the Middle East[1,2]. Thanks to its longevity and its facility for vegetative propagation, the olive tree spread early along the entire perimeter of the Mediterranean Sea[3] (**Figure 1**). Archaeological discoveries also reveal the extraction, commerce, and consumption of olive oil in the main civilizations throughout the Mediterranean region. In addition, botanists speculate that olive tree began to be cultivated almost at the same time in several places in the Mediterranean region by selecting seeds from the related wild species *Olea europaea* var. oleaster and/or *Olea chrysophylla*[4–6]. Whatever the origin of the olive tree, Mediterranean societies developed cultivars from which the fruits were directly eaten and also pressed for oils for culinary, medicinal, ceremonial, and energy uses.

(1) Zohary, D.; Spiegel-Roy, P. Beginnings of fruit growing in the old world. *Science.* **1975**, *187*, 319–327.
(2) Weiss, E. "Beginnings of fruit growing in the old world"–two generations later. *Isr. J. Plant Sci.* **2015**, *62*, 75–85.
(3) Rallo, L.; Barranco, D.; Caballero, J. M.; Del Río, C.; Martín, A.; Tous, J.; Trujillo, I. *Variedades de olivo en España*, Mundi-Pren.; Rallo, L., Barranco, D., Caballero, J. M., Del Río, C., Martín, A., Tous, J., Trujillo, I., Eds.; Junta de Andalucía, MAPA: Madrid, 2005.
(4) Zohary, D. The wild genetic resources of the cultivated olive. *Acta Hortic.* **1994**, *356*, 62–65.
(5) Angiolillo, A.; Mencuccini, M.; Baldoni, L. Olive genetic diversity assessed using amplified fragment length polymorphisms. *Theor. Appl. Genet.* **1999**, *98*, 411–421.
(6) Muzzalupo, I.; Perri, E. Genetic characterization of olive germplasm by molecular markers. *Eur. J. Plant Sci. Biotechnol.* **2008**, *2*, 60–68.

Figure 1. Geographical distribution of olive cultivation around the Mediterranean Sea.

1.2. Civilizations around the olive tree

Since antiquity the olive tree has persisted in the Mediterranean landscape, influencing the daily life and habits of its populations. Thus, the culture of the olive tree and its products has a profound mark on all the major civilizations that have dominated this area.

Olive tree is strongly present in the Greek mythology. Legend tells that the Greek god of the sea Poseidon and the Greek goddess of wisdom Athena competed with each other to offer the best gift to humanity. Poseidon offered a horse and Athena offered an olive tree (**Figure 2**). It was decided that the olive tree was the most valuable gift and Athena was rewarded for her great gift by becoming the patroness of the Greece's most powerful city, Athens[7]. Honoring the olive as a "holy tree", the Greeks believed that the tree bore divine power and supernatural qualities. Olive leaves were used to cover the dead as a sign of respect and divine protection. Young women seeking to be blessed with a healthy pregnancy often reposed in the shade of an olive tree. Olive oil was also used in ancient Greek

(7) Florman, B.; Kestler, J. Introduction to classical mythology. http://www.litcharts.com/lit/mythology (*accessed Sep 22, 2015*).

rituals, such as sacrifices made to the gods and, in the original Olympic Games, where the oil was used to rub the athletes' bodies before the games.

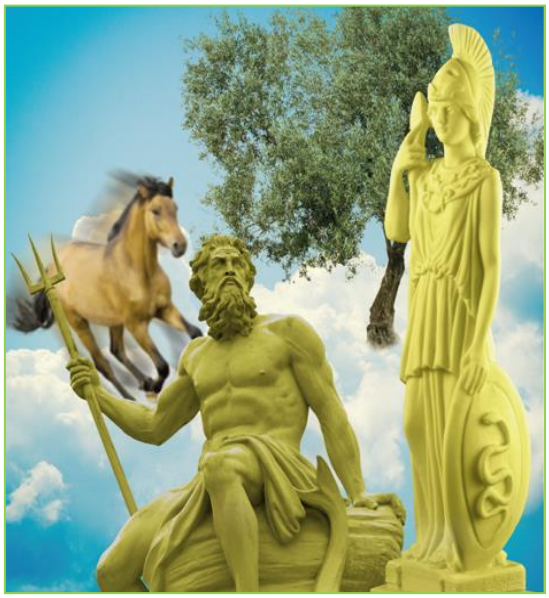

Figure 2. Poseidon and Athena offering their gifts to humanity.

Greek symbolism associated with olive was adopted by the ancient Romans, who had also venerated this plant. For the Romans, the cult of the goddess Athena became the cult of the goddess Minerva[8], and olive leaves combined with laurel leaves were plaited to crown the brave and heroic. As the Roman Empire grew, olive oil became a major trade commodity and commerce grew to an unprecedented scale for the ancient world. In war times, defeated armies carried olive branches to indicate surrender while victorious armies brandished the olive branch to proclaim peace. Indeed, the triumphant legions were honored with olives, and Roman emperors were crowned with wreaths of golden olive leaves and anointed with olive oil[9].

(8) Stoughton, H. L. *Favorite Greek myths*; Yesterday's Classics, 2008; Vol. 44.
(9) Bartolini, G.; Petruccelli, R. *Classification, origin, diffusion and history of the olive*; Tindall, H., Ed.; Nations, Food and Agriculture Organization of the United, 2002.

In ancient Egypt, it was not possible to cultivate olive trees due to climatic conditions, but evidence has been found of intense trade from Syria and Palestine. Nevertheless, it was believed that the creation of olive trees and the knowledge to produce olive oil were gifts from goddess Isis. Also, to the Egyptians, olive oil was highly valued for many purposes, such as cooking, medicine, lighting lamps and even for religious ceremonies. Furthermore, olives have been found among the food items intended for the afterlife in many pharaonic tombs[10].

For the ancient Hebrews, the olive tree was praised for its fecundity and its evergreen endurance. Indeed, the *Old Testament* features an olive leaf in the story of Noah's survival of the flood, and the dove holding an olive branch in its mouth has become a universal symbol of peace.

The Greco-Roman heritage of the olive tree was also incorporated into Christian traditions. In fact, in the *New Testament* the Cross of Jesus Christ is reported to have been made of olive wood. Once again the tree of the Cross revived the old myth of this tree as the "center of the world"[9]. The oil from this sacred tree has also been cited as a symbol of goodness and purity, and it has been used up to the present in anointing ceremonies in the Greek Orthodox Church.

In Muslim world, olives are mentioned in the *Qur'an* seven times and their health benefits have been propounded in prophetic medicine. The *Qur'an* praises the olive with the expression "*mubarakatin zaytoonatin*" which describes the olive as being "plentiful, sacred, auspicious, providing countless blessings".

(10) Miljković, I.; Gašparec-Skočić, L.; Milat, V.; Strikić, F.; Oplanić, M.; Bjeliš, M. *Olive and Olive oil a gift god to the Croats*; Gašparec-Skočić, L., Milat, V., Marijan, R., Strikić, F., Tratnik, M., Eds.; Mavi d.o.o. Hrvastski centar za poljoprivredu, hranu i selo: Zagreb, 2011.

1.3. Overall importance of the crop and production areas

Aside from its place in legends and symbolism, the olive tree has contributed to shaping landscapes and has greatly determined the agro-economy of the olive-growing countries, including industrial economies based on olive by-products. Over the past decade, food and agribusiness systems have undergone rapid internationalization, and the olive oil industry has been no exception. The steady increase of olive oil in worldwide production and the relative stability of its consumption on major traditional markets have prompted olive-oil dealers to seek growth opportunities abroad. Factors such as the expansion of income in both developed and emerging countries, the shift in dietary habits towards healthier products, and the rise in the prestige of the Mediterranean diet (MD) beyond the Mediterranean region, have all encouraged exporters to enter foreign and new (non-producer) markets[11]. Today, olive oil is the second most important oil crop worldwide after palm oil, olive cultivation exceeding 10 million hectares, the majority concentrated in the traditional Mediterranean area[12]. The mean of total olive oil world production for the last six recent seasons has been 2,951,800 tons. Mediterranean countries account for some 98% of world production, about 72% are produced in the European Union alone, with Spain being the main producer (62%), followed by Italy (21%) and Greece (13.5%). Turkey, Syria, Tunisia as well as Morocco are also important olive oil producers (20.3%)[13]. Regarding table olives, the average of the production in the world for last six years was 2,425,800 tons, of which the European Union was again the main producer (30.5%), followed by Egypt (16.7%), Turkey (15.5%), Syria (5.7%) and Morocco (4.1%)[14]. In addition, more than 70% of olive oil and table olives produced globally are consumed in the Mediterranean area; but its demand

(11) Mili, S. Olive oil marketing on non-traditional markets: Prospects and strategies. *New Medit* **2006**, *5*, 27–37.
(12) FAO. FAOSTAT online database http://faostat.fao.org (*accessed Sep 23, 2015*).
(13) IOC. International Olive Council http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures (*accessed Nov 20, 2014*).
(14) IOC. International Olive Council-Olives de table-Table olives. http://www.internationaloliveoil.org/estaticos/view/132-world-table-olive-figures (*accessed Sep 23, 2015*).

is rapidly increasing in other countries, called the new olive world (**Figure 3**). For example, the consumption of olive oil and table olives in USA has exceeded 9% for the last six years, followed by Brazil (2%), Japan (1.5%) and Canada (1.5%). In countries such as Russia, the consumption of table olives grew from 4,000 tons in 1990 to 75,000 tons in 2014, and in Brazil from 41,000 to 114,000 tons[15].

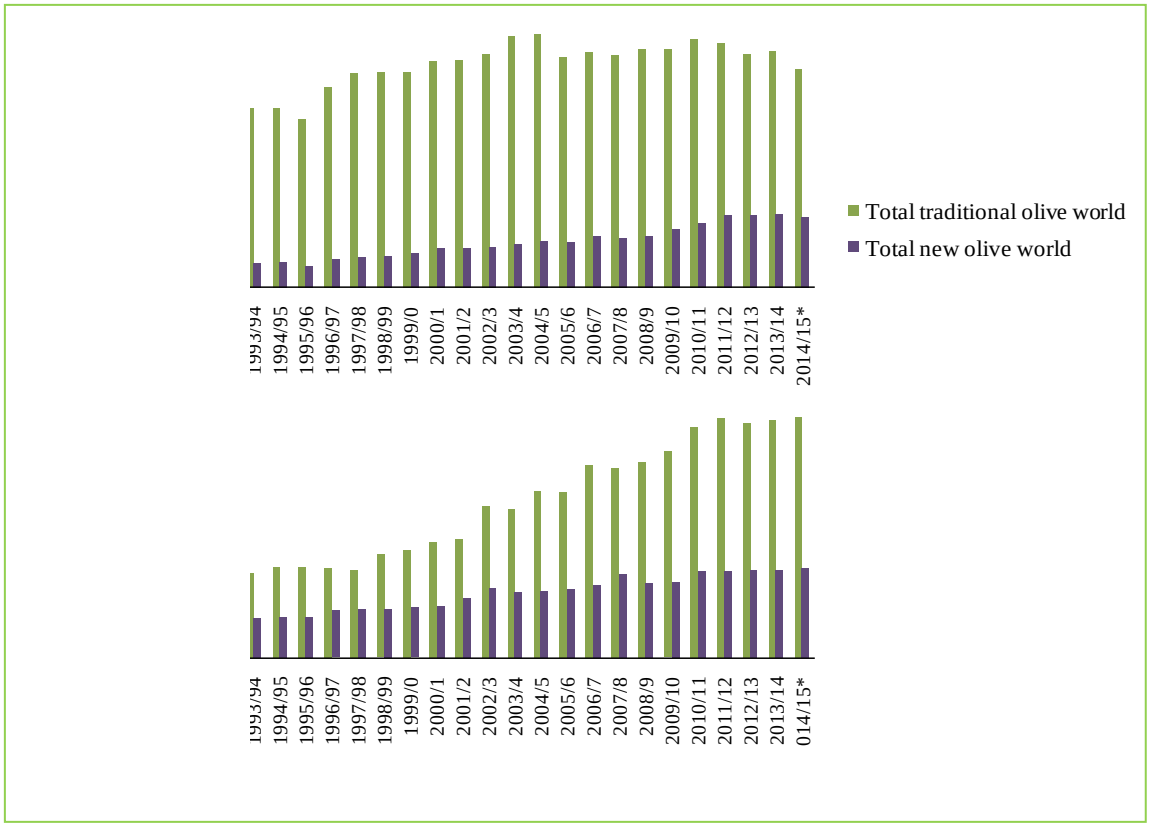

Figure 3. World consumption of olive oil and table olive. * *Provisional data*[15].

1.4. Olive cultivars and production systems

Olive cultivars can be considered as varieties of unknown origin, originating mostly from empirical selections made by growers from naturally cross-bred genotypes over many centuries and propagated from cutting or by grafting. A

(15) IOC. International Olive Council-Consumption. http://www.internationaloliveoil.org/estaticos/view (*accessed Sep 23, 2015*)

few cultivars appear over widespread areas, whereas the most others are highly localized[16]. Approximately 2000 olive cultivars are known in the world[17] with 262 different cultivars identified as being grown in Spain[18].

Overall, there are three broad types of plantation: low-input traditional orchards, intensive farming, and super-intensive modern cultivation. Low-input traditional orchards (80-120 trees/ha) are often of ancient origin and frequently planted on terraces. They are managed with little or no chemical application and their labor input is high. Intensive farming (200-600 trees/ha) shares certain similarities with traditional orchards, but management is more intensive. A greater quantity of artificial fertilizers and pesticides are applied together with more intensive weed control and soil-management techniques. Tree density may be greater and irrigation as well as mechanical harvesting is often used. Super-intensive modern cultivation relies on smaller tree cultivars planted at high densities of (1600-2000 trees/ha). Intensive and highly mechanized systems are implemented, requiring irrigation to create a humid micro-climate that boosts olive-tree growth[19]. More than 100,000 hectares around the world are under this kind of management.

1.5. Olive by-products

By-products derived from olive trees and olive-oil extraction, are generally known as "olive by-products". The different olive by-products catalogued are mainly olive leaves and olive cake (including pulp, skin, stone and water), and olive mill waste water (**Figure 4**). In 1985, a review on the use of olive by-products in ruminant feeding, which included general guidelines for their use in

(16) Baldoni, L.; Belaj, A. Olive. In *oil crops, handbook of plant breeding*; Vollmann, J., Rajcan, I., Eds.; Springer Dordrecht Heidelberg: London New York, **2010**; pp 397–421.
(17) Cavagnaro, P.; Juárez, J.; Bauza, R.; Masuelli, M. Discriminación de variedades de olivo a través del uso de caracteres morfológicos y de marcadores moleculares. *Agriscientia* **2001**, *XVIII*, 27–35.
(18) Barranco, D.; Rallo, L. Olive cultivars in Spain. *Horttechnology* **2000**, *10*, 107–110.
(19) Camarsa, G.; Gardner, S.; Jones, W.; Eldridge, J.; Hudson, T.; Thorpe, E.; O'Hara, E. *Life among the olives: Good practice in improving environmental performance in the olive oil sector*; Luxembourg, 2010.

practice, was published by FAO[20]. The utilization of olive-industry by-products as organic amendments, raw or stabilized through the aerobic fermentation, have been widely reported because they show good efficiency, in terms of fertility as well as chemical, physical and microbiological characteristics of the soils, raising crops productivity[21]. Moreover, olive leaves are a major source of energy, offering a potential of 567,702,107 Kcal per year to be exploited in Andalusia alone, when olive by-products are used as energy biomass[22]. Currently, there are calls to incorporate by-products such as leaves to supplement functional foods, medicines, cosmetics, and pharmaceutical products[23,24].

(20) Sansoucy, R.; Alibes, X.; Berge, P.; Martilotti, F.; Nefzaoui, A. Olive by-products for animal feed http://www.fao.org/docrep/003/x6545e/x6545e00.htm (*accessed Sep 22, 2015*).

(21) Araújo, M.; Pimentel, F.; Alves, R. C.; Oliveira, M. B. P. P. Phenolic compounds from olive mill wastes: health effects, analytical approach and application as food antioxidants. *Trends Food Sci. Technol.* **2015**, *45*, 200–211.

(22) López, J. A. C.; Heras, T. P.; Gordillo, T. M. *Potencial energético de los subproductos de la industria olivarera en Andalucía*; Secretaría General Del Medio Rural Y la Producción Ecológica Junta de Andalucia, 2010.

(23) Guinda, Á.; Pérez-Camino, M. C.; Lanzón, A. Supplementation of oils with oleanolic acid from the olive leaf (O*lea europaea*). *Eur. J. Lipid Sci. Technol.* **2004**, *106*, 22–26.

(24) Briante, R.; Patumi, M.; Terenziani, S.; Bismuto, E.; Febbraio, F.; Nucci, R. *Olea europaea* L. leaf extract and derivatives: antioxidant properties. *J. Agric. Food Chem.* **2002**, *50*, 4934–4940.

Figure 4. Olive cultivation and oil extraction by-products[22,25,26].

(25) Molina-Alcaide, E.; Yáñez-Ruiz, D. Potential use of olive by-products in ruminant feeding: A review. *Anim. Feed Sci. Technol.* **2008**, *147*, 247–264.

(26) Alfano, G.; Lustrato, G.; Lima, G.; Ranalli, G. Present and future perspectives of olive residues composting in the Mediterranean Basin (CompMed). *Dyn. soil, Dyn. plant* **2009**, *3*, 39–56.

2. Therapeutic and medicinal uses of *O. europaea* products and by-products

The olive is valuable not only as food provider, but has long served in the production of soap, and as base for perfumes, and as a fuel for lamps. Moreover, it has a number of traditional and contemporary uses in medicine for a wide range of ailments in various countries. Its bark, fruits, leaves, wood, seeds, and oil are used in different forms, alone or sometimes in combination with other substances.

Historically, olive oil has mainly been used therapeutically and it has been extremely important in general hygiene and care of the body. Massages with oil have been used to soften the skin, relax the muscles, and were considered "a fountain of youth"[9,27]. Historically, beauty, including healthy hair, was also attributed to olive oil. In addition, the ancient Greeks used oil, probably extracted from oleaster, for making perfumes. It formed the basis for producing perfumes and salves when mixed with different essences, such as those from laurel, myrtle, citron, and rose.

Olive oil has also been used in medicine since ancient times and throughout the Middle Ages. Hippocrates on many occasions cited olive oil and referred to its pharmaco-therapeutic uses, highlighting its astringent and antiseptic effects. Dioscorides contended that the oil from unripe olives should be used in unguents and as an emollient and laxative in cases of colic biliary or nephritic calculi. Olive oil has also been used as a component in poultices and ointments for skin lesions, and to treat headaches and scalp disorders. Pliny the Elder described a form of olive that, when retained in the mouth preserved the whiteness of teeth and cured diseased gums[9]. In traditional Arab and Persian medicine, olive oil has been used as a laxative, diuretic, purgative, liver protector, cholagogue, and as

(27) Golzari, S. E.; Valizadeh, L.; Zamanzadeh, V.; Bazzazi, A. Neonatal care and breast feeding in medieval Persian literature : Hakim Esmail Jorjani (1042-1137AD) and the treasure of king Khwarazm : A Review. *Life Sci. J.* **2013**, *10*, 115–120.

prevention of hair loss[28,29]. Maimonides recommended olive oil for the treatment of snakebites[30]. Today, olive oil is still frequently used in folk medicine throughout the Mediterranean area.

Olive leaves have also been widely used in traditional medicine as herbal tea for its health-enhancing qualities, or prepared as a decoction for coughs[28]. In the 1800s, infusions were used to fight malaria[31]. According to Tunisian folk medicine, olive leaves are recommended in a wide range of ailments, including inflammatory disorders, bacterial infections, and hypertension, although modes of preparation and administration vary. Earache is cured by using olive leaves in hot olive oil with salt. Olive-leaf juice, despite its irritation, is recommended for curing trachoma. Also, chewing olive leaves is used to relieve toothaches and to treat lip irritation while the decoction of the leaves, used as a liquid mouthwash, is used for treating aphtous stomatitus, gingivitis, and glossitis[32].

(28) Mikaili, P.; Shayegh, J.; Sarahroodi, S. Pharmacological properties of herbal oil extracts used in Iranian traditional medicine. *Adv. Environ. Biol.* **2012**, *6*, 153–158.
(29) Shoja, M. M.; Tubbs, R. S.; Bosmia, A. N.; Fakhree, M. A. A.; Jouyban, A.; Balch, M. W.; Loukas, M.; Khodadoust, K.; Khalili, M.; Eknoyan, G. Herbal diuretics in medieval Persian and Arabic medicine. *J. Altern. Complement. Med.* **2015**, *21*, 309–320.
(30) Rosner, F. The life of Moses Maimonides, a prominent medieval physician. *Einstein J. Biol. Med.* 2002, 19, 125–128.
(31) Barrett, L. *Olive leaf extract the Mediterranean healing herb*; Stepaniak, J., Ed.; Healthy living public, 2015.
(32) Kuete, V. *Medicinal Plant Research in Africa Pharmacology and Chemistry*; Kuete, V., Ed.; Elsevier insights: London, 2013.

3. Main bioactive compounds in *O. europaea* products and by-products

The MD represents the dietary pattern traditionally consumed by the populations bordering the Mediterranean Sea and has been widely reported to promote health and a better quality of life. Since the first study providing data from "the seven countries"[33], a large mass of research provides evidence for the health benefits of the constituents of this diet. Considered as a major basis of the MD, the olive and its derivatives have stimulated greater interest in olive products, prompting wide nutritional, pharmacological, and chemical research, revealing different bioactive compounds in different matrices of olive tree. Regarding the olive products and by-products derived from olive tree, this PhD thesis focuses on one of the main by-products, i.e. olive leaves, as well as olive fruit and olive oil as products of the olive tree.

3.1. Main bioactive compounds in olive leaves

The bioactive ingredients in olive leaves have attracted the interest of scientists in the food, medical, and cosmetic sectors. In fact, several studies have demonstrated that olive leaves contain numerous phytochemical compounds with a number of healthful properties. The bioactive compounds most reported are tocopherols, triterpenoids, pigments, and phenolic compounds. Moreover, triterpene oleanolic and betulinic acids and triterpenols sitosterol, α and β-amyrin, uvaol, and erythrodiol in addition to fatty acids have been found to be the main components of the chloroform-soluble epicuticular waxes of olive leaves[34]. Synergistic activity between or among classes of bioactive ingredients in leaves has been extensively suggested in literature[35].

(33) Keys, A.; Menotti, A.; Karvonen, M. J.; Aravanis, C.; Blackburn, H.; Buzina, R.; Djordjevic, B. S.; Dontas, A. S.; Fidanza, F.; Keys, M. H. The diet and 15-year death rate in the seven countries study. *Am. J. Epidemiol.* **1986**, *124*, 903–915.

(34) Bianchi, G.; Vlahov, G.; Anglani, C.; Murelli, C. Epicuticular wax of olive leaves. *Phytochemistry* **1992**, *32*, 49–52.

(35) Tsimidou, M. Z.; Papoti, V. T. Bioactive ingredients in olive leaves. In *olives and olive oil in health and disease prevention*; 2010; pp 349–356.

Figure 5 summarizes the most important bioactive compounds reviewed in olive leaves.

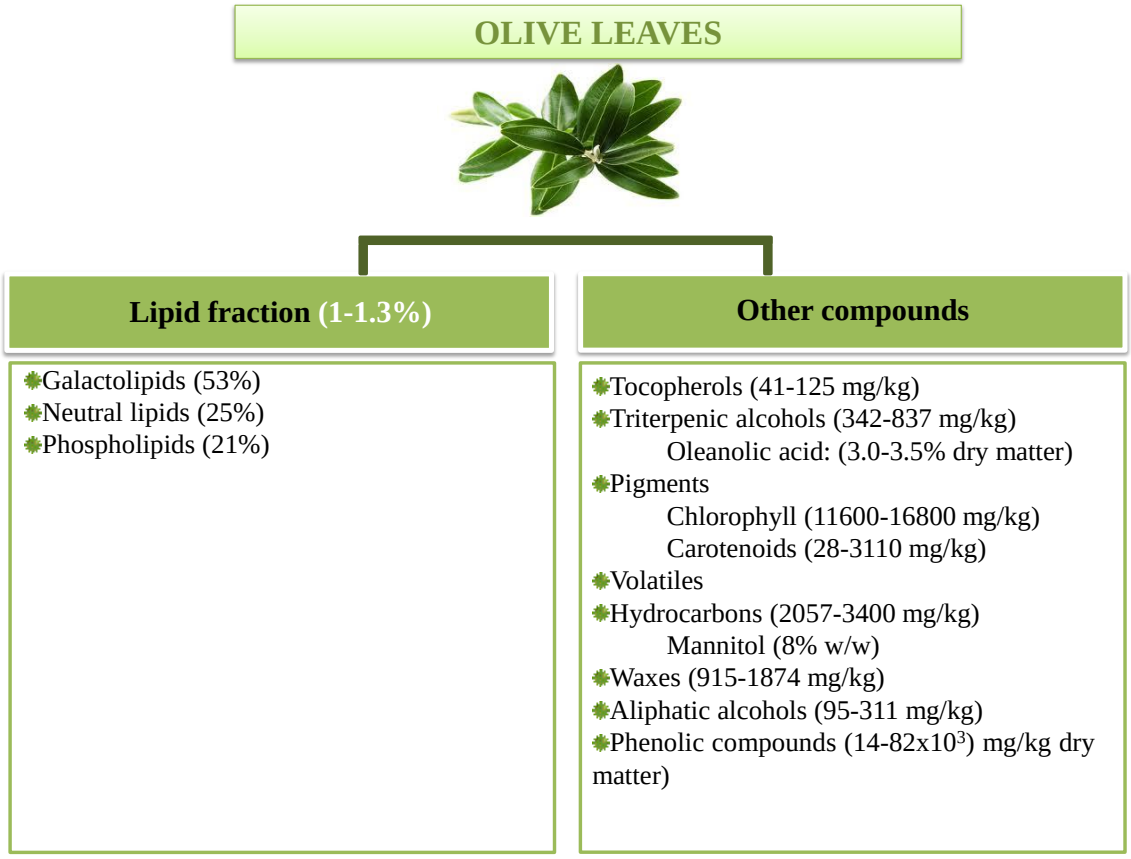

Figure 5. Main bioactive compounds in olive leaves.

Tocopherols (vitamin E) constitute a group chemically characterized as 6-hydroxychroman derivatives bearing an isoprenoid unit. The group is represented by two types of compounds: four tocopherols (*α, β, γ,* and *δ*) and four tocotrienols (*α, β, γ,* and *δ*), which differ in the presence of double bonds in the isoprenoid unit of the latter type[36]. Considered the main representative of this group in olive leaves, *α*-tocopherols [37]are well-known to exhibit a protective role in lipid peroxidation of membrane lipids, lipoproteins, and depot fats, thus

(36) Dilis, V.; Trichopoulou, A. Mediterranean diet and olive oil consumption-estimations of daily intake of antioxidants from virgin olive oil and olives. In *olive oil: minor constituents and health*; Boskou, D., Ed.; CRC press: 2008; pp 201–210.

(37) Lee, O.-H.; Lee, B.-Y.; Lee, J.; Lee, H.-B.; Son, J.-Y.; Park, C.-S.; Shetty, K.; Kim, Y.-C. Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. *Bioresour. Technol.* **2009**, *100*, 6107–6113.

protecting against atherosclerosis, inducing apoptosis in tumor cells, and modulating oncogenes[38].

Triterpenes. Oleanolic acid content prevails with regard to erythrodiol, uvaol, ursolic, and maslinic acids in olive leaves[35]. The non-enzymatic antioxidant activities of oleanolic acid and ursolic acid in a liposome system have been found to surpass even α-tocopherol activity under certain conditions[39]. Both acids as well as erythrodiol, uvaol, and maslinic acid reportedly benefit human health, having antihypertensive, antiatherosclerotic, antioxidant, cardiotonic or antidysrhythmic properties [40,41]

Pigments. Chlorophyll and carotenoids, naturally abundant in olive leaves, are directly involved in light-harvesting processes in photosynthesis. Evidence of the functional properties of chlorophyll-derived products has been reported from their beneficial effects on odor suppression and wound healing[42]. The most carotenoid present in leaves, β-carotene, is well known to have antioxidant activity in atherogenesis and cancer[43].

Volatiles. The chemical composition of the volatile fractions from leaves includes mainly aldheydes, 2-decenal-(E), benzene acetaldehyde, 2-undecenal,

(38) Gómez-Caravaca, A. M.; Verardo, V.; Caboni, M. F. Chromatographic techniques for the determination of alkyl-phenols, tocopherols and other minor polar compounds in raw and roasted cold pressed cashew nut oils. *J. Chromatogr. A* **2010**, *1217*, 7411–7417.

(39) Yin, M. C.; Chan, K. C. Nonenzymatic antioxidative and antiglycative effects of oleanolic acid and ursolic acid. *J. Agric. Food Chem.* **2007**, *55*, 7177–7181.

(40) Guinda, Á.; Castellano, J. M.; Santos-Lozano, J. M.; Delgado-Hervás, T.; Gutiérrez-Adánez, P.; Rada, M. Determination of major bioactive compounds from olive leaf. *LWT - Food Sci. Technol.* **2015**, *64*, 431–438.

(41) Sánchez Avila, N.; Priego Capote, F.; Luque de Castro, M. D. Ultrasound-assisted extraction and silylation prior to gas chromatography-mass spectrometry for the characterization of the triterpenic fraction in olive leaves. *J. Chromatogr. A.* **2007**, *1165*, 158–165.

(42) Humphery, A. M. Chlorophyll as a color and functional ingredient. *J. Food Sci.* **2004**, *69*, C422–C425.

(43) Tabera, J.; Guinda, Á.; Ruiz-Rodríguez, A.; Señoráns, F. J.; Ibáñez, E.; Albi, T.; Reglero, G. Countercurrent supercritical fluid extraction and fractionation of high-added-value compounds from a hexane extract of olive leaves. *J. Agric. Food Chem.* **2004**, *52*, 4774–4779.

valencen and ethyl oleate, (E)-2-hexenal, (E,E)-a-farnesene, b-caryophyllene, (E)-b-damascenone, (E,Z)-2,4-hexadienal, nonanal and (E)-b-damascone[44]. The antibacterial and antifungal activities of the volatile fractions from fresh and dried leaves have been reported by Brahmi et al.[45].

Carbohydrates. Mannitol is the main characteristic sugar of olive leaves and its biosynthesis is generally well known in throughout the family Oleaceae. Mannitol offers olive leaves a series of properties with advantageous applications in the food and pharmaceutical industries. Thanks to its sweetening potency (equivalent to 70% of that of sucrose) with low caloric value (2 kcal/g), it does not cause caries and its metabolism in humans does not depend on insulin, making it suitable for consumption by diabetics. Additionally, it has healthful effects as an antioxidant[40,46].

Phenolic compounds. Extracted from olive leaves have provided chemists with numerous synthetic challenges. Because of their high antioxidant capacity, phenolic compounds in olive leaves may have promote beneficial effects on human health. Moreover, due to the richness of valuable phenolics in olive leaves, several studies have focused on the composition of olive leaves in terms of phenolic compounds. Given the importance of these compounds and given that phenolic compounds constitute the core of this thesis; greater information about them will be revealed in the following sections.

(44) Taamalli, A. Characterization of polyphenols in Tunisian olive with anticancer capacity using liquid chromatography coupled to mass spectrometry, University of Granada, 2012.
(45) Brahmi, F.; Flamini, G.; Issaoui, M.; Dhibi, M.; Dabbou, S.; Mastouri, M.; Hammami, M. Chemical composition and biological activities of volatile fractions from three Tunisian cultivars of olive leaves. *Med. Chem. Res.* **2011**, *21*, 2863–2872.
(46) Guinda, Á. Use of solid residue from the olive industry. *Grasas y aceites* **2006**, 57, 107–115.

3.2. Main bioactive compounds in olive fruit

Fresh olives are not edible because of the presence of a bitter glucoside, oleuropein. However, they are considered a highly functional food either for their direct consumption as table olives or indirect consumption as olive oil. Phenolic compounds make up 2-3% of olive flesh and are one of the major compounds responsible of the bioactive functionality of olive fruits. Further information about these compounds is provided in the present thesis (paragraph 4). The other functionnal and nutritional bioactive compounds are principally phytosterols, proteins, triterpenic acids, squalene, fibers, fatty acids, etc.[47]. Representative bioactive compounds in olive fruits are given in **Figure 6.**

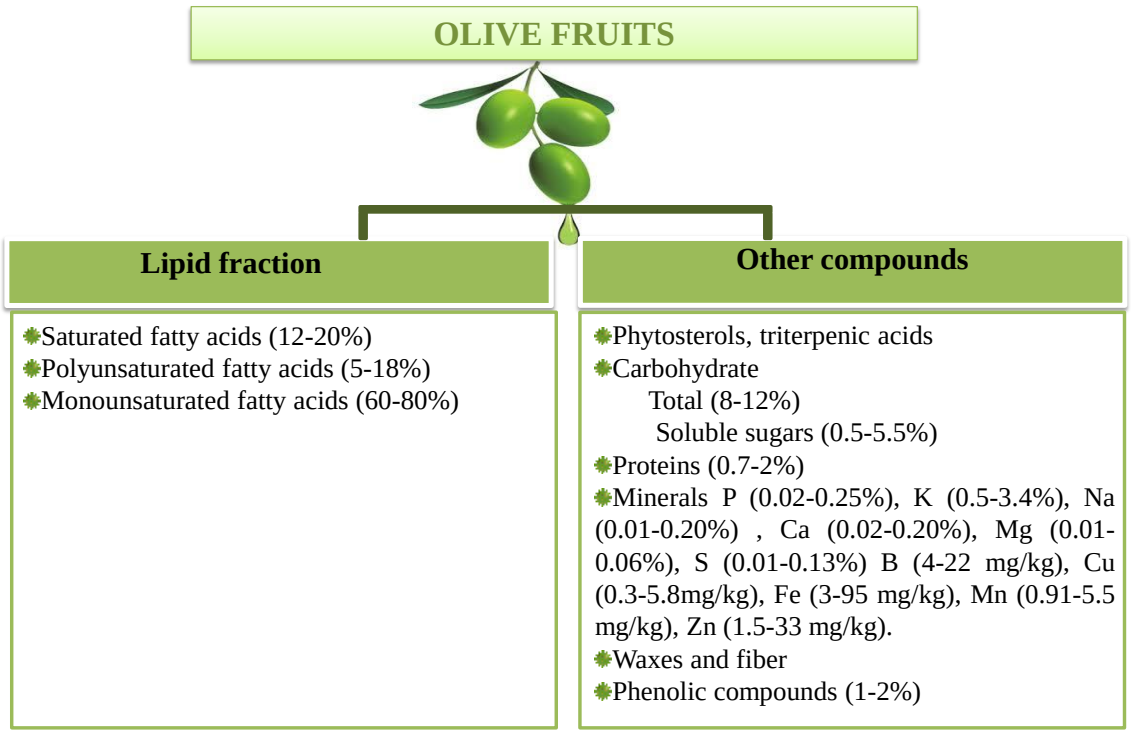

Figure 6. Main bioactive compounds in olive fruits[48].

(47) Boskou, D. Characteristics of the olive tree and olive fruit. In o*live oil chemistry and technology*; AOCS Press, 2006; pp 13–19

(48) Kailis, S.; Harris, D. *Producing table olives*, Kailis, G.; Landlinks press, 2007.

Phytosterols are structurally similar to the body's cholesterol and when consumed they compete with cholesterol for absorption in the digestive system. As a result, they are considered functional ingredients, and therefore high dietary intake might have a positive impact on health. However, the concentration in olive fruits is too low for a significant effect. The consumption of 1.5-2.0 g per day is claimed to exert a hypocholesterolemic effect[49].

Proteins. Olive fruits contain low levels of soluble and insoluble proteins. Major amino acids in raw olives include arginine, alanine, aspartic acid, glutamic acid, and glycine. Other amino acids such histidine, lysine, methionine phenylalanine, turosine are also present[50]. Protein content is an important part of the nutritional value of the olive fruits[51].

Triterpenes. These are represented by the triterpenic acids maslinic and oleanolic acids in olive fruits[52]. Both acids are considered to be important bioactive compounds that potentially benefit human health. Interest in their pharmacological potential focuses on inflammation, cancer cardiovascular pathology and vasorelaxation[49].

Squalene is an unsaturated terpene widely distributed in nature. It is believed to favor human health by exerting a chemopreventive effect in some types of cancer and it is beneficial for patients' with heart disease and diabetes[53].

(49) Boskou, D.; Camposeo, S.; Clodoveo, M. L. Table olives as sources of bioactive compounds. In *olive and olive oil bioactive constituents*; Boskou, D., Ed.; AOCS Press, 2015; pp 217–259.
(50) Manoukas, A. G.; Mazomenos, B.; Patrinou, M. A. Amino acid compositions of three varieties of olive fruit. *J. Agric. Food Chem.* **1973**, *21*, 215–217.
(51) Montealegre, C.; Esteve, C.; García, M. C.; García-Ruiz, C.; Marina, M. L. Proteins in olive fruit and oil. *Crit. Rev. Food Sci. Nutr.* **2014**, *54*, 611–624.
(52) Guinda, A.; Rada, M.; Delgado, T.; Gutiérrez-Adánez, P.; Castellano, J. M. Pentacyclic triterpenoids from olive fruit and leaf. *J. Agric. Food Chem.* **2010**, *58*, 9685–9691.
(53) Ronco, A. L.; Stéfani, E. De. Squalene : A multi-task link in the crossroads of cancer and aging. *Funct. Foods Heal. Dis.* **2013**, *3*, 462–476.

Fiber consists mainly of pectin, hemicellulose, cellulose, and lignin. The bioactivity of these compounds is due principally to their propensity to reduce the absorption of cholesterol and the glucose in type-2 diabetes, to increase satiety, protect stomach mucosa, and to have a laxative effect[49].

Fatty acids. Most of lipid fraction in olive fruits consists of triacylglycerols (98%), which are esters derived from the union of glycerol and three fatty acids, generally unsaturated. The proportions of the more commonly occurring triacylglycerols in olive flesh are: OOO (40-60%), POO (12-20%), OOL (12.5-20%), SOO (3-7%), and POL (5.5-7%) (O=oleic acid; P=palmitic acid; S=stearic acid and L=linoleic acid)[48]. Olive fruits contain a high amount of oleic acid well known as mono-unsaturated fatty acid (MUFA). The positive effect of that high-MUFA diets exert on a variety of health conditions, including cardiovascular disease and cancer, has widely been reported[54–56].

3.3. Main bioactive compounds in extra-virgin olive oil

Extra-virgin olive oil (EVOO) is an integral ingredient of the MD and accumulating evidence suggests that its health benefits include reducing risk factors of coronary heart disease, preventing several types of cancers, and aiding immune and inflammatory responses. Olive oil appears to be an example of a functional food, with varied components that may contribute to its overall therapeutic characteristics. Bioactive compounds in EVOO include several families of chemicals such as fatty acids, phospholipids, phytosterols,

(54) Pérez-Jiménez, F.; Ruano, J.; Perez-Martinez, P.; Lopez-Segura, F.; Lopez-Miranda, J. The influence of olive oil on human health: Not a question of fat alone. *Mol. Nutr. Food Res.* **2007**, *51*, 1199–1208.

(55) De la Torre, R. Bioavailability of olive oil phenolic compounds in humans. *Inflammopharmacology* **2008**, *16*, 245–247.

(56) López-Miranda, J.; Pérez-Jiménez, F.; Ros, E.; De Caterina, R.; Badimón, L.; Covas, M. I.; Escrich, E.; Ordovás, J. M.; Soriguer, F.; Abiá, R.; et al. Olive oil and health: Summary of the II international conference on olive oil and health consensus report, Jaén and Córdoba (Spain) 2008. *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 284–294.

triterpenoids, and phenolic compounds[57]. Representative bioactive compounds among those typically classified in EVOO are presented in **Figure 7**.

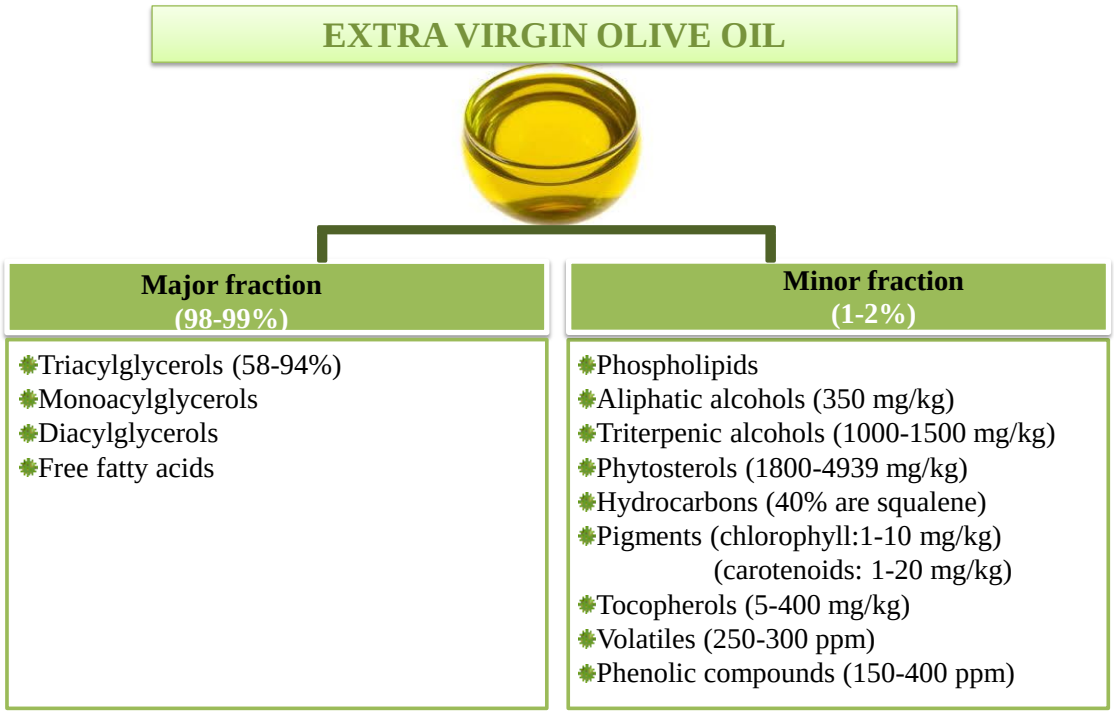

Figure 7. Main bioactive components of EVOO.

Fatty acids. As in olive fruits, the most abundant triacylglycerols found in EVOO are OOO (43.5 %), POO (18.4 %), OOL (6.8 %), POL (5.9 %) and SOO (5.1 %) (O=oleic acid; P=palmitic acid; S=stearic acid and L=linoleic acid)[58]. It is well known that the healthful properties of EVOO are attributed to a high proportion of monounsaturated fatty acids. Epidemiological and experimental studies attribute the beneficial effect of olive oil consumption to the presence of monounsaturated fatty acids (MUFAs), mainly oleic acid. Recent findings have shown that high intake of monounsaturated fat can induce a wide range of

(57) Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr. A.* **2004**, *1054*, 113–127.

(58) Fedeli, E. Lipids of olives. *Prog. Chem. Fats Other Lipids* **1977**, *15*, 57–74.

biological effects on the cardiovascular system[59], on homeostasis, and on plasma cholesterol[54].

Phospholipids are found in small quantities in freshly produced olive oils, their concentration being lower with the age of the oil. However, their beneficial functionality for all body cells and their character as antioxidants are well reported[60].

Phytosterols are tetracyclic compounds biosynthesized from squalling. They are important constituents of olive oil and constitute the major proportion of its unsaponifiable fraction. These compounds reportedly have hypocholesterolemy[61], anti-inflammatory, and anti-carcinogenic effects, and the amount of sterols in particular oil may be used to identify its origin as well as its purity [62]. The main sterols found in olive oil are *β*-sistosterol, *Δ*-5-avenasterol, campesterol, and stigmasterol[63].

Compounds such as chlorophylls, *β*-carotene, *α*-tocopherols, squalene, triterpenes (oleanolic acid, maslinic acid, uvaol, and erythrodiol), volatiles, and phenolic compounds are present also in EVOO. Their biological activities in leaves and fruits are also widely reported in EVOO. They are biologically active with anti-abortive, anti-cariogenic, anti-hepatotoxic, anti-inflammatory, cancer-

(59) Pérez-Jiménez, F.; Lista, J. D.; Pérez-Martínez, P.; López-Segura, F.; Fuentes, F.; Cortés, B.; Lozano, A.; López-Miranda, J. Olive oil and haemostasis: A review on its healthy effects. *Public Health Nutr.* **2006**, *9*, 1083–1088.

(60) Montealegre, C.; Verardo, V.; Gómez-Caravaca, A. M.; García-Ruiz, C.; Marina, M. L.; Caboni, M. F. Molecular characterization of phospholipids by high-performance liquid chromatography combined with an evaporative light scattering detector, high-performance liquid chromatography combined with mass spectrometry, and gas chromatography combined with a flame ionization detector in different oat varieties. *J. Agric. Food Chem.* **2012**, *60*, 10963–10969.

(61) Plat, J.; Mensink, R. P. Plant stanol and sterol esters in the control of blood cholesterol levels: Mechanism and safety aspects. *Am. J. Cardiol.* **2005**, *96*, 15–22.

(62) Lukić, M.; Lukić, I.; Krapac, M.; Sladonja, B.; Piližota, V. Sterols and triterpene diols in olive oil as indicators of variety and degree of ripening. *Food Chem.* **2013**, *136*, 251–258.

(63) Quiles, J.; Ramírez-Tortosa, M.; Yaqoob, P. *Olive oil and health*; Quiles, J. L., Ramírez-Tortosa, M. C., Yaqoob, P., Eds.; Cromwell Press, 2006.

preventive, cardiotonic, diuretic, hepatoprotective, and uterotonic properties[36,49,64,65].

Phenolic compounds in EVOO are probably the compounds most intensely studied in the scientific community over the last two decades. Oleocanthal, for example is one of most discussed, credited with anti-inflammatory properties similar to those of ibuprofen[66]. More details about these compounds are provided in the present thesis (Paragraph 4).

(64) Sánchez-Quesada, C.; López-Biedma, A.; Warleta, F.; Campos, M.; Beltrán, G.; Gaforio, J. J. Bioactive properties of the main triterpenes found in olives, virgin olive oil, and leaves of *Olea europaea*. *J. Agric. Food Chem.* **2013**, *61*, 12173–12182.

(65) Villar, V. H.; Vögler, O.; Barceló, F.; Gómez-Florit, M.; Martínez-Serra, J.; Obrador-Hevia, A.; Martín-Broto, J.; Ruiz-Gutiérrez, V.; Alemany, R. Oleanolic and maslinic acid sensitize soft tissue sarcoma cells to doxorubicin by inhibiting the multidrug resistance protein MRP-1, but not P-glycoprotein. *J. Nutr. Biochem.* **2014**, *25*, 429–438.

(66) Lucas, L.; Russell, A.; Keast, R. Molecular mechanisms of inflammation. Anti-inflammatory benefits of virgin olive oil and the phenolic compound oleocanthal. *Curr. Pharm. Des.* **2011**, *17*, 754–768.

4. Phenolic compounds in olive leaves, fruit and oil

Phenolic compounds are a large class of secondary plant metabolites possessing an aromatic ring bearing one or more hydroxyl groups, including their functional derivatives. An initial classification of phenolic compounds could be based on the number of phenol units in the molecule dividing them into simple phenols and polyphenols. Thus, these compounds may be classified into different groups depending upon the number of phenol rings that they bear and on the structural elements that bind these rings to one[67].

Structurally, despite their extreme variety, phenolic compounds come from a common carbon-skeleton building block: a C6–C3 phenylpropanoid unit. The biosynthesis process leads to a wide range of plant phenols: cinnamic acids (C6–C3), benzoic acids (C6–C1), flavonoids (C6–C3–C6), proanthocyanidins [(C6–C3–C6)n], coumarins (C6–C3), stilbenes (C6–C2–C6), lignans (C6–C3–C3–C6), and lignins[(C6–C3)n][68].

In olive products and by-products, the main families of phenolic compounds so far described include: simple phenols (phenolic alcohols and phenolic acids), flavonoids, secoiridoids, and lignans (**Figure 8**).

(67) Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. Polyphenols: Food sources and bioavailability. *Am. J. Clin. Nutr*. **2004**, *79*, 727–747.

(68) Lamuela-Raventós, R. M.; Vallverdú-Queralt, A.; Jáuregui, O.; Martínez-Huélamo, M.; Quifer-Rada, P. Improved characterization of polyphenols using liquid chromatography. In *Polyphenols in plants: Isolation, purification and extract preparation*; Watson, R. R., Ed.; Academic press Elsevier, 2014; pp 261–292.

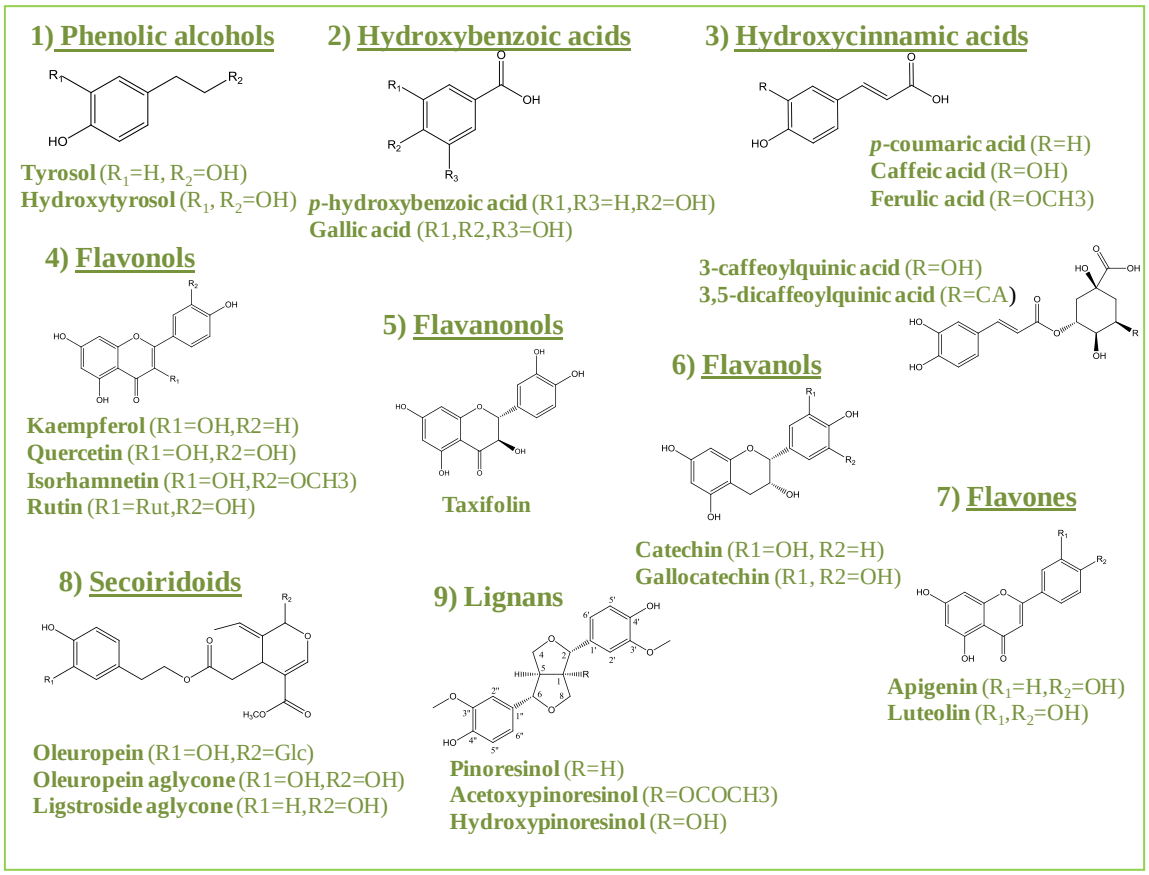

Figure 8. Examples of phenolic compounds present in olive fruit, EVOO and olive leaves. Adapted from Contreras-Gámez et al.[69]. *CA, caffeic acid; Glc, glucose; Rut, rutinose.*

4.1. Simple phenols

Simple phenols, which include phenolic acids and phenolic alcohols, are responsible for the sensory and organoleptic attributes (taste and astringency) as well as the antioxidant proprieties of olive oil[70].

(69) Contreras Gámez, M.M; Rodríguez-Pérez, C.; García Salas, P.; Segura Carretero, A. Polyphenols from the Mediterranean diet: Structure, analysis and health evidence. In *Occurrence, structure, biosynthesis, and health benefits based on their evidences of medicinal phytochemicals in vegetables and fruits. Volume 1.*; Motohashi, N., Ed.; Nova Sicence: New, 2014; pp 141–209.

(70) Bendini, A.; Cerretani, L.; Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Lercker, G. Phenolic molecules in virgin olive oils: A survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules* **2007**, *12*, 1679–1719.

Phenolic acids are secondary aromatic plant metabolites that are widely spread throughout the plant kingdom. They are represented by three major subclasses: hydroxybenzoic acids (C6-C1), hydroxyphenylacetic acids (C6-C2), and hydroxycinnamic acids (C6-C3). Further, some authors have suggested phenolic acids as potential markers for the olive cultivar and harvest time[71]. Overall, it has been reported that gallic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, and sinapic acids are the major phenolic acids found in EVOO[72,73], whereas verbascoside is the main phenolic acid in olive leaves and fruits[74,75].

On the other hand, phenolic alcohols identified in olive products and by-products belong to phenylethanoids subclass with carbon skeleton (C6-C2), represented mainly by tyrosol and hydroxytyrosol.

The main classes of simple phenols are presented in olive fruits, olive oil, and olive leaves. However, glycoside forms of tyrosol and hydroxytyrosol have only been only identified in olive leaves and fruits. **Table 1** summarizes the main simple phenols present in olive leaves, fruit, and oil.

(71) Rivas, A.; Sanchez-Ortiz, A.; Jimenez, B.; García-Moyano, J.; Lorenzo, M. L. Phenolic acid content and sensory properties of two Spanish monovarietal virgin olive oils. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 621–630.

(72) Alu'datt, M. H.; Rababah, T.; Ereifej, K.; Gammoh, S.; Alhamad, M. N.; Mhaidat, N.; Kubow, S.; Johargy, A.; Alnaiemi, O. J. Investigation of natural lipid-phenolic interactions on biological properties of virgin olive oil. *J. Agric. Food Chem.* **2014**, *62*, 11967–11975.

(73) Gambacorta, G.; Faccia, M.; Trani, A.; Lamacchia, C.; Gomes, T. Phenolic composition and antioxidant activity of Southern Italian monovarietal virgin olive oils. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 958–967.

(74) Charoenprasert, S.; Mitchell, A. Factors influencing phenolic compounds in table olives (*Olea europaea*). *J. Agric. Food Chem.* **2012**, *60*, 7081–7095.

(75) Ahmad-Qasem, M. H.; Cánovas, J.; Barrajón-Catalán, E.; Carreres, J. E.; Micol, V.; García-Pérez, J. V. Influence of olive leaf processing on the bioaccessibility of bioactive polyphenols. *J. Agric. Food Chem.* **2014**, *62*, 6190–6198.

Table 1. Main simple phenols in olive leaves,fruit, and oil.

Class	Subclass	Phenolic compounds	Leaves	Fruits	Oil
Phenolic acids	*Hydroxybenzoic acids*	*p*-hydroxybenzoic acid	**X**	**X**	**X**
		Gallic acid	**X**	**X**	**X**
		Protocatechuic acid	**X**	**X**	**X**
		Vanillic acid	**X**	**X**	**X**
		Homovanillic acid	**X**	**X**	**X**
		Vanillin	**X**	**X**	**X**
		Syringic acid	**X**	**X**	**X**
	Hydroxyphenylacetic acids	Hydroxyphenylacetic acid	**X**	**X**	**X**
		Phenylacetic acid	**X**	**X**	**X**
		Rosmarinic acid	**X**		
	Hydroxycinnamic acids	*p*-coumaric acid	**X**	**X**	**X**
		Chlorogenic acid	**X**	**X**	**X**
		Caffeic acid	**X**	**X**	**X**
		Ferulic acid	**X**	**X**	**X**
		Sinapic acid	**X**	**X**	**X**
		Cinnamic acid	**X**	**X**	**X**
		Verbascoside	**X**	**X**	
		3-caffeoylquinic acid	**X**	**X**	
		3,5-dicaffeoylquinic acid	**X**	**X**	
Phenolic alcohols	*Phenylethanoids*	Tyrosolglucoside	**X**	**X**	
		Hydroxytyrosolglucoside	**X**	**X**	
		Tyrosol	**X**	**X**	**X**
		Tyrosol acetate		**X**	**X**
		Hydroxytyrosol	**X**	**X**	**X**
		Hydroxytyrosol acetate		**X**	**X**
		3,4-dihydroxyphenylglycol		**X**	

4.2. Flavonoids

The basic flavonoid structure is formed by two aromatic rings linked by a three-carbon bridge. They are formed *via* condensation of a phenylpropane (C6–C3) compound with the participation of three molecules of malonyl coenzyme A, this leading to the formation of chalcones that subsequently cyclize under acidic conditions. The major classes of flavonoids differ mainly in the degree of oxidation of the three-carbon bridge. The major flavonoids present in olive products and by-products are anthocyanins, flavonols, flavanonols, flavanols, and flavones (**Table 2**). In general, anthocyanins appear in colored vegetables and have strong antioxidant activity[69]. They are present in olive fruits and are frequently account for the purple-black colors of olive fruits during ripening

process. Furthermore, rutin, luteolin 7-*O*-glucoside, apigenin and luteolin are reported to be the main flavonoids identified in olive leaves[76–78] and fruits[79,80].

Several authors have reported that flavonoids such as luteolin and apigenin are also the main flavonoids in olive oil[81,82]. Luteolin may originate from rutin or luteolin-7-*O*-glucoside, and apigenin from apigenin glucoside, by the enzymatic activities occurring during the oil-extraction process[83,84].

(76) Goulas, V.; Papoti, V. T.; Exarchou, V.; Tsimidou, M. Z.; Gerothanassis, I. P. Contribution of flavonoids to the overall radical scavenging activity of olive (*Olea europaea* L.) leaf polar extracts. *J. Agric. Food Chem.* **2010**, *58*, 3303–3308.

(77) Heimler, D.; Pieroni, M.; Tattini, A.; Cimato, A. Determination of flavonoids , flavonoid glycosides and biflavonoids in *Olea europaea* L. leaves. *Chromatogrphia* **1992**, *33*, 369–373.

(78) Taamalli, A.; Arráez-Román, D.; Ibañez, E.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Optimization of microwave-assisted extraction for the characterization of olive leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS2. *J. Agric. Food Chem.* **2012**, *60*, 791–798.

(79) Yorulmaz, A.; Poyrazoglu, E. S.; Ozcan, M. M.; Tekin, A. Phenolic profiles of Turkish olives and olive oils. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 1083–1093.

(80) Vinha, A. F.; Ferreres, F.; Silva, B. M.; Valentão, P.; Gonçalves, A.; Pereira, J. A.; Oliveira, M. B.; Seabra, R. M.; Andrade, P. B. Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): Influences of cultivar and geographical origin. *Food Chem.* **2005**, *89*, 561–568.

(81) Carrasco-Pancorbo, A.; Neusüss, C.; Pelzing, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. CE- and HPLC-TOF-MS for the characterization of phenolic compounds in olive oil. *Electrophoresis* **2007**, *28*, 806–821.

(82) Ouni, Y.; Taamalli, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Zarrouk, M. Characterisation and quantification of phenolic compounds of extra-virgin olive oils according to their geographical origin by a rapid and resolutive LC–ESI-TOF MS method. *Food Chem.* **2011**, *127*, 1263–1267.

(83) Frankel, E.; Bakhouche, A.; Lozano-Sánchez, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Literature review on production process to obtain extra virgin olive oil enriched in bioactive compounds. Potential use of byproducts as alternative sources of polyphenols. *J. Agric. Food Chem.* **2013**, *61*, 5179–5188.

(84) Gómez-Rico, A.; Inarejos-García, A. M.; Salvador, M. D.; Fregapane, G. Effect of malaxation conditions on phenol and volatile profiles in olive paste and the corresponding virgin olive oils (*olea europaea* L. cv. Cornicabra). *J. Agric. Food Chem.* **2009**, *57*, 3587–3595.

Table 2. Flavonoids in olive leaves, fruits and oil.

Class	Subclass	Phenolic compounds	Leaves	Fruits	Oil
Flavonoids	*Anthocyanins*	Cyanidin-3-*O*-glucoside		**X**	
		Cyanidin-3-*O*-rutinoside		**X**	
	Flavanonols	Taxifolin	**X**	**X**	**X**
	Flavones	Luteolin	**X**	**X**	**X**
		Apigenin	**X**	**X**	**X**
		Diosmetin	**X**	**X**	**X**
		Luteolin glucoside/diglucoside	**X**	**X**	
		Apigenin glucoside	**X**	**X**	
		Apigenin rutinoside	**X**	**X**	
		Diosmetin glucoside	**X**	**X**	
		Chrysoeriol glucoside	**X**	**X**	
	Flavonols	Catechin	**X**	**X**	**X**
		Gallocatechin		**X**	
		Kaempferol	**X**		**X**
		Kaempferol-3-glucoside	**X**		
		Quercetin	**X**	**X**	**X**
		Quercetin rhamnoside	**X**	**X**	
		Rutin	**X**	**X**	**X**
	Flavanones	Hesperidin	**X**		

4.3. Lignans

Lignans are dimers of phenylpropanoid (C6–C3) units linked by the central carbons of their side chains. Among the matrices under study, olive oil was the one that presented the highest quantities of these compounds. Lignans such as pinoresinol and its derivatives (acetoxypinoresinol, hydroxypinoresinol) are among the constituents of the olive oil phenol fraction, and they have been demonstrated to contribute to the stability of olive oil[85,86]. Furthermore, syringaresinol has also been detected in olive oil[87]. However, few references

(85) Dais, P.; Boskou, D. Detection and quantification of phenolic compounds in olive oil, olives, and biological fluids. *In olive oil minor constituents and health*; Boskou, D., Ed.; CRC Press, 2008; pp 55–107.

(86) Gómez-Caravaca, A. M.; Verardo, V.; Bendini, A.; Gallina-Toschi, T. From wastes to added value by-products: An overview on chemical composition and healthy properties of bioactive compounds of olive oil chain by-products. *In virgin olive oil: production, composition, uses and benefits for man*; Leonardis, A. De, Ed.; Nova publishers: 2014; pp 301–334.

(87) Christophoridou, S.; Dais, P. Detection and quantification of phenolic compounds in olive oil by high resolution 1H nuclear magnetic resonance spectroscopy. *Anal. Chim. Acta* **2009**, *633*, 283–292.

have reported the presence of such lignans in olive leaves[88–90]. Indeed, olive fruits show small amounts of lignans compared to EVOO[91,92] (**Table 3**).

Table 3. Lignans in olive leaves, fruits and oil.

Class	Phenolic compounds	Leaves	Fruits	Oil
Lignans	Pinoresinol	X	X	X
	Acetoxypinoresinol	X	X	X
	Hydroxypinoresinol			X
	Syringaresinol	X		X

4.5. Secoiridoids

Secoiridoids are usually derived from oleosides, which are characterized by an exocyclic 8,9-olefinic functionality, and they are exclusive to the Oleaceae family (**Figure 8,** above). Secoiridoids are not necessarily phenolic compounds but include a phenolic moiety in their structure as a result of esterification[93,94].

(88) Taamalli, A.; Arráez-Román, D.; Barrajón-Catalán, E.; Ruiz-Torres, V.; Pérez-Sánchez, A.; Herrero, M.; Ibañez, E.; Micol, V.; Zarrouk, M.; Segura-Carretero, A.; et al. Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells. *Food Chem. Toxicol.* **2012**, *50*, 1817–1825.

(89) Japón-Luján, R.; Luque-Rodríguez, J. M.; Luque de Castro, M. D. Dynamic ultrasound-assisted extraction of oleuropein and related biophenols from olive leaves. *J. Chromatogr. A* **2006**, *1108*, 76–82.

(90) Herrero, M.; Temirzoda, T. N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. *J. Chromatogr. A* **2011**, *1218*, 7511–7520.

(91) Goulas, V.; Charisiadis, P.; Gerothanassis, I.; Manganaris, G. Classification, biotransformation and antioxidant activity of olive fruit biophenols: A review. *Curr. Bioact. Compd.* **2012**, *8*, 232–239.

(92) Obied, H. K.; Karuso, P.; Prenzler, P. D.; Robards, K. Novel secoiridoids with antioxidant activity from Australian olive mill waste. *J. Agric. Food Chem.* **2007**, *55*, 2848–2853.

(93) Quirantes-Piné, R.; Lozano-Sánchez, J.; Herrero, M.; Ibáñez, E.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC-ESI-QTOF-MS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts. *Phytochem. Anal.* **2012**, *24*, 213–223.

(94) Ranalli, A.; Contento, S.; Lucera, L.; Di Febo, M.; Marchegiani, D.; Di Fonzo, V. Factors affecting the contents of iridoid oleuropein in olive leaves (*Olea europaea* L.). *J. Agric. Food Chem.* **2006**, *54*, 434–440.

The secoiridoids group represents the major phenolic contents in olive leaves and are represented mainly by oleuropein, oleuropein aglycone, demethyloleuropein, ligstroside, oleuroside, methoxyoleuropein and secolagonoside[78,95–97]. Oleuropein and ligstroside remain the most significant secoiridoids in olive leaves[95,96,98].

Likewise, secoiridoids are structurally very complex in olive fruits[74]. Oleuropein and demethyloleuropein, the main secoiridoids in olive fruits[99,100], cause the intense fruit bitterness, particularly, oleuropein[101]. Derivatives of oleuropein found in olive fruits include oleoside-11-methyl, oleuropein aglycone, and oleuroside **(Table 4)**. Moreover, it has been suggested that demethyloleuropein may be a varietal marker because of its high selectivity among cultivars[99,102].

In olive oil, specifically virgin olive oil, it has been reported that secoiridoids together with tyrosol and hydroxytyrosol, and their derivatives, make up around 90% of the total phenolics[55]. Furthermore, the bitterness of virgin olive oil has also been attributed to secoiridoids derivatives[103]. Overall, the most abundant

(95) Kiritsakis, K.; Kontominas, M. G.; Kontogiorgis, C.; Hadjipavlou-Litina, D.; Moustakas, A.; Kiritsakis, A. Composition and antioxidant activity of olive leaf extracts from Greek olive cultivars. *J. Am. Oil Chem. Soc.* **2010**, *87*, 369–376.
(96) Fu, S.; Arráez-Roman, D.; Segura-Carretero, A.; Menéndez, J. A.; Menéndez-Gutiérrez, M. P.; Micol, V.; Fernández-Gutiérrez, A. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal. Bioanal. Chem.* **2010**, *397*, 643–654.
(97) Arioti, A. K.; Hatzopoulou, A. C.; Ilia, A. R. B.; Iakopoulos, G. L.; Tavrianakou, S. S.; Kaltsa, H. S. Novel secoiridoid glucosides in *Olea europaea* leaves suffering from boron deficiency. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1898–1903.
(98) Benavente-garcía, O.; Castillo, J.; Lorente, J.; Ortun, A. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem.* **2000**, *68*, 457–462.
(99) Sivakumar, G.; Briccoli Bati, C.; Uccella, N. HPLC-MS Screening of the antioxidant profile of Italian olive cultivars. *Chem. Nat. Compd.* **2005**, *41*, 588–591.
(100) Servili, M.; Baldioli, M.; Selvaggini, R.; Macchioni, A.; Montedoro, G. Phenolic compounds of olive fruit: One and two-dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit. *J. Agric. Food Chem.* **1999**, *47*, 12–18.
(101) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Accumulation of oleuropein derivatives maturation. *Phytochemistry* **1989**, *28*, 67–69.
(102) Esti, M.; Cinquanta, L.; La Notte E. Phenolic compounds in different olive varieties. *J. Agric. Food Chem.* **1998**, *46*, 32–35.
(103) Boskou, D. Phenolic compounds in olives and olive oil. *In olive oil: minor constituents and health*; 2009; pp 11–44.

phenolic compounds in virgin olive oil are aglycone compounds deriving from secoiridoids present in olive fruit. Such transformation is reported to be the result primarily of many enzymes released during the pressing and malaxation steps of olive milling, mainly polyphenol oxidase, which could be responsible for the indirect oxidation of secoiridoids, and the *β*-glucosidase, which could participate in the production of phenol aglycones such as the deacetoxyoleuropein aglycone, oleuropein aglycone and their isomers by hydrolysis of the oleuropein and demethyloleuropein[57,104,105]. Notably, secoiridoid derivatives of hydroxytyrosol and tyrosol and elenolic acid, e.g. aglycone forms of oleuropein (3,4-DHPEA-EA) and ligstroside (*p*-HPEA-EA) and their aldehydic forms are reported in olive oil. Another important group derived from secoiridoids are the dialdehydic forms of elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA or DOA) and tyrosol (*p*-HPEA-EDA, DLA or oleocanthal) (**Table 4**).

(104) Sánchez de Medina, V.; Priego-Capote, F.; de Castro, M. D. L. Characterization of monovarietal virgin olive oils by phenols profiling. *Talanta* **2015**, *132*, 424–432.

(105) Artajo, L.S.; Romero, M.P.; Suárez, M.; Motilva, M.J. Partition of phenolic compounds during the virgin olive oil industrial extraction process. *Eur. Food Res. Technol.* **2007**, *225*, 617–625.

Table 4. Secoiridoids in olive leaves, fruits and oil.

Class	Phenolic compounds	Leaves	Fruits	Oil
Secoiridoids	Oleuropein	X	X	
	Oleuropein glucoside	X	X	
	Oleuropein aglycone	X	X	
	10-hydroxy-oleuropein	X	X	X
	Methoxyoleuropein	X	X	
	Ligstroside	X	X	
	Ligstroside aglycone	X	X	X
	Demethyloleuropein	X	X	
	Elenolic acid	X	X	
	Oleoside	X	X	
	Oleoside methyl-ester	X	X	
	Oleoside dimethyl-ester	X	X	
	Oleoside methyl ester-7-epiloganin	X	X	
	Secolagonoside	X	X	
	Nuzhenide		X	
	3,4-DHPEA-EA			X
	p-HPEA-EA			X
	3,4-DHPEA-EDA (DOA)			X
	p-HPEA-EDA (Oleocanthal)			X
	Aldehydic form of oleuropein aglycone			X
	Aldehydic form ligstroside aglycone			X

5. Biosynthesis and biotransformation of phenolic compounds in olive leaves, fruit and oil

Plant metabolism can be divided into primary pathways that are found in all cells and deal with manipulating a uniform group of basic compounds, and secondary pathways that occur in specialized cells and produce a wide variety of unique compounds. The primary pathways deal with the metabolism of carbohydrates, lipids, proteins, and nucleic acids and act through the many-step reactions of glycolysis, the tricarboxylic acid cycle, and the pentose phosphate shunt, together with lipid, protein, and nucleic acid biosynthesis. In contrast, the secondary metabolites (e.g. terpenes, alkaloids, phenylpropanoids, lignin, flavonoids, coumarins, and related compounds) are produced by the shikimic, malonic, and mevalonic acid pathways, and the methylerythritol phosphate pathway[106] (**Figure 9**).

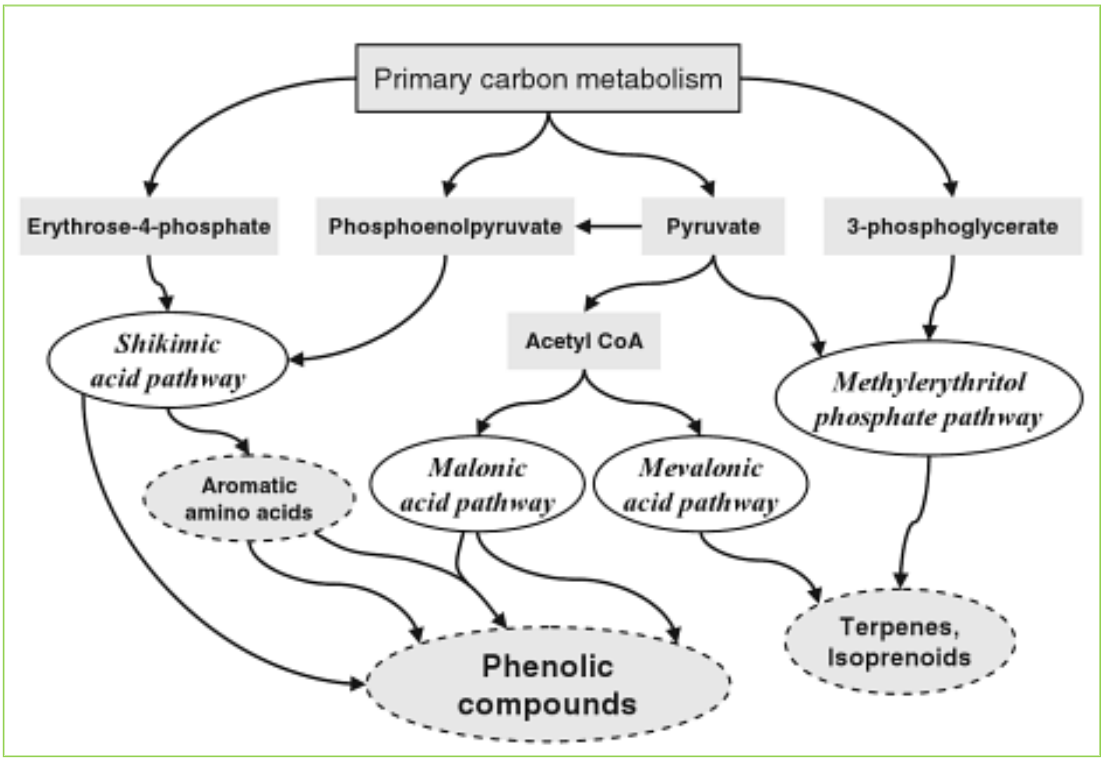

Figure 9. Diagram of the major biosynthetic pathways giving rise to secondary metabolism[106].

(106) De la Rosa, L. A.; Alvarez-Parrilla, E.; Gonzalez-Aguilar, G. A. *Fruit and vegetable phytochemicals chemistry, nutritional value, and stability*; De la Rosa, L. A., Alvarez-Parrilla, E., Gonzalez-Aguilar, G. A., Eds.; Wiley-Blackwell, 2010.

The key enzyme in phenolic biosynthesis is phenylalanine ammonia lyase (PAL), which starts the biosynthesis of a wide range of phenylpropanoid secondary compounds, including lignin and flavonoids. PAL catalyzes the non-oxidative stereo specific elimination of ammonia from phenylalanine (or tyrosine) to yield trans-cinnamate. This enzyme is highly sensitive to environmental conditions, and especially to stresses such as temperature, wounding, and ultraviolet light. Particularly, PAL activity greatly varies according to the degree of fruit ripening[107].

Few references in the literature report the biosynthesis of phenolic compounds in *O. europaea*. Some authors have attributed this process to the low rate of water uptake by plants in this genus and to the corresponding difficulty of conducting biosynthetic studies. However, water uptake is more rapid in plants of the genera *Fraxinus* and *Syringa*, and much of the detailed knowledge of biosynthesis in *O. europaea* has been inferred from studies of these genera[108].

5.1. Biosynthesis of simple phenols

Simple phenols are produced in plants *via* the shikimate pathway and phenylpropanoid metabolism. In fact, the non-oxidative glycolysis of glucose yields phosphoenol-pyruvate and erythrose-4-phosphate; both constitute the initial reactants of shikimic acid. The resulting phenylalanine represents the initial substrate of the general phenylalanine metabolism[107]. This metabolism involves the participation of compounds having a phenol ring with a side chain of 3-carbon atoms (C6-C3). These reactions lead to the synthesis of cinnamic acids,

(107) El Riachy, M.; Priego-Capote, F.; León, L.; Rallo, L.; Luque de Castro, M. D. Hydrophilic antioxidants of virgin olive oil. Part 2: Biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 692–707.

(108) Obied, H. K.; Prenzler, P. D.; Ryan, D.; Servili, M.; Taticchi, A.; Esposto, S.; Robards, K. Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from *Olea europaea* L. *Nat. Prod. Rep.* **2008**, *25*, 1167–1179.

benzoic acids, and simple phenols, which may act as precursors for the synthesis of other highly complex compounds [109].

Verbascoside is the most complex simple phenol in *O. europaea*. Despite the intense interest in verbascoside for medical purposes, its biosynthetic pathway remains to be fully elucidated. In fact, the early steps are known, but several downstream intermediates, key enzymes, and their corresponding genes remain to be discovered. Current knowledge of the pathway, which is based on feeding experiments with stable isotope-labeled precursors, has been proposed (**Figure 10**). Its biosynthesis begins with the generation of phenylalanine and tyrosine precursors by the shikimate pathway[110]. The hydroxytyrosol moiety of verbascoside is biosynthesized from tyrosine either through tyramine and/or dopamine, whereas its caffeoyl moiety is synthesized from phenylalanine *via* cinnamate pathway. Dopamine is incorporated into verbascoside through oxidation to the corresponding aldehyde, reduction to the alcohol, and finally, *β*-glycosylation[111].

(109) Ryan, D.; Antolovich, M.; Prenzler, P.; Robards, K.; Lavee, S. Biotransformations of phenolic compounds in *Olea europaea* L. *Sci. Hortic. (Amsterdam).* **2002**, *92*, 147–176.
(110) Alipieva, K.; Korkina, L.; Orhan, I. E.; Georgiev, M. I. Verbascoside-A review of its occurrence, (bio) synthesis and pharmacological significance. *Biotechnol. Adv.* **2014**, *32*, 1065–1076.
(111) Saimaru, H.; Orihara, Y. Biosynthesis of acteoside in cultured cells of *Olea europaea*. *J. Nat. Med.* **2010**, *64*, 139–145.

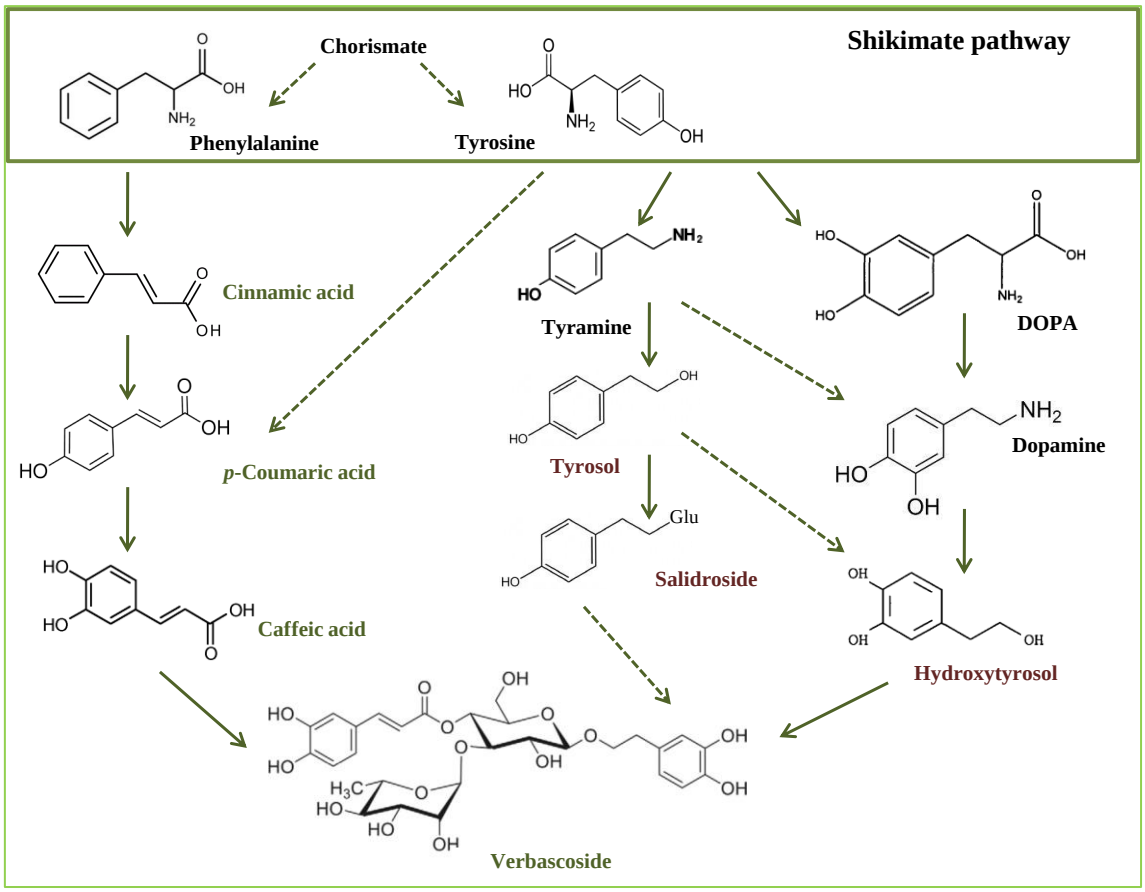

Figure 10. Tentative pathway of verbascoside biosynthesis, as proposed (by Alipieva et al.[110]).

5.2. Biosynthesis of flavonoids and lignans

The flavonoids originate from phenylalanine *via* the phenylpropanoid biosynthetic pathway. This metabolic pathway, unique to plants, is responsible for the biosynthesis of a number of products, including flavonoids and lignans. In fact, this pathway has been described by Sperry and Smith[112] as follows: phenylalanine, entering the pathway at the end of the shikimate pathway, is converted first to *trans*-cinnamic acid, and then *via* oxidation to *p*-coumaric acid. The first branch point of this pathway utilizes various enzymes that can convert the newly formed *p*-coumaric acid *via* reduction of the carboxylic acid moiety to

(112) Sperry, J.; Smith, A. B. Chemical synthesis of diverse phenolic compounds isolated from olive oils. In *olives and olive oil in health and disease prevention*; Preedy, V. R., Watson, R. R., Eds.; Academic press Elsevier, 2010; pp 1439–1464.

p-coumaryl alcohol or derivatives such as coniferyl alcohol and sinapyl alcohol through the oxidation of the phenyl ring. The resultant alcohols make up the biosynthetic precursors to the lignan family. Cyclization provides access to the flavonoid skeleton and in turn to the vast family of flavonoids (**Figure 11**).

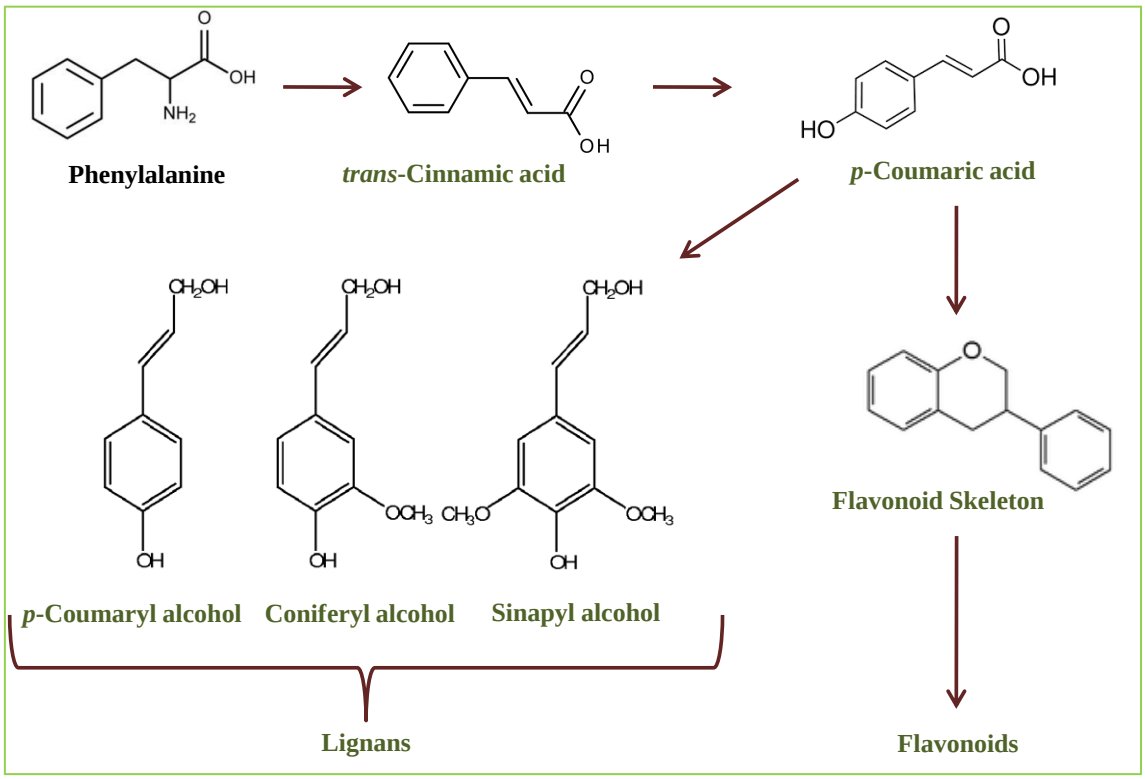

Figure 11. Pathway of lignans and flavonoids biosynthesis, as proponed (by Sperry and Smith[112]).

5.3. Biosynthesis of secoiridoids

Secoiridoids are derived from iridoids by opening of the cyclopentane ring of the iridoids. Iridoids are monoterpenes characterized by a bicyclic fused ring system comprising a 6-membered heterocyclic ring fused to a cyclopentane ring. Although two known routes for the production of iridoids exist, Oleaceae species are characterized by the presence of iridoids derived only from the pathway

proposed by Jensen et al.[113], namely the biosynthesis of deoxyloganic acid from iridodial *via* iridotrial[108] (**Figure 12**).

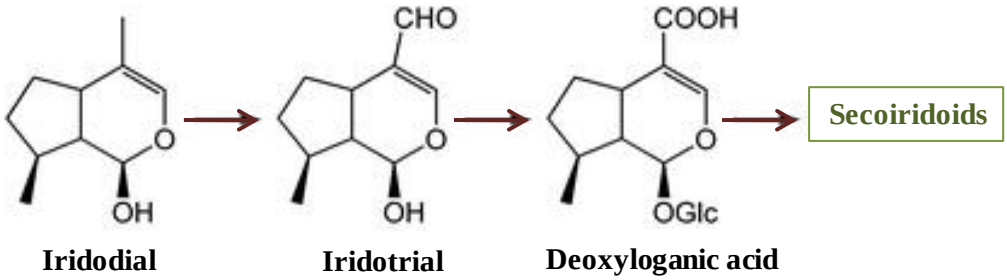

Figure 12. Biosynthetic pathway of the common precursor deoxyloganic acid (by Jensen et al.[113]).

It has been proposed that 7-epiloganic/7-epiloganin acids are the key intermediates in the biosynthesis of most of the secoiridoids, termed oleosidic secoiridoids or also oleosides. This involves pathways designated as 1d and 1e by Jensen et al.[113] (**Figure 13**). In fact, the 1d pathway from 7-epiloganic acid has been established to be the probable precursor for secologanoside and, thus, also their derivatives. The second pathway (1e), from 7-epiloganin, has demonstrated been to be the precursor of oleoside and 10-hydroxyoleoside derivatives. The initial steps of 1e pathway are similar to those of route 1d, but in this case methyl esters seem to be utilized instead of the acids.

(113) Jensen, S. R.; Franzyk, H.; Wallander, E. Chemotaxonomy of the oleaceae: Iridoids as taxonomic markers. *Phytochemistry* **2002**, *60*, 213–231.

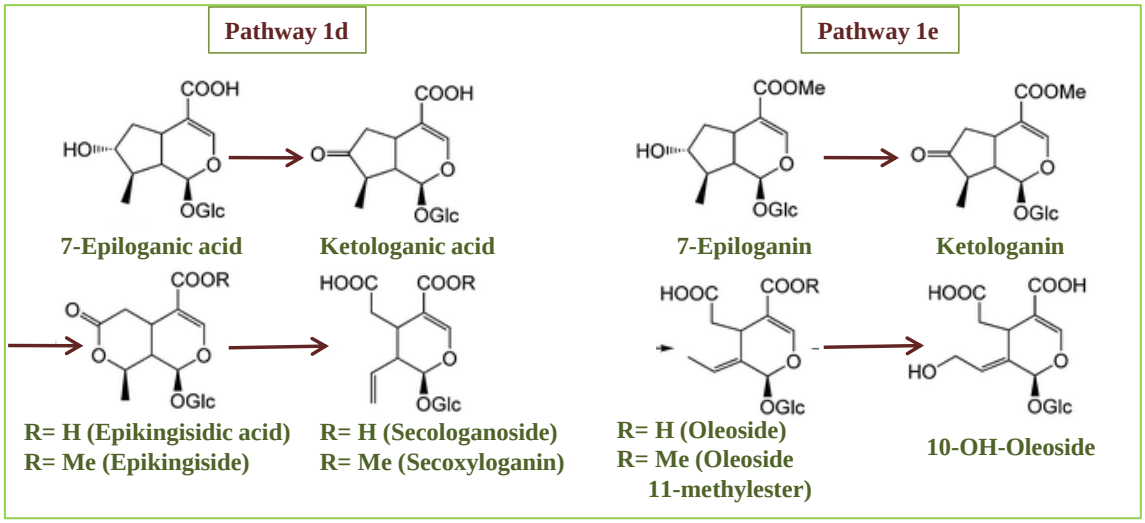

Figure 13. Biosynthetic pathways leading to formation of oleosides. These pathways are identified as routes 1d and 1e by Jensen et al.[113].

5.4. Biotransformation of phenolic compounds

Studies on *O. europaea* have generally concentrated on a single tissue, such as olive leaf or fruit, and hence metabolic relationships between different parts of the tree have not been elucidated. It remains unclear whether transport between the compartments involves movement of precursor compounds (non-phenolic or simple phenols) or the intact complex phenolic species[109]. There are many difficulties associated with metabolic studies of phenolic compounds, principally because the metabolites isolated from a natural source are not necessarily the metabolites that are present in the living tissue. The extraction and purification process could trigger chemical changes because of the exposure of phenolic compounds to oxygen, solvents, and changes in pH. Moreover, different metabolites may be produced in response to microbial infection, so that the spectrum of metabolites is often characteristic of the health status of the organism. In this context, the dynamic state of the fruits should be considered[107,109].

6. Factors affecting phenolic composition in olive leaves, fruit and oil

One of the most intriguing aspects of the metabolism of phenolic compounds concerns the rapid changes that can take place in their synthesis rates. Evidence of the occurrence of a specific phenolic only during specific times of the year, only in certain cell types, or only in response to particular stimuli implies considerable regulation over their synthesis and further metabolism. Thus, the absence of a phenolic in a given plant material would not suggest that the plant was incapable of synthesizing it. The profound capability of plants to manufacture phenolic compounds in response to the stimuli suggests that synthesis must be subject to strict control under most conditions[114]. Changes occurring in the phenolic composition of olive leaves and olive fruits regarding different factors have been extensively reported in the literature. In addition, the changes that occur in the olive fruits automatically affect the phenolic composition in their correspond olive oil[115,116]. **Figure 14** ummarizes the main factors affecting phenolic composition in olive leaves, fruits, and oil.

(114) Runeckles, V.; Conn, E. *Metabolism and regulation of secondary plant products-recent advances in phytochemistry*; V.C, R., Conn, E., Eds.; Academic Press, 1974.

(115) Salvador, M. D.; Aranda, F.; Fregapane, G. Influence of fruit ripening on 'Cornicabra' virgin olive oil quality: A study of four successive crop seasons. *Food Chem.* **2001**, *73*, 45–53.

(116) Kaliora, A. C.; Artemiou, A.; Giogios, I.; Kalogeropoulos, N. The impact of fruit maturation on bioactive microconstituents, inhibition of serum oxidation and inflammatory markers in stimulated PBMCs and sensory characteristics of 'Koroneiki' virgin olive oils from Messenia, Greece. *Food Funct.* **2013**, *4*, 1185–1194.

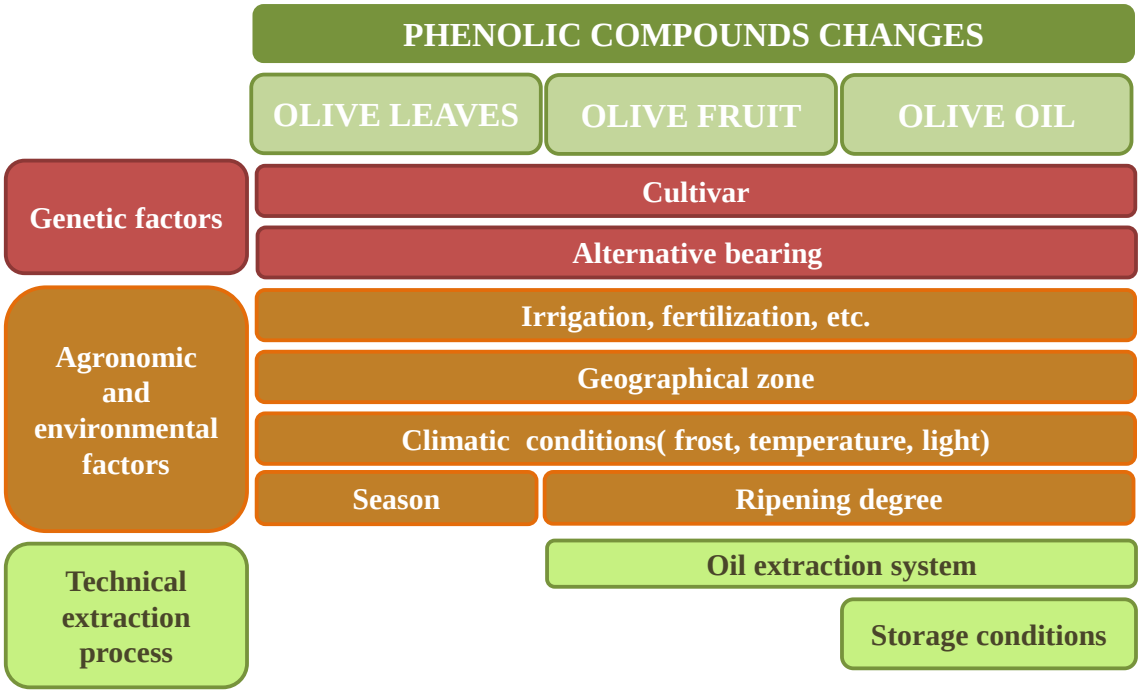

Figure 14. The main factors affecting phenolic composition in olive leaves, fruits, and oil.

6.1. Genetic factors

Overall, several studies examine the effect of the cultivar on phenolic compounds in olive[91,117]. Phenolic compounds from olive leaves have been used as chemotaxonomic markers[118,119]. In olive fruit, the cultivar is a determinant factor of phenol variation[120]; moreover, it has been found that, in general, small-fruit cultivars are characterized by a high oleuropein content compared to large-fruit

(117) Ortega-García, F.; Peragón, J. Phenol metabolism in the leaves of the olive tree (*Olea europaea* L.) cv. Picual, Verdial, Arbequina, and Frantoio during ripening. *J. Agric. Food Chem.* **2010**, *58*, 12440–12448.

(118) Luque De Castro, M. D.; Capote Priego, F. Extraction of oleuropein and related phenols from olive leaves and branches. In *olives and olive oil in health and disease prevention*; Elsevier, 2010; pp 259–273.

(119) Japón-Luján, R.; Ruiz-Jiménez, J.; De Castro, M. D. L. Discrimination and classification of olive tree varieties and cultivation zones by biophenol contents. *J. Agric. Food Chem.* **2006**, *54*, 9706–9712.

(120) Petridis, A.; Therios, I.; Samouris, G. Genotypic variation of total phenol and oleuropein concentration and antioxidant activity of 11 Greek olive cultivars (*Olea europaea* L.). *HortScience* **2012**, *47*, 339–342.

cultivars during developmental stages[121]. The strong influence of cultivar on phenolic compounds in olive oil has not been excluded[122,123].

Olive tree has genetically highly alternating fruit production. The expression of alternate bearing in olive involves a wide range of changes in activation and repression of endogenous metabolic pathways[124]. Evidence of the impact of alternate bearing on levels of phenolic compounds in leaves has been reported. In fact, phenolic compounds such as chlorogenic acid, *p*-coumaric acids, hydroxytyrosol, oleuropein, and oleuroside have shown an increase in the 'on' season (high fruiting seasons) compared to the 'off' season (low fruiting seasons) in leaves[125,126]. In contrast to the situation in olive leaves, in EVOO the concentration of phenolic compounds is reportedly higher during 'off' years[127].

6.2. Environmental and agronomic factors

Various studies have analyzed the effect of cultivation practices and environment on qualitative and quantitative composition of olive phenolic content, mainly the effect of irrigation, fertilization, geographical zone, altitude, climatic conditions, ripening degree, etc..

(121) Bianchi, G. Lipids and phenols in table olives. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 229–242.
(122) Rigane, G.; Ayadi, M.; Boukhris, M.; Sayadi, S.; Bouaziz, M. Characterisation and phenolic profiles of two rare olive oils from southern Tunisia: 'Dhokar' and 'Gemri-Dhokar' cultivars. *J. Sci. Food Agric.* **2013**, *93*, 527–534.
(123) Hashempour, A.; Ghazvini, R. F.; Bakhshi, D.; Aliakbar, A.; Papachatzis, A.; Kalorizou, H. Characterization of virgin olive oils (*Olea europaea* L.) from three main Iranian cultivars, 'Zard', 'Roghani' and 'Mari' in Kazeroon region. *Biotechnol. Biotechnol. Equip.* **2010**, *24*, 2080–2084.
(124) Lavee, S. Biennial bearing in olive (*Olea europaea*). *Ann. Ser. Hist. Nat.* **2007**, *17*, 101–112.
(125) Ryan, D.; Prenzler, P. D.; Lavee, S.; Antolovich, M.; Robards, K. Quantitative changes in phenolic content during physiological development of the olive (*Olea europaea*) cultivar 'Hardy 's Mammoth'. *J. Agric. Food Chem.* **2003**, *51*, 2532–2538.
(126) Mert, C.; Barut, E.; Ipek, A. Quantitative seasonal changes in the leaf phenolic content related to the alternate-bearing patterns of olive (*Olea europaea* L. cv. Gemlik). *J. Agric. Sci. Technol.* **2013**, *15*, 995–1006.
(127) Barone, E.; Gullo, G.; Zappia, R.; Inglese, P. Effect of crop load on fruit ripening and olive oil (*Olea europea* L.) quality. *J. Hortcultural Sci.* **1994**, *69*, 67–73.

Phenolic compounds (mainly oleuropein and derivatives) have been shown to significantly increase in olive leaves[128–130], fruits[91] , and oil[131] when irrigation deficiency is applied as water-saving strategy. Furthermore, the variability due to planting systems has proved to have a heavy impact also on olive oil phenolic composition[132].

The addition of mineral nutrients such as urea, nitrogen, copper, manganese and zinc have been demonstrated to significantly increase some phenolic compounds (tyrosol, catechin, oleuropein) in olive leaves[133]. Meanwhile, a general deficit of boron in olive tree has been found to increase the concentration of phenolics such as quercetin rutinoside, *p*-coumaric acid, luteolin glucoside, apigenin glucoside, and apigenin rutinoside, in olive leaves[134]. The application of foliar fertilization containing a mixture of boron, manganese, magnesium, and sulfur, has been found to cause a significant decrease in total phenolic compound contents in olive fruits[135]. Similar results have been reported in olive oil, when the same

(128) Bacelar, E. A.; Santos, D. L.; Moutinho-Pereira, J. M.; Gonçalves, B. C.; Ferreira, H.F.;Correia, C. M. Immediate responses and adaptative strategies of three olive cultivars under contrasting water availability regimes: Changes on structure and chemical composition of foliage and oxidative damage. *Plant Sci.* **2006**, *170*, 596–605.

(129) Ennajeh, M.; Vadel, A. M.; Khemira, H. Osmoregulation and osmoprotection in the leaf cells of two olive cultivars subjected to severe water deficit. *Acta Physiol. Plant.* **2009**, *31*, 711–721.

(130) Petridis, A.; Therios, I.; Samouris, G.; Koundouras, S.; Giannakoula, A. Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (*Olea europaea* L.) cultivars. *Plant Physiol. Biochem.* **2012**, *60*, 1–11.

(131) Tovar, M. J.; Motilva, M. J.; Romero, M. P. Changes in the phenolic composition of virgin olive oil from young trees (*Olea europaea* L. cv. Arbequina) grown under linear irrigation strategies. *J. Agric. Food Chem.* **2001**, *49*, 5502–5508.

(132) El Riachy, M.; Priego-Capote, F.; Rallo, L.; Luque-de Castro, M. D.; León, L. Phenolic composition of virgin olive oils in cultivars for narrow hedgerow olive orchards. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 800–810.

(133) Del Río, J. A.; Báidez, A. G.; Botía, J. M.; Ortuño, A. Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against *Phytophthora sp*. *Food Chem.* **2003**, *83*, 75–78.

(134) Liakopoulos, G.; Karabourniotis, G. Boron deficiency and concentrations and composition of phenolic compounds in *Olea europaea* leaves: A combined growth chamber and field study. *Tree Physiol.* **2005**, *25*, 307–315.

(135) Tekaya, M.; Mechri, B.; Cheheb, H.; Attia, F.; Chraief, I.; Ayachi, M.; Boujneh, D.; Hammami, M. Changes in the profiles of mineral elements, phenols, tocopherols and soluble carbohydrates of olive fruit following foliar nutrient fertilization. *LWT - Food Sci. Technol.* **2014**, *59*, 1047–1053.

foliar fertilization was applied[136]. Other studies report that total phenolic contents significantly fell in the olive oil as the N concentration (provided by fertilization) rose in the fruit[137–140].

On the other hand, several studies have demonstrated the effect of geographical on phenolic compounds contents in olive products. In fact, it has been found that as the geographical altitude decreases so do the contents in phenolic compounds in olive leaves[141]. The variation in phenol contents has also been noted at different latitudes[142,143]. Likewise, the evaluation of 13 different geographical zones in relation 23 phenolic compounds in 'Chemlali' olive oil has reported strong evidence of the latitude effect on olive oil phenolic contents[144]. However, authors found that, unlike olive leaves, virgin olive oils from fruits collected at low altitudes have higher amounts of phenolic compounds[145].

(136) Tekaya, M.; Mechri, B.; Bchir, A.; Attia, F.; Cheheb, H.; Daassa, M.; Hammami, M. Effect of nutrient-based fertilisers of olive trees on olive oil quality. *J. Sci. Food Agric.* **2013**, *93*, 2045–2052.

(137) Fernández-Escobar, R.; Beltrán, G.; Sánchez-Zamora, M. A.; García-Novelo, J.; Aguilera, M. P.; Uceda, M. Olive oil quality decreases with nitrogen over-fertilization. *HortScience* **2006**, *41*, 215–219.

(138) Dag, A.; Ben-David, E.; Kerem, Z.; Ben-Gal, A.; Erel, R.; Basheer, L.; Yermiyahu, U. Olive oil composition as a function of nitrogen, phosphorus and potassium plant nutrition. *J. Sci. Food Agric.* **2009**, *89*, 1871–1878.

(139) Erel, R.; Kerem, Z.; Ben-Gal, A.; Dag, A.; Schwartz, A.; Zipori, I.; Basheer, L.; Yermiyahu, U. Olive (*Olea europaea* L.) tree nitrogen status is a key factor for olive oil quality. *J. Agric. Food Chem.* **2013**, *61*, 11261–11272.

(140) Chouliaras, V.; Tasioula, M.; Chatzissavvidis, C.; Therios, I.; Tsabolatidou, E. The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity, fruit maturation, leaf nutritional status and oil quality of the olive (*Olea europaea* L.) cultivar 'Koroneiki'. *J. Sci. Food Agric.* **2009**, *89*, 984–988.

(141) Bilgin, M.; Şahin, S. Effects of geographical origin and extraction methods on total phenolic yield of olive tree (*Olea europaea*) leaves. *J. Taiwan Inst. Chem. Eng.* **2013**, *44*, 8–12.

(142) Brahmi, F.; Mechri, B.; Dhibi, M.; Hammami, M. Variation in antioxidant activity and phenolic content in different organs of two Tunisian cultivars of *Olea europaea* L. *Acta Physiol. Plant.* **2014**, *36*, 169–178.

(143) Di Donna, L.; Mazzotti, F.; Salerno, R.; Tagarelli, A.; Taverna, D.; Sindona, G. Characterization of new phenolic compounds from leaves of *Olea europaea* L . by high-resolution tandem mass spectrometry. *Wiley Intersci.* **2007**, *21*, 3653–3657.

(144) Taamalli, A.; Arráez Román, D.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Classification of 'Chemlali' accessions according to the geographical area using chemometric methods of phenolic profiles analysed by HPLC-ESI-TOF-MS. *Food Chem.* **2012**, *132*, 561–566.

(145) Aparicio, R.; Luna, G. Characterisation of monovarietal virgin olive oils. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 614–627.

Climatic conditions such as low temperatures can also have a strong effect on the phenolic contents in olive leaves. Several studies have shown a general burst of phenolic content in winter and a decline in summer[146,147]. Other studies have reported greater flavonoid content (particularly, luteolin glucoside) in the spring period[148]. Furthermore, a prolonged exposure of olive leaves to light reportedly strongly augments the flavonoid content. This is because flavonoids act as a barrier against damaging UV radiation owing to their adsorption maxima in the region[77,149–151].

Climatic conditions can, however, harm olive fruit and oil. In fact, a sharp fall in the concentration of secoiridoid derivatives and 3,4-DHPEA-AC has been noted in the oils extracted after frost damage to the olive fruit[152,153]. Also, oleuropein concentration in leaves reportedly spiked after olive-tree exposure to cold stress[154]. This is because oleuropein may protect against oxidative damage induced by freezing.

(146) Fabbri, A.; Galaverna, G.; Ganino, T. Polyphenol composition of olive leaves with regard tocultivar , time of collection and shoot type. *Acta Hortic.* **2008**, *791*, 459–464.
(147) Papoti, V. T.; Tsimidou, M. Z. Impact of sampling parameters on the radical scavenging potential of olive (*Olea europaea* L.) leaves. *J. Agric. Food Chem.* **2009**, *57*, 3470–3477.
(148) Heimler, D.; Cimato, A.; Alessandri, S.; Sani, G.; Pieroni, A. Seasonal trend of flavonoids in olive (*Olea europaea* L.) leaves. *Agric. Mediterr.* **1996**, *126*, 205–209.
(149) Liakoura, V.; Stefanou, M.; Manetas, Y.; Cholevas, C.; Karabourniotis, G. Trichome density and its UV-B protective potential are affected by shading and leaf position on the canopy. *Environ. Exp. Bot.* **1997**, *38*, 223–229.
(150) Melgar, J. C.; Guidi, L.; Remorini, D.; Agati, G.; Degl'innocenti, E.; Castelli, S.; Camilla Baratto, M.; Faraloni, C.; Tattini, M. Antioxidant defences and oxidative damage in salt-treated olive plants under contrasting sunlight irradiance. *Tree Physiol.* **2009**, *29*, 1187–1198.
(151) Liakopoulos, G.; Stavrianakou, S.; Karabourniotis, G. Trichome layers versus dehaired lamina of *Olea europaea* leaves: Differences in flavonoid distribution, UV-absorbing capacity, and wax yield. *Environ. Exp. Bot.* **2006**, *55*, 294–304.
(152) Morelló, J. R.; Motilva, M. J.; Ramo, T.; Romero, M. P. Effect of freeze injuries in olive fruit on virgin olive oil composition. *Food Chem.* **2003**, *81*, 547–553.
(153) Poerio, A.; Bendini, A.; Cerretani, L.; Bonoli-Carbognin, M.; Lercker, G. Effect of olive fruit freezing on oxidative stability of virgin olive oil. *Eur. J. Lipid Sci. Technol.* **2008**, *110*, 368–372.
(154) Ortega-García, F.; Peragón, J. The response of phenylalanine ammonia-lyase, polyphenol oxidase and phenols to cold stress in the olive tree (*Olea europaea* L. cv.Picual). *J. Sci. Food Agric.* **2009**, *89*, 1565–1573.

The degree of fruit ripening is also a key to phenolic composition changes both in fruits and in the resulting oil[155]. At the beginning of ripening, oleuropein is the most abundant compound in olive fruits, its concentration reaching up to 14% of the dry matter of young fruit. As ripening progresses, oleuropein progressively degrades, producing other compounds such as hydroxytyrosol[91]. In addition, the decline in the oleuropein concentration is replaced with a significant rise in verbascoside. Olive fruit ripening has consequently been deemed the most important factor associated with olive oil[83,156]. Indeed, fruit harvested early renders olive oil with high contents in phenolic compounds and high oxidative stability.

6.3. Olive-oil extraction process and storage

Olive oil phenolic content is greatly affected by the manufacturing process. The type of mill used for pressing and centrifugation plays an important role in phenolic composition. The type of mill defines the extractability of phenols from olive fruits to oil. Notably, studies have shown that a monovarietal virgin olive oil produced by the three-phase pressing system contains lower amounts of phenols than the same oil extracted by cold press[145]. In addition, olive oil produced by centrifugation has lower phenol content because this procedure uses large amounts of warm water, which substantially lowers the phenolic content, since these compounds are hydrophilic[91]. Malaxation has been recognized as one of the most critical points in the mechanical extraction process for virgin olive oil, since it causes considerable loss of phenolic compounds[157]. In fact, malaxing conditions, such as time, temperature, and the composition of the atmosphere in

(155) Damak, N.; Bouaziz, M.; Ayadi, M.; Sayadi, S.; Damak, M. Effect of the maturation process on the phenolic fractions, fatty acids, and antioxidant activity of the 'Chétoui' olive fruit cultivar. *J. Agric. Food Chem.* **2008**, *56*, 1560–1566.

(156) Gambacorta, G.; Faccia, M.; Previtali, M. A.; Pati, S.; Notte, E. L.; Baiano, A. Effects of olive maturation and stoning on quality indices and antioxidant content of extra virgin oils (cv. Coratina) during storage. *J. Food Sci.* **2010**, *75*, C229–C235.

(157) Clodoveo, M. L. Malaxation: Influence on virgin olive oil quality. Past, present and future–An overview. *Trends Food Sci. Technol.* **2012**, *25*, 13–23.

contact with the olive paste, can influence the activity of the enzymes responsible for the oxidative degradation of phenolic compounds[157].

During storage, olive oil phenols undergo qualitative and quantitative alterations due to hydrolytic decomposition and oxidation. Thus, it has widely been claimed that the role of phenolic compounds on the oil quality and stability is pivotal. These compounds tend to decrease in oil over the storage time although the content of some compounds such as phenolic acids (*p*-coumaric, vanillic acid, and vanillin) have been found to remain almost constant during storage[158,159]. The main changes in the phenolic compounds present in virgin olive oil during storage have been associated with the hydrolysis of the secoiridoids aglycones[108,160]. Some practices, such as veiled virgin olive oil filtration could cause a high loss in oil stability during storage, due to the decrease in total phenol content[160,161].

Furthermore, metal contact, temperature, light and packaging are the main factors affecting the phenol composition of olive oil[162]. These factors could negatively affect the phenol levels in olive oil during storage time, leading to the partial or complete deterioration of the oil sensorial quality. For instance, the contact of oil with metal such iron (during extraction or storage) affects the stability of oleuropein, hydroxytyrosol, 3,4-DHPEA-EA and 3,4-DHPEA-EDA due to the metal-chelating activity of these compounds[163]. Moreover, in another study, total phenolic compounds have shown a sharp decrease at 20ºC and in PET packing,

(158) Suárez, M.; Romero, M. P.; Ramo, T.; Motilva, M. J. Stability of a phenol-enriched olive oil during storage. *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 894–903.
(159) Morelló, J. R.; Motilva, M. J.; Tovar, M. J.; Romero, M. P. Changes in commercial virgin olive oil (cv. Arbequina) during storage, with special emphasis on the phenolic fraction. *Food Chem.* **2004**, *85*, 357–364.
(160) Brenes, M.; García, A.; García, P.; Garrido, A. Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. *J. Agric. Food Chem.* **2001**, *49*, 5609–5614.
(161) Tsimidou, M. Z.; Georgiou, A.; Koidis, A.; Boskou, D. Loss of stability of "veiled" (cloudy) virgin olive oils in storage. *Food Chem.* **2005**, *93*, 377–383.
(162) Vitaglione, P.; Savarese, M.; Paduano, A.; Scalfi, L.; Fogliano, V.; Sacchi, R. Healthy virgin olive oil : A matter of bitterness healthy virgin olive oil : A matter of bitterness. *Crit. Rev. Food Sci. Nutr.* **2013**, *55*, 1808–1818.
(163) Paiva-Martins, F.; Gordon, M. H. Interactions of ferric ions with olive oil phenolic compounds. *J Agric Food Chem* **2005**, *53*, 2704–2709.

this highlighting again that olive oil storage at low temperatures (suitably at 4ºC), and in special packing (Tetra-Brik container) could preserve the phenolic compounds in oil and consequently lengthen its shelf life[164].

(164) Samaniego-Sánchez, C.; Oliveras-López, M. J.; Quesada-Granados, J. J.; Villalón-Mir, M.; Serrana, H. L. G. Alterations in 'Picual' extra virgin olive oils under different storage conditions. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 194–204.

7. Bioactivities of olive phenolic compounds

Free radicals are molecules or atoms with an unpaired electron that are in permanent reactivity looking for another electron to fulfill a pair. They can be a natural by-product of cellular metabolism, but can also be generated by the external action of some factors such UV radiation, toxic substances, microbial attacks, among others[165]. Free radicals have deleterious effects on cellular membranes and internal structures, for example provoking cardiovascular disease or cancer or impairing immune function by altering the body's metabolism.

Fortunately, a complex natural antioxidant system exists in the biological systems, which prevents damage by pro-oxidants. Indeed, biological systems have evolved with endogenous defense mechanisms, mainly antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase, which metabolize toxic oxidative intermediates[166]. Moreover, exogenous factors are also evolved and are provided primarily by the diet; these are antioxidants and mainly phenolic compounds, which protect against several diseases. A brief review about the main diseases prevented by phenolic compounds of olive products are presented below.

7.1. Chronic inflammation

Inflammation is an essential part of the body's attempt at self-protection. However, chronic inflammation is a long-term inflammation, which can ensue particularly from an autoimmune response to a self antigen or from a chronic persistent irritant. In fact, macrophages are then recruited together with T-cells. The by-products of macrophage activation are toxic agents such as reactive oxygen species (ROS), reactive nitrogen species, elastase 5, cathepsin G, and

(165) Sumpio, B. E.; Cordova, A. C.; Berke-Schlessel, D. W.; Qin, F.; Chen, Q. H. Green tea, the "Asian paradox," and cardiovascular disease. *J. Am. Coll. Surg.* **2006**, *202*, 813–825.
(166) Singh, R. P.; Sharad, S.; Kapur, S. Free radicals and oxidative stress in neurodegenerative diseases: Relevance of dietary antioxidants. *J. Indian Acad. Clin. Med.* **2004**, *5*, 218–225.

proteinase 3. Although effective at killing pathogenic agents, these toxic effectors also inflict damage on host tissues[66] and could lead to serious diseases such as asthma, chronic sinusitis, chronic active hepatitis or Crohn's disease.

It is well documented that phenolic compounds derived from olive products have significant anti-inflammatory capacity[66,167–170]. For example, recent *in vitro* and *in vivo* evidence has pointed to the anti-inflammatory properties of hydroxytyrosol, demonstrating its ability to influence the release of superoxide anions (O_2^-) and the expression of cyclooxygenase2 (COX2) in human monocytes[171]. In addition, phenolic compounds in olive leaf have also been found to suppress messenger RNA expression of pro-inflammatory cytokines[172].

7.2. LDL oxidation and endothelial dysfunction

The oxidation of plasma low-density lipoprotein (LDL) into the oxidized form (oxLDL) by free radicals is proatherogenic, pro-inflammatory, and highly immunogenic. They play a key role in the development of atherosclerosis and coronary heart diseases[173]. This is because oxLDL has cytotoxic properties that can promote endothelial injury and could also act as a chemo attractant for circulating monocytes, leading to their increased accumulation within plaques.

(167) Bogani, P.; Galli, C.; Villa, M.; Visioli, F. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis* **2007**, *190*, 181–186.
(168) Miles, E. A.; Zoubouli, P.; Calder, P. C. Differential anti-inflammatory effects of phenolic compounds from extra virgin olive oil identified in human whole blood cultures. *Nutrition* **2005**, *21*, 389–394.
(169) Muñoz, A.; Costa, M. Nutritionally mediated oxidative stress and inflammation. *Oxid. Med. Cell. Longev.* **2013**, *2013*, 1–12.
(170) El, S. N.; Karakaya, S. Olive tree (*Olea europaea*) leaves: Potential beneficial effects on human health. *Nutr. Rev.* **2009**, *67*, 632–638.
(171) Rosignoli, P.; Fuccelli, R.; Fabiani, R.; Servili, M.; Morozzi, G. Effect of olive oil phenols on the production of inflammatory mediators in freshly isolated human monocytes. *J. Nutr. Biochem.* **2013**, *24*, 1513–1519.
(172) Liu, Y.-N.; Jung, J.-H.; Park, H.; Kim, H. Olive leaf extract suppresses messenger RNA expression of proinflammatory cytokines and enhances insulin receptor substrate 1 expression in the rats with streptozotocin and high-fat diet-induced diabetes. *Nutr. Res.* **2014**, *34*, 450–457.
(173) Rice-Evans, C.; Miller, N.; Paganga, G. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* **1997**, *2*, 152–159.

Moreover, oxLDL has also been reported to inhibit the egression of macrophages from plaques[165] (**Figure 15**).

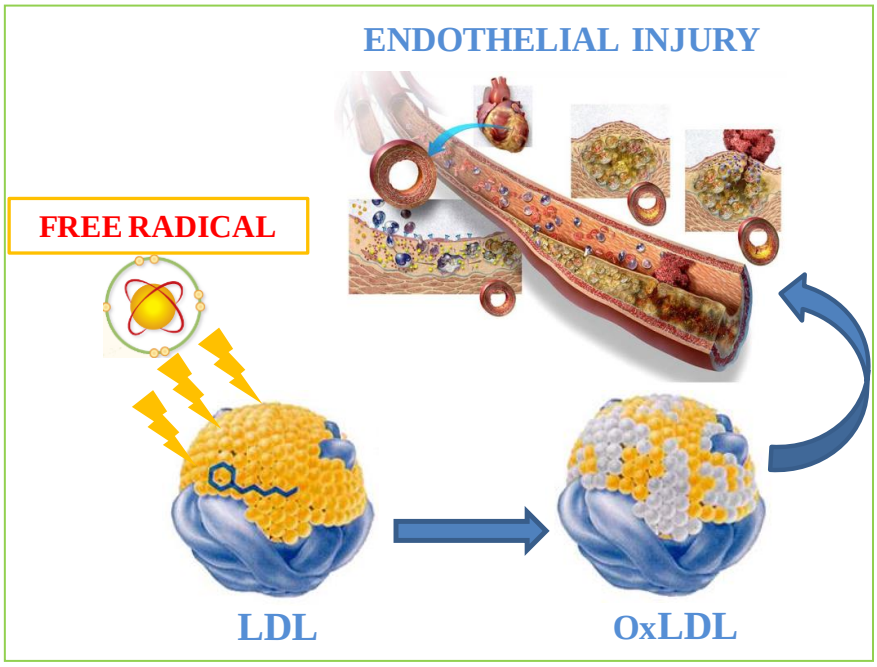

Figure 15. The effect of free radical on LDL oxidation and atherosclerosis.

Phenolic compounds from olive products have been shown to possess substantial cardiovascular-protective qualities, owing mainly to the orthodiphenolic structure of hydroxytyrosol and oleuropein that confers an especially strong antioxidant property[174,175]. The mode of action of those phenols could be summarized principally as the chelating of free metal ions, such as copper and iron, and also the scavenging of free radicals[176]. The effects of oleuropein on LDL susceptibility to copper-mediated oxidation *in vitro* have been reported by Visioli and Galli[177]. In addition, several *in vitro* and *in vivo* studies revealing the

(174) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* **1996**, *20*, 933–956.
(175) Wang, L.; Geng, C.; Jiang, L.; Gong, D.; Liu, D.; Yoshimura, H.; Zhong, L. The anti-atherosclerotic effect of olive leaf extract is related to suppressed inflammatory response in rabbits with experimental atherosclerosis. *Eur. J. Nutr.* **2008**, *47*, 235–243.
(176) Huang, C. L.; Sumpio, B. E. Olive oil, the Mediterranean diet, and cardiovascular health. *J. Am. Coll. Surg.* **2008**, *207*, 407–416.
(177) Visioli, F.; Galli, C. Oleuropein protects low density lipoprotein from oxidation. *Life Sci.* **1994**, *55*, 1965–1971.

efficient protection of hydroxytyrosol against LDL oxidation have extensively been reviewed in the literature[178,179]. The most relevant study reported was the Eurolive study, performed in 200 healthy subjects from five European countries. This study is considered the largest clinical study demonstrating that olive phenols lower plasma oxLDL and provide solid evidence for the antioxidant activity of olive phenols. Consequentially, the doses of olive phenolics showing an effect on plasma oxidized LDL ranged from 4 to 20 mg per day[178].

7.3. Cancer

Cell oxidation is one of the major risks in the formation of cancer: the more susceptible the cell is to oxygen, the greater the risk of cancer. Over the last few years, a number of experimental studies have provided evidence of a remarkable role of reactive oxygen species (ROS) in mediating the development of oxidative stress. In fact, DNA damage by ROS has been implicated in mutagenesis, oncogenesis, and aging, among other degenerative processes[180] (**Figure 16**).

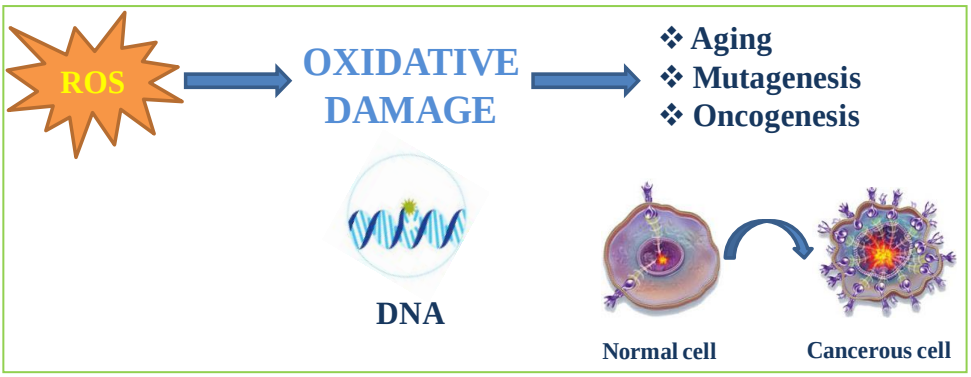

Figure 16. Schematic representation of cell oxidative damage.

(178) Raederstorff, D. Antioxidant activity of olive polyphenols in humans: A review. *Int. J. Vitam. Nutr. Res.* **2009**, *79*, 152–165.
(179) Frankel, E. N. E. Nutritional and biological properties of extra virgin olive oil. *J. Agric. Food Chem.* **2011**, *59*, 785–792.
(180) Cárdeno, A.; Sánchez-Hidalgo, M.; Alarcón-de-la-Lastra, C. An up-date of olive oil phenols in inflammation and cancer: molecular mechanisms and clinical implications. *Curr. Med. Chem.* **2013**, *20*, 4758–4776.

In vitro studies with individual phenols or whole olive oil/leaf phenolic extracts have suggested that olive phenols are capable of significantly affecting the overall process of carcinogenesis by their abilities to inhibit the cell cycle, cell proliferation or oxidative stress, improve the efficacy of detoxification enzymes, induce apoptosis, and stimulate the immune system[180–182].

Owing to the strong antioxidant potency of olive phenols such as oleuropein, hydroxytyrosol, and flavonoids, it has been demonstrated that these phytochemicals inhibit cancer and endothelial cell proliferation at low micro-molar concentrations[183]. This inhibition consists of slowing or preventing the oxidation of other molecules that help to limit the oxidative damage by acting directly on ROS or by stimulating endogenous defense systems[180,184].

Accordingly, complex phenolic compounds in olive oil efficiently inhibit proliferation and induce apoptotic cell death in human-derived breast-cancer cell lines bearing high levels of the tyrosinekinase receptor HER2, an oncoprotein found in human-breast carcinomas[88,185]. In addition, olive-leaf phenolic extracts were found to inhibit cell proliferation of human breast adenocarcinoma (MCF-7), human urinary-bladder carcinoma (T-24) and bovine-brain capillary endothelial (BBCE)[181]. Hydroxytyrosol has shown to be protective effects against induced oxidative stress by scavenging several free-radical species in different

(181) Fabiani, R.; Rosignoli, P.; De Bartolomeo, A.; Fuccelli, R.; Servili, M.; Montedoro, G. F.; Morozzi, G. Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. *J. Nutr.* **2008**, *138*, 1411–1416.

(182) Goulas, V.; Exarchou, V.; Troganis, A. N.; Psomiadou, E.; Fotsis, T.; Briasoulis, E.; Gerothanassis, I. P. Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. *Mol. Nutr. Food Res.* **2009**, *53*, 600–608.

(183) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(184) Anter, J.; Fernández-Bedmar, Z.; Villatoro-Pulido, M.; Demyda-Peyras, S.; Moreno-Millán, M.; Alonso-Moraga, A.; Muñoz-Serrano, A.; Luque de Castro, M. D. A pilot study on the DNA-protective, cytotoxic, and apoptosis-inducing properties of olive-leaf extracts. *Mutat. Res.* **2011**, *723*, 165–170.

(185) Lozano-Sánchez, J.; Segura-Carretero, A.; Menendez, J. A.; Oliveras-Ferraros, C.; Cerretani, L.; Fernández-Gutiérrez, A. Prediction of extra virgin olive oil varieties through their phenolic profile. Potential cytotoxic activity against human breast cancer cells. *J. Agric. Food Chem.* **2010**, *58*, 9942–9955.

cell lines, such as epithelial intestinal cells (Caco-2)[186], melanoma cells (M14)[187], human hepatoma (HepG2) cells[188]. Further, the inhibitory activity on human promyelocytic leukemia HL-60 cells[181], as well as human breast cancer MCF-7[189] and colon cancer cells (HT-29)[190] have also been demonstrated. Notably, lignans such as pinoresinol appears to have a pro-apoptotic activity sufficient to inhibit the proliferation of HL60 cells[191]. Flavonoids such as apigenin and luteolin have extensively been studied due to their presence in a variety of other plants, and to their effect in a large number of carcinoma cell lines[192].

In *in vivo* animal studies, oleuropein when administered orally to mice rapidly and completely induced tumor regression[193]. Similarly, after the oral administration of olive leaf extract, oleuropein reportedly prevented chronic ultraviolet B radiation-induced skin damage and carcinogenesis in hairless mice by inhibiting increases in skin thickness and reducing the skin elasticity, and skin carcinogenesis and tumor growth[194]. Likewise, an inhibition of 4-nitroquinoline1-

(186) Cells, C.; Manna, C.; Galletti, P.; Cucciolla, V.; Moltedo, O.; Leone, A.; Zappia, V. Biochemical and molecular roles of nutrients the protective effect of the olive oil polyphenol (3,4-Dihydroxyphenyl)-ethanol counteracts reactive oxygen metabolite–induced cytotoxicity. *J. Nutr.* **1997**, *127*, 286–292.

(187) Angelo, S. D.; Ingrosso, D.; Migliardi, V.; Sorrentino, A.; Donnarumma, G.; Baroni, A.; Masella, L.; Antonietta, M.; Zappia, M.; Galletti, P. Hydroxytyrosol , a natural antioxidant from olive oil , prevents protein damage induced by long-wave ultraviolet radiation in melanoma cells. **2005**, *38*, 908–919.

(188) Goya, L.; Mateos, R.; Bravo, L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. *Eur. J. Nutr*. **2007**, *46*, 70–78.

(189) Han, J.; Talorete, T. P. N.; Yamada, P.; Isoda, H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. *Cytotechnology* **2009**, *59*, 45–53.

(190) Obied, H. K.; Prenzler, P. D.; Konczak, I.; Rehman, A.-U.; Robards, K. Chemistry and bioactivity of olive biophenols in some antioxidant and antiproliferative *in vitro* bioassays. *Chem. Res. Toxicol.* **2009**, *22*, 227–234.

(191) Servili, M.; Sordini, B.; Esposto, S.; Urbani, S.; Veneziani, G.; Di Maio, I.; Selvaggini, R.; Taticchi, A. Biological activities of phenolic compounds of extra virgin olive oil. *Antioxidants* **2014**, *3*, 1–23.

(192) Kampa, M.; Pelekanou, V.; Notas, G.; Castanas, E. Olive oil phenols, basic cell mechanisms, and cancer. In *olive oil: minor constituents and health*; 2009; pp 129–172.

(193) Hamdi, H. K.; Castellon, R. Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 769–778.

(194) Kimura, Y.; Sumiyoshi, M. Olive leaf extract and its main component oleuropein prevent chronic ultraviolet B radiation-induced skin damage and carcinogenesis in hairless mice. *J. Nutr. Dis.* **2009**, *11*, 2079–2086.

oxide (4-NQO)-induced F433 rat tongue carcinogenesis by oleuropein ingestion has recently been reported[195].

7.4. Diabetes

Diabetes is a major health concern for humans. It is documented that high blood glucose (hyperglycemia) is a major cause of nervous-system damage. Apoptosis could be proposed as a possible mechanism for high glucose-induced neural dysfunction and cell death in both *in vitro* and *in vivo* studies[196]. Oleuropein aglycone has been shown to protect against type-2 diabetes. In particular, oleuropein aglycone supplementation to high-fat diet in mice reversed liver-weight gain together with rises in hepatic and plasma-lipid levels[197]. *In vitro* incubation of the high glucose-induced cell damage (NGF-treated pheochromocytoma (PC12)) cells with phenolic olive-leaf extract has revealed that the extract inhibited high glucose-induced neural damage and suppressed diabetes-induced thermal hyperalgesia. The mechanisms of these effects may be due, at least in part, to reduced neuronal apoptosis. Moreover, *in vivo*, olive-leaf extract has been demonstrated to attenuate thermal hyperalgesia in diabetic rats[196].

7.5. Neurodegenerative diseases

Neurodegenerative disorders such as Alzheimer's and Parkinson's diseases represent a growing problem in our aging societies, primarily because there is a

(195) Grawish, M. E.; Zyada, M. M.; Zaher, A. R. Inhibition of 4-NQO-induced F433 rat tongue carcinogenesis by oleuropein-rich extract. *Med. Oncol.* **2011**, *28*, 1163–1168.

(196) Kaeidi, A.; Esmaeili-Mahani, S.; Sheibani, V.; Abbasnejad, M.; Rasoulian, B.; Hajializadeh, Z.; Afrazi, S. Olive (*Olea europaea* L.) leaf extract attenuates early diabetic neuropathic pain through prevention of high glucose-induced apoptosis: *in vitro* and *in vivo* studies. *J. Ethnopharmacol.* **2011**, *136*, 188–196.

(197) Casamenti, F.; Grossi, C.; Rigacci, S.; Pantano, D.; Luccarini, I.; Stefani, M. Oleuropein aglycone : A possible drug against degenerative conditions. *In vivo* evidence of its effectiveness against alzheimer's disease. *J. Alzheimer's Dis.* **2015**, *45*, 679–688.

higher prevalence of both diseases with age[198]. Alzheimer's disease is characterized by massive neuronal cell and synapse loss at specific sites, by extracellular deposition of amyloid-β peptide into senile plaques as well as by intracellular accumulation of tau proteins as neuro-fibrillary tangles and neuropil threads[199].

Studies in rodents suggest that diet supplementation with oral phenol-rich components of EVOO, including oleuropein aglycone and its glycoside and/or one of its derivatives, improves learning and behavioral problems associated with aging and disease[197,199,200]. Likewise, olive-oil phenols may exert an effect on anxiety-associated behavior, possibly by modulating the expression level of GR, a gene involved in the defense mechanisms against oxidative stress[201]. Indeed, oleuropein aglycone treatment seems to combat amyloid-β peptide neurotoxicity by reducing the plaque load. However, the mechanism by which the olive-oil phenols exert their neuro-protective effects is not completely clear[191].

Parkinson's disease is characterized by a progressive and selective loss of dopaminergic neurons in the substantia nigra. Conjugates such as the 5-S-cysteinyl-dopamine, possess strong neurotoxicity and may contribute to the underlying progression of the pathology[180]. In the context of Parkinson's disease, in addition to the neuroprotection afforded by flavonoids, phenolic compounds such as caffeic acid, and tyrosol has also been shown to protect against 5-S-cysteinyl-dopamine and peroxynitrite neurotoxicity *in vitro*[202].

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(198) Harwood, J.; Aparicio, R. *Handbook of olive oil. Analysis and properties*; Harwood, J., Aparicio, R., Eds.; Springer science+business media, 2000.

(199) Grossi, C.; Ed Dami, T.; Rigacci, S.; Stefani, M.; Luccarini, I.; Casamenti, F. Employing Alzheimer disease animal models for translational research: Focus on dietary components. *Neurodegener. Dis.* **2014**, *13*, 1–4.

(200) Grossi, C.; Rigacci, S.; Ambrosini, S.; Ed Dami, T.; Luccarini, I.; Traini, C.; Failli, P.; Berti, A.; Casamenti, F.; Stefani, M. The polyphenol oleuropein aglycone protects TgCRND8 mice against Aß plaque pathology. *PLoS One* **2013**, *8*, 1–13.

(201) Pitozzi, V.; Jacomelli, M.; Zaid, M.; Luceri, C.; Bigagli, E.; Lodovici, M.; Ghelardini, C.; Vivoli, E.; Norcini, M.; Gianfriddo, M.; et al. Effects of dietary extra-virgin olive oil on behaviour and brain biochemical parameters in ageing rats. *Br. J. Nutr.* **2010**, *103*, 1674–1683.

(202) Vauzour, D.; Rodriguez-Mateos, A.; Corona, G.; Oruna-Concha, M. J.; Spencer, J. P. E.

8. Determination of phenolic compounds in olive leaves, fruit and oil

8.1 Extraction of phenolic compounds in olive leaves, fruit and oil

Liquid-liquid extraction (LLE) and solid-liquid extraction (SLE) procedures are the most common extraction methods used prior to analyzing phenolic compounds. Solvents frequently used for these kinds of extractions include alcohols (methanol, ethanol), acetone, diethyl ether, and ethyl acetate[203]. However, in some cases phenolic acids (highly polar) cannot be extracted using pure organic solvents, because the use of alcohol-water or acetone-water mixtures is highly recommendable. An optimized sample-preparation method using LLE has improved the results with separation techniques for analyzing phenolic compounds[204].

Furthermore, solid-phase extraction (SPE) is another technique for sample preparation which has replaced conventional extraction methods. SPE is a rapid and sensitive sample-preparation technique that has successfully replaced many laborious conventional extraction methods[205].

In the last decade, new extraction techniques have started to replace conventional ones. Remarkably, techniques such as pressurized and supercritical fluid extraction (PLE and SFE, respectively), microwave-assisted extraction (MAE) or ultrasound-assisted extraction (UAE) have recently been reported[68,88,89,93,206,207].

Polyphenols and human health: Prevention of disease and mechanisms of action. *Nutrients* **2010**, *2*, 1106–1131.

(203) Antolovich, M.; Prenzler, P.; Robards, K.; Ryan, D. Sample preparation in the determination of phenolic compounds in fruits. *Analyst* **2000**, *125*, 989–1009.

(204) Rodríguez-Delgado, M. A.; Malovaná, S.; Pérez, J. P.; Borges, T.; García Montelongo, F. J. Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection. *J. Chromatogr. A.* **2001**, *912*, 249–257.

(205) Tura, D.; Robards, K. Sample handling strategies for the determination of biophenols in food and plants. *J. Chromatogr. A.* **2002**, *975*, 71–93.

(206) Capote, F. P.; Marinas, A.; Japo, R. Liquid chromatography/triple quadrupole tandem mass spectrometry with multiple reaction monitoring for optimal selection of transitions to evaluate nutraceuticals from olive-tree materials. *Wiley Intersci.* **2008**, *22*, 855–864.

(207) Xynos, N.; Papaefstathiou, G.; Gikas, E.; Argyropoulou, A.; Aligiannis, N.; Skaltsounis,

These techniques are considered less time consuming and more environmentally friendly as they require smaller volumes of solvents[205].

8.1.1. Extraction of olive leaves phenolic compounds

Growing interest in olive leaves phenols has given rise to the adoption of many extraction systems used for plant phenols in order to achieve the most efficient procedure. To stabilize the by-product and to avoid quality losses or undesirable degradation during storage and transportation, the immediate dehydration of olive leaves is the key step in post-harvest processing. Many leaf-conservation methods have been described, such as traditional drying, hot and freeze-air drying, microwave oven or the application of ultrasound[208].

Conventional extraction techniques such as SLE are based mostly on the use of heat and/or agitation to accelerate the rate of mass transfer to the suitable solvent. However, these are generally time consuming and inefficient[209]. Notably, the combination of SLE with new techniques such as ultrasound-assisted extraction has given rise to ultrasound-assisted solid-liquid extraction (USLE), which has raised efficiency in comparison to ultrasound bath and agitation (33% and 80% enhancement of oleuropein, respectively)[209]. Nevertheless, the authors claim that the conventional method is still effective for recovering secoiridoids and flavonoids from olive leaves[95,210]. Moreover, the combination of SLE with instruments such as ultrasound bath and Ultra-Turrax blender, significantly

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A.L. Design optimization study of the extraction of olive leaves performed with pressurized liquid extraction using response surface methodology. *Sep. Purif. Technol.* **2014**, *122*, 323–330.

(208) Abaza, L.; Taamalli, A.; Nsir, H.; Zarrouk, M. Olive tree (*Olea europaea* L.) leaves: Importance and advances in the analysis of phenolic compounds. *Antioxidants* **2015**, *4*, 682–698.

(209) Rahmanian, N.; Jafari, S. M.; Wani, T. A. Bioactive profile, dehydration, extraction and application of the bioactive components of olive leaves. *Trends Food Sci. Technol.* **2015**, *42*, 150–172.

(210) Silva, S.; Gomes, L.; Leitao, F.; Coelho, A. V.; Boas, L. V. Phenolic compounds and antioxidant activity of *Olea europaea* L. fruits and leaves. *Food Sci. Technol. Int.* **2006**, *12*, 385–396.

shortened the extraction time while providing good phenolic recovery[125,211]. The latter extraction method was adopted for extracting olive leaves phenols in the present thesis.

Boiling dried leaves in water for extracting oleuropein and verbascoside reportedly gave 96 and 94% recoveries of these compounds, respectively, when compared with the methanol extract[212]. However, the solvent most widely used to extract olive leaves phenolics remains methanol or aqueous methanol mixtures. In fact, the presence of attached sugar tends to render the phenolic compounds more water soluble, and the combination of the above solvents with water are thus better solvents for glycosides[213]. By contrast, less polar aglycones such as flavanones and highly methoxylated flavonols tend to be more soluble in non-aqueous solvents. Thus, methanol/water (80:20, v/v) was found to be the most recommended mixture for olive leaves extracts with high phenol recovery[212,214], and with high levels of flavonoids[213], compared to ethanol or acetone.

8.1.2. Extraction of olive fruits phenolic compounds

Recovery of the phenolic compounds from olive fruits is more challenging compared to leaves and oil, as the fruits represent a less homogeneous sample and has higher enzyme activity. Hence, olive fruits require good conservation and more sample handling, such as filtration or centrifugation to remove solid components, to help avoid the alteration of the phenolics[215,216]. Thus, different

(211) Ryan, D.; Antolovich, M.; Herlt, T.; Prenzler, P. D.; Lavee, S.; Robards, K. Identification of phenolic compounds in tissues of the novel olive cultivar 'Hardy's Mammoth'. *J. Agric. Food Chem.* **2002**, *50*, 6716–6724.

(212) Malik, N. S. A.; Bradford, J. M. Recovery and stability of oleuropein and other phenolic compounds during extraction and processing of olive (*Olea europaea* L.) leaves. *J. Food, Agric. Environ.* **2008**, *6*, 8–13.

(213) Abaza, L.; Ben Youssef, N.; Manai, H.; Mahjoub Haddada, F.; Methenni, K.; Zarrouk, M. 'Chétoui' olive leaf extracts: Influence of the solvent type on phenolics and antioxidant activities. *Grasas y Aceites* **2011**, *62*, 96–104.

(214) Rafiee, Z.; Jafari, S. M.; Alami, M.; Khomeiri, M. Antioxidant effect of microwave-assisted extracts of olive leaves on sunflower oil. *J. Agric. Sci. Technol.* **2012**, *14*, 1497–1509.

(215) Ryan, D.; Robards, K. Phenolic compounds in olives. *Analyst* **1998**, *123*, 31R–44R.

sample treatments have been developed in the literature to achieve phenolic compounds extraction from olive fruits. Olive fruit phenolic compounds have been studied mainly by extraction of freeze-dried or fresh drupes/pulp immediately after picking[216–220]. Sample preparation by freeze drying and powdering the olive fruits with the aid of liquid nitrogen is a typical procedure that allows good preservation of phenolic compounds[216,221]. Likewise, lyophilized olive pulp reportedly causes less hydrolysis of oleuropein and oleuropein and ligstroside[219]. Often, 2% of metabisulfite, a powerful preservative, has been added to inhibit polyphenoloxidase and lipoxygenase activities during during the extraction[215,221]. Indeed, some authors reported the addition of 20 mg/L of sodium diethyldithiocarbamate (DIECA) for the same purpose[218].

The next step to be considered before starting phenolic compound extraction from olive fruits is to remove the lipid fraction and pigments. Many apolar solvents have been used in literature for this purpose, mainly hexane, petroleum ether, ethyl ether[203,215,221]. Thus, the most widely used extraction procedure for extracting olive fruit phenolic compounds has been SLE using solvents such as

(216) Ryan, D.; Robards, K.; Lavee, S. Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. *J. Chromatogr. A* **1999**, *832*, 87–96.

(217) Brenes, M.; Rejano, L.; Garcia, P.; Sanchez, A.; Garrido, A. Biochemical changes in phenolic compounds during Spanish-style green olive processing. *J. Agric. Food Chem.* **1995**, 994, 2702–2706.

(218) Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. High-performance liquid chromatography evaluation of phenols in olive fruit, virgin olive oil, vegetation waters, and pomace and 1D-and 2D-nuclear magnetic resonance characterization. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873–882.

(219) Rios, J. J.; Gutiérrez-Rosales, F. Comparison of methods extracting phenolic compounds from lyophilised and fresh olive pulp. *LWT - Food Sci. Technol.* **2010**, *43*, 1285–1288.

(220) Morelló, J.R.; Romero, M.P.; Motilva, M.J. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from ‘Arbequina’ , ‘Farga’ , and ‘Morrut’ Cultivars. *J. Agric. Food Chem.* **2004**, *52*, 6002–6009.

(221) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Importance and evolution of phenolic compounds in olive during growth and maturation. *J. Agric. Food Chem.* **1986**, *34*, 823–826.

methanol, ethanol or mixtures of either of the two with water in order to recover a wide range of phenols with diverse structures in fresh pulp[220,222–224].

Few reports describe the use of other new extraction techniques in the case of olive fruits. In fact, the use of USLE has recently been reported by Jerman et al.[225]. This method offers greater simplicity and efficiency, providing results of high selectivity, precision, and sensitivity. Likewise, SPE has been reported to give high recovery rates of phenolic compounds from olive drupes[218,226].

8.1.3. Extraction of olive oil phenolic compounds

Techniques such as SFE, MAE, PLE or SPE, among others, have also been reported for olive oil phenolic extraction[227]. However, SPE and LLE are the most widely used[228,229]. Sample preparation and concentration *via* SPE can be achieved in a one-step extraction. Thus, different types of SPE cartridges have been tested to maximize the recovery of phenolic compounds from olive oil[230]. For instance,

(222) Romani, A.; Mulinacci, N.; Pinelli, P.; Vincieri, F. F.; Cimato, A. Polyphenolic content in five Tuscany cultivars of *Olea europaea* L. *J. Agric. Food Chem.* **1999**, *47*, 964–967.
(223) Romero, C.; Garcia, P.; Brenes, M.; García, A.; Garrido, A. Phenolic compounds in natural black Spanish olive varieties. *Eur. Food Res. Technol.* **2002**, *215*, 489–496.
(224) Sousa, C.; Gouvinhas, I.; Barreira, D.; Carvalho, M. T.; Vilela, A.; Lopes, J.; Martins-Lopes, P.; Barros, A. I. 'Cobrançosa' olive oil and drupe: Chemical composition at two ripening stages. *J. Am. Oil Chem. Soc.* **2014**, *91*, 599–611.
(225) Jerman, T.; Trebše, P.; Mozetič Vodopivec, B. Ultrasound-assisted solid liquid extraction (USLE) of olive fruit (*Olea europaea*) phenolic compounds. *Food Chem.* **2010**, *123*, 175–182.
(226) Ryan, D.; Robards, K.; Lavee, S. Changes in phenolic content of olive during maturation. *Int. J. Food Sci. Technol.* **1999**, *34*, 265–274.
(227) Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Gallina-Toschi, T.; Fernández-Gutiérrez, A. Analytical determination of polyphenols in olive oils. *J. Sep. Sci.* **2005**, *28*, 837–858.
(228) Suárez, M.; Macià, A.; Romero, M.-P.; Motilva, M.-J. Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. *J. Chromatogr. A* **2008**, *1214*, 90–99.
(229) Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E.; Agrarie, I.; Costanzo, V. S. Simple and hydrolyzable phenolic compounds in virgin olive oil. 1. Their extraction , separation , and quantitative and semiquantitative evaluation by HPLC. *J. Agric. Food Chem.* **1992**, *40*, 1571–1576.
(230) Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Lercker, G.; Toschi, T. G. L iquid–liquid and solid-phase extractions of phenols from virgin olive oil and their separation by chromatographic and electrophoretic methods. *J. Chromatogr. A* **2003**, *985*, 425–433.

For instance, the use of C_{18} cartridges for the isolation of phenolic compounds from EVOO has shown good results mainly in terms of simplicity and speed of the procedure[231]. However, in a more recent study, the comparison of different types of cartridges C_{18}, Diol and Sax, has highlighted the efficiency of Diol cartridges over the others in extracting the polar fraction from non-polar matrices[232].

The LLE technique, though relatively time consuming, offers efficient and precise results[233]. LLE is based simply on the phase shift of the phenolic fraction from a fatty media (olive oil) to an aqueous phase (usually mixtures of methanol or ethanol/water at different percentages)[203]. The best solvent for the complete recovery of phenolics from olive oil remains[234–240]. However, the best results were reported to be obtained using methanol/water (80:20 v/v)[215,241].

(231) Pirisi, F. M.; Cabras, P.; Cao, C. F.; Migliorini, M.; Muggelli, M. Phenolic compounds in virgin olive oil. 2. Reappraisal of the extraction, HPLC separation, and quantification procedures. *J. Agric. Food Chem.* **2000**, *48*, 1191–1196.

(232) Gómez Caravaca, A. M.; Carrasco Pancorbo, A.; Cañabate Díaz, B.; Segura Carretero, A.; Fernández Gutiérrez, A. Electrophoretic identification and quantitation of compounds in the polyphenolic fraction of extra-virgin olive oil. *Electrophoresis* **2005**, *26*, 3538–3551.

(233) Mahugo Santana, C.; Sosa Ferrera, Z.; Esther Torres Padrón, M.; Juan Santana Rodríguez, J. Methodologies for the extraction of phenolic compounds from environmental samples: New approches. *Molecules* **2009**, *14*, 298–320.

(234) Gómez-Alonso, S.; Mancebo-Campos, V.; Salvador, M. D.; Fregapane, G. Evolution of major and minor components and oxidation indices of virgin olive oil during 21 months storage at room temperature. *Food Chem.* **2007**, *100*, 36–42.

(235) Baiano, A.; Gambacorta, G.; Terracone, C.; Previtali, M. A.; Lamacchia, C.; La Notte, E. Changes in phenolic content and antioxidant activity of Italian extra-virgin olive oils during storage. *J. Food Sci.* **2009**, *74*, C177–C183.

(236) Reboredo-Rodríguez, P.; González-Barreiro, C.; Cancho-Grande, B.; Simal-Gándara, J. Quality of extra virgin olive oils produced in an emerging olive growing area in north-western Spain. *Food Chem.* **2014**, *164*, 418–426.

(237) Ceci, L. N.; Carelli, A. A. Characterization of monovarietal Argentinian olive oils from new productive zones. *J. Am. Oil Chem. Soc.* **2007**, *84*, 1125–1136.

(238) Torres, M. M.; Maestri, Damián, M. Chemical composition of 'Arbequina' virgin olive oil in relation to extraction and storage conditions. *J. Sci. Food Agric.* **2006**, *10*, 2311–2317.

(239) Kesen, S.; Kelebek, H.; Selli, S. Characterization of the volatile, phenolic and antioxidant properties of monovarietal olive oil obtained from cv. Halhali. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1685–1696.

(240) Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Phenolic compounds and squalene in olive oils: The concentration and antioxidant potential of total phenols, simple phenols, secoiridoids, lignansand squalene. *Food Chem. Toxicol.* **2000**, *38*, 647–659.

(241) Segura-Carretero, A.; Carrasco-Pancorbo, A.; Bendini, A.; Cerretani, L.; Fernández-Gutiérrez, A. Analytical determination of polyphenols in olive oil. In *olives and olive oil*

8.2. Analytical separation techniques for determining phenolic compounds in olive leaves, fruit and oil

The phenolic fraction of olive products can be analyzed in different ways: the simple total quantitative determination is carried out by a colorimetric method, Folin-Ciocalteu being the most common[242]. The separation of individual phenolic compounds has commonly been performed by liquid chromatography (LC), although gas chromatography (GC)[89,243–245] and capillary electrophoresis (CE)[246–249] are also used.

Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) whereas the other (the mobile phase) moves in a definite

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in health and disease prevention; Preedy, V. R., Watson, R. R., Eds.; Academic Press, 2010; Vol. 28, pp 509–523.

(242) Centro, C.; Sacconi, V. L.; Florence, S. F. Minor polar compounds in extra virgin olive oil : Correlation between HPLC-DAD-MS and the Folin-ciocalteu spectrophotometric method. *J. Agric. Food Chem.* **2014**, *62*, 826–835.

(243) Angerosa, F.; D'Alessandro, N.; Corana, F.; Mellerio, G. Characterization of phenolic and secoiridoid aglycons present in virgin olive oil by gas chromatography-chemical ionization mass spectrometry. *J. Chromatogr. A.* **1996**, *736*, 195–203.

(244) Bouaziz, M.; Grayer, R. J.; Simmonds, M. S. J.; Damak, M.; Sayadi, S. Identification and antioxidant potential of flavonoids and low molecular weight phenols in olive cultivar 'Chemlali' growing in Tunisia. *J. Agric. Food Chem.* **2005**, *53*, 236–241.

(245) Owen, R. W.; Mier, W.; Giacosa, A.; Hull, W. E.; Spiegelhalder, B.; Bartsch, H. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin. Chem.* **2000**, *46*, 976–988.

(246) Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Cerretani, L.; Bendini, A.; Fernández-Gutiérrez, A. Use of capillary electrophoresis with UV detection to compare the phenolic profiles of extra-virgin olive oils belonging to Spanish and Italian PDOs and their relation to sensorial properties. *J. Sci. Food Agric.* **2009**, *89*, 2144–2155.

(247) Godoy-Caballero, M. D. P.; Galeano-Díaz, T.; Isabel Acedo-Valenzuela, M. Simple and fast determination of phenolic compounds from different varieties of olive oil by nonaqueous capillary electrophoresis with UV-visible and fluorescence detection. *J. Sep. Sci.* **2012**, *35*, 3529–3539.

(248) Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. A simple and rapid electrophoretic method to characterize simple phenols, lignans, complex phenols, phenolic acids, and flavonoids in extra-virgin olive oil. *J. Sep. Sci.* **2006**, *29*, 2221–2233.

(249) Priego-Capote, F.; Ruiz-Jiménez, J.; Luque De Castro, M. D. Fast separation and determination of phenolic compounds by capillary electrophoresis-diode array detection: Application to the characterisation of alperujo after ultrasound-assisted extraction. *J. Chromatogr. A* **2004**, *1045*, 239–246.

direction[250]. Solutes present in the sample usually have differential partitioning or interactions with the mobile and stationary phases. Because the stationary phase is the fixed phase, the solutes having stronger interactions with the stationary phase will tend to move slower (have longer retention times) than others having lower or no interactions with the stationary phase, which will tend to move faster. Therefore, chromatographic separations are a consequence of differential migration of solutes (**Figure 17**). As mentioned above, among the different types of chromatography, high-performance liquid chromatography has been the most widely used chromatographic techniques for the determination of phenolic compounds in plant matrices and, particularly, in *O. europaea* products.

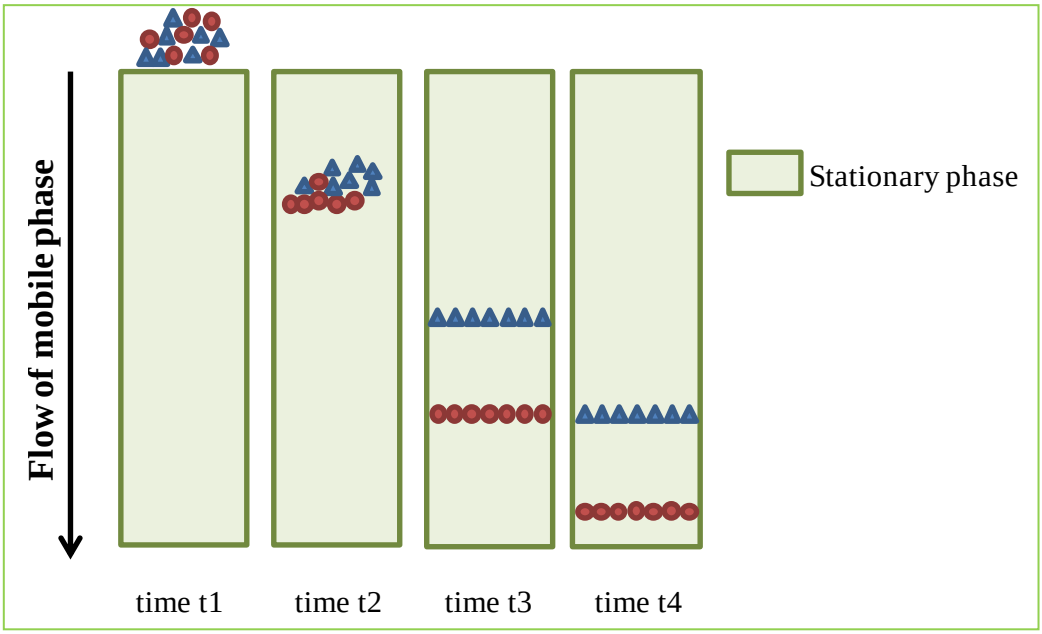

Figure 17. Representation of the separation of two components by chromatography.

8.2.1. Liquid chromatography (LC)

Liquid chromatography (LC) is a chromatographic technique where the mobile phase is a liquid and the stationary phase can be a liquid or a solid phase. The great power of LC resides in the combination of a wide range of possible mobile-

(250) IUPAC. Nomenclatur for chromatography (IUPAC Recommendations 1993). *Pure Appl. Chem.* **1993**, *65*, 819–872.

phase properties together with the choice of numerous, significantly different kinds of stationary phases and a wide variety of detectors. As result, LC in fact refers to multiple combinations, many with more than one name. For instance, one of the primary classification schemes of LC is by overall physical shape of the stationary phase, such as column chromatography (CC), thin-layer chromatography (TLC) and capillary liquid chromatography (CLC). Other names arise based on the direction flow of the mobile phase: ascending chromatography, descending chromatography and flat-bed chromatography. Classification is also based on the efficiency of the separations, such as high-performance liquid chromatography (HPLC) or high-performance thin-layer chromatography (HPTLC)[251]. The types of LC also are named after the general type of interaction between the stationary phase and the solutes in the eluent. The classifications are then called the normal-phase (NPLC), the reversed-phase (RPLC), ion-exchange (IELC), and size-exclusion chromatography (SELC)[252].

Overall, RPLC is the most commonly used modality used because it is highly stable and efficient. This kind of chromatography is based on a nonpolar stationary phase. The most popular column-packing material is octadecylsilyl silica (ODS-C18), in which silica is covalently modified by C18 functional group. In RPLC the mobile phase is more polar than the stationary phase; water and water-miscible organic solvents such as methanol, acetonitrile and tetrahydrofuran are commonly used[252].

Numbers of chromatographic modalities have been developed to identify and quantify specific phenolics in olive fruits, oil, and by-products[77,230,232,253]. Nevertheless, one of the most extensively used chromatographic modes to

(251) Rubinson, K. a.; Rubinson, J. F. Liquid chromatography. In *contemporary instrumental analysis*; Kenneth A., Ed.; Prentice-Hall, Inc, 2000; pp 628–671.
(252) Mansoor, M. A. Liquid chromatography. *Encyclopedia of Life Sciences* **2002**, 1–3.
(253) Carrasco-Pancorbo, A.; Nevedomskaya, E.; Arthen-Engeland, T.; Zey, T.; Zurek, G.; Baessmann, C.; Deelder, A. M.; Mayboroda, O. A. Gas chromatography/atmospheric pressure chemical ionization-time of flight mass spectrometry: Analytical validation and applicability to metabolic profiling. *Anal. Chem.* **2009**, *81*, 10071–10079.

determine olive phenolic compounds is reversed-phase high performance liquid chromatography (RPHPLC). The major advantage of HPLC over other chromatographic techniques for phenolic separation is that it provides high resolution and a sensitive quantitative analysis in the same operation and requires a simple sample treatment.

8.2.2. High-performance liquid chromatography (HPLC)

HPLC is characterized by the use of high pressure to push a mobile phase solution through a column of stationary phase, allowing the separation of complex mixtures with high resolution. This resolution depends on the extent of the interactions between the components of the sample and the mobile phase. Therefore, the separations can be improved by the selection of the proper mobile phase (solvent) and stationary phase. The multiple choices possible to perform a separation render HPLC a highly versatile technique for separating a wide number of different chemical compounds.

For HPLC the instrumentation includes different essential units, mainly: pump, injector, column, detector, and data system. A typical configuration of a HPLC system is shown in **Figure 18** and the main HPLC components are described in **Table 5**.

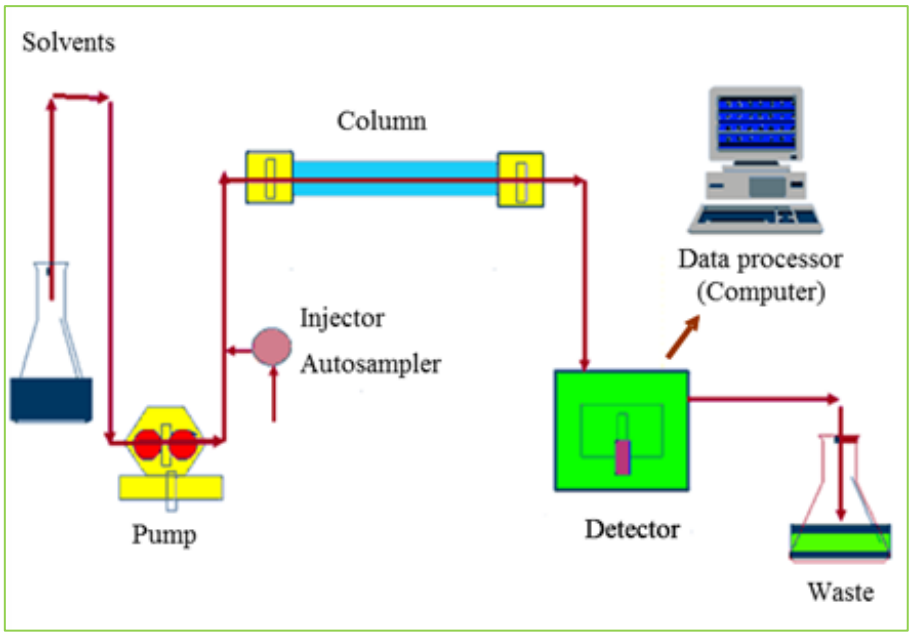

Figure 18. Configuration of a typical HPLC system.

Table 5. The main components of an HPLC system.

System component	Description
Mobile phase reservoir	Stores the mobile phase required for analysis
Degasser	Degasses the mobile phase
Pump	Solvent delivery system, enables the flow of the mobile phase through the system
Injector	Sample delivery system, injects the sample into the system
Column compartment	Used to control the temperature of the column
Detector	Detects each component of the separated mixture after they are eluted from the column
Data processor	Converts the data from the detector into meaningful results
Waste	Collection of the liquid waste

HPLC coupled to different detection systems has widely been accepted as the main tool for identification, structural characterization, and quantitative analysis of phenolic compounds in olive leaves, fruits and oil.

The separation of the components is strongly affected by the type and the composition of the mobile phase. In fact, different solvents are used for different types of HPLC. For RPHPLC, the solvent is normally a mixture of water and a polar organic solvent. In general, gradient elution has usually been necessary to recognize the complexity of the olive phenolic profile although isocratic elution has been successful for particular applications in olive leaves and oil[254–257]. Gradient elutions vary from laboratory to laboratory, and thus the literature provides many descriptions of gradients for the best separation of compounds. In general, the gradient is commonly started by 90-95% of the phase A (formic or acetic acidified water), and 5-10% of the phase B (methanol, acetonitrile, etc.), the gradient is kept for a time and then gradually changes during the analysis until providing the best separation of compounds. Finally, the column is usually equilibrated up to the initial condition[78,104,258,259].

Numerous mobile phases have been used, although water has always been combined with an organic solvent which is miscible with water in all proportions. Increasing the proportion of the organic solvent in the mobile phase will reduce the retention time of the analyte. Consequently, binary systems consisting of water and a less-polar solvent (methanol, acetonitrile) are the most common

(254) Akasbi, M.; Shoeman, D. M.; Saari Csallany, A. High-performance liquid chromatography of selected phenolic compounds in olive oils. *J. Am. Oil Chem. Soc.* **1993**, *70*, 367–370.

(255) Pirisi, F. M.; Angioni, A.; Cabras, P.; Garau, V. L.; Sanjust di Teulada, M. .; Karim dos Santos, M.; Bandino, G. Phenolic compounds in virgin olive oils I. Low-wavelength quantitative determination of complex phenols by high-performance liquid chromatography under isocratic elution. *J. Chromatogr. A* **1997**, *768*, 207–213.

(256) Altinyay, Ç.; Altun, M. L. HPLC analysis of oleuropein in *Olea europaea* L. *J. Fac. Pharm. Ankara* **2006**, *35*, 1–11.

(257) Savournin, C.; Baghdikian, B.; Elias, R.; Dargouth-kesraoui, F.; Boukef, K.; Balansard, G. Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *J. Agric. Food Chem.* **2001**, *49*, 618–621.

(258) Bajoub, A.; Carrasco-Pancorbo, A.; Ajal, E. A.; Ouazzani, N.; Fernández-Gutiérrez, A. Potential of LC-MS phenolic profiling combined with multivariate analysis as an approach for the determination of the geographical origin of north Moroccan virgin olive oils. *Food Chem.* **2015**, *166*, 292–300

(259) Uylaşer, V. Changes in phenolic compounds during ripening in ‘Gemlik’ variety olive fruits obtained from different locations. *CyTA - J. Food* **2015**, *13*, 167–173.

mobile phases[260–263]. Acids such as acetic, formic, and perchloric acid are usually added to water to maintain a constant acid concentration during gradient runs[260,264,265]. Lowering the pH partly helps to improve the resolution, and indeed it has been demonstrated that a weakly acidic mobile phase suppresses ionization of the most polar compounds such as phenolic acids, and as a result enhances the separation in a reversed-phase column[266]. Furthermore, the flow of the mobile phase and the temperature affect the relative retention of different analytes in the column and also the resolution of the whole analysis. Increasing the flow involves a reduction of the analysis duration and improves the resolution, although it directly increases the pump pressure. It bears mentioning that raising the temperature resolves the pressure problem, since at high temperatures the solvent becomes less viscous, and the eluent flows faster. Notably, the analysis is more reproducible when the temperature remains constant during the analysis time.

RP columns are reportedly the most commonly used for all olive-matrix phenols, offering better reproducibility and separation of polar compounds[215,227,267]. Thus,

(260) De la Torre-Carbot, K.; Jauregui, O.; Gimeno, E.; Castellote, A. I.; Lamuela-Ravento, R. M.; López-Sabater, M. C. Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction HPLC-DAD , and HPLC-MS/MS. *J. Agric. Food Chem.* **2005**, *53*, 4331–4340.

(261) Bakhouche, A.; Lozano-Sánchez, J.; Bengana, M.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Time course of Algerian 'Azeradj' extra-virgin olive oil quality during olive ripening. *Eur. J. Lipid Sci. Technol.* **2015**, *117*, 389–397.

(262) Taamalli, A.; Arráez-Román, D.; Zarrouk, M.; Valverde, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. The occurrence and bioactivity of polyphenols in Tunisian olive products and by-products: A review. *J. Food Sci.* **2012**, *77*, 83–92.

(263) Garcia, B.; Coelho, J.; Costa, M.; Pinto, J.; Paiva-Martins, F. A simple method for the determination of bioactive antioxidants in virgin olive oils. *J. Sci. Food Agric.* **2013**, *93*, 1727–1732.

(264) Bayram, B.; Esatbeyoglu, T.; Schulze, N.; Ozcelik, B.; Frank, J.; Rimbach, G. Comprehensive analysis of polyphenols in 55 extra virgin olive oils by HPLC-ECD and their correlation with antioxidant activities. *Plant foods Hum. Nutr.* **2012**, *67*, 326–336.

(265) Taamalli, A.; Abaza, L.; Arráez Román, D.; Segura Carretero, A.; Fernández Gutiérrez, A.; Zarrouk, M.; Nabil, B. Y. Characterisation of phenolic compounds by HPLC-TOF/IT/MS in buds and open flowers of 'Chemlali' olive cultivar. *Phytochem. Anal.* **2013**, *24*, 504–512.

(266) Harnly, J. M.; Bhagwat, S.; Lin, L.-Z. Profiling methods for the determination of phenolic compounds in foods and dietary supplements. *Anal. Bioanal. Chem.* **2007**, *389*, 47–61.

(267) Laura Capriotti, A.; Cavaliere, C.; Crescenzi, C.; Foglia, P.; Nescatelli, R.; Samperi, R.; Laganà, A. Comparison of extraction methods for the identification and quantification of

a nonpolar octadecylsilane (C_{18}) bonded phase has widely been used as the stationary phase[88,95,259,268]. although a pentafluorophenyl-modified silica gel (PFP) column also reportedly provides satisfactory results for olive-oil phenols[269].

Moreover, a wide variety of column dimensions and specifications are used[215,227]. The preferred columns are 100 to 250 mm in length, with 2-4.6 mm inner diameter and 1.8-5 μm particle size but shorter and narrower columns with small particle sizes would be preferred to attain better resolution and reduce the analysis time[265,270]. Newly developed stationary phases with fused-core particles have been introduced, giving high chromatographic efficiency and resolution, shorter analysis times, and keener sensitivity at lower operating pressures[271]. Likewise, columns with100 to 250 mm in length, 2-4.6 mm inner diameter and 1.8 to 3 μm particle sizes have been reported for separating phenolics from olive leaves[208]. However, regarding the analysis of olive-fruit phenolics, it has been reported that columns are usually longer than the ones normally used for olive-oil phenolic compounds. The Spherisorb ODS-2 (250×4.6 mm, 5 μm) analytical column has commonly served for fruits applications[272].

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polyphenols in virgin olive oil by ultra-HPLC-QTOF mass spectrometry. *Food Chem.* **2014**, *158*, 392–400.

(268) Reboredo-Rodríguez, P.; Rey-Salgueiro, L.; Regueiro, J.; González-Barreiro, C.; Cancho-Grande, B.; Simal-Gándara, J. Ultrasound-assisted emulsification-microextraction for the determination of phenolic compounds in olive oils. *Food Chem.* **2014**, *150*, 128–136.

(269) Dierkes, G.; Krieger, S.; Du, R.; Bongartz, A.; Schmitz, O. J.; Hayen, H. High-performance liquid chromatography−mass spectrometry profiling of phenolic compounds for evaluation of olive oil bitterness and pungency. *J. Agric. Food Chem.* **2012**, *60*, 7597–7606.

(270) Selvaggini, R.; Servili, M.; Urbani, S.; Esposto, S.; Taticchi, A.; Monterdero, G. F. Evaluation of phenolic compounds in virgin olive oil by direct injection in high-performance liquid chromatography with fluorometric detection. *J. Agric. Food Chem.* **2006**, *54*, 2832–2838.

(271) Bayram, B.; Ozcelik, B.; Schultheiss, G.; Frank, J.; Rimbach, G. A validated method for the determination of selected phenolics in olive oil using high-performance liquid chromatography with coulometric electrochemical detection and a fused-core column. *Food Chem.* **2013**, *138*, 1663–1669.

(272) Gómez-Caravaca, A. M.; Lozano-Sánchez, J.; Contreras Gámez, M. d. M.; Segura Carretero, A.; Taamalli, A. Bioactive phenolic compounds from *Olea europaea*: A challenge for analytical chemistry. In *olive and olive oil bioactive constituents*; Boskou, D., Ed.; AOCS Press, 2015; pp 261–298.

8.2.3. Detectors

HPLC coupled to different detection systems (UV-Vis, mass spectrometry, NMR, etc.) has widely been accepted as the main tool for identification, structural characterization, and quantitative analysis of phenolic compounds in olive leaves[211], fruits, and oil[272]. The choice of the detector for each application is based on the nature and properties of the analytes to be determined, the required sensitivity and type of desired information (structural, quantitative, qualitative, etc.). The ideal characteristics of an HPLC detector are high sensitivity, good stability, linearity, short response time, reliability, non-destructiveness, ease of use, and low dead volume. Thus, for the analysis of phenolic compounds, the most widely used are UV-Vis detectors such as the diode-array detector (DAD), and mass spectrometry (MS). Both kinds of detectors have been used in this thesis.

a. DAD

A DAD detector measures the concentration of the bands of compounds as they elute from the column and pass through the detector flow cell, by transducing the analyte signal into an electrical signal. The DAD has multiple photodiode arrays to provide information over a wide range of wavelengths at one time, this being a merit of the DAD. Its robustness makes DAD the best option to quantify compounds and it can also help to distinguish subclasses of phenolic compounds[266]. Therefore, the International Olive Council (IOC) proposed the hyphenation HPLC-DAD to denote the official method for analyzing phenolic compounds in olive oil, and it included the maximum absorbance values of 27 different phenolic compounds on the basis of the data provided by the UV detector[273]. In addition, it has been reported that 280 nm is a useful wavelength for routine analysis of most olive oil phenolics because they present maximum

(273) International olive council. *Determination of biophenols in olive oils by HPLC*; 2009; Vol. COI/T.20/D.

absorption at this wavelength; however, 240 nm is used for secoiridoids and their derivatives, 310-320 nm for hydroxyl cinnamic acids, and 350 nm for flavonoids[263,274,275].

b. Mass-spectrometry

Mass spectrometry (MS) relies on the formation of gas-phase ions (positively or negatively charged) that can be isolated electrically (or magnetically) based on their mass-to-charge ratio (*m/z*). This method can provide valuable information about the analytes, including their structure, purity, and composition. The response in MS clearly depends on the interface technology and mass analyzer used, as well as the analytical conditions of the separation method[70]. However, it is probably the most versatile and comprehensive analytical technique currently available to chemists and biochemists[276].

The principle of MS involves three basic steps. The first is ionization, which converts analyte molecules or atoms into gas-phase ionic species. This step requires the removal or addition of an electron or proton(s). The excess energy transferred during an ionization event may break the molecule into characteristic fragments. The next step is the separation and mass analysis of the molecular ions and their charged fragments on the basis of their *m/z* (mass-to-charge) ratios. And, finally, the ion current due to these mass-separated ions is measured, amplified, and displayed in the form of a mass spectrum. The first two steps are carried out under high vacuum, which allows ions to move freely in space without colliding or interacting with other species. Collisions may lead to

(274) Bakhouche, A.; Lozano-Sánchez, J.; Beltrán-Debón, R.; Joven, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Phenolic characterization and geographical classification of commercial 'Arbequina' extra-virgin olive oils produced in southern Catalonia. *Food Res. Int.* **2013**, *50*, 401–408.

(275) Godoy-Caballero, M. P.; Acedo-Valenzuela, M. I.; Galeano-Díaz, T. Simple quantification of phenolic compounds present in the minor fraction of virgin olive oil by LC–DAD–FLD. *Talanta* **2012**, *101*, 479–487.

(276) Chhabil, D. Basics of mass spectrometry. In *fundamentals of contemporary mass spectrometry*; John Wiley and sons, 2007; pp 3–14.

fragmentation of the molecular ions and may also produce a different species through ion-molecule reactions. These processes can reduce sensitivity, increase ambiguity in the measurement, and decrease resolution. In addition, the atmospheric background can introduce interference[276].

Thus, a mass spectrometer consists of three basic parts: an ion source, a mass analyzer, and a detector system **(Figure 19)**.

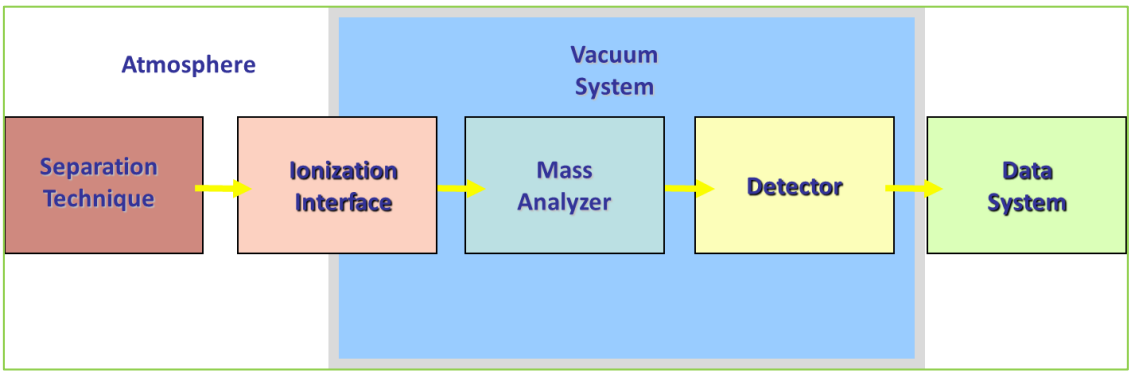

Figure 19. Mass-spectrometer diagram.

In the ion source, the samples are ionized prior to analysis in the mass spectrometer. A variety of ionization techniques can be used for mass spectrometry. The most important considerations are the internal energy transferred during the ionization process and the physico-chemical properties of the analyte to be ionized. Some ionization techniques are energetically costly and cause extensive fragmentation. Other techniques are gentler and produce ions only of the molecular species[277]. Among these, a soft ionization source such as electrospray ionization (ESI) has been used in the present thesis.

(277) Hoffmann, E. de; Stroobant, V. *Mass spectrometry-priniples and applications.*; Hoffmanan, E. de., Ed.; Jhon Wiley and Sons, 2007; Vol. 29.

Electrospray ionization

Electrospray ionization (ESI) has revolutionized the use of MS, enabling researchers to easily study organic substances such as phenolic compounds. The basics of ESI could be summarized as following: an aerosol spray consisting of fine droplets created when a high electric potential is applied to a needle containing a solution with a polar solvent **(Figure 20)**. The spray process can be pneumatically or ultrasonically assisted. A drying bath gas or thermal desolvation method is used to eliminate clustering as the droplets are cooled by supersonic expansion. The droplets are induced into the vacuum region through an orifice or skimmer. The vacuum interface consists of pumping stages and ion optics designed to maximize ion transmission; collision induced-dissociation (CID) in the vacuum interface aids in breaking up solvent clusters and providing a means for generating fragment ions, which are often structurally significant.

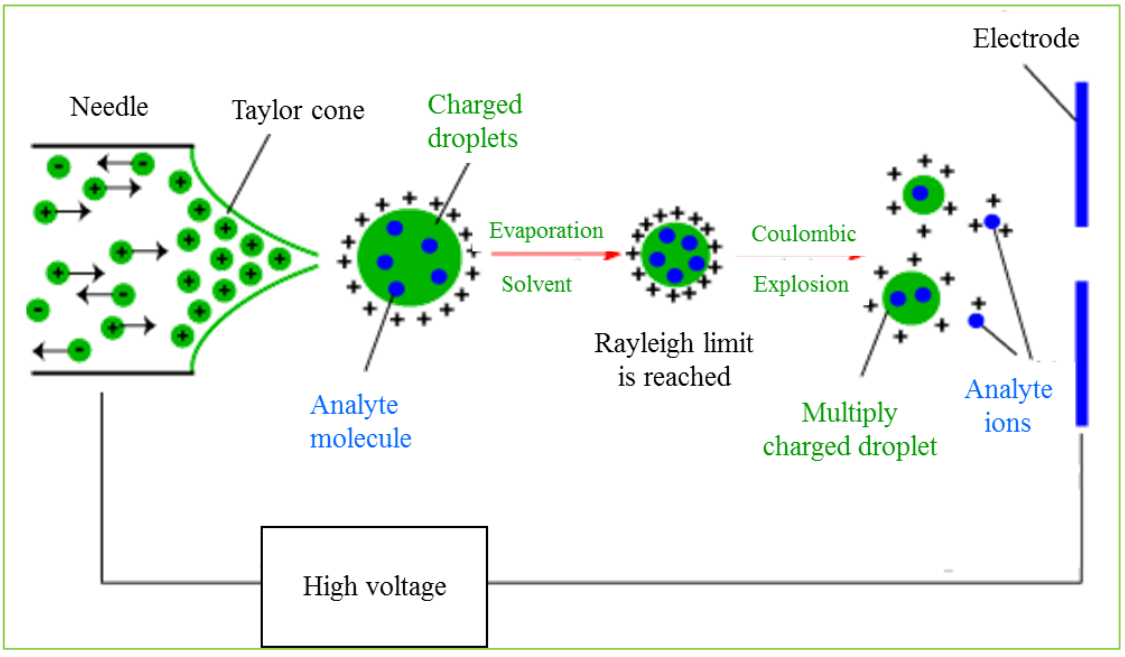

Figure 20. Electrospray formation process.

The advantage of the ESI method is its gentleness and its capacity to multiply charged ions in addition to its wide action range, from very low mass to

extraordinary high. In fact, the ESI-MS combination is used almost exclusively today to detect nonvolatile polar and thermally labile phenolics at very low concentrations[70,278]. Both positive and negative ionizations are applied. Although phenolic compounds from olive-related matrices are detected with a greater sensitivity in HPLC-ESI-MS in the negative-ionization mode, the results from positive and negative ion modes could be complementary.

Mass analyzer

Once the gas-phase ions have been produced, they need to be separated according to their masses and, then, determined. A mass analyzer is the part of the instrument in which ions are separated based on their *m/z* values. Similar to ionization process in terms of the available methodologies, numerous systems can isolate ions based on their *m/z*. Today, the development of MS analyzers, such as time-of-flight (TOF-MS), quadrupole time-of-flight (QTOF-MS), and ion trap (IT-MS) detectors have enhanced the attractiveness of MS for the identification of phenolic compounds[266]. These analyzers vary in terms of size, price, resolution, mass range, and the ability to perform tandem mass-spectrometry experiments (MS/MS).

In reference to TOF-MS, the mass analyzer used in this thesis, this ion-separation methodology can be considered one of the simplest. In fact, TOF relies simply on the free flight of the ionized molecules in a tube of 1-2 m in length, before reaching the detector (**Figure 21**). For instance, two ions that are formed at the same time with the same charge but different masses, the ion with the lowest mass will reach the detector first. The main advantage of a TOF analyzer is that all ions formed will eventually reach the detector (unlike quadrupole or sector instruments). TOF technology presents numerous advantages, such as high mass

(278) Boskou, D. Phenolic compounds in olives and olive oil. In *olive oil: minor constituents and health*; Boskou, D., Ed.; 2008.

resolution, high mass accuracy, theoretically unlimited mass range, and relatively low cost.

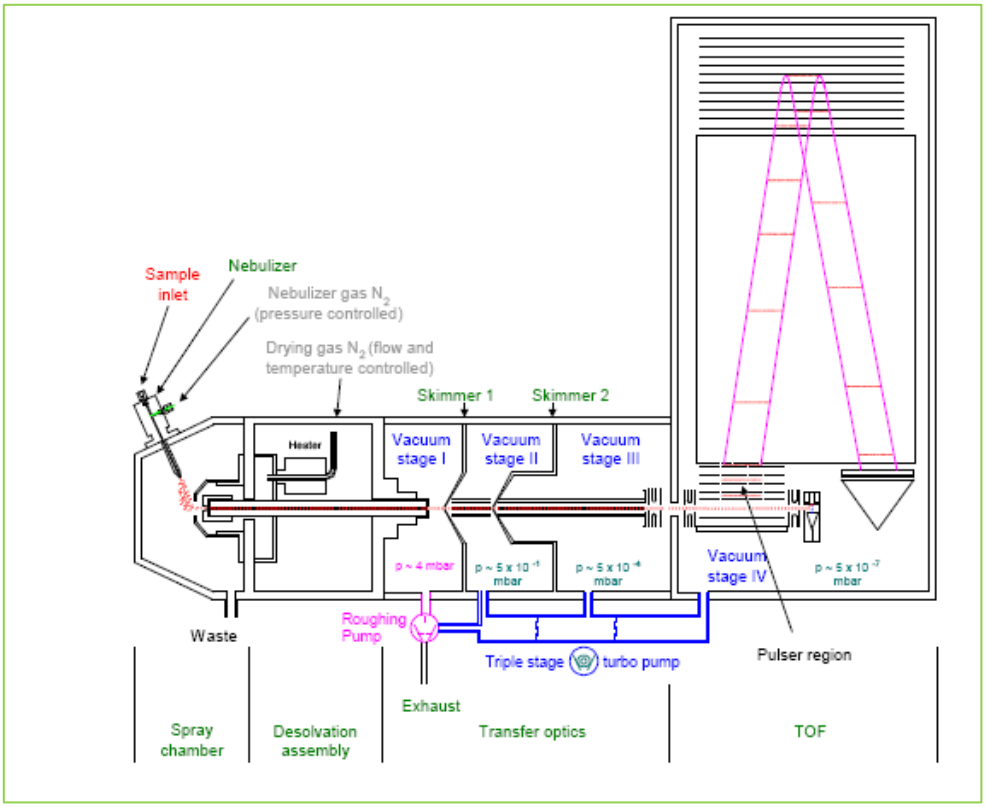

Figure 21. Schematic diagram of aTOF analyzer.

8.3. Applications of HPLC-DAD-ESI-MS for the analysis of phenolic compounds in *O. europaea*

Data giving by ESI-MS can provide either positive identification, by matching the characteristics of the analytical peaks to those of standards or well-characterized plant materials reported in the literature, or provisional identification, based on structural information for the compound subunits. In addition, the extracted ion chromatogram (EIC) provided allows the differentiation of compounds when there are overlapping peaks[70]. However, the main disadvantage of MS is that repeatability and reproducibility are normally worse than using UV/Vis or DAD detectors[272]. Consequently, the online coupling of the HPLC-DAD with ESI-MS constitutes a huge step in the analysis of

phenolic compounds in olive fruit, oil, and by-products. Notably, the combination of data found in literature with DAD spectra and accurate mass data from TOF enables the characterization of phenolic compounds for which no commercial standards are available[70,96,279–283].

For example, the applicability of HPLC-DAD-ESI-TOF-MS by Fu et al.[96], to identify phenolic compounds in olive leaf extracts, has screened 54 phenolic compounds. Among these compounds, many isomers of secoiridoids and flavonoids were greatly separated and identified, and some were reported in olive leaves sources for the first time. Using the same technology, Taamalli et al.[78] have for the first time in olive leaves revealed the presence of a large number of phenolic compounds from different classes; among these the secoiridoid 2″-methoxyoleuropein, the flavonoids diosmetin and its isomer, luteolin diglucoside isomer, and luteolin rutinoside isomer.

HPLC-MS in the positive and negative ion modes have been used to characterize phenolic compounds in the fruits of Italian olive cultivars[216]. This methodology confirmed the presence of oleuropein as the major phenolic in olive fruits. Other compounds, namely, hydroxytyrosol-4-*β*-*D*-glucoside, have been detected for the first time by HPLC-ESI-MS in the negative ion mode in olive fruit[284]. Besides,

(279) Fu, S.; Segura-Carretero, A.; Arráez-Román, D.; Menéndez, J. A; De La Torre, A.; Fernández-Gutiérrez, A. Tentative characterization of novel phenolic compounds in extra virgin olive oils by rapid-resolution liquid chromatography coupled with mass spectrometry. *J. Agric. Food Chem.* **2009**, *57*, 11140–11147.

(280) Bakhouche, A.; Lozano-Sánchez, J.; Ballus, C. A.; Bendini, A.; Gallina-Toschi, T.; Fernández-Gutiérrez, A.; Segura-Carretero, A. A new extraction approach to correct the effect of apparent increase in the secoiridoid content after filtration of virgin olive oil. *Talanta* **2014**, *127*, 18–25.

(281) Bouaziz, M.; Jemai, H.; Khabou, W.; Sayadi, S. Oil content, phenolic profiling and antioxidant potential of Tunisian olive drupes. *J. Sci. Food Agric.* **2010**, *90*, 1750–1758.

(282) Rigane, G.; Salem, R. Ben; Sayadi, S.; Bouaziz, M. Phenolic composition, isolation, and structure of a new deoxyloganic acid derivative from ‘Dhokar’ and ‘Gemri-Dhokar’ olive cultivars. *J. Food Sci.* **2011**, *76*, 965–973.

(283) Botsoglou, E.; Govaris, A.; Fletouris, D.; Iliadis, S. Olive leaves (*Olea europea* L.) and α-tocopheryl acetate as feed antioxidants for improving the oxidative stability of *α*-linolenic acid-enriched eggs. *J. Anim. Physiol. Anim. Nutr. (Berl).* **2013**, *97*, 740–753.

(284) Romero, C.; Brenes, M.; García, P.; Garrido, A. Hydroxytyrosol 4-*β*-D-glucoside, an important phenolic compound in olive fruits and derived products. *J. Agric. Food Chem.*

HPLC-DAD-ESI-MS have been successfully used to report the origin and distribution between pulp and stone of lignans in olive fruits[285].

Finally, the application HPLC-DAD-ESI-TOF-MS in olive oil is the most widely reported in literature. For instance, the sensitivity, together with mass accuracy and true isotopic pattern of the TOF-MS, enabled the identification of a broad series of hitherto unknown phenolic compounds in EVOO[81]. Furthermore, the combination of data found in literature with DAD spectra and accurate mass data from TOF makes it possible to characterize phenolic compounds for which no commercial standards are available, e.g. phenolic products formed during storage of EVOO[286]. A recent study in 88 commercial EVOOs has strongly confirmed once again that HPLC-DAD-MS currently cannot be easily replaced by a simpler test to identify and quantify single phenolic compounds fraction in EVOO[242].

2002, *50*, 3835–3839.

(285) Oliveras López, M. J.; Innocenti, M.; Ieri, F.; Giaccherini, C.; Romani, A.; Mulinacci, N. HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian *Olea europaea* L. fruits. *J. Food Compos. Anal.* **2008**, *21*, 62–70.

(286) Lozano-Sánchez, J.; Bendini, A.; Quirantes-Piné, R.; Cerretani, L.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Monitoring the bioactive compounds status of extra-virgin olive oil and storage by-products over the shelf life. *Food Control* **2013**, *30*, 606–615.

EXPERIMENTAL PART

CHAPTER 1. Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits

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Review

Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits

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Abstract

Olive leaves have always aroused an important interest, especially for folk medicine. Polyphenols contained in olive leaves have played an important role to this end, because they have demonstrated to be responsible for their anti-carcinogenic, anti-inflammatory, and antimicrobial proprieties. Olive leaves have common phenolics with other plants, but they also contain phenolics belonging to the secoiridoids family (exclusive to the Oleaceae family). Chemical, agronomical and medicinal researches have contributed together to highlight the interest in the use of olive leaves as a potential source of phenolic compounds for the production of functional food and nutraceuticals. The aim of this review is to provide a guideline summarizing the great information available about phenolic compounds of olive leaves. Therefore, from one side, it has been reported the availability of leaves as by-products, a brief description of the main phenolics identified in leaves, as well as the main analytical methods used for their extraction and determination. From another side, the effects of abiotic and biotic factors on the phenolic compound content in leaves have also been exposed for the first time, and finally, an overview of the main research studies dealing with the beneficial effects of olive leaves phenolic compounds has been included.

Keywords: Olive leaves, phenolic compounds, biotic and abiotic factors, health benefits.

1. Introduction

The olive tree (*Olea europaea* L.) is one of the oldest known cultivated plants. It is usually native to Mediterranean countries and its cultivation has spread globally during the past two decades due to the healthiness attributed to the consumption of olive oil. More than 8 million ha of olive trees are cultivated worldwide; almost 98% of them are in the Mediterranean basin[1]. Spain is the country with the largest olive orchard acreage and the highest number of olive trees[2]. The estimated total world production of olive oil in 2014/2015 accounts for 2.39 million tons, among which 1.53 million tons are produced by the European Union and its main destination is human consumption[3]. In addition to olive oil, olive trees are also cultivated for table olive production. Table olives and olive oil are two of the most representative foods of the traditional Mediterranean diet[4].

By-products derived from olive trees and olive oil extractions are generally known as "olive by-products"[5]. A high number of by-products and residues derived from both olive tree cultivation and the olive processing industry are obtained yearly; most of them have no practical applications. Olive leaves, one of these by-products, can be found in large amounts in olive oil industries. Leaves represent 10% of the weight of olives collected for oil extraction[6]. Furthermore, they also accumulate in large volumes on farms during the pruning of the trees[7]. It has been estimated that pruning produces 25 kg of by-products (twigs and leaves) per tree annually. A typical olive tree pruning lot includes leaves (approximately 25% by weight), thin branches (approximately 50% by weight), and thick branches or wood (approximately 25% by weight), although the proportions may vary depending on culture conditions, tree age, production and/or local pruning practice. In the Mediterranean region, residual biomass from olive tree pruning yield ranges from 1 to 5 and from 4 to 11 t/ha, for Spanish and Italian orchards, respectively[8], making of residues a huge, cheap, and unexploited source of energy or chemicals. Olive leaves are usually burned or ground

together with the remainder of the olive tree pruning by-products, i.e., branches[9] and are then directly thrown away as by-products, potentially causing environmental damage and wasting a resource[10].

Thus, this residue is a very abundant vegetable material with increasing cost for producers due to their removal, storage and elimination. Nevertheless, olive leaves are a potential source of phenolic compounds[11,12].

The interest of olive leaves, as a matrix rich in antioxidants, have increased with the aim to be further use in food and food supplements. In food industry, there is an increasing interest in producing functional foods for their health beneficial. The incorporation of such extracts in food industry may contribute to the health benefit of the consumers significantly and also to prolong the shelf life of food products[13]. Enrichment of oils with olive leaves, olive leaf extract as well as with the main secoiridoid compound (oleuropein) has been reported in literature[14]. Moreover, the enrichment of refined olive and refined olive-pomace oils with oleuropein, oleuropein aglycone and hydroxytyrosol rich extracts has proven to inhibit the deterioration of oil rancidity by improving stability[13].

Olive leaf extracts have been recently marketed as dietary product[15]. Commercial products in the form of herbal teas or food supplements are available all over the world, as complete dried leaves, powder, extracts or tablets[16]. It has been shown that encapsulation of olive leaf extracts with the aid of β-cyclodextrin increase the aqueous solubility of the polyphenolic residue from olive leaf[17].

Therefore, the valorization of this by-product is needed since, in many cases, the wasted by-products can yield similar or even higher contents of bioactive compounds than the final product does[18]. As a result, these bioactive compounds can be used as an important source to produce nutraceuticals or to be included in functional food thanks to their potential health benefits.

2. Phenolic compounds in olive leaves

Phenolic compounds or polyphenols are defined as secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants. These compounds, one of the most widely occurring groups of phytochemicals, are of considerable physiological and morphological importance in plants[19], and are of considerable interest for human diet due to their antioxidant properties[20]. Structurally, despite their extreme variety, polyphenols possess a common carbon skeleton building block: the C6–C3 phenylpropanoid unit. Biosynthesis by this pathway leads to a wide range of plant phenols: cinnamic acids (C6–C3), benzoic acids (C6–C1), flavonoids (C6–C3–C6), proanthocyanidins [(C6–C3–C6)n], coumarins (C6–C3), stilbenes (C6–C2–C6), lignans (C6–C3–C3–C6) and lignins [(C6–C3)n][21].

Olive leaves contain a large variety of phenolic derivatives, and consist of simple phenols (the most common and important low-molecular weight phenolic compounds), flavonoids (flavones, flavanones, flavonols, flavanols), and secoiridoids (**Figure 1**). Hydroxytyrosol has been widely described as one of the main components of simple phenols in olive leaves[22-27]. Flavonoids are one of the most common and widely distributed group of olive leaves polyphenols[28,29] and consist of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle[30]. They can be present in the aglycone form (quercetin, apigenin, luteolin, diosmetin) or in the glycosylated form (quercetin-7-*O*-rutinoside, luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucoside, luteolin-5-*O*-glucoside)[31-33].

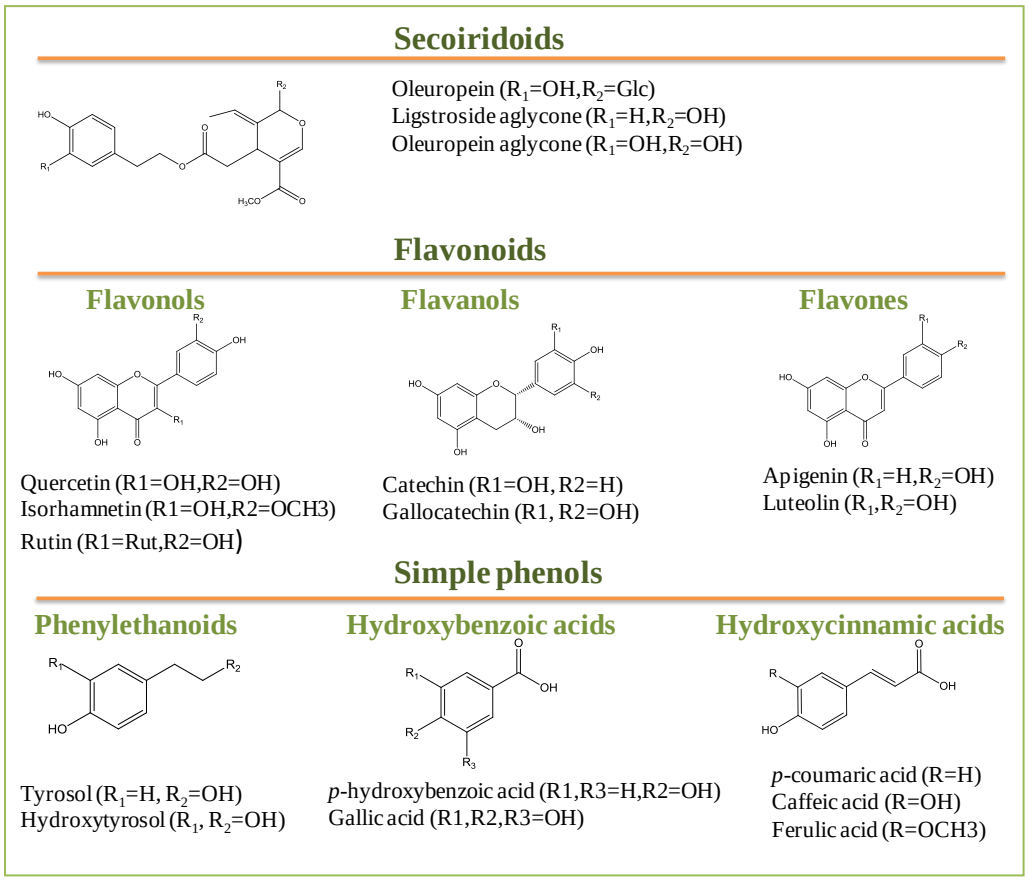

Figure 1. Examples of phenolic compounds from olive leave. Glc (Glucose); Rut (Rutinose).

However, secoiridoids, which are a subclass of iridoids (monoterpene derivatives with an iridane ring) derived from the cleavage of the cyclopentane ring at the 7, 8 bond containing phenol moieties, are restricted to the Oleaceae family and are the main family of compounds contained in olive leaves[11,34-36]. Among them, oleuropein is the main phenolic compound in olive leaves[22,24,37]. In addition to their diversity, phenolic compounds are found in olive leaves at different concentration levels. The quantitative determination has widely been reported in scientific researches. The ranges of individual phenolic compounds contents in the literature are reviewed in **Table 1**.

Table 1. Concentration levels of main phenolic compounds in olive leaves.

Class	Phenolic compounds	Range (mg/kg dry extract)	Ref.	Range (mg/kg dry leaf)	Ref.	Range (mg/kg fresh leaf)	Ref.
Secoiridoids	Oleuropein aglycone	$14.8x10^3$	[38]	170-280	[39]		
	Oleuropein glucoside	6600	[38]	$430\text{-}16.4x10^3$	[39–41]		
	Demethyloleuropein	2300	[38]	1340-6380			
	Oleuropein	$6.97x10^3$- $441x10^3$	[38]	$24.7\text{-}143.2x10^3$	[39–49]	236.14-8610	[35,50,5]
	Ligstroside	12400	[38]	600-3840	[39,41]		
	Oleuroside			2010-7000	[39,46]		
	Methoxyoleuropein			870-2190	[39]		
	Oleoside	10800	[38]	390	[39]		
	Secologanoside	7300	[38]	1820-3680	[39]		
Flavonoids	***Flavones***						
	Luteolin			10.1-5600	[39,41,44,46,52,53]		
	Luteolin glucoside	507-10500	[6,38]	$85.2\text{-}11.1x10^3$	[39,40,44,46,48,49,52,53]		
	Luteolin diglucoside			0.0-121.4	[52]		
	Luteolin rutinoside			67-2700	[39–41,53]		
	Apigenin	1-480	[6,38]	4.6-339.5	[44,52]		
	Apigenin glucoside	12-680	[6,38]	122.7-1261.3	[41,46,49,52,53]		
	Apigenin diglucoside			90-480	[40]		
	Apigenin rutinoside			7.3-1130	[39,40,44,46,48,49,52,53]		
	Diosmetin	1-37	[6]	traces -350.8	[52]		
	Chrysoeriol-7-*O*- glucoside			580-840	[39]		

Class	Phenolic compounds	Range (mg/kg dry extract)	Ref.	Range (mg/kg dry leaf)	Ref.	Range (mg/kg fresh leaf)	Ref.
	Flavonols						
	Rutin			13.8-3500	[39,42,46,52]		
	Quercetin rutinoside			654-1210	[53]		
	Quercetrin	1-129	[6]				
	Flavan3-ols						
	Catechin			0.8-64.2	[44]		
Simple phenols	***Simple phenols***						
	Tyrosol			90-660	[45]	8.2-410.74	[35,51]
	Tyrosol glucoside			860-1280	[39]		
	Hhydroxytyrosol	30.8-11400	[6,38,54]	2.1-1120	[44,45]	11.94-479.28	[35,51]
	Hydroxytyrsol glucoside			340-790	[39]		
	Phenolic aldehyde						
	Vanillin			1.3-8.2	[42]		
	Phenolic acids						
	Vanillic acid			12.8-110.1	[44]		
	Caffeic acid	1-60	[6]	1.4-4.5	[42]	1350-22.190x10^3	[50]
	Gallic acid			7.4-55.8	[44]		
	Cinnamic acid			5.4-44.5	[44]		
	Hydroxycinnamic acid					5040-32.69x10^3	[50]
	Syringic acid	174-447	[15]	5.2-13.7	[55]		
	Ferulic acid			7-91.4	[44,53]		

Class	Phenolic compounds	Range (mg/kg dry extract)	Ref.	Range (mg/kg dry leaf)	Ref.	Range (mg/kg fresh leaf)	Ref.
	Verbascoside	$29x10^3$	[38]	300-$18.6x10^3$	[39–41,46,49]		
	Isoverbascoside	17200	[38]				
	Cholorogenic acid			3.4-3.8	[44]	6140-$70.71x10^3$	[50]
	Protocatechuic acid			2.3-61.0	[44]		
	Hydroxyphenylacetic acid			14.7-45.7	[44]		
	Other compounds						
	Elenolic acid					99.6-662.92	[35,51]
	Elenolic acid glucoside	5600	[38]				
	Elenolic acid diglucoside			270-1370	[39]		

3. Determination of phenolic compounds in olive leaves

Extraction is one of the most important steps in sample pre-treatment for polyphenols analysis. Generally, it is a separation process where the distribution of the analyte (in this case, a phenolic compound) between two immiscible phases is made in order to arrive at the appropriate distribution coefficient[56]. A great number of extractions procedures have been developed to determine phenolic compounds fraction in olive leaves. The most commonly extraction system used has been the solid–liquid extraction (SLE) by maceration of the olive leaves in a solvent. Common extraction solvents used for olive leaves are methanol, ethanol, acetone, ethyl acetate, and diethyl ether, as well as aqueous alcohol mixtures as the usual solvents for polyphenols' extraction[25,39,57].

Nowadays, the application of SLE is slowly starting to decline because of the big necessity to low the costs by reducing solvent consumption, and to accelerate the extraction process. Thus, other modern extraction and isolation techniques have been used as alternative. These modern techniques include: microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), and ultrasound-assisted extraction (UAE) **(Table 2)**.

The MAE has gained much attention in plants and particularly in olive leaves analytical chemistry for its major advantages including short extraction time, low-energy requirement, high extraction efficiency, and minimum degradation of target components[58,59].

The PLE, is a technique which uses conventional solvents and performs a fully automated extraction under constant pressure and various controllable parameters like temperature, static extraction time, extraction cycles etc.[60]. The use of organic solvents at high pressures and temperatures above their normal boiling point enables to achieve fast and efficient extraction of the analytes from solid matrices. The PLE technique limits the use of organic solvents, hereby making

possible the use of solvent allowed for alimentary uses such as water and ethanol, while obtaining higher extraction yields and faster extraction processes[11].

The SFE is more environmentally friendly, avoiding the use of large amounts of toxic solvents, as well as being rapid, automatable. The intrinsic low viscosity and high diffusivity of supercritical CO_2 has permitted faster and more efficient separation, and relatively clean extracts. In addition, the absence of light and air during extraction reduces the degradation of analytes that occur in traditional extraction techniques[21].

The UAE has been proved to be drastically faster and more efficient than conventional extraction in olive leaves[61]. This is because this method is a powerful aid in accelerating various steps of the analytical process. In fact, it facilitates and speeds up operations such as the extraction, the homogenization, and various others[56] (**Table 2**).

The comparison between those sophisticated techniques applied on olive leaves, showed that MAE is the auxiliary energy that requires shorter extraction time, meanwhile UAE needs less solvent than the others. SPE shows intermediate values in extraction time as well as in the percentage of ethanol in the solvent mixture[62]. On the other hand, Taamalli et al.[27] reported that each technique was more adequate than others for the extraction of each particular class of compounds. In fact, MAE and conventional extraction showed to be more efficient for extracting more polar compounds such as oleuropein derivatives, apigenin rutinoside and luteolin glucoside. However, SFE was the best extraction procedure for apigenin and diosmetin.

The analysis of phenolic compounds in olive leaves was first elaborated by spectrophotometric techniques; the most used method was Folin-Ciocalteu for the determination of total phenolic compounds. However, the identification of single phenolic compounds present in olive leaves is only possible performing a

previous separation of the compounds present in the samples[63]. The use of gas chromatography (GC) and nuclear magnetic resonance (NMR) have been reported as possible techniques for phenolics characterization in olive leaves[15,32]. NMR spectroscopy has found interesting application in the analysis of complex mixtures without previous separation of the individual components in the mixture, but it has been increasingly recognized for its non- invasiveness, rapidity and sensitivity to a wide range of compounds in one single measurement[64]. GC although needs reagents derivatizing as samples pre-treatments, it has the advantages of lower detection limits and better separations[65].

Nevertheless, high/ultra performance liquid chromatography (HPLC/UPLC) coupled to diode-array detection (DAD) and/or coupled to mass spectrometry (MS) is the most used to quantify and characterize phenolic compounds olive leaves **(Table 2)**. This powerful analytical technique provides shorter times of analysis, acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical mixtures.

Table 2. Main extraction systems and analytical methods used to determine phenolic compounds in olive leaf.

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
Solid-liquid extraction with different combinations of solvents (acetone, methanol, ethanol, dichloromethane, ethyl acetate, petroleum ether, dichloromethane) and/or water.			
Thin-layer chromatography	10 Italian cultivars	luteolin, luteolin-7-glucoside, rutin, quercitrin, chlorogenic acid	29
Atmospheric Pressure Ionization Tandem Mass Spectrometry (APCI-MS)	'Cassanese'	oleuropein, ligstroside, disaccharide of hydroxytyrosol	66
HPLC-DAD	5 Spanish cultivars	oleuropein, hydroxytyrosol, luteolin-7-glucoside,apigenin-7-glucoside, verbascoside, tyrosol, vanillic acid, diosmetin-7-glucoside, caffeic acid, luteolin, rutin, diosmetin, vanillin, catechin	22
HPLC-DAD	14 French cultivars	rutin, verbascoside, luteolin 7-glucoside, apigenin 7-glucoside, oleuropein, oleuroside, coumarin	46
HPLC-DAD-ESI-TOF-MS and NMR	'Hardy's Mammoth'	apigenin, apigenin 7-glucoside, apigenin 7-rutinoside, caffeic acid, caffeoylquinic acid, *p*-coumaric acid, demethyloleuropein, elenolic acid, elenolic acid glucosides, hesperidin, homovanillic acid, hydroxytyrosol glucoside, hydroxytyrosol, ligstroside, luteolin, luteolin 4-glucoside, luteolin 7-glucoside, luteolin 7-rutinoside, oleuropein, oleuroside, tyrosolglucoside, tyrosol,vanillic acid, verbascoside	32
Gas Chromatography detector and Flame Ionization Detector (GC-FIL)	'Moraiolo'	tyrosol, hydroxytyrosol, syringic acid, gallic acid, ferulic acid, oleuropein, oleuropeinaglycon	15
HPLC- DAD	'Hardy's Mammoth'	tyrosol, hydroxytyrosol, 3,4-DHPEA-DEDA, oleuropein, oleuroside, luteolin 7-*O*-glucoside, luteolin 4´-*O*-glucoside, luteolin glucoside, caffeic acid, caffeoylquinic acid.	67
HPLC-DAD-MS	10 Greek cultivars	oleuropein, tyrosol, hydroxytyrosol, elenolic acid	51
HPLC-DAD	23 Portuguese cultivars	luteolin 7,4'-*O*-diglucoside; luteolin 7-*O*-glucoside; rutin; apigenin 7-*O*-rutinoside, apigenin 7-*O*-glucoside; luteolin 4´-*O*-glucoside; luteolin; apigenin; diosmetin	52

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
HPLC-DAD-MS	‘Arbequina’	oleuroepin, luteolin-7-*O*-glucoside, verbascoside, luteolin-4-*O*-glucoside, hesperidin	68
HPLC- Spectrophotometer and High-Resolution Gas Chromatographic (HRGC)	Seven Italian cultivars	oleuropein	35
HPLC-APCI-MS	11 Italian cultivars	hydroxytyrosol, rutin, verbascoside, luteolin-7-glucoside, luteolin-4’-*O*-glucoside, oleuropein, oleuropein aglycon, ligstroside aglycon	69
NMR and HPLC-DAD	‘ Manaki ’	secologanoside, oleuropein, oleoside dimethyl-ester, 6’-e-*p*-coumaroyl-secologanoside, 6’-*O*-[(2e)-2,6-dimethyl-8-hydroxy- 2-octenoyloxy] secologanoside	70
HPLC-DAD	‘Picual’	hydroxytyrosol, apigenin 7-glucoside, oleuropein, oleuropein aglycon	71
HPLC-DAD-MS	Five Italian cultivars	hydroxytyrosol glucoside, oleoside, oleoside 11-methyl ester, ligstroside, verbascoside	72
HPLC-DAD	‘Cobrançosa’	caffeic acid, verbascoside, rutin, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein	73
HPLC-DAD	Seven Spanish cultivars	hydroxytyrosol, tyrosol, hydroxytyrosol acetate, 3,4-DHPEA-EDA, oleuropein, 3,4-DHPEA-EA 4-HPEA-EDA	71
HPLC-DAD	Not cited	rutin, luteolin-7-glucoside, verbascoside, apigenin-7-glucoside hydroxytyrosol, tyrosol, catechin, caffeic acid, vanillic acid, vanillin, rutin, luteolin-7-glucoside, verbascoside, apigenin-7-glucoside, diosmetin-7-glucoside, oleuropein, luteolin	25
Capillary Electrophoresis-ESI-TOF-MS	‘ Hojiblanca’, ‘Manzanilla’	tyrosol, hydroxytyrosol acetate, hydroxytyrosol, ligstroside aglycon, oleuropein aglycon, oleuropein, apigenin-7-glucoside, dimethyloleuropein, vanillic acid, coumaric acid, caffeic acid, rutin, verbascoside, oleoside , diosmetin, apigenin, luteolin	75
HPLC-DAD	‘Koroneiki’	luteolin diglucoside, rutin, luteolin glucoside, luteolin rutinoside, apigenin rutinoside, luteolin glucoside, oleuropein	76
HPLC-DAD	Not cited	oleuropein, verbascoside, luteolin-7-*O*-glucoside, luteolin-4-*O*-glucoside	48
HPLC-DAD-MS	11 Greek cultivars and ‘Picual’ ‘Frantoio’	oleuropein, luteolin 7-*O*-glucoside, luteolin, 4´-*O*-glucoside, luteolin, luteolin glucosides, verbascoside.	77

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
HPLC –DAD	Not cited	caffeic acid, vanillin, rutin, oleuropein, catechin	42
HPLC-DAD, HPLC-Fluorescence Detector (FLD)	'Chondrolia', 'Chalkidiki' 'Koroneiki'	apigenin, apigenin 7-glucoside, apigenin 7-rutinoside, caffeic acid, caffeoylquinic acid, *p*-coumaric acid, demethyloleuropein, elenolic acid, elenolic acid glucosides, hesperidin, homovanillic acid, hydroxytyrosol glucoside, hydroxytyrosol, ligstrosideluteolin, luteolin 4-glucoside, luteolin 7-glucoside, luteolin 7-rutinoside, oleuropein, oleuroside, tyrosol glucoside, tyrosol,vanillic acid, verbascoside	79
HPLC-MS-ESI	'Picholine'	oleuropein, oleuroside, ligstroside, verbascoside and isomers, luteolin-7-*O*-glucoside, luteolin-glucoside and isomers, oleuropein, oleuroside, ligstroside, quercetin, diosmetin aglycon and isomers	31
HPLC -DAD	'Picual', 'Verdial', 'Arbequina', 'Frantoio'	hydroxytyrosol, tyrosol, oleuropein	45
HPLC-MS	'Koroneiki', 'Megaritiki' 'Kalamon'	secologanoside, dimethyloleuropein, oleuropein diglucoside, luteolin-7-*O*-glucoside, rutin, oleuropein, oleuroside, quercetin, ligstroside, verbascoside	37
HPLC-DAD-MS	11 Greek cultivars and 'Picual', 'Frantoio'	hydroxytyrosolglucoside, hydroxytyrosol, verbascoside, luteolin 7-*O*-glucoside, luteolin 4-*O*-glucoside, oleuropein, oleuropein derivative, luteolin	80
HPLC-DAD-ESI-TOF- MS and ESI-IT-MS^2	Not cited	acetic acid glucoside, demethyloleuropein, 10-hydroxy-oleuropein, nüzhenide, ligstroside aglycon, ligstroside, oleuropein derivative, oleuropein diglucoside, oleuropein diglucoside and isomers, oleuropein aglycon and isomers, oleuropein, oleuropein aglycon derivative, oleuroside, oleoside, secologanoside, elenolic acid glucoside and isomers, deacetoxy 10-hydroxy- oleuropeinaglycon, rutin, taxifolin, apigenin-7-glucoside, chrysoeriol-7-*O*-glucoside, luteolin, quercetin, apigenin, luteolin glucoside and isomers, lignans, syringaresinol, hydroxytyrosol, hydroxytyrosol glucoside, cinnamic acid derivatives, acteoside	24
HPLC-DAD	Not cited	hydroxytyrosol, tyrosol, luteolin-7-*O*-glucoside, verbascoside, apigenin-7-*O*-glucoside, oleuropein	81
HPLC and Mid-Infrared Spectroscopy.	Six Tunisian cultivars	oleuropein	82

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
HPLC-DAD	Six Italien cultivars	catechin, rutin, verbascoside, luteolin-7-glucoside, luteolin-7-rutinoside, luteolin-3-*O*-glucoside, luteolin-4-*O*-glucoside, luteolin, diosmetin-7-glucoside, diosmetin, apigenin-7-rutinoside, apigenin-7-glucoside, oleuropein.	83
HPLC-DAD	'Gaidourelia', 'Kalamon', 'Koroneiki', 'Megaritiki'	hydroxytyrosol, oleuropein	84
HPLC-DAD-ESI-TOF- MS	'Kalamon'	oleuropeosides (oleuropein and verbascoside), flavones (luteolin, apigenin-7-o-glucoside, luteolin-7-*O*-glucoside and luteolin-4-*O*-glucoside) and flavonols (rutin)	85
HPLC-DAD-MS	'Chemlali', 'Nebjmel'	gallic acid, hydroxytyrosol, chlorogenic acid, protocatechuic acid, hydroxyphenylacetic acid, 4-hydroxybenzoic acid, catechin, oleuropein, *p*-coumaric acid, ferulic acid, rosmarinic acid, vanillic acid, m-coumaric acid, *O*-coumaric acid, phenlyacetic, cinnamic acid, luteolin, apigenin, 3-hydroxybenzoic acid	44
HPLC-DAD-ESI-TOF- MS	'Sikitita', 'Arbequina', 'Picual'	oleoside, hydroxytyrosol-hexose and isomers, secologanoside and isomers, tyrosolglucoside, elenolic acid glucoside and isomers, oleuropein aglycon, luteolin diglucoside, luteolin glucoside, demethyloleuropein, rutin, luteolin rutinoside, luteolin glucoside and isomers, verbascoside, apigenin rutinoside, oleuropein diglucoside and isomers, chrysoeriol-7-*O*-glucoside, methoxyoleuropein and isomers, oleuropein and isomers, oleuroside, ligstroside, luteolin.	39
HPLC-DAD-ESI-TOF- MS	'Arbosana', 'Arbequina', 'Picual', 'Sikitita', 'Changlot Real', 'Koroneiki'	oleoside,hydroxytyrosol-hexose and isomers, secologanoside and isomers, tyrosolglucoside, elenolic acid glucoside and isomers, oleuropein aglycon, luteolin diglucoside, luteolin glucoside, demethyloleuropein, rutin, luteolin rutinoside, luteolin glucoside and isomers, apigenin rutinoside, oleuropein diglucoside and isomers, chrysoeriol-7-*O*-glucoside, methoxyoleuropein and isomers, oleuropein and isomers, oleuroside, ligstroside, luteolin.	86

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
HPLC-DAD-MS	'Chetoui', 'Chemchali'	six hydroxybenzoic acids (gallic, protocatechuic, 4-hydroxybenzoic, 3-hydroxybenzoic, vanillic, cinnamic acids), eight hydroxycinnamic acids (chlorogenic, caffeic, ferulic, *p*-, *m*- and *O*-coumaric, cinnamic and rosmarinic acids), phenolic alcohol (hydroxy- tyrosol), three flavonoids (catechin, luteolin and apigenin), phenolic acids (hydroxyphenylacetic and phenylacetic acids), one secoiridoid (oleuropein)	55
Microwave-assisted extraction (MAE)			
HPLC-Triple quadrupole mass detector (QQQ)-MS	'Picual'	hydroxytyrosol, verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein, luteolin, apigenin, diosmetin	59
HPLC-DAD-MS	'Picual', 'Arbequina', 'Lechín'	verbascoside, luteolin-7-glucoside, apigenin-7-glucoside, oleuropein	87
HPLC ESI-TOF-MS and ESI-IT-MS^2	'El Hor'	quinic acid, secologanoside, vanillin, hydroxytyrosol, elenolic acid glucoside and isomers, oleuropein aglycon derivative, luteolin diglucoside, luteolin diglucoside and isomers, 2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl) ac glucoside, rutin, luteolin rutinoside and isomers, 10-hydroxy-oleuropein, luteolin glucoside and isomers, oleuropein glucoside, apigenin rutinoside, syringaresinol, diosmin and isomer, taxifolin, apigenin-7-glucoside, chryseriol-7-*O*-glucoside, 2″-methoxyoleuropein and isomers, oleuropein and isomers, luteolin, quercetin, pinoresinol, acetoxypinoresinol, apigenin.	58
Pressurized Liquid Extraction (PLE)			
HPLC–DAD	Not cited	verbacoside, apigenin-7-glucoside, luteolin-7-glucoside, oleuropein	62

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
HPLC–ESI-MS, UPLC–MS^2	'Hojiblanca'	5-hydroxymethylfurfural, hydroxytyrosolglucoside, hydroxytyrosol, oleoside, tyrosol, coumaroyl derivative, elenolic acid glucoside, luteolin diglucoside, rutin, luteolin rutinoside, 10-hydroxyoleuropein , verbascoside, luteolin-7-glucoside, oleuropein diglucoside and isomers, apigenin rutinoside, hydroxytyrosol acetate, luteolin glucoside, oleuropein and isomer, oleuroside, oleuropein derivative, ligstroside, luteolin, apigenin, diosmetin	6
HPLC-ESI-QTOF-MS	'Hojiblanca'	quinic acid, oleoside/secologanoside, hydroxytyrosol, *p*-hydroxybenzoic acid, elenolic acid diglucoside, *p*-coumaric acid, vanillin, oleoside methyl ester 7-epiloganin, 7-epiloganin, elenolic acid glucoside, luteolin-7,4-*O*-diglucoside, hydroxyoleuropein, luteolin-7-*O*-rutinoside, rutin, verbascoside, hydroxytyrosol acetate, luteolin-7-*O*-glucoside, oleuropein diglucoside and isomers, apigenin-7-*O*-rutinoside, luteolin-4-*O*-glucoside, luteolin-3-*O*-glucoside, oleuropein and isomers, oleuroside, lucidumoside and isomers, 6'-*O*-[2,6-dimethyl-8-hydroxy-2-octenoyloxy] secologanoside, ligstroside, luteolin	11
Supercritical Fluid Extraction (SFE)			
Mass Spectrometric Screening	Not cited	tyrosol, hydroxybenzoic acid, cinnamicacid, hydroxytyrosol, protocatechuic acid, caffeic acid, homovanillic acid, syringic acid, elenolic acid 4-methoxytectochysin, caftaric acid, cirsimaritin, fertaric acid, chlorogenic acid, ligstroside, oleuropein.	57
Supercritical Fluid, Pressurized liquid Extraction+ Microwave-Assisted Extraction			

Analytical technique	Olive leaf cultivar	Phenolic compounds described	Ref.
HPLC-ESI-TOF-MS/IT-MS^2	Six Tunisian cultivars	quinic acid, secologanoside, vanillin, hydroxytyrosol, elenolic acid glucoside and isomers, oleuropeinaglycon derivative, luteolindiglucoside, luteolindiglucoside and isomers, 2-(2-ethyl-3-hydroxy-6-propionylcyclohexyl) ac glucoside, rutin, luteolinrutinoside and isomers , 10-hydroxy-oleuropein, luteolin glucoside and isomers, oleuropeinglucoside, apigeninrutinoside, syringaresinol, diosmin and isomer, taxifolin, apigenin-7-glucoside, chryseriol-7-*O*-glucoside, 2″-methoxyoleuropein and isomers, oleuropein and isomers, luteolin, quercetin, pinoresinol, acetoxypinoresinol, apigenin	27
	Ultrasound Assisted Extraction (USAE)+ Solid-Liquid Extraction		
HPLC-DAD and GC-MS	Not cited	verbacoside, luteolin-7-glucoside, 3, apigenin-7-glucoside, 4, oleuropein.	61
HPLC-DAD- MS^2	‘Serrana’	caffeoyl ,oleuropein, verbascoside, luteolin-7-*O*-glucoside and isomer, apigenin-6,8-diglucoside, luteolin-7-*O*-rutinoside, oleuropein glucoside, apigenin rutinoside, apigenin-7-*O*-glucoside, luteolin	40
HPLC-DAD- MS^2	‘Serrana’	verbacoside, oleuropein glucoside, oleuropein, luteolin glucoside, luteolin-7-*O*-rutinoside, apigenin-7-*O*-glucoside, apigenin rutinoside, apigenin-6,8-diglucoside, apigenin rutinoside, caffeoyl, luteolin	41

4. Influence of abiotic and biotic factors in olive leaf phenolic compounds

Phenolic compounds may be divided into two classes: preformed phenolics that are synthesized during the normal development of plant tissues, and induced phenolics that are synthesised by plants in response to physical injury, infection or when plants are stressed by elicitors such as heavy metal-salts, UV-irradiation, temperature, etc. Induced phenolics may also be constitutively synthesized, but, additionally, their synthesis is often enhanced under different factors of stress[88-90]. These factors, named abiotic and biotic factors, qualitatively and quantitatively change the phenolic compounds composition of olive leaves[49,77,91-93]. The abiotic factors are non-living chemical and physical parts of the environment that affect living organisms and the functioning of ecosystems such as soil, water, air, temperature, moisture, light etc. In contrast, biotic factors are any living component that affects another organism. Biotic components usually comprise plants, fungi, and bacteria, as well as animals and human influences.

4.1. Abiotic factors

4.1.1. Hydric deficiency

Olive is known to be a drought-tolerant tree[94]. The ability of olive to acclimatise to water availability includes alterations at the leaf associated with morphological, anatomical and physiological characteristics[95-97]. Besides, adaptations to water availability also involve changes in foliar chemistry. In fact, under such environmental conditions, phenolic compounds produced in leaves increase[84,95,97]. Oleuropein, the main phenolic compound of olive leaves, increases in response to water stress. This highlights its importance in the olive antioxidant defence mechanism. Oleuropein and other secondary metabolites such as hydroxytyrosol and verbascoside possess an ideal chemistry for free radical scavenging actively acting as plant antioxidants[84,98].

4.1.2. Salinity

Salinity is one of the major abiotic stresses affecting plant productivity because of its negative effects on plant growth, ion balance and water relations. Salinity produces oxidative stress in olive plants[99]. Several reports have signalled changes in the content and activity of different components of the antioxidant defence system in olive leaves in response to salt stress[99-101]. Actually, the salt tolerance of olive trees results from the interaction between different mechanisms leading to the activation of water uptake, showing a high evolution of the anti-oxidative enzyme activities and an accumulation of the antioxidant defence system in different parts of the plant, especially in leaves[101,102]. Petridis et al.[103] demonstrated that oleuropein was the main phenolic compound involved in the olive tree protection against salinity stress in leaves. The high level of oleuropein may serve as a glucose-reservoir for osmo-regulation or high energy-consuming processes required for plant adaptation to salinity. Moreover, it was shown that salinity stress associated with high sunlight revealed an enhancement of the biosynthesis of other phenolic compounds, particularly flavonoids[104].

4.1.3. Fertilization

The effect of nutriments on olive oil phenolic compounds contents has been widely studied[105-109]. However, relatively few studies have been focused on the effects of mineral nutrition on olive leaf phenolic compounds. Del Río et al.[110] reported the polyphenol enhancing effect following the application of nutrient solution 'Brotomax' which contains urea nitrogen, copper, manganese and zinc. After spraying it on olive trees, several phenolic compounds (tyrosol, catechin, oleuropein) increased in leaves and in other parts of the olive tree. Besides, a study carried out by Liakopoulos and Karabourniotis[53] reported that boron (H_3BO_3) deficiency in olive trees enhanced the phenolic concentration of quercetin 3-*O*-rutinoside, *p*-coumaric acid, luteolin 7-*O*-glucoside, apigenin 7-*O*-

glucoside and apigenin 7-*O*-rutinoside, and decreased the concentration of quercetin aglycone in olive leaves.

4.1.4. Geographical zone

Phenolic content, phenolic profile and, thus, the antioxidant activities of olive leaves show consistent variability among locations. Olive leaves cultivated in the North of Tunisia contained higher total phenol content and higher antioxidant activity than olive leaves from the South of Tunisia, indicating an important impact of the region on their antioxidant content[55]. Samples of 'Carolea' cultivar, cultivated in three different geographical zones (Rende, Mirto and Spoleto, Italy), showed as well significant differences in terms of phenolics and could be classified in three groups depending on their geographical origin[92]. Similar results were found in a study performed using two Greek olives leaves cultivars grown in three different locations in Greece[111]. According to phenolic compounds contents (namely, oleuropein, verbacoside, apigenin-7-glucoside, and luteolin-7-glucoside) in olive leaves, a very good discrimination among samples of 'Arbequina' cultivar cultivated in six different geographical zones in Spain was also achieved by Japón-Luján et al.[49]. A study carried out by Bilgin and Şahin[91] demonstrated that total phenolic levels in olive leaves declined as the geographical altitude decreases. The main cause of this behavior could be related to the climatic conditions changing with geographical zone. Indeed, phenolic compounds tend to decrease in the leaves of the trees cultivated in windy and humid air (near to the sea level) and tend to increase in high altitude with terrestrial and Mediterranean climate, where annual temperature differences are very high[91].

4.1.5. Sampling time

The effect of sampling time has been widely studied in olive leaves polyphenols. In effect, an increase of flavonoid content (luteolin-4′-glucoside) in the spring

period has been reported by Heimler et al.[112]. This could be correlated with the general increase of biological activities at the renewal of the leaves vegetative cycle. Moreover, the main determinant of oleuropein concentration in 'Leccino' olive leaves has appeared to be the sampling period, with winter being the period during which it presented the maximum amount[113]. Similarly, discrepancies in the level of oleuropein in 'Chondrolia Chalkidikis' and 'Koroneiki' leaves have been detected over six months of study between June and November, in which the highest value was registered in October[77]. The increase of oleuropein and total phenol concentrations in 'Picual' leaves have also been demonstrated during ripening fruit (from August to November). The increase was associated with an augumentation of the polyphenol protein oxidase (PPO) content and activity in leaves. In addition, the wide distribution of PPO in leaves (epidermis, parenchyma and phloem companion cells of leaves) was consistentwith an important function of this enzyme in the normal metabolism of leaves, probably related to the protection of the plant against such agents as oxidants, pathogens or ultraviolet light[114].

Di Donna et al.[92] have obtained, by the principal components analysis for leaves of 'Carolea' cultivar, a clear separation of three clusters representing three harvest periods (March–April, July and January). Precisely, going from March–April to January, an increase in the concentration of a number of compounds (hydroxytyrosol glucoside, oleoside, oleoside 11-methyl ester, verbascoside, angustifolioside A, angustifolioside B, saturated oleuropein, dimethyloleuropein, oleuropein and ligstroside) was noticed, whereas a decrease of methoxytyrosol glucoside and 2-methoxyhydroxytyrosol glucoside was detected. Besides, a decrease of verbascoside and hydroxytyrosol glucoside concentrations was detected in leaves harvested in July. Interestingly, the level of total phenols and 28 individual phenolic compounds examined in olive leaves of six cultivars grown under the same conditions have been found at their highest values in the cold season[86]. Similar results for other cultivars and in different zones of cultivation have been reported in other studies[44,50,115,116]. The increase of phenolic

compounds during the cold season could be related to the increase in the activity of L-phenylalanine ammonia lyase (PAL). This enzyme is responsible for the phenilpropanoid pathway and it seems that cold influences its activity[117].

4.1.6. Light exposition

Light seems to be one of the abiotic factors affecting phenolic compounds of olive leaves, especially flavonoids compounds. In fact, protection against ultraviolet B (UV-B) radiation may be afforded by flavonoids and other phenolics, acting as a barrier against damaging UV radiation owing to their adsorption maxima in the UV region[29,104,118,119]. In an experiment carried out by Heimler et al.[120], a comparison of olive leaves flavonoid contents between olive trees grown in greenhouse and in open-air field, brought out the involvement of luteolin and luteolin-7-glucoside in protection against UV radiation. The increase of those flavonoids was higher in open-air fields than in the greenhouse. Besides, leaves in full sunlight had a significantly greater concentration of oleuropein and flavonoids than shade leaves. The light-induced increase in flavonoid concentration was mostly due to dihydroxy B-ring-substituted flavonoids (quercetin glycosides and luteolin 7-*O*-rutinoside). However, other flavonoid glycosides, namely luteolin 4-*O*-glucoside and apigenin 7-*O*-glycosides, were unresponsive to changes in sunlight irradiance[104]. Flavonoids seem to exhibit their protection against radiation through two different mechanisms: a simple screen effect and a radical scavenging pigments effect[104,120]. A study of the flavonoids distribution between two parts of olive leaf: the dehaired lamina and the isolated trichome layers of the abaxial leaf surface specified that the increase observed in quercetin and quercetin 3-*O*-rhamnoside, induced by leaves light exposure, seems to be located exclusively in the trichome layer[119]. Flavonoids serve their antioxidant functions and may have contributed to counter heat-stress-induced oxidative load more in sun than in shade leaves[121].

4.1.7. Frost stress

Phenolic compounds conjointly with other strategies were reported to have a role in cold-hardiness in olive trees[122]. The phenolic compounds were proven to have the ability to scavenge free radicals and inhibit the membrane lipid peroxidation of plants[123]. Oleuropein in particular was demonstrated to be the major phenolic compound responsible for this effect. In fact, Ortega-García and Peragón[124] reported a large augmentation in oleuropein concentration in leaves after olive exposure to cold stress. The high oleuropein concentration may be related to its antioxidant capacity and, therefore, oleuropein may offer protection against oxidative damage induced by freezing. In the same way, various anti-oxidative enzymes and proteins were then associated with cold-acclimation or freezing tolerance in olive leaves tissues.

4.2. Biotic factors

4.2.1. Fungi

The production of phenolic compounds in olive tree (phloem, xylem or roots) has widely been studied. Changes in levels of tyrosol, catechin, quercetin, luteolin, verbascoside and oleuropein have been revealed, demonstrating their involvement in defensive reactions against fungi such as *Verticillium dahlia*, *Phytophtora sp*. and *Neurospora crassa*[88,93,125,126]. Nevertheless, few works have studied the involvement of olive leaf phenols. Rahioui et al.[127] have shown that four families of polyphenols: hydroxycinnamic derivatives, oleuropein derivatives, tyrosol derivatives and flavonol monoglucosides, were remarkably responsible for olive tree resistance to the leaf-spot disease caused by *Fusicladium oleagineum* (=*Spilocaea oleagina*). In their study, they registered large levels of those compounds in resistant cultivars and low levels in susceptible cultivars. Similarly, resistance of olive to *F. oleagineum* was related to multi factorial phenolic components suggesting a polygenic resistance in olive

leaves. In fact, resistance degree was related positively to tyrosol derivatives, oleuropein and rutin contents and negatively to verbascoside and apigenin contents. Then, tyrosol and its derivatives in olive leaves have been demonstrated to be related to constitutive resistance, whereas oleuropein and rutin were in relation to induced resistance[128].

4.2.2. Bacteria

The *in vitro* antimicrobial activity of olive leaf phenolic compounds has been widely reported[73,129-131]. The anti-bacterial effect of olive leaves has been correlated with the presence of olive phenolic compounds such as the dialdehydic form of decarboxymethyl elenolic acid either free (EDA), linked to tyrosol (TyEDA) or hydroxytyrosol (HyEDA)[130]. Hence, antimicrobial studies have been carried out not only for human health, but also for agricultural pest control. The natural resistance of olive tree is attributed, in part, to the existence of a physical barrier of oleanolic acid crystals at the leaf surface and to the production of secoiridoid glucosides, oleuropein and ligstroside antimicrobial derivatives[132]. Olive knot disease, commonly called tuberculosis, is characterized by the development of galls on *O. europaea* stems as a result of infection by *Pseudomonas savastanoi* pv. *Savastanoi*[133]. The study of the changes in phenolic composition induced by tuberculosis infection in olive trees revealed a considerable enhancement of verbascoside in leaves, probably because it is implicated in the defense mechanisms of olive trees against tuberculosis disease[134].

4.2.3. Genotypes

Studies performed on phenolic compounds in olive leaves demonstrate that genotypes are one of the most important factors which contribute to differences in quantitation of phenolic compounds[35,45,46,49,51,77,92,113,135,136]. In fact, the content in major phenolic compounds of olive leaves has been used in many studies as

chemo-taxonomicmarkers. Statistical models have allowed data treatment and, a great discrimination of cultivars[49,86,137]. Studies carried out in 110 genotypes generation Filial 1 hybrid (F1), resulting from cross-breeding between the susceptible cultivar 'Picholine marocaine' and the resistant cultivar 'Picholine du Languedoc' to *F. oleagineum*, showed no qualitative differences between cultivars in terms of phenolic compounds. However, phenolic contents and resistance degree of cultivars to fungus were strongly correlated. Resistant and highly resistant genotypes have revealed clear differences from the highly susceptible genotypes in terms of the contents of chlorogenic acid, luteolin-7-glucoside, flavonol monoglucoside 2 and verbascoside and its derivatives[128]. In addition, it was demonstrated that highly susceptible genotypes contain the lowest contents of tyrosol and its derivatives, and oleuropein and its derivatives[128]. In a similar study, authors treated seven cultivars infested by F. oleagineum; it appeared that the resistant cultivars were richer in some phenolic compounds than the susceptible cultivars and intermediate cultivars[127]. An identification approach of bacterial protein markers in olive trees infected by *P. savastanoi* bacteria showed that biochemical responses (including phenolic compounds) in 'Galega' and 'Cordovil de Serpa' to *P. savastanoi* were distinct and likely to be genotype-dependent[138].

4.2.4. Leaves age

The age of leaves at sampling could change quantitatively and qualitatively the phenolic compounds in leaves. The first study dealing with polyphenols in relationship with leaves age was carried out by Ryan and co-workers[32,67]. In those studies, new season leaves (soft leaves that have not reached full cuticular development) and old season leaves were reported. Old season leaves showed higher levels of 5-caffeoylquinic acid, caffeic acid, luteloin glucoside and oleuroside than new leaves. However, generally oleuropein levels were higher in new leaves than old ones. Likewise, a comparison between oleuropein contents in three leaves stages (young, average age and old leaves) revealed that the

contents of oleuropein were markedly affected by the age factor, which was quite higher in young leaves. The average age leaves and old leaves displayed markedly lower contents of oleuropein. This suggests that oleuropein is gradually degraded with the leaves progressive aging, due probably to biochemical degradation pathways[35]. In another study, oleuropein was found to be higher in mature leaves than in new and old ones[77], whereas the very old were found to contain higher amounts of luteolin glucosides and verbascoside[68,77].

4.2.5. Alternate bearing patterns of olive

Evidence of the impact of alternate bearing on levels of phenol accumulation in leaves from one season to the next was provided by patterns of some phenolic compounds concentration changes in 'Hardy's Mammoth'. Overall, hydroxytyrosol, oleuropein, and oleuroside levels all increased in the high load season but showed either little variation (hydroxytyrosol) or a decrease (oleuropein and oleuroside) in the low load season. Also, concentrations of chlorogenic acid in high load season leaves decreased dramatically between seasons[67]. The data obtained were consistent with the findings of Lavee et al.[139]. Mert et al.[50] found that phenolic compound quantitative changes were related to alternate bearing in the leaves of 'Gemlik' olive cultivar. Consequently, in the "on" year (the year in which the production is high), the levels of chlorogenic and *p*-coumaric acids were high, whereas the abundance of other phenolic compounds, such as caffeic acid, 3-hydroxcinnamic acid and scopolin was low. In contrast, during the "off" year (the year in which the production is low), the chlorogenic acid and *p*-coumaric acid levels were at low levels, whereas the levels of the other phenolics (caffeic acid, 3-hydroxycinnamic acid and scopolin) were high. The oleuropein level did not fluctuate notably in either year.

5. Health potential of olive leaf phenolic compounds

The evergreen olive tree has been treasured for many centuries. The olive fruit, its oil, and the leaves of the olive tree have a rich history of nutritional, medicinal, and ceremonial uses[140]. Historically, olive leaf extract has been widely used as a folk remedy for combating fever and other diseases[22]; today, it is also known to have various health enhancing properties. Several studies have shown that olive leaf extract exhibits a large spectrum of *in vitro* and *in vivo* properties, including antioxidant activity[141,142], radio-protective effects[141], anti- proliferative effect on leukaemic cells by inducing apoptosis1[43–145], cytotoxic activity against human breast cancer cells[24,27], anti-HIV[146], anti-fungal[147], gastroprotective[148] activities, attenuation of diabetic neuropathic pain[149] and amelioration of gentamicin nephrotoxicity[150]. In a recent research, olive leaf extract suppressed messenger RNA expression of proinflammatory cytokines, enhanced IRS-1 signalling, and improved dyslipidaemia in rats with type 2 diabetes induced by a high-fat diet and streptozotocin[151]. Several biological properties such as antioxidant, anti-atherosclerotic, hypoglycaemic, and cardioprotective effects of olive leaves have been reviewed in some works in the literature[16,152]. These potential health benefits of olive leaves are mostly related to low molecular weight antioxidants such as polyphenols. In **Table 3** are presented some health potential of olive leaf phenolic compounds with their attributed mechanisms.

Oleuropein, the major constituent of secoiridoid class in olive leaves, has been shown to reduce free fatty acid-induced lipogenesis *via* lowered extracellular signal-regulated kinase activation in hepatocytes[153]. Moreover, it exhibited antioxidant effects on intestine mucosal damage induced by absolute ethanol[154]. Platelet function at increasing concentrations of oleuropein (5.4 mg/mL, 16.2 mg/mL, 27.0 mg/mL, 37.8 mg/mL, and 54.0 mg/mL in olive leaf extract) was investigated through measures of platelet aggregation and ATP release from activated platelets[155]; the results showed that oleuropein inhibited *in vitro* platelet activation in healthy, non-smoking males. Other researches revealed the efficacy

of oleuropein against UV-B irradiation and showed that oleuropein presents a soothing effect stronger than the protective one in the treatment of UVB-induced erythema[156]. Further researches demonstrated the anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells[157]. The results of the MTT assay showed that 200 μg/mL of oleuropein or 50 μg/mL of hydroxytyrosol remarkably reduced cell viability of MCF-7 cells. Furthermore, oleuropein showed a high protective power against tamoxifen toxicity (a medication used as anti-neoplastic drug for the treatment of breast cancer, it often induces menopausal symptoms) by reducing its toxicity by 9-fold and was proved to be a strong free radical scavenger, markedly inhibiting the formation of 8-hydroxydeoxyguanosine[158]. Oleuropein and its semi-synthetic peracetylated derivatives have also demonstrated anti-proliferative and antioxidant effects on two human breast cancer cell lines (MCF-7 and T-47D)[159]. Indeed, treatment with increasing doses of oleuropein for 72 h caused a dose-dependent inhibition of cell proliferation in T-47D cells, with a maximal reduction evident at 100 μM (about 40%). Stronger significant effects were observed with oleuropein acetylated derivatives. In the presence of oleuropein (10 and 100 μM), the generation of reactive oxygen species (ROS) was significantly reduced and ROS decrease was greater with the peracetylated compounds used at 10 μM in MCF-7 cells. In another study, oleuropein and its hydrolysates-rich extracts have been documented for the antidiabetic, hypolipidaemic and antioxidant activities[160,161].

Lipid-lowering and antioxidant effects of hydroxytyrosol and its triacetylated derivative recovered from olive tree leaves were investigated by the examination of serum lipid levels, thiobarbituric acid-reactive substances (TBARS) level in cholesterol-fed rats (as an indicator of lipid peroxidation) and the activity of superoxide dismutase (SOD) as well as that of catalase (CAT)[162]. The obtained results suggested that the hypolipidaemic effect of triacetylated hydroxytyrosol and hydroxytyrosol might be due to their abilities to lower serum total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol

(LDL-C) levels as well as to their antioxidant activities preventing the lipid peroxidation process. In a study on breast cancer, hydroxytyrosol rich extract from olive leaves was shown to modulate cell cycle progression in MCF-7 human breast cancer cells[26]. A dose-dependent growth inhibition of MCF-7 cells was observed due to the cell cycle arrest in the G0/G1 phase.

Not only were individual phenolic compounds evaluated for biological properties, but both individual and mixtures of phenolics of olive leaf extracts were tested for antioxidant and antimicrobial activities[163]. The results showed that both the individual and combined phenolics exhibited good radical scavenging abilities, and also revealed superoxide dismutase (SOD)-like activity. In terms of antimicrobial activity, both oleuropein and caffeic acid showed inhibition effects against micro-organisms. Oleuropein (800 μg/disc) exhibited a strong growth inhibition effect (23.5mm) against Salmonella enteritidis, whereas a moderate growth inhibition effect (9.8–10.4 mm) was observed against *Bacillus cereus*, *Escherichia coli* and *S. enteritidis* due to caffeic acid (800 μg/disc). Furthermore, the antimicrobial effect of the combined phenolics was significantly higher than those of the individual phenolics[163]. In another study, olive leaf extracts consisting of oleuropein (19.8%), luteolin-7-*O*-glucoside (0.04%), apigenin-7-*O*-glucoside (0.07%), quercetin (0.04%) and caffeic acid (0.02%) decreased lipid peroxidation in the liver of rats exposed to cold restraint stress. Superoxide dismutase and catalase enzyme activity were increased in liver tissue homogenates. The obtained results indicate that olive leaf exhibits a potent anti-oxidative activity at the level of the liver[164]. The same mixture (oleuropein (19.8%), luteolin-7-*O*-glucoside (0.04%), apigenin-7-*O*-glucoside (0.07%), quercetin (0.04%) and caffeic acid (0.02%) showed a protective effect on the peripheral blood leukocytes. This effect was assessed from the ability of the extract to attenuate formation of DNA lesions induced by adrenaline for six healthy subjects[142].

Another olive leaf extract composed of oleuropein (356 mg/g), tyrosol (3.73 mg/g), hydroxytyrosol (4.89 mg/g), and caffeic acid (49.41 mg/g) was assayed in an animal study on the inhibitory effect of olive leaf extract on Gentamicin-induced nephrotoxicity. The results showed that olive leaf extract ameliorates gentamycin nephrotoxicity *via* antioxidant activity, increases renal glutathione content, and increases renal antioxidant enzymes activity, except for glutathione peroxidase[150]. Goulas et al.[23] also tested an olive leaf extract composed of oleuropein, hydroxytyrosol, hydroxytyrosol acetate, luteolin, luteolin-7-*O*-glucoside, and luteolin-*O*-glucoside and they observed an inhibition of the proliferation of cancer and endothelial cells (Human endothelial cells from bovine brain, MCF-7 cells and T-24 cells (human urinary bladder carcinoma)).

In a study on the growth inhibition and differentiation of human leukaemia hl-60 cells, the flavonoid apigenin-7-*O*-glucoside of the olive leaf extractwas reported to be mainly responsible for the HL-60 differentiation and oleuropein showed to exert an influence over this differentiation[143]. Furthermore, *in vitro* anti-inflammatory activity was tested on human keratinocytes NCTC 2544 with different concentrations (1, 10 and 100 mg/mL) of aromadendrine (dihydrokaempferol). The results showed a decrease in the expression of membrane molecules (ICAM-1) and the release of inflammatory-soluble factors (MCP-1 and IL-8) induced by IFN-γ and histamine in normal keratinocytes[165].

Many different illnesses such as cardiovascular disease, cancer, and neurological and endocrinological disorders have been related to oxidative stress, which can be either a cause or a consequence of the diseae[166]. An appropriate equilibrium between oxidation and antioxidants is fundamental to life. In general, free radical scavenging and antioxidant activity of phenolics mainly depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, and is also affected by other factors, such as glycosylation of aglycones, other H-donating groups (-NH,-SH), etc.[167].

Benavente-García et al.[22] found that for oleuropeosides and the other phenols present in olive leaf extract, it is the *O*-dihydroxy (catechol) structure present in their moieties which mainly confers antioxidant properties to these compounds. Moreover, the antioxidant activity of oleuropein is mainly due to the hydroxytyrosol moiety in its structure. Its ability to scavenge the ABTS•+ radical cation is lower than that of hydroxytyrosol due to the increased molecular weight[141]. In the latter study rutin, catechin and luteolin were found to be the most active flavonoids[141]. Studying the radioprotective effect of olive leaf extract, the authors highlighted the importance of the B-ring catechol structure in the flavonoids (rutin, catechin, luteolin, and luteolin-7-*O*-glucoside) and substituted phenols (hydroxytyrosol, verbascoside, caffeic acid, etc.) present in its composition.

As can be seen, most of the observed biological activities of olive leaf extracts have been attributed to secoiridoids and, particularly, oleuropein. However, other compounds have also been shown to be responsible for part of the bioactivity of olive leaves or, at least, for synergistically reinforcing those actions. This fact has led to the presentation of new patents of functional foods, nutraceuticals and cosmetics based on phenolic olive leaf extracts. Investigations on the structure-bioactivity relationships of olive leaf phenolic compounds are scarce and, thus, it would be important to perform further research studies in this direction.

Table 3. Health potential effects of phenolic compounds from olive leaves.

		Compounds	Subjects/ cell models	Effects	Mechanisms	Ref.
In vivo	**Animal studies**	Oleuropein 24.54%, hydroxytyrosol 1.46%, the flavone-7-glucosides of luteolin and apigenin (1.38 and 1.37% respectively) and verbascoside (1.11%), diosmetin-7-glucoside luteolin, diosmetin, rutin, catechin, vanillin, vanillic acid, caffeic acid	Adult male Swiss mice 9–12 weeks old. whole-body irradiated with a single dose of 48 cGy	Antioxidant and radioprotective effects	Flavonols, flavan-3-ols and flavones with catechol structure are the most efficient quenchers for $ABTS^{\bullet+}$ radical cation. Hydroxyl radical (OH) scavenging capacity	141
		Oleuropein	Adult male Sprague–Dawley rats, weighting 180 and 220 g, divided randomly into four equal groups as follows: control, ethanol, oleuropein (12 mg/kg body weight for 10 consecutive days), and oleuropein plus ethanol (12 mg/kg body weight for 10 consecutive days)	Antioxidant effects on intestine mucosal damage induced by absolute ethanol	Significant increases in the levels of antioxidant enzymes such as GPx and CAT for oleuropein and oleuropein plus ethanol groups in comparison with ethanol-treated rats, whereas oleuropein treatment abolished the increase in TBARS concentration. The antioxidant effect of oleuropein results from its ability to scavenge ROS, produced by ethanol, which initiate lipid peroxidation	154
		Oleuropein and its hydrolysates-(hydroxytyrosol) rich extracts	Adult male Wistar rats. Diabetes in Wistar rats was induced by intraperitoneal injections of alloxan	Antidiabetic and antioxidant effects	Increase of antioxidant enzyme expressions and/or activities.	160

	Compounds	Subjects/ cell models	Effects	Mechanisms	Ref.
	Hydroxytyrosol and its triacetylated derivative	Wistar rats fed a standard laboratory diet or a cholesterol-rich diet	Lipid-lowering and antioxidant effects	Inhibition of the absorption of dietary cholesterol in the intestine or its production by the liver or stimulation of the biliary secretion of cholesterol and cholesterol excretion in the feces	162
	Olive leaf extracts consisting of oleuropein (19.8%), luteolin-7-*O*-glucoside (0.04%), apigenin-7-*O*-glucoside (0.07%), quercetin (0.04%) and caffeic acid (0.02%)	Twenty-four male Wistar rats exposed to cold restraint stress. The olive leaf extract group received olive leaf extract (80 mg kg-1 daily, i.g.) dissolved in distilled water for 14 day	Olive leaf extract modulates cold restraint stress-induced oxidative changes in rat liver	Synchronization of antioxidant enzymes and inhibited lipid peroxidation in liver	168
	Oleuropein (356 mg/g), tyrosol (3.73 mg/g), hydroxytyrosol (4.89 mg/g), and caffeic acid (49.41 mg/g)	Thirty-five Sprague-dawley rats: 5 groups to receive saline; gentamicin, 100 mg/kg/d; and gentamicin plus OLE in 3 different doses (25 mg/kg/d, 50 mg/ kg/d, and 100 mg/kg/d, once daily for 12 days	Amelioration of gentamycin nephrotoxicity	Inhibition of lipid peroxidation, enhancing renal glutathione content, and antioxidant enzymes activity	150

		Compounds	Subjects/ cell models	Effects	Mechanisms	Ref.
	Human studies	Oleuropein	Ten healthy female subjects 20–30 years old, having skin Fitzpatrick types II and III	Soothing effect in the treatment of UVB-induced erythema	The mechanism of action of the lenitive efficacy of oleuropein is not actually fully understood. The activity could be related to the property of oleuropein that exhibits inhibitory effects on 'reactive nitrogen species' including the radical nitric oxide (NO·)	[156]
In vitro	**Animal studies**	Oleuropein	Normal mouse hepatocyte FL83B cells. HepG2 and FL83B cells	Decrease of the number and size of lipid droplets in FFA-treated cells and reduced intracellular triglyceride accumulation	Reduction of FFA-induced extracellular signal-regulated kinase activation but had no effect on c-JunN-terminal kinase or AKT activation	[153]
	Human study	Oleuropein	Whole blood of 11 healthy male volunteers	Inhibition of platelet activation in healthy, non-smoking males	Ability to scavenge H_2O_2, which is produced during the arachidonic acid metabolism cascade,which leads to platelet aggregation	[155]
		Oleuropein and hydroxytyrosol	Human breast cancer cell line MCF-7	Apoptotic cell death of human breast cancer MCF-7 cells	Significant block of G1 to S phase transition manifested by the increase of cell number in G0/G1 phase.	[157]

	Compounds	Subjects/ cell models	Effects	Mechanisms	Ref.
	Oleuropein and its semi-synthetic peracetylated derivatives	Human breast cancer cell lines (MCF-7 and T-47D)	Anti-proliferative and antioxidant effects. The peracetylated compounds exerted higher antiproliferative effects than oleuropein	An arrest of cell cycle progression, associated with a strong antioxidant activity	[159]
	Hydroxytyrosol rich extract	MCF-7 human breast cancer cells	A dose-dependent growth inhibition of MCF-7 cells	Cell cycle arrest in the G1 phase. down-expression of Pin1 which in turn decreased the level of cyclin D1	[26]
	Aromadendrine (dihydrokaempferol)	Human keratinocytes NCTC 2544	Anti-inflammatory activity	Decrease markedly the expression of membrane molecules (ICAM-1) and the release of inflammatory-soluble factors (MCP-1 and IL-8) induced by IFNγ and histamine in normal keratinocytes NCTC 2544	[165]
	Oleuropein (19.8%), luteolin-7-*O*-glucoside (0.04%), apigenine-7-*O*-glucoside (0.07%), quercetin (0.04%) and 0.02% of caffeic acid	Peripheral blood leukocytes from six healthy volunteers (4 female and 2 male subjects)	Protective effect on the peripheral blood leukocytes against adrenaline induced DNA damage	Synergistic activation of several molecular mechanisms such as ROS scavenging and increasing the antioxidant capacity of cells.	[142]
	Oleuropein, hydroxytyrosol, hydroxytyrosol acetate, luteolin, luteolin-7-*O*-glucoside, and luteolin-*O*-glucoside	Human endothelial cells from bovine brain, MCF-7 cells and T-24 cells (human urinary bladder carcinoma)	Antiproliferative effect against cancer and endothelial cells	Inhibition of the cell proliferation	[23]

		Compounds	Subjects/ cell models	Effects	Mechanisms	Ref.
		Apigenin-7-glucoside, oleuropein	Human HL-60 cells	Apigenin-7-glucoside of the olive leaf extract was mainly responsible for the HL-60 differentiation and oleuropein showed to exert an influence over this differentiation	Growth inhibition of HL-60 cells treated with the olive leaf extracts can be attributable to differentiation-mediated cell cycle arrest and/or apoptosis	[143]

6. Conclusions

Olive growth is a commodity of great importance in Mediterranean countries and in new oleicole countries. Olive and olive oil processing give rise to a substantial quantities of leaves which are considered as phenolics-rich by-products. Consequently, very powerful extraction and analytical tools have been used to identify and quantify more than 30 phenolic compounds in more than 50 cultivars from Spain, Italy, Greece, Tunisia, France, Portugal and Australia. Phenolic compounds have been found to vary qualitatively and quantitavely depending on several factors such as hydric deficiency, salinity, fertilization, geographical zone, period of the year and climatic conditions. Likewise, others biotic factors such as fungi and bacteria attacks, genotype, load bearing, and leaves age affect remarkably the content of these compounds in leaves. This is the first time that abiotic and biotic factors that influence phenolic in olive leaves have been reviewed. Nevertheless, it is of great importance to take into account these factors when leaves are used as a source of phenolic compounds, because they can predict which family or compounds are available in the moment of sampling. Indeed, the huge number of researches related to the valuable effect of olives leaves phenolic compounds on health in last decade should encourage the industry to the valorization of olive leaves as a source of antioxidant to produce medicines, cosmetics, nutraceuticals and to develop functional foods.

References

(1) Peralbo-Molina, Á.; Luque de Castro, M. D. Potential of residues from the Mediterranean agriculture and agrifood industry. *Trends Food Sci. Technol*. **2013**, 32, 16–24.

(2) IOC. International Olive Council http://www.internationaloliveoil.org/estaticos/view/136-country-profiles *(accessed Nov 20, 2014)*.

(3) IOC. International Olive Council http://www.internationaloliveoil.org/estaticos/view/131-world-olive-oil-figures *(accessed Nov 20, 2014)*.

(4) Obied, H. K.; Prenzler, P. D.; Omar, S. H.; Ismael, R.; Servili, M.; Esposto, S.; Taticchi, A.; Selvaggini, R.; Urbani, S. Pharmacology of olive biophenols. *In Advances in Molecular Toxicology; Fishbein, J. C., Ed.; Advances in Molecular Toxicology*; Elsevier, 2012; pp 195–242.

(5) Molina-Alcaide, E.; Yáñez-Ruiz, D. Potential use of olive by-products in ruminant feeding: A review. *Anim. Feed Sci. Technol*. **2008**, 147, 247–264.

(6) Herrero, M.; Temirzoda, T. N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. *J. Chromatogr. A*. **2011**, 1218, 7511–7520.

(7) Govaris, A.; Botsoglou, E.; Moulas, A.; Botsoglou, N. Effect of dietary olive leaves and rosemary on microbial growth and lipid oxidation of turkey breast during refrigerated storage. *S. Afr. J. Anim. Sci*. **2010**, 40, 145–155.

(8) Spinelli, R.; Picchi, G. Industrial harvesting of olive tree pruning residue for energy biomass. *Bioresour. Technol*. **2010**, 101, 730–735.

(9) Romero-García, J. M.; Niño, L.; Martínez-Patiño, C.; Álvarez, C.; Castro, E.; Negro, M. J. Biorefinery based on olive biomass. State of the art and future trends. *Bioresour. Technol*. **2014**, 159, 421–432.

(10) Xie, P.; Huang, L.; Zhang, C.; You, F.; Zhang, Y. Reduced pressure extraction of oleuropein from olive leaves (*Olea europaea* L.) with ultrasound assistance. *Food Bioprod. Process*. **2015**, 93, 29–38.

(11) Quirantes-Piné, R.; Lozano-Sánchez, J.; Herrero, M.; Ibáñez, E.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC-ESI-QTOF-MS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts. *Phytochem. Anal*. **2012**, 24, 213–223.

(12) Rahmanian, N.; Jafari, S. M.; Wani, T. A. Bioactive profile, dehydration, extraction and application of the bioactive components of olive leaves. *Trends Food Sci. Technol*. **2015**, 42, 150–172.

(13) Bouaziz, M.; Fki, I.; Jemai, H.; Ayadi, M.; Sayadi, S. Effect of storage on refined and husk olive oils composition: Stabilization by addition of natural antioxidants from 'Chemlali' olive leaves. *Food Chem*. **2008**, 108, 253–262.

(14) Erbay, Z.; Icier, F. The Importance and potential uses of olive leaves. *Food Rev. Int*. **2010**, 26, 319–334.

(15) Briante, R.; Patumi, M.; Terenziani, S.; Bismuto, E.; Febbraio, F.; Nucci, R. *Olea europaea* L. leaf extract and derivatives: Antioxidant properties. J. *Agric. Food Chem*. **2002**, 50, 4934–4940.

(16) Tsimidou, M. Z.; Papoti, V. T. Bioactive ingredients in olive leaves. *In olives and olive oil in health and disease prevention*; 2010; pp 349–356.

(17) Mourtzinos, I.; Salta, F.; Yannakopoulou, K.; CHiou, A.; Karathanos, V. Encapsulation of olive leaf extract in *β*-Cyclodextrin. *J. Agric. Food Chem*. **2007**, 55, 8088–8094.

(18) Ayala-Zavala, J. F.; Vega-Vega, V.; Rosas-Domínguez, C.; Palafox-Carlos, H.; Villa-Rodriguez, J. A.; Siddiqui, M. W.; Dávila-Aviña, J. E.; González-Aguilar, G. A. Agro-industrial potential of exotic fruit by-products as a source of food additives. *Food Res. Int*. **2011**, 44, 1866–1874.

(19) Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem*. **2006**, 99, 191–203.

(20) Petti, S.; Scully, C. Polyphenols, oral health and disease: A review. *J. Dent*. **2009**, 37, 413–423.

(21) Lamuela-Raventós, R. M.; Vallverdú-Queralt, A.; Jáuregui, O.; Martínez-Huélamo, M.; Quifer-Rada, P. Improved characterization of polyphenols using liquid chromatography. *In polyphenols in plants: Isolation,*

purification and extract preparation; Watson, R. R., Ed.; Academic press Elsevier, 2014; pp 261–292.

(22) Benavente-garcía, O.; Castillo, J.; Lorente, J.; Ortun, A. Antioxidant activity of phenolics extracted from *Olea europaea* L . leaves. *Food Chem*. **2000**, 68, 457–462.

(23) Goulas, V.; Exarchou, V.; Troganis, A. N.; Psomiadou, E.; Fotsis, T.; Briasoulis, E.; Gerothanassis, I. P. Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. *Mol. Nutr. Food Res*. **2009**, 53, 600–608.

(24) Fu, S.; Arráez-Roman, D.; Segura-Carretero, A.; Menéndez, J. A.; Menéndez-Gutiérrez, M. P.; Micol, V.; Fernández-Gutiérrez, A. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal. Bioanal. Chem*. **2010**, 397, 643–654.

(25) Altıok, E.; Bayçın, D.; Bayraktar, O.; Ülkü, S. Isolation of polyphenols from the extracts of olive leaves (*Olea europaea* L.) by adsorption on silk fibroin. *Sep. Purif. Technol*. **2008**, 62, 342–348.

(26) Bouallagui, Z.; Han, J.; Isoda, H.; Sayadi, S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food Chem. Toxicol*. **2011**, 49, 179–184.

(27) Taamalli, A.; Arráez-Román, D.; Barrajón-Catalán, E.; Ruiz-Torres, V.; Pérez-Sánchez, A.; Herrero, M.; Ibañez, E.; Micol, V.; Zarrouk, M.; Segura-Carretero, A.; et al. Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells. *Food Chem. Toxicol*. **2012**, 50, 1817–1825.

(28) Abaza, L.; Ben Youssef, N.; Manai, H.; Mahjoub Haddada, F.; Methenni, K.; Zarrouk, M. 'Chétoui' olive leaf extracts: Influence of the solvent type on phenolics and antioxidant activities. *Grasas y Aceites* **2011**, 62, 96–104.

(29) Heimler, D.; Pieroni, M.; Tattini, A.; Cimato, A. Determination of flavonoids, flavonoid glycosides and biflavonoids in *Olea europaea* L. leaves. *Chromatogrphia* **1992**, 33, 369–373.

(30) Škerget, M.; Kotnik, P.; Hadolin, M.; Hraš, A. R.; Simonič, M.; Knez, Ž. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. *Food Chem*. **2005**, 89, 191–198.

(31) Laguerre, M.; López Giraldo, L. J.; Piombo, G.; Figueroa-Espinoza, M. C.; Pina, M.; Benaissa, M.; Combe, A.; Rossignol Castera, A.; Lecomte, J.; Villeneuve, P. Characterization of olive-leaf phenolics by ESI-MS and evaluation of their antioxidant capacities by the CAT assay. *J. Am. Oil Chem. Soc*. **2009**, 86, 1215–1225.

(32) Ryan, D.; Antolovich, M.; Herlt, T.; Prenzler, P. D.; Lavee, S.; Robards, K. Identification of phenolic compounds in tissues of the novel olive cultivar 'Hardy ’ s Mammoth'. *J. Agric. Food Chem*. **2002**, 50, 6716–6724.

(33) Briante, R.; La Cara, F.; Febbraio, F.; Patumi, M.; Nucci, R. Bioactive derivatives from oleuropein by a biotransformation on *Olea europaea* leaf extracts. *J. Biotechnol*. **2002**, 93, 109–119.

(34) Ye, J.; Wijesundera, C.; Shi, M. Effects of agronomic and oil processing conditions on natural antioxidative phenolics in olive (*Olea europaea* L.). *Austin J. Nutr. Food Sci*. **2014**, 2, 1–8.

(35) Ranalli, A.; Contento, S.; Lucera, L.; Di Febo, M.; Marchegiani, D.; Di Fonzo, V. Factors affecting the contents of iridoid oleuropein in olive leaves (*Olea europaea* L.). *J. Agric. Food Chem*. **2006**, 54, 434–440.

(36) Pérez-Trujillo, M.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Parella, T. Separation and identification of phenolic compounds of extra virgin olive oil from *Olea Europaea* L. by HPLC-DAD-SPE-NMR/MS. Identification of a new diastereo isomer of the aldehydic form of oleuropein aglycone. *J. Agric. Food Chem*. **2010**, 58, 9129–9136.

(37) Kiritsakis, K.; Kontominas, M. G.; Kontogiorgis, C.; Hadjipavlou-Litina, D.; Moustakas, A.; Kiritsakis, A. Composition and antioxidant activity of

olive leaf extracts from Greek olive cultivars. *J. Am. Oil Chem*. Soc. **2010**, 87, 369–376.

(38) Quirantes-Piné, R.; Zurek, G.; Barrajón-Catalán, E.; Bäßmann, C.; Micol, V.; Segura-Carretero, A.; Fernández-Gutiérrez, A. A metabolite-profiling approach to assess the uptake and metabolism of phenolic compounds from olive leaves in SKBR3 cells by HPLC–ESI-QTOF-MS. *J. Pharm. Biomed. Anal.* **2013**, 72, 121–126.

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

(40) Ahmad-Qasem, M. H.; Cánovas, J.; Barrajón-Catalán, E.; Micol, V.; Cárcel, J. A.; García-Pérez, J. V. Kinetic and compositional study of phenolic extraction from olive leaves (var. *Serrana*) by using power ultrasound. *Innov. Food Sci. Emerg. Technol*. **2013**, 17, 120–129.

(41) Ahmad-Qasem, M. H.; Cánovas, J.; Barrajón-Catalán, E.; Carreres, josé E.; Micol, V.; García-Pérez, J. V. Influence of olive leaf processing on the bioaccessibility of bioactive polyphenols. *J. Agric. Food Chem*. **2014**, 62, 6190–6198.

(42) Lee, O.-H.; Lee, B.-Y.; Lee, J.; Lee, H.-B.; Son, J.-Y.; Park, C.-S.; Shetty, K.; Kim, Y.-C. Assessment of phenolics-enriched extract and fractions of olive leaves and their antioxidant activities. *Bioresour. Technol*. **2009**, 100, 6107–6113.

(43) Tayoub, G.; Sulaiman, H.; Hassan, A. H.; Alorfi, M. Determination of oleuropein in leaves and fruits of some Syrian olive varieties. *Int. J. Med. Aromat. Plants* **2012**, 2, 428–433.

(44) Brahmi, F.; Mechri, B.; Dhibi, M.; Hammami, M. Variations in phenolic compounds and antiradical scavenging activity of *Olea europaea* leaves and fruits extracts collected in two different seasons. *Ind. Crops Prod.* **2013**, 49, 256–264.

(45) Ortega-García, F.; Peragón, J. Phenol metabolism in the leaves of the olive tree (*Olea europaea* L.) cv. Picual, Verdial, Arbequina, and Frantoio during ripening. *J. Agric. Food Chem*. **2010**, 58, 12440–12448.

(46) Savournin, C.; Baghdikian, B.; Elias, R.; Dargouth-kesraoui, F.; Boukef, K.; Balansard, G. Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *J. Agric. Food Chem*. **2001**, 49, 618–621.

(47) Afaneh, I.; Yateem, H.; Al-rimawi, F. Effect of olive leaves drying on the content of oleuropein. *Am. J. Anal. Chem*. **2015**, 6, 246–252.

(48) Malik, N. S. A.; Bradford, J. M. Recovery and stability of oleuropein and other phenolic compounds during extraction and processing of olive (*Olea europaea* L.) leaves. *J. Food, Agric. Environ*. **2008**, 6, 8–13.

(49) Japón-Luján, R.; Ruiz-Jiménez, J.; De Castro, M. D. L. Discrimination and classification of olive tree varieties and cultivation zones by biophenol contents. *J. Agric. Food Chem*. **2006**, 54, 9706–9712.

(50) Mert, C.; Barut, E.; Ipek, A. Quantitative seasonal changes in the leaf phenolic content related to the alternate-bearing patterns of olive (*Olea europaea* L. cv. Gemlik). *J. Agric. Sci. Technol*. **2013**, 15, 995–1006.

(51) Agalias, A.; Melliou, E.; Mitaku, S.; Gikas, E.; Tsarbopoulos, A. Quantitation of oleuropein and related metabolites in decoctions of *Olea europaea* leaves from ten Greek cultivated varieties by HPLC with Diode Array Detection (HPLC-DAD). J. *Liq. Chromatogr. Relat. Technol*. **2005**, 28, 1557–1571.

(52) Meirinhos, J.; Silva, B.; Valentao, P.; Seabra, R.; Pereira, J.; Dias, A.; Andrade, P.; Ferreres, F. Analysis and quantification of flavonoidic compounds from portuguese olive (*Olea europaea* L.) leaf cultivars. *Nat. Prod. Res.* **2005**, 19, 189–195.

(53) Liakopoulos, G.; Karabourniotis, G. Boron deficiency and concentrations and composition of phenolic compounds in *Olea europaea* leaves: A combined growth chamber and field study. *Tree Physiol*. **2005**, 25, 307–315.

(54) Lalas, S.; Athanasiadis, V.; Gortzi, O.; Bounitsi, M.; Giovanoudis, I.; Tsaknis, J.; Bogiatzis, F. Enrichment of table olives with polyphenols extracted from olive leaves. *Food Chem*. **2011**, 127, 1521–1525.

(55) Brahmi, F.; Mechri, B.; Dhibi, M.; Hammami, M. Variation in antioxidant activity and phenolic content in different organs of two Tunisian cultivars of *Olea europaea* L. *Acta Physiol. Plant*. **2014**, 36, 169–178.

(56) Garcia-Salas, P.; Morales-Soto, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Phenolic-compound-extraction systems for fruit and vegetable samples. *Molecules* **2010**, 15, 8813–8826.

(57) Le Floch, F.; Tena, M. T.; Ríos, A.; Valcárcel, M. Supercritical-fluid extraction of phenol compounds from olive leaves. *Talanta* **1998**, 46, 1123–1130.

(58) Taamalli, A.; Arráez-Román, D.; Ibañez, E.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Optimization of microwave-assisted extraction for the characterization of olive leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS^2. *J. Agric. Food Chem*. **2012**, 60, 791–798.

(59) Capote, F. P.; Marinas, A.; Japo, R. Liquid chromatography/triple quadrupole tandem mass spectrometry with multiple reaction monitoring for optimal selection of transitions to evaluate nutraceuticals from olive-tree materials. *Wiley Intersci*. **2008**, 22, 855–864.

(60) Xynos, N.; Papaefstathiou, G.; Gikas, E.; Argyropoulou, A.; Aligiannis, N.; Skaltsounis, A.-L. Design optimization study of the extraction of olive leaves performed with pressurized liquid extraction using response surface methodology. *Sep. Purif. Technol*. **2014**, 122, 323–330.

(61) Japón-Luján, R.; Luque-Rodríguez, J. M.; Luque de Castro, M. D. Dynamic ultrasound-assisted extraction of oleuropein and related biophenols from olive leaves. J. *Chromatogr. A.* **2006**, 1108, 76–82

(62) Japón-Luján, R.; Luque de Castro, M. D. Superheated liquid extraction of oleuropein and related biophenols from olive leaves. J. *Chromatogr. A* **2006**, 1136, 185–191.

(63) Gómez-Caravaca, A. M.; Verardo, V.; Bendini, A.; Gallina-Toschi, T. From wastes to added value by-products: An overview on chemical composition and healthy properties of bioactive compounds of olive oil chain by-products. *In virgin olive oil: Production, composition, uses and benefits for man*; Leonardis, A. De, Ed.; Nova publishers: New York, 2014; pp 301–334.

(64) Christophoridou, S.; Dais, P. Detection and quantification of phenolic compounds in olive oil by high resolution 1H nuclear magnetic resonance spectroscopy. *Anal. Chim. Acta* **2009**, 633, 283–292.

(65) Saitta, M.; Di Bella, G.; Turco, V. Lo; La Torre, G. L.; Dugo, G. Low-level free phenols in Sicilian olive oils. *In olives and olive oil in health and disease prevention*; Victor R. Preedy and Ronald Ross Watson, Ed.; Elsevier Inc., 2010; pp 187–200.

(66) Nino, A. De; Lombardo, N.; Perri, E.; Procopio, A.; Ra, A.; Sindona, G. Direct identication of phenolic glucosides from olive leaf extracts by atmospheric pressure ionization tandem mass spectrometry. *J. Mass Spectrom*. **1997**, 32, 533–541.

(67) Ryan, D.; Prenzler, P. D.; Lavee, S.; Antolovich, M.; Robards, K. Quantitative changes in phenolic content during physiological development of the olive (*Olea europaea*) cultivar 'Hardy ’s Mammoth'. *J. Agric. Food Chem*. **2003**, 51, 2532–2538.

(68) Malik, N. S. A.; Bradford, J. M. Changes in oleuropein levels during differentiation and development of floral buds in 'Arbequina' olives. *Sci. Hortic. (Amsterdam)*. **2006**, 110, 274–278.

(69) Silva, S.; Gomes, L.; Leitao, F.; Coelho, A. V.; Boas, L. V. Phenolic compounds and antioxidant activity of *Olea europaea* L. fruits and leaves. *Food Sci. Technol. Int*. **2006**, 12, 385–396.

(70) Karioti, A.; Chatzopoulou, A.; Bilia, A. R.; Liakopoulos, G.; Stavrianakou, S.; Skaltsa, H. Novel secoiridoid glucosides in *Olea europaea* leaves suffering from boron deficiency. *Biosci. Biotechnol. Biochem.* **2006**, 70, 1898–1903.

(71) Rada, M.; Guinda, Á.; Cayuela, J. Solid/liquid extraction and isolation by molecular distillation of hydroxytyrosol from *Olea europaea* L. leaves. *Eur. J. Lipid Sci. Technol*. **2007**, 109, 1071–1076.

(72) Di Donna, L.; Mazzotti, F.; Salerno, R.; Tagarelli, A.; Taverna, D.; Sindona, G. Characterization of new phenolic compounds from leaves of *Olea europaea* L. by high-resolution tandem mass spectrometry. *Wiley Intersci.* **2007**, 21, 3653–3657.

(73) Pereira, A. P.; Ferreira, I. C.; Marcelino, F.; Valentão, P.; Andrade, P. .; Seabra, R.; Estevinho, L.; Bento, A.; Pereira, J. A. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. Cv. Cobrançosa) leaves. *Molecules* 2**007**, 12, 1153–1162.

(74) Paiva-Martins, F.; Correia, R.; Félix, S.; Ferreira, P.; Gordon, M. H. Effects of enrichment of refined olive oil with phenolic compounds from olive leaves. *J. Agric. Food Chem*. **2007**, 55, 4139–4143.

(75) Arráez-román, D.; Sawalha, S.; Segura-carretero, A.; Menendez, J.; Fernández-gutiérrez, A. Identification of phenolic compounds in olive leaves using CE-ESI-TOF-MS. *Agro. Food Ind. hi-tech* **2008**, 19, 18–22.

(76) Mylonaki, S.; Kiassos, E.; Makris, D. P. Optimisation of the extraction of olive (*Olea europaea*) leaf phenolics using water/ethanol-based solvent systems and response surface methodology. *Anal. Bioanal. Chem*. **2008**, 392, 977–985.

(77) Papoti, V. T.; Tsimidou, M. Z. Impact of sampling parameters on the radical scavenging potential of olive (*Olea europaea* L.) leaves. *J. Agric. Food Chem.* **2009**, 57, 3470–3477.

(78) Ortega-García, F.; Blanco, S.; Peinado, M. Á.; Peragón, J. Phenylalanine ammonia-lyase and phenolic compounds in leaves and fruits of *Olea europaea* L. cv. Picual during ripening. *J. Sci. Food Agric*. **2009**, 89, 398–406.

(79) Papoti, V. T.; Tsimidou, M. Z. Looking through the qualities of a fluorimetric assay for the total phenol content estimation in virgin olive oil, olive fruit or leaf polar extract. *Food Chem*. **2009**, 112, 246–252.

(80) Goulas, V.; Papoti, V. T.; Exarchou, V.; Tsimidou, M. Z.; Gerothanassis, I. P. Contribution of flavonoids to the overall radical scavenging activity of olive (*Olea europaea* L.) leaf polar extracts. *J. Agric. Food Chem*. **2010**, 58, 3303–3308.

(81) Hayes, J. E.; Allen, P.; Brunton, N.; O'Grady, M. N.; Kerry, J. P. Phenolic composition and *in vitro* antioxidant capacity of four commercial phytochemical products: Olive leaf extract (*Olea europaea* L.), lutein, sesamol and ellagic acid. *Food Chem*. **2011**, 126, 948–955.

(82) Aouidi, F.; Dupuy, N.; Artaud, J.; Roussos, S.; Msallem, M.; Perraud Gaime, I.; Hamdi, M. Rapid quantitative determination of oleuropein in olive leaves (*Olea europaea*) using mid-infrared spectroscopy combined with chemometric analyses. *Ind. Crops Prod*. **2012**, 37, 292–297.

(83) Scognamiglio, M.; D'Abrosca, B.; Pacifico, S.; Fiumano, V.; De Luca, P. F.; Monaco, P.; Fiorentino, A. Polyphenol characterization and antioxidant evaluation of *Olea europaea* varieties cultivated in Cilento National Park (Italy). *Food Res. Int*. **2012**, 46, 294–303.

(84) Petridis, A.; Therios, I.; Samouris, G.; Koundouras, S.; Giannakoula, A. Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (*Olea europaea* L.) cultivars. *Plant Physiol. Biochem*. **2012**, 60, 1–11.

(85) Botsoglou, E.; Govaris, A.; Fletouris, D.; Iliadis, S. Olive leaves (*Olea europea* L.) and α-tocopheryl acetate as feed antioxidants for improving the oxidative stability of α-linolenic acid-enriched eggs. *J. Anim. Physiol. Anim. Nutr*. (Berl). **2013**, 97, 740–753.

(86) Talhaoui, N.; Gómez-Caravaca, A. M.; Roldán, C.; León, L.; De la Rosa, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages. *J. Agric. Food Chem*. **2015**, 63, 1722–1729.

(87) Japón-Luján, R.; Luque de Castro, M. D. Liquid-liquid extraction for the enrichment of edible oils with phenols from olive leaf extracts. *J. Agric. Food Chem*. **2008**, 56, 2505–2511.

(88) Lattanzio, V.; Lattanzio, V. M. .; Cardinali, A.; Amendola, V. Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. *Phytochem. Adv. Res*. **2006**, 23–67.

(89) Clérivet, A.; Alami, I.; Breton, F.; Garcia, D.; Sanier, C. Les composés phénoliques et la résistance des plantes aux agents pathogènes. *Acta Bot. Gall*. **1996**, 143, 531–538.

(90) Treutter, D. Significance of flavonoids in plant resistance: A review. *Environ. Chem. Lett*. **2006**, 4, 147–157.

(91) Bilgin, M.; Şahin, S. Effects of geographical origin and extraction methods on total phenolic yield of olive tree (*Olea europaea*) leaves. J. *Taiwan Inst. Chem. Eng*. **2013**, 44, 8–12.

(92) Di Donna, L.; Mazzotti, F.; Naccarato, A.; Salerno, R.; Tagarelli, A.; Taverna, D.; Sindona, G. Secondary metabolites of *Olea europaea* leaves as markers for the discrimination of cultivars and cultivation zones by multivariate analysis. *Food Chem*. **2010**, 121, 492–496.

(93) Markakis, E. A.; Tjamos, S. E.; Antoniou, P. P.; Roussos, P. A.; Paplomatas, E. J.; Tjamos, E. C. Phenolic responses of resistant and susceptible olive cultivars induced by defoliating and nondefoliating *Verticillium dahliae* pathotypes. *Am. Phytopathol. Soc*. **2010**, 94, 1156–1162.

(94) Tubeileh, A.; Bruggeman, A.; Turkelboom, F. Effect of water harvesting on growth of young olive trees in degraded Syrian dryland. *Environ. Dev. Sustain*. **2008**, 11, 1073–1090.

(95) Bacelar, E. A.; Santos, D. L.; Moutinho-Pereira, J. M.; Gonçalves, B. C.; Ferreira, H. F.; Correia, C. M. Immediate responses and adaptative strategies of three olive cultivars under contrasting water availability regimes: Changes on structure and chemical composition of foliage and oxidative damage. *Plant Sci*. **2006**, 170, 596–605.

(96) Lo Gullo, M.; Salleo, S. Different strategies of drought resistance in three Mediterranean sclerophyllous trees growing in the same environmental conditions. *New Phytol*. **1988**, 108, 267–276.

(97) Ennajeh, M.; Vadel, A. M.; Khemira, H. Osmoregulation and osmoprotection in the leaf cells of two olive cultivars subjected to severe water deficit. *Acta Physiol. Plant.* **2009**, 31, 711–721.

(98) Blokhina, O.; Virolainen, E.; Fagerstedt, K. V. Antioxidants, oxidative damage and oxygen deprivation stress: A review. *Ann. Bot*. **2003**, 91, 179–194.

(99) Valderrama, R.; Corpas, F. J.; Carreras, A.; Gomez-Rodriguez, M. V; Chaki, M.; Pedrajas, J. R.; Fernandez-Ocana, A.; Del Rio, L. A.; Barroso, J. B. The dehydrogenase-mediated recycling of NADPH is a key antioxidant system against salt-induced oxidative stress in olive plants. *Plant, Cell Environ*. **2006**, 29, 1449–1459.

(100) Ben Ahmed, C.; Magdich, S.; Ben Rouina, B.; Boukhris, M.; Ben Abdullah, F. Saline water irrigation effects on soil salinity distribution and some physiological responses of field grown 'Chemlali' olive. *J. Environ. Manage*. **2012**, 113, 538–544.

(101) Sofo, A.; Bartolomeo, D.; Xiloyannis, C.; Masia, A. Antioxidant defences in olive trees during drought stress : Changes in activity of some antioxidant enzymes. *Func. Plant Biol*. **2005**, 32, 45–53.

(102) Ben Ahmed, C.; Ben Rouina, B.; Sensoy, S.; Boukhriss, M.; Ben Abdullah, F. Saline water irrigation effects on antioxidant defense system and proline accumulation in leaves and roots of field-grown olive. J. *Agric. Food Chem*. **2009**, 57, 11484–11490.

(103) Petridis, A.; Therios, I.; Samouris, G.; Tananaki, C. Salinity-induced changes in phenolic compounds in leaves and roots of four olive cultivars (*Olea europaea* L.) and their relationship to antioxidant activity. Environ. *Exp. Bot*. **2012**, 79, 37–43.

(104) Melgar, J. C.; Guidi, L.; Remorini, D.; Agati, G.; Degl'innocenti, E.; Castelli, S.; Camilla Baratto, M.; Faraloni, C.; Tattini, M. Antioxidant defences and oxidative damage in salt-treated olive plants under contrasting sunlight irradiance. *Tree Physiol*. **2009**, 29, 1187–1198.

(105) Tekaya, M.; Mechri, B.; Bchir, A.; Attia, F.; Cheheb, H.; Daassa, M.; Hammami, M. Effect of nutrient-based fertilisers of olive trees on olive oil quality. *J. Sci. Food Agric*. **2013**, 93, 2045–2052.

(106) Dag, A.; Ben-David, E.; Kerem, Z.; Ben-Gal, A.; Erel, R.; Basheer, L.; Yermiyahu, U. Olive oil composition as a function of nitrogen, phosphorus and potassium plant nutrition. *J. Sci. Food Agric*. **2009**, 89, 1871–1878.

(107) Erel, R.; Kerem, Z.; Ben-Gal, A.; Dag, A.; Schwartz, A.; Zipori, I.; Basheer, L.; Yermiyahu, U. Olive (*Olea europaea* L.) tree nitrogen status is a key factor for olive oil quality. *J. Agric. Food Chem*. **2013**, 61, 11261–11272.

(108) Fernández-Escobar, R.; Beltrán, G.; Sánchez-Zamora, M. A.; García-Novelo, J.; Aguilera, M. P.; Uceda, M. Olive oil quality decreases with nitrogen over-fertilization. *HortScience* **2006**, 41, 215–219.

(109) Chouliaras, V.; Tasioula, M.; Chatzissavvidis, C.; Therios, I.; Tsabolatidou, E. The effects of a seaweed extract in addition to nitrogen and boron fertilization on productivity, fruit maturation, leaf nutritional status and oil quality of the olive (*Olea europaea* L.) cultivar 'Koroneiki'. *J. Sci. Food Agric*. **2009**, 89, 984–988.

(110) Del Río, J. A.; Báidez, A. G.; Botía, J. M.; Ortuño, A. Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against *Phytophthora sp*. *Food Chem*. **2003**, 83, 75–78.

(111) Giannakopoulou, E.; Mitsopoulos, G.; Hagidimitriou, M.; Papageorgiou, V.; Komaitis, M. Influence of cultivar, harvesting season and geographical origin on phenolic content in leaves of Greek olive cultivars. *Acta Hortic*. **2011**, 924, 437–444.

(112) Heimler, D.; Cimato, A.; Alessandri, S.; Sani, G.; Pieroni, A. Seasonal trend of flavonoids in olive (*Olea europaea* L.) leaves. *Agric. Mediterr*. **1996**, 126, 205–209.

(113) Fabbri, A.; Galaverna, G.; Ganino, T. Polyphenol composition of olive leaves with regard to cultivar , time of collection and shoot type. *Acta Hortic*. **2008**, 791, 459–464.

(114) Ortega-garcía, F.; Blanco, S.; Peinado, M. Á.; Pergarón, J. Polyphenol oxidase and its relationship with oleuropein concentration in fruits and leaves of olive (*Olea europaea*) cv.'Picual' trees during fruit ripening. *Tree Physiol*. **2008**, 28, 45–54.

(115) Hashemi, P.; Delfan, B.; Ghiasvand, A. R.; Alborzi, M.; Raeisi, F. A study of the effects of cultivation variety, collection time, and climate on the amount of oleuropein in olive leaves. *Acta Chromatogr*. **2010**, 22, 133–140.

(116) Brahmi, F.; Mechri, B.; Dabbou, S.; Dhibi, M.; Hammami, M. The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. *Ind. Crops Prod*. **2012**, 38, 146–152.

(117) Hura, K.; Rapacz, M.; Hura, T.; Żur, I.; Filek, M. The effect of cold on the response of Brassica napus callus tissue to the secondary metabolites of Leptosphaeria maculans. *Acta Physiol. Plant.* **2015**, 37, 1-13.

(118) Liakoura, V.; Stefanou, M.; Manetas, Y.; Cholevas, C.; Karabourniotis, G. Trichome density and its UV-B protective potential are affected by shading and leaf position on the canopy. *Environ. Exp. Bot*. **1997**, 38, 223–229.

(119) Liakopoulos, G.; Stavrianakou, S.; Karabourniotis, G. Trichome layers versus dehaired lamina of *Olea europaea* leaves: Differences in flavonoid distribution, UV-absorbing capacity, and wax yield. *Environ. Exp. Bot*. **2006**, 55, 294–304.

(120) Heimler, D.; Cimato, A.; Sani, G.; Pieroni, A.; Galardi, C.; Romani, A. Flavonoids from olive leaves (*Olea europea* L.) as affected by light. *J. Commod. Sci*. **2002**, 41, 31–39.

(121) Remorini, D.; Melgar, J. C.; Guidi, L.; Degl'Innocenti, E.; Castelli, S.; Traversi, M. L.; Massai, R.; Tattini, M. Interaction effects of root-zone

salinity and solar irradiance on the physiology and biochemistry of *Olea europaea*. *Environ. Exp. Bot*. **2009**, 65, 210–219.

(122) Cansev, A.; Gulen, H.; Eris, A. Cold-hardiness of olive (*Olea europaea* L.) cultivars in cold-acclimated and non-acclimated stages: Seasonal alteration of antioxidative enzymes and dehydrin-like proteins. *J. Agric. Sci*. **2008**, 147, 51–61.

(123) Hashempour, A.; Ghasemnezhad, M.; Fotouhi Ghazvini, R.; Sohani, M. M. The physiological and biochemical responses to freezing stress of olive plants treated with salicylic acid. Russ. *J. Plant Physiol*. **2014**, 61, 443–450.

(124) Ortega-García, F.; Peragón, J. The response of phenylalanine ammonia-lyase, polyphenol oxidase and phenols to cold stress in the olive tree (*Olea europaea* L. cv. Picual). *J. Sci. Food Agric*. **2009**, 89, 1565–1573.

(125) Antoniou, P. P.; Markakis, E. a.; Tjamos, S. E.; Paplomatas, E. J.; Tjamos, E. C. Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae. Eur. J. Plant Pathol.* **2008**, 122, 549–560.

(126) Báidez, A. G.; Gómez, P.; Del Río, J. A.; Ortuño, A. Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *J. Agric. Food Chem*. **2007**, 55, 3373–3377.

(127) Rahioui, B.; Zine El Aabidine, A.; Baissac, Y.; El Boustani, E.; Khadari, B.; Jay-Allemand, C.; El Modafar, C. Phenolic compounds of olive tree leaves and their relationship with the resistance to the leaf-spot disease caused by *Spilocaea oleaginea*. *Am. J. Agric. Environ. Sci*. **2009**, 5, 204–214.

(128) El Aabidine, A. Z.; Baissac, Y.; Moukhli, A.; Jay-Allemand, C.; Khadari, B.; El Modafar, C. Resistance of olive tree to *Spilocaea oleagina* is mediated by the synthesis of phenolic compounds. *Int. J. Agric. Biol*. **2010**, 12, 61–67.

(129) Marsilio, V.; Lanza, B. Characterisation of an oleuropein degrading strain of *Lactobacillus plantarum*. Combined effects of compounds present in olive fermenting brines (phenols, glucose and NaCl) on bacterial activity. *J. Sci. Food Agric*. **1998**, 76, 520–524.

(130) Medina, E.; De Castro, A.; Romero, C.; Ramírez, E.; Brenes, M. Effect of antimicrobial compounds from olive products on microorganisms related to health , food and agriculture. *Microb. Pathog. Strateg. Combat. them Sci. Technol. Educ*. **2013**, 2, 1087–1094.

(131) Markin, D.; Duek, L.; Berdicevsky, I. *In vitro* antimicrobial activity of olive leaves. *Mycoses* **2003**, 46, 132–136.

(132) Kubo, A.; Lunde, C. S.; Kubo, I. Antimicrobial activity of the olive oil flavor compounds. *J. Agric. Food Chem*. **1995**, 43, 1629–1633.

(133) Hatzopoulos, P.; Banilas, G.; Giannoulia, K.; Gazis, F.; Nikoloudakis, N.; Milioni, D.; Haralampidis, K. Breeding , molecular markers and molecular biology of the olive tree. *Eur. J. Lipid Sci. Technol*. **2002**, 104, 574–586.

(134) Cayuela, J. A.; Rada, M.; Rios, J. J.; Albi, T.; Guinda, A. Changes in phenolic composition induced by *Pseudomonas savastanoi pv. Savastanoi* infection in olive tree : Presence of large amounts of verbascoside in nodules of tuberculosis disease. *J. Agric. Food Chem*. 2**006**, 54, 5363–5368.

(135) Ben Salah, M.; Abdelmelek, H.; Abderraba, M. Medicinal chemistry study of phenolic composition and biological activities assessment of olive leaves from different varieties grown in Tunisia. *Med. Chem*. **2012**, 2, 107–111.

(136) Petridis, A.; Therios, I.; Samouris, G. Genotypic variation of total phenol and oleuropein concentration and antioxidant activity of 11 Greek olive cultivars (*Olea europaea* L .). *HortScience* **2012**, 47, 339–342.

(137) Luque De Castro, M. D.; Capote Priego, F. Extraction of oleuropein and related phenols from olive leaves and branches. *In olives and olive oil in health and disease prevention*; Elsevier, 2010; pp 259–273.

(138) Campos, A.; Da Costa, G.; Coelho, A. V.; Fevereiro, P. Identification of bacterial protein markers and enolase as a plant response protein in the infection of *Olea europaea subsp. europaea* by *Pseudomonas savastanoi pv. savastanoi*. *Eur. J. Plant Pathol*. **2009**, 125, 603–616.

(139) Lavee, S.; Avidan, N.; Pierik, R. L. Chlorogenic Acid: An independent morphgenesis regulator or a cofactor. *Acta Hortic*. **1994**, 381, 405–412.

(140) Soni, M. G.; Burdock, G. A.; Christian, M. S.; Bitler, C. M.; Crea, R. Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. *Food Chem. Toxicol*. **2006**, 44, 903–915.

(141) Castillo, J. J.; Alcaraz, M.; Benavente-García, O. Antioxidant and radioprotective effects of olive leaf extract. *In olives and olive oil in health and disease prevention*; Preedy, V. R., Watson, R. R., Eds.; Elsevier Inc., 2010; pp 951–958.

(142) Cabarkapa, A.; Zivković, L.; Zukovec, D.; Djelić, N.; Bajić, V.; Dekanski, D.; Spremo-Potparević, B. Protective effect of dry olive leaf extract in adrenaline induced DNA damage evaluated using *in vitro* comet assay with human peripheral leukocytes. *Toxicol. Vitr*. **2014**, 28, 451–456.

(143) Abaza, L.; Talorete, T. P. N.; Yamada, P.; Kurita, Y.; Zarrouk, M.; Isoda, H. Induction of growth inhibition and differentiation of human leukemia HL-60 cells by a Tunisian 'Gerboui' olive leaf extract. *Biosci. Biotechnol. Biochem.* **2007**, 71, 1306–1312.

(144) Fares, R.; Bazzi, S.; Baydoun, S. E.; Abdel-Massih, R. M. The antioxidant and anti-proliferative activity of the Lebanese *Olea europaea* extract. *Plant foods Hum. Nutr*. **2011**, 66, 58–63.

(145) Samet, I.; Han, J.; Jlaiel, L.; Sayadi, S.; Isoda, H. Olive (*Olea europaea*) leaf extract induces apoptosis and monocyte/macrophage differentiation in human chronic myelogenous leukemia K562 cells: insight into the underlying mechanism. *Oxid. Med. Cell. Longev*. **2014**, 2014, 1–16.

(146) Lee-Huang, S.; Zhang, L.; Lin Huang, P.; Chang, Y.-T.; Huang, P. L. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell

gene expression by HIV-1 infection and OLE treatment. *Biochem. Biophys. Res. Commun.* **2003**, 307, 1029–1037.

(147) Korukluoglu, M.; Sahan, Y.; Yigit, A.; Karakas, R. Antifungal activity of olive leaf (*Olea europaea* L.) extracts from the Trilye Region of Turkey. *Ann. Microbiol*. **2006**, 56, 359–362.

(148) Dekanski, D.; Janicijevic-Hudomal, S.; Tadic, V.; Markovic, G.; Arsic, I.; Mitrovic, D. Phytochemical analysis and gastroprotective activity of an olive leaf extract. *J. Serbian Chem. Soc*. **2009**, 74, 367–377.

(149) Kaeidi, A.; Esmaeili-Mahani, S.; Sheibani, V.; Abbasnejad, M.; Rasoulian, B.; Hajializadeh, Z.; Afrazi, S. Olive (*Olea europaea* L.) leaf extract attenuates early diabetic neuropathic pain through prevention of high glucose-induced apoptosis: *in vitro* and *in vivo* studies. *J. Ethnopharmacol*. **2011**, 136, 188–196.

(150) Tavafi, M.; Ahmadvand, H.; Toolabi, P. K. Inhibitory Effect of olive leaf extract on gentamicin-induced nephrotoxicity in rats. *Iran. J. Kidney Dis.* **2012**, *6*, 25–32.

(151) Liu, Y.-N.; Jung, J.-H.; Park, H.; Kim, H. Olive leaf extract suppresses messenger RNA expression of proinflammatory cytokines and enhances insulin receptor substrate 1 expression in the rats with streptozotocin and high-fat diet-induced diabetes. *Nutr. Res*. **2014**, 34, 450–457.

(152) N El, S.; Karakaya, S. Olive tree (*Olea europaea*) leaves: Potential beneficial effects on human health. *Nutr. Rev*. **2009**, 67, 632–638.

(153) Hur, W.; Kim, S. W.; Lee, Y. K.; Choi, J. E.; Hong, S. W.; Song, M. J.; Bae, S. H.; Park, T.; Um, S.-J.; Yoon, S. K. Oleuropein reduces free fatty acid-induced lipogenesis *via* lowered extracellular signal-regulated kinase activation in hepatocytes. *Nutr. Res*. **2012**, 32, 778–786.

(154) Alirezaei, M.; Dezfoulian, O.; Sookhtehzari, A.; Asadian, P.; Khoshdel, Z. Antioxidant effects of oleuropein versus oxidative stress induced by ethanol in the rat intestine. *Comp. Clin. Path*. **2013**, 23, 1359–1365.

(155) Singh, I.; Mok, M.; Christensen, A.M.; Turner, A. H.; Hawley, J. A. The effects of polyphenols in olive leaves on platelet function. *Nutr. Metab. Cardiovasc. Dis*. **2008**, 18, 127–132.

(156) Perugini, P.; Vettor, M.; Rona, C.; Troisi, L.; Villanova, L.; Genta, I.; Conti, B.; Pavanetto, F. Efficacy of oleuropein against UVB irradiation: Preliminary evaluation. *Int. J. Cosmet. Sci*. **2008**, 30, 113–120.

(157) Han, J.; Talorete, T. P. N.; Yamada, P.; Isoda, H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. *Cytotechnology* **2009**, 59, 45–53.

(158) Jamshed, F.; Ahmad, W.; Emdadul Haque, A. T. M.; Saad, A.; Al-Jassabi, S. Ameliorative role of oleuropein extracted from olive leaf on tamoxifen-induced hepatic 8-hydroxydeoxyguanosine in DNA of Balb/C mice. *World Appl. Sci. J*. **2014**, 30, 765–769.

(159) Bulotta, S.; Corradino, R.; Celano, M.; D'Agostino, M.; Maiuolo, J.; Oliverio, M.; Procopio, A.; Iannone, M.; Rotiroti, D.; Russo, D. Antiproliferative and antioxidant effects on breast cancer cells of oleuropein and its semisynthetic peracetylated derivatives. *Food Chem*. **2011**, 127, 1609–1614.

(160) Jemai, H.; El Feki, A.; Sayadi, S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. *J. Agric. Food Chem*. **2009**, 57, 8798–8804.

(161) Jemai, H.; Bouaziz, M.; Fki, I.; El Feki, A.; Sayadi, S. Hypolipidimic and antioxidant activities of oleuropein and its hydrolysis derivative-rich extracts from 'Chemlali' olive leaves. *Chem. Biol. Interact*. **2008**, 176, 88–98.

(162) Jemai, H.; Fki, I.; Bouaziz, M.; Bouallagui, Z.; El Feki, A.; Isoda, H.; Sayadi, S. Lipid-lowering and antioxidant effects of hydroxytyrosol and its triacetylated derivative recovered from olive tree leaves in cholesterol-fed rats. *J. Agric. Food Chem*. **2008**, 56, 2630–2636.

(163) Lee, O.-H.; Lee, B.-Y. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. *Bioresour. Technol*. **2010**, 101, 3751–3754.

(164) Dekanski, D.; Janićijević-hudomal, S.; Ristić, S.; Radonjić, N. V; Petronijević, N. D.; Piperski, V.; Mitrović, D. M. Attenuation of cold restraint stress-induced gastric lesions by an olive. *Gen. Physiol. Biophys*. **2009**, 28, 135–142.

(165) Venditti, A.; Serrilli, A. M.; Rizza, L.; Frasca, G.; Cardile, V.; Bonina, F. P.; Bianco, A. Aromadendrine, a new component of the flavonoid pattern of *Olea europaea* L. and its anti-inflammatory activity. *Nat. Prod. Res*. 2013, 27, 340–349.

(166) Cornelli, U. Antioxidant use in nutraceuticals. *Clin. Dermatol*. **2009**, 27, 175–194.

(167) Cai, Y.; Luo, Q.; Sun, M.; Corke, H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci*. **2004**, 74, 2157–2184.

(168) Dekanski, D.; Ristic, S.; Radonjic, N.; Petronijevic, N.; Dekanski, A.; Mitrovic, D. Olive leaf extract modulates cold restraint stress-induced oxidative changes in rat liver. *J. Serbian Chem. Soc*. **2011**, 76, 1207–1218.

CHAPTER 2. Determination of phenolic compounds of 'Sikitita' olive leaves by HPLC-DAD-TOF MS. Comparison with its parents 'Arbequina' and 'Picual' olive leaves.

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Determination of phenolic compounds of 'Sikitita' olive leaves by HPLC-DAD-TOF-MS. Comparison with its parents 'Arbequina' and 'Picual' olive leaves

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Abstract

'Sikitita' is a new olive cultivar developed in Spain by crossing the cultivars 'Picual' and 'Arbequina'. The phenolic fraction of olive leaves (*Olea europaea* L.), is purported to have good anti-oxidative properties which help to prevent several health problems. To our knowledge, no studies are available on the phenolic fraction of 'Sikitita' olive leaves or any other new cultivar developed from breeding. Thus, the identification and quantification of the phenolic fraction of 'Sikitita' olive leaves by HPLC-DAD-MS were studied and compared with those of its parent cultivars. The three cultivars were grown under the same agronomic and environmental conditions in the same orchard. The quantification was performed using HPLC-DAD, whereas qualitative data were acquired using HPLC-MS. It was thus possible to identify 30 different compounds, two of which have been tentatively characterized for the first time in olive leaves of Spanish cultivars. Significant differences between cultivars were observed for almost all the compounds. Results for 'Sikitita' olive leaves presented a higher degree of similarity with respect to 'Picual' than to 'Arbequina'. Further work will monitor the time course of phenolic compounds over the growth period.

Keywords: Olive leaves, Phenolic compounds, HPLC-DAD-TOF-MS, 'Sikitita' cultivar

1. Introduction

The relationship between diet and health has given rise to intense research in bioactive compounds in foods. Olive oil, olive leaves, and the by-products of the olive industry appear to be essential components and may be partially responsible for health promoting properties observed among the Mediterranean population, due to its antioxidant and anti-inflammatory effects[1]. While olive oil is well known for its flavor and health benefits, olive leaves have been used traditionally as a folk remedy for combating fevers and other diseases. Recently, different studies have demonstrated the anti-hypertensive[2], anticarcinogenic[3], anti-inflammatory, hypoglycemic, antimicrobial, and hypo-cholesterolemic effects of olive leaves[4], all these positive effects appearing to be at least partly related to an anti-oxidative action[5,6], related primarily to low-molecular-weight polyphenols such as oleuropein. Accordingly, olive leaves could be used not only in medicines, cosmetics, and pharmaceuticals, but they can also be used to improve the shelf life of foods and to develop functional foods. In fact, olive leaves have been mixed with overripe olives before processing to produce oils with a more marked flavor and a higher resistance to oxidation[7], or they have been used directly as supplements for oils[8]. Also, phenolic extracts of olive leaves have been obtained to perform tablets which are commercially available as dietetic products and/or food integrators[9].

The most important classes of phenolic compounds in olive leaf include phenolic acids, phenolic alcohols, flavonoids and secoiridoids. Several studies have explored the presence of a high number of phenolic compounds in olive leaves, notably: hydroxytyrosol, rutin, verbascoside, luteolin-7-glucoside, luteolin-4'-glucoside, oleuropein, oleuropein aglycone, and ligstroside aglycone[10,11]. Oleuropein is generally the most prominent phenolic compound in olive cultivars[12], and it is easily extracted as part of the phenolic fraction of olive fruits, leaves and seeds, but it has not been reported in virgin olive oils[10,11].

The phenolic profile of olive, generally, and olive leaf, specifically, is known to be affected, among other things, by geographical origin and cultivar[13]. Recently, many studies have examined olive leaves from various cultivars using different separation techniques, e.g. reverse-phase HPLC with diode-array detection with or without coupling to mass spectrometry (MS)[10,14,15].

'Sikitita' ('Chiquitita' in USA) is a new olive cultivar developed in Spain by crossing 'Picual' and 'Arbequina', two cultivars with high productivity and oil content from different geographical origins: 'Arbequina' from Catalonia (NE Spain) and, 'Picual' from Andalusia (Spain)[16]. 'Sikitita' was released and protected in 2008, and planted for the first time in commercial orchards in 2009-2011[17]. This cultivar was adapted specifically to hedgerow olive growing due to its low vigor and weeping habit. The fruit and oil characteristics as well as resistance to biotic stresses of this new cultivar resemble those reported for its male parent 'Arbequina'[16]. 'Sikitita' olive oil has also been the object of phenolic studies1[18,19], but so far, no study is available on the leaf phenolic profile.

This work was designed to identify and quantify, for the first time, the phenolic compounds in leaves from the cultivar 'Sikitita' and to compare the results with those of its parents, 'Picual' and 'Arbequina'.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade and used as received. Methanol was purchased from Panreac (Barcelona, Spain) and HPLC-grade acetonitrile was purchased from Labscan (Dublin, Ireland). Acetic acid was of analytical grade (assay >99.5%) and purchased from Fluka (Switzerland).Water was purified by using a Milli-Q system (Millipore, Bedford, MA, USA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, and apigenin were

purchased from SigmaeAldrich (St. Louis, MO, USA), and oleuropein from Extrasynthese (Lyon, France). The stock solutions containing these analytes were prepared in methanol. All the solutions were stored in a dark flask at -20 ºC until used.

2.2. Samples

Olive leaves of cultivars 'Arbequina', 'Picual', and 'Sikitita' grown under the same agronomical and environmental conditions in a common olive orchard at "IFAPA Centro Alameda del Obispo", Cordoba (Spain), were used. Leaf samples were collected from three trees of each cultivar from different parts of the trees in mid-June, immediately transferred to the laboratory, and dried outdoors. Finally, samples were stored at -80 ºC until used. All the leaves collected from the same tree were pooled in a unique sample. Three replicates of each sample were executed.

Dry leaves (0.5 g) were crushed and extracted *via* Ultra-Turrax IKA T18 basic with 10 mL of MeOH/H_2O (80/20). After that, the sample was placed in an ultrasonic bath (10 min) and centrifuged at 1000 g for 10 min. Then, the supernatant was removed, and the extraction was repeated twice. The supernatants were collected and the extracts were then evaporated and reconstituted with 2mL of MeOH/H_2O (50/50). Samples were picked from 3 cultivars, 3 trees per cultivars and three replicates from each three, which was 18 samples per cultivars, 54 samples in total.

2.3. Determination of phenolic compounds by HPLC-DAD-ESI-TOF-MS

Analyses of the phenolic fraction of olive leaves were performed on an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA) consisting of a vacuum degasser, autosampler, and a binary pump equipped with a Poroshell 120 ECC18 analytical column (4.6 × 100 mm, 2.7 μm)

from Agilent Technologies. The mobile phases used were water with acetic acid (1%) (phase A) and acetonitrile (phase B), and the solvent gradient changed according to the following conditions: 0 min, 5% B; 4 min, 9% B; 7 min,12% B; 8 min,15% B; 9min,16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 28% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 35 min, 100% B; 37 min, 5% B.

The flow rate used was set at 0.8 mL/min throughout the gradient. The column temperature was maintained at 25ºC, and the injection volume was 2.5 µL. The UV spectra were recorded from 200 to 600 nm, whereas the chromatograms were registered at 240, 280, and 330 nm. The effluent from the HPLC column was split using a T type phase separator before introducing it into the mass spectrometer (split ratio 1:3).

The HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The HPLC-MS was conducted following the method of Gómez-Caravaca et al.[20].

2.4. Evaluation of radical-scavenging activity by DPPH assay

The scavenging activity was evaluated using the DPPH according to Goulas et al.[21] with some modifications. 20 µL of each sample were mixed with 980 µL of 0.2 mM solution of DPPH in methanol, and the absorbance of the mixture was measured after 30 min incubation time in the dark at 517 nm. Different concentrations of sample were evaluated and the % of free radical scavenging activity was determined as follows:

% scavenging activity= 100 - [(Absorbance of sample - Absorbance of blank) × 100/Absorbance of control]

A Synergy Mx microplate reader (BioTek Instruments, VT, USA) was used for measurements. EC50 values are referred to the lower concentration of sample required to reach the 50% of the antioxidant activity.

2.5. Statistical analysis

One-way analysis of variance, ANOVA (Tukey's honest significant-difference multiple comparison) was evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA); *p* values lower than 0.05 were considered statistically significant. All chemical analyses were made in triplicate, and the analytical data were used for statistical comparisons. Pearson's linear correlations, at the $p<0.05$ level, was also evaluated using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Chromatographic profile and identification of phenolic compounds

The method used was a modified version of one proposed by Gómez-Caravaca et al.[20]. The best separation conditions are reported in Materials and Methods. In the present work, a total of 30 phenolic compounds were tentatively identified in all samples, mainly phenolic compounds such as: simple phenols, secoiridoids, flavonoids and other compounds. The tentatively identified phenolic compounds are summarized in **Table 1** in negative ionization mode, including retention times, experimental and calculated *m/z*, molecular formula, errors, sigma values, together with their proposed identities. Also, the base peak chromatogram (BPC) of olive leaf, displayed in **Figure 1**, resulted from the optimal-gradient-elution program and the optimal MS conditions in negative ionization mode. The

compounds were identified by interpreting their mass spectra determined *via* TOF-MS and taking into account the data reported in the literature. All these results were complemented with the UV spectra provided by DAD, which gave additional information about the family of compounds, in terms of the absorbance bands. However quantification was performed using HPLC-DAD data.

The HPLC-DAD-TOF-MS analyses of the olive-leaf extracts revealed the following compounds already reported in olive leaf (**Table 1**): peaks 1, 4, and 7, at 4.25, 5.00, and 8.86 min presented the same molecular mass at *m/z* 389.1. Peak 1 was identified as oleoside[11,15,22-24], and peaks 4 and 7 were proposed to be secologanoside (isomer of oleoside) by comparing their molecular formula, the fragments obtained, and the order of elution in the literature[22,23]. Peaks 2 and 3 (RT 4.65 min and 4.85 min, respectively) gave a molecular mass of *m/z* 315.1085 and they were tentatively identified as hydroxytyrosol-hexose (isomer a and b), according to the molecular formula provided for their mass and, corroborated by their fragment ion at *m/z* 153, corresponding to hydroxytyrosol after the loss of a sugar moiety (162 Da)[10,15,22]. Peak 5 (RT 6.64 min) was tentatively described as tyrosol-glucoside; it presented a molecular mass of *m/z* 299.1107 and a fragment at *m/z* 137.0552, which coincided with the *m/z* of tyrosol due to the loss of a sugar moiety[10,11,15,22,25]. Another hydroxytyrosol derivative was identified at 15.60 min and *m/z* 623.1922 (peak 17) as verbascoside[10,11,22,24,26]. Peak 6 (RT 8.77 min) and peak 8 (RT 10.27 min) at *m/z* 403.1223 and 403.1248, respectively, were identified as isomers of elenolic acid glucoside, as reported in literature[10,15,24,25]. The presences of other isomeric forms of elenolic acid glucoside were tentatively identified in peaks 9 and 12 (RT 10.40 and 12.24 min, respectively)[10,15,22].

The following secoiridoids previously found in olive leaf were also confirmed in our sample[10,15,22,24]: Oleuropein aglycone (peak 10, at 11.16 min and *m/z* 377.1403), demethyloleuropein (peak 13, at 13.85 min and *m/z* 525.1556),

oleuropein glucoside and its isomers (peaks 19, 20, 21, at 17.23 min, 17.46 min and 17.80 min, respectively, and *m/z* 701), oleuropein and its isomer (peaks 26 and 27 at 19.47 min and 20.09 min, respectively, and *m/z* 539), oleuroside (peak 28, at 20.44 min and *m/z* 539.1770) and ligstroside (peak 29 at 20.09 min and *m/z* 523.1776). Another two secoiridoids were also detected at peaks 24 and 25 (at 19.01 min and 19.16 min, respectively) showing the same *m/z* at 569. These compounds were tentatively identified as 2”-methoxyoleuropein and its isomer. This secoiridoid glucoside was previously reported in other genera of the family Oleaceae, such as *Jasminum*[27], and for the first time in olive leaves by Taamalli et al.[26]. However, to our knowledge, the present study is the first available in which this 2”-methoxyoleuropein compound and its isomer have been identified in Spanish cultivars.

Flavonoids, another important group of compounds in olive leaves, have also been characterized in the extracts. The peaks of the flavonoids were between 11.74 and 21.81 min of the chromatogram. According to the bibliography, 8 flavonoids were identified. Peak 11, at 11.74 min and *m/z* 609.1386, showed a fragment at *m/z* 447 that resulted from the loss of a glucose moiety (162 Da).

Table 1. Phenolic compounds identified in an olive leaf extract by HPLC–ESI-TOF, including retention time, *m/z* experimental and calculated molecular formula, sigma value, and some fragments.

Peak	RT (min)	UV max (nm)	*m/z* experimental	*m/z* calculated	Tolerance (ppm)	Error (ppm)	mili Sigma	Fragments	Molecular Formula	Compounds
1	4.25	227	389.1081	389.1089	10	2.2	6	183,209,227	$C_{16}H_{22}O_{11}$	Oleoside
2	4.65	227,277	315.1073	315.1085	10	3.9	8.6	123,153	$C_{14}H_{20}O_8$	Hydroxytyrosol-hexose isomer a
3	4.85	227,279	315.1078	315.1085	10	2.4	8.2	123,153	$C_{14}H_{20}O_8$	Hydroxytyrosol-hexose isomer b
4	5.00	234	389.1086	389.1089	10	0.8	1.5	183,209,227	$C_{16}H_{22}O_{11}$	Secologanoside isomer a
5	6.64	228	299.1107	299.1102	15	11.4	12.9	129,137	$C_{14}H_{20}O_7$	Tyrosol glucoside
6	8.77	237	403.1223	403.1246	10	5.8	7.2		$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer a
7	8.88	235	389.1093	389.1089	10	0.8	3.1	183,209,227	$C_{16}H_{22}O_{11}$	Secologanoside isomer b
8	10.27	238	403.1248	403.1246	10	0.6	1.1		$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer b
9	10.40	235,325	403.1249	403.1246	10	0.7	2.4		$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer c
10	11.16	235,271,336	377.1403	377.1453	20	13.2	2.9	153,197	$C_{16}H_{26}O_{10}$	Oleuropein aglycon
11	11.74	248,267,335	609.1386	609.1461	15	12.3	14.9	447	$C_{27}H_{30}O_{16}$	Luteolin-diglucoside
12	12.24	237	403.1255	403.1246	10	2.2	4.9	179,223,371	$C_{17}H_{24}O_{11}$	Elenolic acid glucoside isomer d
13	13.85	235,281	525.1556	525.1614	20	11.0	11.3		$C_{24}H_{30}O_{13}$	Demethyloleuropein
14	14.42	253,352	609.1394	609.1461	10	4.9	3.5	179,301	$C_{27}H_{30}O_{16}$	Rutin
15	14.65	253,348	593.1452	593.1512	15	10.1	6.2		$C_{27}H_{30}O_{15}$	Luteolin rutinoside
16	15.35	253,347	447.0937	447.0933	10	0.8	1.5		$C_{21}H_{20}O_{11}$	Luteolin glucoside isomer a
17	15.60	234,329	623.1922	623.1981	10	2.2	1.1	315,461	$C_{29}H_{36}O_{15}$	Verbascoside
18	16.96	237,266,336	577.1527	577.1563	10	6.3	3.6		$C_{27}H_{30}O_{14}$	Apigenin rutinoside
19	17.23	237,282,332	701.2265	701.2298	10	4.7	16.6		$C_{31}H_{42}O_{18}$	Oleuropein glucoside isomer a
20	17.46	240,349	701.2212	701.2298	10	6.8	7		$C_{31}H_{42}O_{18}$	Oleuropein glucoside isomer b
21	17.8	235,277	701.2222	701.2298	10	6.6	1.9		$C_{31}H_{42}O_{18}$	Oleuropein glucoside isomer c
22	17.95	247,268,336	447.0882	447.0933	10	5.2	2.2		$C_{21}H_{20}O_{11}$	Luteolin glucoside isomer b
23	18.35	250,347	461.1085	461.1089	10	1.1	2.8		$C_{22}H_{22}O_{11}$	Chrysoeriol-7-*O*-glucoside
24	19.01	236,280	569.1758	569.1876	10	6.1	17.1		$C_{26}H_{34}O_{14}$	2″-Methoxyoleuropein isomer a
25	19.16	236,280	569.1762	569.1876	20	17.1	14.6		$C_{26}H_{34}O_{14}$	2″-Methoxyoleuropein isomer b
26	19.47	242,280	539.1736	539.1770	10	5.3	1.6		$C_{25}H_{32}O_{13}$	Oleuropein isomer a
27	20.09	236,280	539.1718	539.1770	10	3.2	2.3		$C_{25}H_{32}O_{13}$	Oleuropein isomer b
28	20.44	234,280	539.1728	539.1770	10	1.3	4.0		$C_{25}H_{32}O_{13}$	Oleuropein/Oleuroside
29	21.34	230,279	523.1776	523.1282	10	2.1	2.3		$C_{25}H_{32}O_{12}$	Ligstroside
30	21.81	237,286	285.0373	285.0405	20	11.1	3.0		$C_{15}H_{10}O_6$	Luteolin

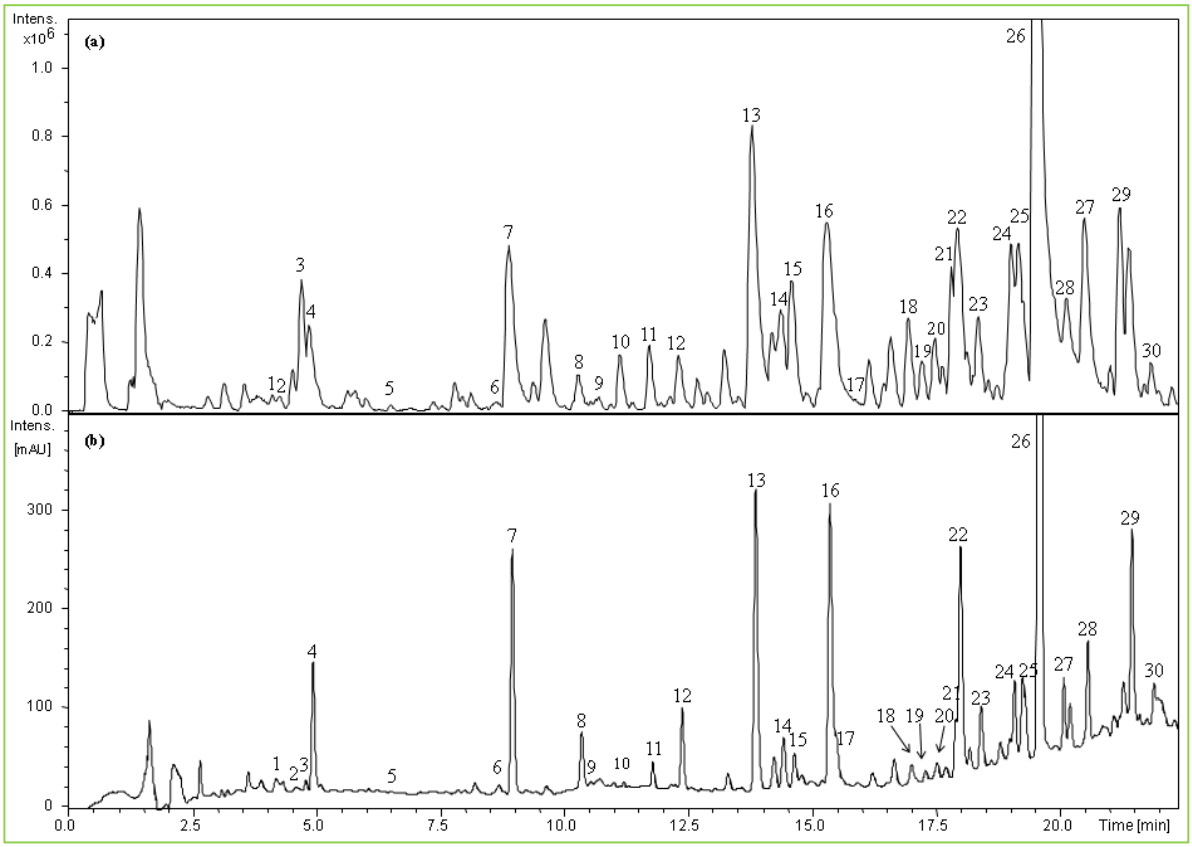

Figure 1. Base peak chromatogram (BPC) (a) and UV chromatogram at 240 nm (b) of 'Sikitita' olive leaf extract obtained by HPLC-DAD-TOF-MS *(See table 1 for identification numbers).*

According to these data and the literature, this peak was described as luteolin-diglucoside[11,22,24,26]. Peak 14 at 14.42 min presented the same *m/z* 609.1394. Although the two peaks presented molecular ions at *m/z* 609 and eluted closely, this peak presented fragments with *m/z* 301 and 179, typical of quercetin; therefore, it was assigned to rutin[10,22,28]. Peak 15, at 14.65 min and *m/z* 593.1452, was tentatively identified as luteolin-rutinoside, as previously proposed by other authors[15,22,24]. Peaks 16 and 22 (at *m/z* 447 and, 15.35 and 17.95 min, respectively) were assigned as luteolin glucoside isomers[11,15,22,24]. Peak 18 (at 16.96 min and *m/z* 577.1527) was tentatively identified as apigenin rutinoside[11,15,22,24]. Peak 23 (at 18.35 min and *m/z* 461.1085) was proposed to be chrysoeriol-7-*O*-glucoside[11,15,22] and peak 30 (at 21.81 min and *m/z* 285.0373) was identified as luteolin[15,22,24].

3.2. Quantification of phenolic compounds

Five standard calibration graphs for the quantification of the phenolic compounds of olive leaves were prepared using five commercial standards (oleuropein, hydroxytyrosol, tyrosol, apigenin, and luteolin). The calibration plots indicated good correlations between peak areas and analyte concentrations, and regression coefficients were higher than 0.990 in all cases. The different parameters of the method are summarized in **Table 2**.

Table 2. Analytical parameters of the method proposed.

Analyte	RSD	LOD (µg/mL)	LOQ (µg/mL)	Calibration range (µg/mL)	Calibration equations	r^2
Apigenin	0.01	0.021	0.071	LOQ-80	y= 7.4833x+6.1314	0.9996
Hydroxytyrosol	1.48	0.126	0.420	LOQ-80	y= 1.2636x-1.513	0.9991
Luteolin	0.77	0.016	0.052	LOQ-80	y= 10.132 x+4.6291	0.9997
Oleuropein	0.34	0.046	0.155	LOQ-1000	y= 3.4325x+13.602	0.9973
Tyrosol	1.36	0.526	1.753	LOQ-80	y= 0.3026x +2.5511	0.9912

The LOQ was determined as the signal-to-noise ratio of 10:1, and the limit of detection (LOD) was determined as signal-to-noise ratio of 3:1. LOD was found to be within the range 0.016-0.526 µg/mL while LOQ was within 0.052-1.753 µg/mL.

Intraday and interday precision was performed to assess the repeatability of the method. An olive-leaf extract was injected (n=6) on the same day (intraday precision) for 3 consecutive days (interday precision, n=18). The highest intraday repeatability of the peak area among all peaks, expressed by the RSD, was 1.03%, whereas the highest interday repeatability among all peaks was 3.6%.

Oleuropein and other secoiridoids were quantified with the calibration curve of oleuropein at λ=240 nm; hydroxytyrosol hexose isomers and verbascoside were quantified with the calibration curve of hydroxytyrosol at λ=280 nm; tyrosol glucoside was quantified with the calibration curve of tyrosol at λ=280 nm;

apigenin rutinoside was quantified with the calibration curve of apigenin at λ=240 nm and luteolin, luteolin-diglucoside and the other flavonoids were quantified with the calibration curve of luteolin at λ=240 nm.

3.3. Evaluation and comparison of the phenolic compounds of 'Sikitita' olive leaves with its parents 'Arbequina' and 'Picual'

Concentrations of the total and individual free phenolic compounds determined in the olive-leaf samples analyzed are reported in **Table 3**. All results are expressed as mg/g dry matter of olive leaves. A one-way analysis of variance indicated significant differences among the three cultivars for most of the compounds (p<0.05) except for oleuropein and oleuroside. Total phenolic compounds of 'Sikitita' olive leaves were estimated in 52.1 mg/g dry weight. This value did not significantly differ from that of phenolic compounds of 'Picual' olive leaves but was significantly lower (16.3%) than that of 'Arbequina' olive leaves.

Oleosides are Oleaceae-specific secoiridoids that are commonly esterified to a phenolic moiety as in oleuropein and ligstroside. In the last decade, considerable effort has been expended on identifying new conjugated oleosides in *Olea europaea*, and their bioactivity due to increasing interest in the potential health benefits of a Mediterranean diet[29]. According to the literature, oleuropein was the most concentrated compound present in the leaves of 'Arbequina', 'Sikitita', and 'Picual' cultivars, at 28%, 33%, and 34% of the total compound concentrations, respectively[15,22,30-32]. The concentration of oleuropein in olive leaves referred to in this work were in general higher than those given by other authors[9,13]; however, the cultivars used and the conditions of leaf sampling reported in those works were different, and this could substantially alter oleuropein levels.

No significant differences in the concentration of oleuropein 'isomer a' were detected among the three cultivars studied. For oleuropein 'isomer b', no statistical differences between 'Picual' and 'Arbequina' were found; however,

both statistically differed from 'Sikitita', reaching values of 20.6% and 11.9% higher in 'Picual' and 'Arbequina', than in 'Sikitita', respectively.

Table 3. Quantification of the identified phenolic compounds in olive leaves of 'Arbequina', 'Sikitita' and 'Picual' cultivars expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at p <0.05.

	Phenolic compounds	'Arbequina'	'Sikitita'	'Picual'
1	**Oleoside**	0.370$^{a.b}$	0.366^{b}	0.399^{a}
2	**Hydroxytyrosol-hexose isomer a**	0.340^{b}	0.438^{a}	0.341^{b}
3	**Hydroxytyrosol-hexose isomer b**	0.469^{b}	0.793^{a}	0.485^{b}
4	**Secologanoside isomer a**	2.376^{a}	2.035^{b}	1.823^{c}
5	**Tyrosol glucoside**	1.278^{a}	0.863^{b}	0.858^{b}
6	**Elenolic acid glucoside isomer a**	1.315^{a}	0.267^{b}	0.693^{c}
7	**Secologanoside isomer b**	3.305$^{a.b}$	3.677^{a}	2.966^{b}
8	**Elenolic acid glucoside isomer b**	1.367^{a}	0.904^{c}	1.036^{b}
9	**Elenolic acid glucoside isomer c**	0.909^{a}	0.186^{c}	0.285^{b}
10	**Oleuropein aglycon**	0.288^{a}	0.134^{c}	0.170^{b}
11	**luteolin-diglucoside**	0.364^{a}	0.201^{c}	0.310^{b}
12	**Elenolic acid glucoside isomer d**	1.133^{b}	1.212$^{a.b}$	1.277^{a}
13	**Demethyloleuropein**	3.922^{b}	6.382^{a}	1.338^{c}
14	**Rutin**	0.651^{a}	0.319^{b}	0.289^{c}
15	**Luteolin rutinoside**	0.491^{a}	0.199^{c}	0.259^{b}
16	**Luteolin glucoside isomer a**	5.724^{a}	3.534^{c}	5.166^{b}
17	**Verbascoside**	4.069^{a}	1.162^{b}	1.127^{b}
18	**Apigenin rutinoside**	0.284^{b}	0.230^{c}	0.386^{a}
19	**Oleuropein glucoside isomer a**	0.572^{b}	0.430^{c}	0.828^{a}
20	**Oleuropein glucoside isomer b**	0.792^{a}	0.680^{b}	0.733$^{a.b}$
21	**Oleuropein glucoside isomer c**	1.083^{a}	0.900^{b}	n.d
22	**Luteolin glucoside isomer b**	1.591^{b}	1.072^{c}	1.744^{a}
23	**Chrysoeriol-7-*O*-glucoside**	0.845^{a}	0.581^{c}	0.749^{b}
24	**2''-Methoxyoleuropein isomer a**	1.128^{a}	1.036^{a}	0.921^{b}
25	**2''-Methoxyoleuropein isomer b**	1.205^{b}	0.870^{c}	2.188^{a}
26	**Oleuropein isomer a**	17.083^{a}	17.460^{a}	18.010^{a}
27	**Oleuropein isomer b**	1.431^{a}	1.279^{b}	1.543^{a}
28	**Oleuropein/Oleuroside**	2.337^{a}	2.110^{a}	2.100^{a}
29	**Ligstroside**	3.845^{a}	3.476$^{a.b}$	3.251^{b}
30	**Luteolin**	0.394^{b}	0.367^{b}	0.497^{a}
	Total	**60.644^{a}**	**52.129^{b}**	**52.579^{b}**

(n.d non determined)

Concerning the other derivatives of oleuropein, the majority of these compounds were more abundant in 'Arbequina' than in 'Sikitita' and 'Picual' leaves. Oleuropein aglycon was the minor compound found in all three cultivars, the content of this compound ranging from 0.26 to 0.47% of total phenolic content.

High level of oleuropein aglycon in olive extract is generally a sign of oleuropein hydrolysis[25,29,33-35]. Therefore, this result indicates that oleuropein did not degrade in the samples analyzed. However, oleuropein aglycon was 53.5% higher in 'Arbequina' and 26.9% higher in 'Picual' than in 'Sikitita' olive leaves. Ligstroside is known to be a result of the esterification between oleoside and tyrosol[33]. Ligstroside content ranged between 3.8 mg/g of dry weight ('Arbequina') and 3.2 mg/g of dry weight ('Picual'). However, the mean values of ligstroside content in 'Sikitita', 'Arbequina' and 'Picual' samples notably overlapped. Concerning the oleuropein glucoside isomers, the concentration of the oleuropein glucoside isomer significantly differed in the three olive leaves cultivars, reaching the highest concentration in 'Picual' olive leaves, while 'Sikitita' leaves presented half the amount of 'Picual', and 'Arbequina' leaves were 30.9% less concentrated than were those of 'Picual'. Oleuropein glucoside isomers b and c concentrations were 16.5% and 20.3% higher in 'Arbequina' than in 'Sikitita' olive leaves; while no differences were noted for the isomer b between 'Picual' and 'Sikitita', and isomer c was not detected in 'Picual' leaves.

'Picual' leaves were also the richest in 2"-methoxyoleuropein. Indeed, the total concentration of the two 2"-methoxyoleuropein isomers was 3.1 mg/g dry weight in 'Picual', as opposed to 2.3 and 1.9 mg/g dry weight in 'Arbequina' and 'Sikitita', respectively.

Elenolic acid glucoside and demethyloleuropein were reported in literature as an obvious route of the degradation of oleuropein[29,36]. A clear and statistically significant difference (p<0.05) was found for demethyloleuropein and elenolic acid glucoside contents among the three cultivars **(Table 3)**. 'Sikitita' leaves presented the highest concentration of demethyloleuropein (6.4 mg/g of dry weight) but this compound showed much lower values in 'Arbequina' and 'Picual' leaf samples (38.5% and 79.0%, respectively). Studies by Servili et al.[36], and confirmed by Obied et al.[29], in olive fruit and olive oil, stipulated that the concentration of demethyloleuropein is cultivar dependent. In contrast of

demethyloleuropein results, the majority of elenolic acid isomers showed a significantly higher content in 'Arbequina' leaves than in 'Sikitita' and 'Picual' **(Table 3)**. Indeed, total elenolic acid isomers (a, b, c, d) were 83.9% and 28.1% higher in 'Arbequina' and 'Picual' leaf samples, respectively, compared with 'Sikitita' ones.

The elenolic acid derivatives, oleoside and secologanoside, are not necessarily phenolic compounds but may include a phenolic moiety as a result of esterification[11]. Significant differences were found among the analyzed samples for oleoside and secologanoside isomers a and b. In fact, 'Picual' samples showed the highest oleoside content (0.395 mg/g of dry weight), although the variation of 'Arbequina' and 'Picual' with respect to 'Sikitita' did not exceed 9%. On the contrary, secologanoside isomers a, and b presented the lowest values in 'Picual' leaf samples. The total content of secologanoside isomers did not show a major difference between 'Arbequina' and 'Sikitita' samples (0.5% decrease in 'Arbequina' from 'Sikitita'), whereas the total content of secologanoside isomers decreased in 'Picual' samples by 16.2% compared with 'Sikitita' samples **(Figure 1, Table 3)**.

Hydroxytyrosol and tyrosol, two of the most important phenolic alcohols present in olive leaf, are of great interest due to their beneficial properties[37]. The total concentration of the two hydroxytyrosol-hexose isomers was significantly higher in 'Sikitita' samples (1.3 mg/g dry weight) than in 'Arbequina' and 'Picual', which were 34.3% and 32.9% lower, respectively **(Figure 1, Table 3)**. With respect to tyrosol glucoside concentration, significant differences appeared among cultivars **(Table 3)**. 'Arbequina' presented the highest tyrosol glucoside value (1.3 mg/g dry weight), this being 48.1% higher than in 'Sikitita' and 'Picual'. Verbascoside, a sugar ester of hydroxytyrosol and caffeic acid and defined as the main hydroxycinnamic derivative in olives by Gómez-Rico et al.[38] was also identified in the olive leaves[37]. In the samples studied, the verbascoside content (4.1 mg/g dry weight) was enormously higher in 'Arbequina' leaves than

in ‘Sikitita’ and ‘Picual’ leaves, which were 79.4% and 80% lower, respectively (**Figure 1**). It should be noted that the partial degradation of oleuropein is responsible for the formation of verbascoside[11,39] which may have occurred in ‘Arbequina’ more than in the other cultivars.

In all of the cultivars studied, luteolin glucoside was the most abundant flavonoid. A similar trend was observed by Meirinhos et al.[40] for some Portuguese cultivars. ‘Arbequina’ samples registered significantly the highest amounts of almost all the flavonoids. In fact, the content in luteolin glucoside isomers a and b (7.3 mg/g dry weight) in ‘Arbequina’ samples was 58.8% higher than the content of this compound in ‘Sikitita’ samples, even though no large differences were found in the content of luteolin glucoside isomers a and b between ‘Arbequina’ and ‘Picual’ leaves (6.9 mg/g dry weight; **Figure 1, Table 3**). Moreover, luteolin diglucoside, rutin, luteolin rutinoside, and chrysoeriol-7-*O*-glucoside showed significantly the highest amounts in ‘Arbequina’ samples (**Table 3**). The exception was rutin, which reached the lowest value in ‘Picual’ samples; the other compounds (luteolin diglucoside, luteolin rutinoside and chrysoeriol-7-*O*-glucoside) were significantly lower in ‘Sikitita’ samples. By contrast, apigenin rutinoside content, which ranged between 0.2 and 0.4 mg/g (dry weight) and luteolin content, ranging from 0.4 to 0.5 mg/g (dry weight) were significantly higher in ‘Picual’ samples. Meanwhile, apigenin rutinoside content was 23.5% higher in ‘Arbequina’ than in ‘Sikitita’ samples and the luteolin content did not significantly differ between ‘Arbequina’ and ‘Sikitita’ samples (**Table 3**).

Generally, significant genotypic differences were recorded among cultivars in terms of the total and specific phenols identified. In contrast to the literature on olive oil, ‘Arbequina’ olive leaves showed the highest concentration of total phenols, verbascoside, most of secoiridoids, and most of flavonoids compared with ‘Picual’ and ‘Sikitita’ olive leaves. Other authors[37] have reported similar

results, concerning tyrosol, hydroxytyrosol and oleuropein, for ‘Picual’ and ‘Arbequina’ olive leaves.

‘Sikitita’ phenol profile was found to be more similar to the ‘Picual’ parent than ‘Arbequina’ one. This was in contrast to the only study on the phenol profile of ‘Sikitita’ is by Garcia-Gonzalez et al.[41] in olive oil, which showed a higher degree of similarity of ‘Sikitita’ and ‘Arbequina’ samples. However, that work included a lower number of phenolic compounds than in the present study.

3.4. Radical scavenging activity by DPPH

The results of the study of the radical scavenging activity by DPPH test showed that ‘Arbequina’ olive leaves had the highest scavenging activity with EC50 of 7.2 μg /mL, followed by ‘Sikitita’ with EC50 of 11.3 μg /mL and ‘Picual’ with EC50 of 12.3 μg /mL. ‘Picual’ and ‘Sikitita’ radical scavenging activities did not significantly differ, while ‘Arbequina’ scavenging activity was significantly higher than them. These results are in agreement with those found for the total phenolic content by HPLC analyses. Moreover a high correlation was showed between radical scavenging activity and total phenolic content by HPLC analyses of olive leaves ($r=-0.9525$; $p<0.05$).

4. Conclusions

A powerful analytical method HPLC-DAD-TOF-MS was used to characterize olive leaves of a new cultivar ‘Sikitita’ and its parent cultivars ‘Picual’ and ‘Arbequina’ making it possible to identify total of 30 compounds in the samples. To our knowledge, this is the first report available that tentatively identifies two of these compounds in olive leaves of Spanish cultivars. Furthermore, the comparison, on the basis of those 30 compounds, among cultivar have shown that ‘Arbequina’ olive leaves presented the highes concentration of total phenols and almost of all single phenols Indeed, our results showed that, generally, the new cultivar ‘Sikitita’ phenol profile is more similar to the ‘Picual’ parent than

'Arbequina one. Finally, these preliminary findings should be tested by further study that takes into consideration the variation of phenolic profile in different periods of the year.

References

(1) Visioli, F.; Galli, C. Biological properties of olive oil phytochemicals biological properties of olive oil Phytochemicals. *Crit. Rev. Food Sci. Nutr*. **2002**, 42, 209–221.

(2) Susalit, E.; Agus, N.; Effendi, I.; Tjandrawinata, R. R.; Nofiarny, D.; Perrinjaquet-Moccetti, T.; Verbruggen, M. Olive (*Olea europaea*) leaf extract effective in patients with stage-1 hypertension: comparison with Captopril. *Phytomedicine* **2011**, 18, 251–258.

(3) Bouallagui, Z.; Han, J.; Isoda, H.; Sayadi, S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food Chem. Toxicol*. **2011**, 49, 179–184.

(4) Lee, O.-H.; Lee, B.-Y. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. *Bioresour. Technol*. **2010**, 101, 3751–3754.

(5) Dekanski, D.; Ristic, S.; Radonjic, N.; Petronijevic, N.; Dekanski, A.; Mitrovic, D. Olive leaf extract modulates cold restraint stress-induced oxidative changes in rat liver. *J. Serbian Chem. Soc*. **2011**, 76, 1207–1218.

(6) Raederstorff, D. Antioxidant activity of olive polyphenols in humans: A review. *Int. J. Vitam. Nutr. Res*. **2009**, 79, 152–165.

(7) Ranalli, A.; Gomes, T.; Delcuratolo, D.; Contento, S.; Lucera, L. Improving virgin olive oil quality by means of innovative extracting biotechnologies. *J. Agric. Food Chem*. **2003**, 51, 2597–2602.

(8) Guinda, Á.; Pérez-Camino, M. C.; Lanzón, A. Supplementation of oils with oleanolic acid from the olive leaf (*Olea europaea*). *Eur. J. Lipid Sci. Technol*. **2004**, 106, 22–26.

(9) Briante, R.; La Cara, F.; Febbraio, F.; Patumi, M.; Nucci, R. Bioactive derivatives from oleuropein by a biotransformation on *Olea europaea* leaf extracts. *J. Biotechnol*. **2002**, 93, 109–119.

(10) Silva, S.; Gomes, L.; Leitao, F.; Coelho, A. V.; Boas, L. V. Phenolic compounds and antioxidant activity of *Olea europaea* L. fruits and leaves. *Food Sci. Technol. Int*. **2006**, 12, 385–396.

(11) Ryan, D.; Antolovich, M.; Prenzler, P.; Robards, K.; Lavee, S. Biotransformations of phenolic compounds in *Olea europaea* L. *Sci. Hortic. (Amsterdam)*. **2002**, 92, 147–176.

(12) Benavente-garcía, O.; Castillo, J.; Lorente, J.; Ortun, A. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem*. **2000**, 68, 457–462.

(13) Ranalli, A.; Contento, S.; Lucera, L.; Di Febo, M.; Marchegiani, D.; Di Fonzo, V. Factors affecting the contents of iridoid oleuropein in olive leaves (*Olea europaea* L.). *J. Agric. Food Chem*. **2006**, 54, 434–440.

(14) Bilgin, M.; Şahin, S. Effects of geographical origin and extraction methods on total phenolic yield of olive tree (*Olea europaea*) leaves. *J. Taiwan Inst. Chem. Eng*. **2013**, 44, 8–12.

(15) Taamalli, A.; Gómez-Caravaca, A. M.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area. *Eur. Food Res. Technol*. **2010**, 231, 965–975.

(16) Rallo, L.; Barranco, D.; De la Rosa, R.; León, L. "Chiquitita" olive. *HortScience* **2008**, 43, 529–531.

(17) Rallo, L.; Barranco, D.; de la Rosa, R.; Leon, L. Advances in the joint UCO-IFAPA olive breeding program (JOBP). *Acta Hortic*. **2011**, 924, 283–290.

(18) El Riachy, M.; Priego-Capote, F.; Rallo, L.; Luque-de Castro, M. D.; León, L. Phenolic composition of virgin olive oils from cross breeding segregating populations. *Eur. J. Lipid Sci. Technol*. **2012**, 114, 542–551.

(19) Garcia-Gonzalez, D.; Tena, N.; Aparicio, R. Quality characterization of the new virgin olive oil Var . 'Sikitita' by phenols and volatile compounds. *J. Agric. Food Chem*. **2010**, 58, 8357–8364.

(20) Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Caboni, M. F. Simultaneous determination of phenolic compounds and saponins in quinoa (*Chenopodium quinoa* Willd) by a liquid chromatography-diode array detection-electrospray ionization-time-of-flight mass spectrometry methodology. *J. Agric. Food Chem*. **2011**, 59, 10815–10825.

(21) Goulas, V.; Exarchou, V.; Troganis, A. N.; Psomiadou, E.; Fotsis, T.; Briasoulis, E.; Gerothanassis, I. P. Phytochemicals in olive-leaf extracts and their antiproliferative activity against cancer and endothelial cells. *Mol. Nutr. Food Res*. **2009**, 53, 600–608.

(22) Fu, S.; Arráez-Roman, D.; Segura-Carretero, A.; Menéndez, J. A.; Menéndez-Gutiérrez, M. P.; Micol, V.; Fernández-Gutiérrez, A. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal. Bioanal. Chem*. **2010**, 397, 643–654.

(23) Obied, H. K.; Bedgood, D. R.; Prenzler, P. D.; Robards, K. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Anal. Chim. Acta* **2007**, 603, 176–189.

(24) Quirantes-Piné, R.; Lozano-Sánchez, J.; Herrero, M.; Ibáñez, E.; Segura-Carretero, A.; Fernández-Gutiérrez, A. HPLC-ESI-QTOF-MS as a powerful analytical tool for characterising phenolic compounds in olive-leaf extracts. *Phytochem. Anal*. **2012**, 24, 213–223.

(25) Cardoso, S. M.; Guyot, S.; Marnet, N.; Lopes-da-Silva, J. A.; Renard, C. M.; Coimbra, M. A. Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J. Sci. Food Agric*. **2005**, 85, 21–32.

(26) Taamalli, A.; Arráez-Román, D.; Ibañez, E.; Zarrouk, M.; Segura-

Carretero, A.; Fernández-Gutiérrez, A. Optimization of microwave-assisted extraction for the characterization of olive leaf phenolic compounds by using HPLC-ESI-TOF-MS/IT-MS^2. *J. Agric. Food Chem*. **2012**, 60, 791–798.

(27) Tanahashi, T.; Sakai, T.; Takenaka, Y.; Nagakura, N.; Chen, C.-C. Structure elucidation of two secoiridoid glucosides from *Jasminum officinale* L. var. *grandiflorum* (L.) KOBUSKI. *Chem. Pharm. Bull*. **1999**, 47, 1582–1586.

(28) Herrero, M.; Temirzoda, T. N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. J. *Chromatogr*. *A* **2011**, 1218, 7511–7520.

(29) Obied, H. K.; Prenzler, P. D.; Ryan, D.; Servili, M.; Taticchi, A.; Esposto, S.; Robards, K. Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from *Olea europaea* L. *Nat. Prod. Rep*. **2008**, 25, 1167–1179.

(30) Hashemi, P.; Delfan, B.; Ghiasvand, A. R.; Alborzi, M.; Raeisi, F. A study of the effects of cultivation variety, collection time, and climate on the amount of oleuropein in olive leaves. *Acta Chromatogr*. **2010**, 22, 133–140.

(31) Luque de Castro, M. D.; Japón-Luján, R. State-of-the-art and trends in the analysis of oleuropein and derivatives. *Trends Anal. Chem*. **2006**, 25, 501–510.

(32) Jemai, H.; El Feki, A.; Sayadi, S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. *J. Agric. Food Chem*. **2009**, 57, 8798–8804.

(33) Gutierrez-Rosales, F.; Romero, M. P.; Casanovas, M.; Motilva, M. J.; Mínguez-Mosquera, M. I. Metabolites involved in oleuropein accumulation and degradation in fruits of *Olea europaea* L.: 'Hojiblanca' and 'Arbequina' varieties. *J. Agric. Food Chem*. **2010**, 58, 12924–12933.

(34) Servili, M.; Esposto, S.; Fabiani, R.; Urbani, S.; Taticchi, A.; Mariucci, F.; Selvaggini, R.; Montedoro, G. F. Phenolic compounds in olive oil:

Antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology* **2009**, 17, 76–84.

(35) Briante, R.; Patumi, M.; Terenziani, S.; Bismuto, E.; Febbraio, F.; Nucci, R. *Olea europaea* L. leaf extract and derivatives: Antioxidant properties. *J. Agric. Food Chem*. **2002**, 50, 4934–4940.

(36) Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. Health and sensory properties of virgin olive oil hydrophilic phenols: Agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr. A* **2004**, 1054, 113–127.

(37) Ortega-García, F.; Peragón, J. Phenol metabolism in the leaves of the olive tree (*Olea europaea* L.) cv. Picual, Verdial, Arbequina, and Frantoio during ripening. *J. Agric. Food Chem*. **2010**, 58, 12440–12448.

(38) Gómez-Rico, A.; Fregapane, G.; Salvador, M. D. Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. *Food Res. Int*. **2008**, 41, 433–440.

(39) Artajo, L.-S.; Romero, M.-P.; Suárez, M.; Motilva, M.-J. Partition of phenolic compounds during the virgin olive oil industrial extraction process. *Eur. Food Res. Technol*. **2007**, 225, 617–625.

(40) Meirinhos, J.; Silva, B.; Valentao, P.; Seabra, R.; Pereira, J.; Dias, A.; Andrade, P.; Ferreres, F. Analysis and quantification of flavonoidic compounds from portuguese olive (*Olea europea* L.) leaf cultivars. *Nat. Prod. Res*. **2005**, 19, 189–195.

(41) García-González, D. L.; Tena, N.; Aparicio, R. Quality characterization of the new virgin olive oil var. 'Sikitita' by phenols and volatile compounds. *J. Agric. Food Chem*. **2010**, 58, 8357–8364.

CHAPTER 3. Chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages

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Chemometric Analysis for the Evaluation of Phenolic Patterns in Olive Leaves from Six Cultivars at Different Growth Stages

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Abstract

Leaves from six important olive cultivars grown under the same agronomic conditions were collected at four different times from June to December and analyzed by high performance liquid chromatography-diode array detector-time-of-flight-mass spectrometry (HPLC-DAD-TOF-MS). Twenty-eight phenolic compounds were identified and quantified. No qualitative differences were detected among leaves. However, for all cultivars, total concentrations of phenolic compounds decreased from June to August, then increased from October on, and reached higher levels again in December. Principal component analysis provided a clear separation of the phenolic content in leaves for different sampling times and cultivars. Hence, the availability of phenolic compounds depends on both the season and the cultivar. June and December seem to be good times to collect leaves as a source of phenolic compounds. December coincides with the harvest period of olives in the Andalusian region. Thus, in December olive leaves could be valorized efficiently as olive by-products.

Keywords: olive leaves, phenolic compounds, HPLC-DAD-TOF-MS, cultivar, sampling time.

1. Introduction

Secondary metabolites such as phenolic compounds play a key role in plants, as defense mechanisms against herbivores and biotic infections[1,2], and also in adaptation to abiotic stress[3]. In fact, many studies strongly support the idea that polyphenols play a significant role in plant tolerance to salinity[4], and a link has also been established between tolerance to oxidative stress induced by water deficit and a rise in the antioxidant concentration in photosynthetic plants[5,6].

The level of phenolics in plants varies extensively; it is affected by many factors that influence phenolic stability, biosynthesis, and degradation. These include genetic and physiological factors as well as environmental factors[7]. Therefore, the effect of phenolic compounds in plants resistance depends upon their respective biological activities, which in turn can be determined by the particular physicochemical environments to which the compounds are exposed (high and low temperature, drought, alkalinity, salinity, UV stress, bacteria, fungi, insects, etc.)[8,9].

The olive tree (*Olea europaea* L.) is one of the oldest and most characteristic crops in the Mediterranean basin, as 95% of the world's surface dedicated to olives is concentrated in this area[10]. Olive trees are considered drought tolerant because trees can survive on shallow soils with little supplemental water beyond winter rainfall that is typical of the Mediterranean climate. This is possible because, as can be observed in several plants of the Mediterranean shrubland biome, the olive tree has developed a series of physiological mechanisms to tolerate drought stress and grow under adverse climatic conditions[11]. The most relevant mechanisms are the regulation of stomata closure and transpiration, the regulation of gas exchange, osmotic adjustment, and regulation of the antioxidant system[11,12]. In addition, some olive cultivars have shown high resistance to diseases such as *Verticillium* wilt (caused by *Verticillium dahliae*) and olive scab (caused by *Fusicladium oleagineum*). Various studies have related this

pathogenic resistance to a multifactorial phenolic component (tyrosol and its derivatives, oleuropein and rutin)[13–15].

Spain is one of the world's leading producer, importer, and exporter country in terms of olives oil and fruit, with a production of 7 820 060 tons. As result of olive processing, a huge quantity of olive byproducts are produced annually; just in the Andalusian region around 277 063 tons of olive stones, 985 552 tons of olive cake, and 432 984 tons of olive leaves and twigs are generated[16]. The use of by-products of this crop has long been part of the agricultural tradition of the country. Olive leaves are one of those byproducts that are used in many areas such as animal food[17] or energetic biomasses[16]. Furthermore, as a great source of antioxidant and bioactive compounds, olive leaves have strong potential to be used in pharmaceutical preparations and as a supplement in the functional food industry[18]. In fact, several studies have been made on the health-promoting potential of olive leaves due to the phenolic compounds they contain[19,20]. Others studies have also been carried out focused on the understanding of the metabolism of some phenolic compounds in olive leaves, or the influence of factors such as water deficit, genetic factor, or seasonal period[21–25].

In a previous work[26], our group reported the phenolic composition of 'Sikitita' ('Chiquitita' in the U.S.), a newly bred olive cultivar[27]. In the present work, an exhaustive number of phenolic compounds and cultivars were considered with the main aim of providing further insights into the evolution of olive leaves phenolic compounds in different olive cultivars during their growth and the olive ripening period under the Andalusian climate. We also highlight the optimal sampling time to use olive leaves as a source of bioactive compounds.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, the reagent used for extracting the phenolic compounds from the olive-leaf samples, was purchased from Panreac (Barcelona, Spain), and HPLC-grade acetonitrile was purchased from Labscan (Dublin, Ireland). The acetic acid used was of analytical grade (assayed at >99.5%) and was purchased from Fluka (Switzerland). Water was purified using a Milli-Q system (Millipore, Bedford, MA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, and apigenin were purchased from Sigma-Aldrich (St. Louis, MO) and oleuropein was from Extrasynthèse (Lyon, France). The stock solutions containing these analytes were prepared in methanol. All chemicals were of analytical reagent grade and used as received. All of the solutions were stored in a dark flask at -20 °C until use.

2.2. Samples

Olive leaves (*Olea europaea* L.) from cultivars 'Arbequina', 'Arbosana', 'Changlot Real', 'Koroneiki', 'Picual', and 'Sikitita' were used in this study. These cultivars were selected as some of the most widely used in new orchards currently in Spain, highly productive, well adapted to modern olive growing techniques, and initially originated in different areas: 'Arbequina' and 'Arbosana' from Catalonia (Spain), 'Changlot Real' from Valencia (Spain), 'Picual' from Andalusia (Spain), 'Koroneiki' (Greece), and 'Sikitita', a new Spanish cultivar from cross-breeding between 'Arbequina' and 'Picual'. All cultivars were grown under the same agronomic and environmental conditions in the same olive orchards located at "IFAPA, Centro Alameda del Obispo" in Córdoba, Spain (37°51'36.5"N 4°47'53.7"W). Samples were processed at four times: mid-June (fruit-set), mid- August, mid-October, and mid-December (fruit-ripening) in 2012. Adult leaves were collected from three individuals of each

cultivar, in 5 year old trees of these cultivars planted at 7 × 5 m spacing and trained as single-trunk vase. Standard cultural practices were followed, with minimal pruning to allow early bearing and irrigation by in-line drips with 2000 m^3/ha per year to avoid water stress of plants. All of the leaves collected from the same tree were pooled in a unique sample, immediately transferred to the laboratory, and dried outdoors. Finally, samples were stored at -80 °C until needed.

2.3. Extraction of phenolic compounds from olive leaves

Sample extraction was performed as described previously by Talhaoui et al[26]. Briefly, dry leaves (0.5 g) were crushed and extracted *via* Ultra-Turrax IKA T18 basic using 30 mL of MeOH/H_2O (80/20). After solvent evaporation, the extracts were reconstituted with 2 mL of MeOH/H_2O (50/50). Three replicates of each sample were processed.

2.4 Determination of phenolic compounds by HPLC-DAD-TOF-MS

Phenolic compounds were separated by a Poroshell 120 EC-C18 analytical column (4.6 × 100 mm, 2.7 μm) from Agilent Technologies, on an Agilent 1200 series rapid resolution liquid chromatograph (Agilent Technologies, CA). The gradient eluent, at a flow rate of 0.8 mL/min, was achieved using the method previously described by Talhaoui et al.[26]. The column temperature was maintained at 25 °C, and the injection volume was 2.5 μL.

The HPLC system with diode-array detection was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Analysis parameters were set using a negative-

ion mode with spectra acquired over a mass range from m/z 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bars. The accurate mass data on the molecular ions were processed through the newest Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas *via* the Smart Formula Editor. The Smart Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula. Peak areas of phenolic compounds were integrated using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics, Bremen, Germany). All phenolic compounds showed good levels for quantification in the various samples on each date of sampling. Five standard calibration graphs were prepared for quantification of the phenolic compounds in the olive leaves using five commercial standards (oleuropein, hydroxytyrosol, tyrosol, apigenin, and luteolin).

2.5. Statistical analysis

All assays were run in triplicate. Values of different results were expressed as the means mg/g olive leaves. Results were tested for statistical significance by one-way ANOVA. Significant statistical differences among treatments ($p< 0.001$) were assessed by Tukey's honest significant-difference multiple comparisons. Statistica 8.0 software (2001, StatSoft, Tulsa, OK) was used for statistical determinations. Principal components analysis (PCA) was elaborated using the MatLab function "princomp ()" version R 2012a.

3. Results and discussion

3.1. Identification and quantification of phenolic compounds

Samples of phenolic extracts from olive leaves picked at different times were analyzed by HPLC with UV-vis and MS detection. The identification of phenolic compounds from samples was carried out as previously reported by Talhaoui et al.[26]. The compounds were identified by interpreting their mass spectra determined *via* TOF-MS and taking into account the data reported in the literature. All of these results were complemented with the UV spectra provided by DAD, which gave additional information about the family of compounds, in terms of the absorbance bands. However, quantification was performed using MS data. Twenty-eight compounds were detected in all of the samples and for all sampling times, except for 'Arbosana' in which tyrosol glucoside was only detected in December (**Table a,** Supporting Information). No qualitative differences in phenolic compounds were detected between leaves from the six different olive cultivars. **Figure 1** shows the base peak chromatogram of 'Arbequina' cultivar in December as a representative example. The calibration plots indicated good correlations between peak areas and analyte concentrations, and regression coefficients were higher than 0.990 in all cases. Sensitivity (RSD, %), linearity (r^2), calibration ranges, calibration curves, and limits of detection and quantification are reported (**Table b**, Supporting Information). The limit of quantification (LOQ) was determined as a signal-to-noise ratio of 10:1, and the limit of detection (LOD) was determined as a signal-to-noise ratio of 3:1. LOD was found to be within the range 0.53−27.63 μg/L, while LOQ was within 1.76−92.10 μg/L. Intraday and interday precisions were developed to assess the repeatability of the method. An olive leaf extract was injected six times on the same day (intraday precision, n=6) for 3 consecutive days (interday precision, n =18). The intraday repeatability of the peak area, expressed by the RSD, was 1.03%, whereas the interday repeatability was 3.6%. Oleuropein and other secoiridoids were quantified with the calibration curve for oleuropein;

hydroxytyrosol-hexose isomers were quantified with the calibration curve for hydroxytyrosol; tyrosol glucoside was quantified with the calibration curve for tyrosol; apigenin rutinoside was quantified with the calibration curve for apigenin and luteolin; and luteolin-diglucoside and the other flavonoids were quantified with the calibration curve for luteolin. All phenolic compound contents are reported as mg of compound/g dry olive leaves (**Tables a, c, d, e, f, g,** Supporting Information).

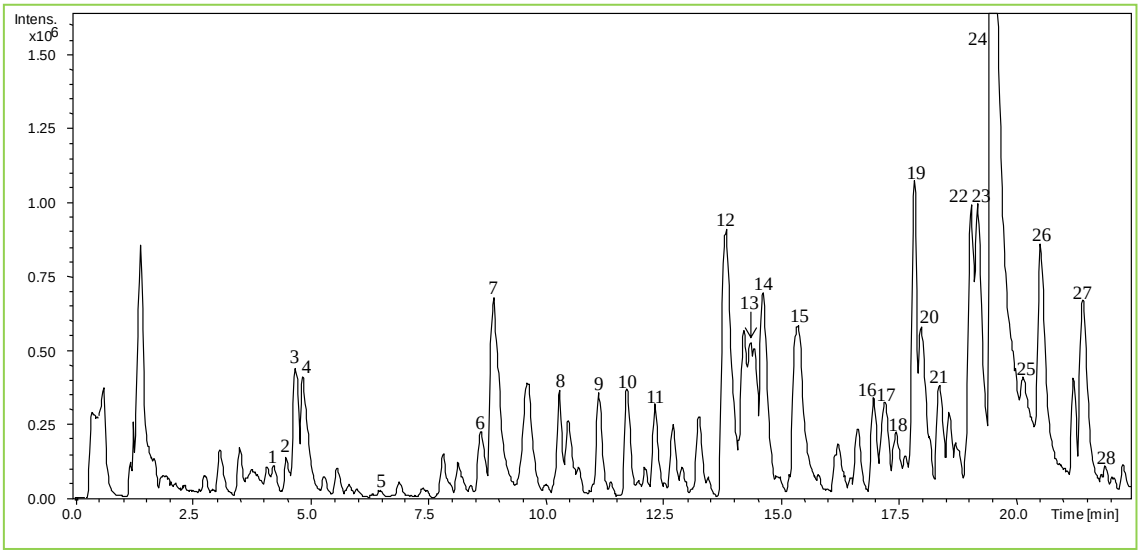

Figure 1. Base peak chromatogram (BPC) of 'Arbequina' olive leaf extract in December, obtained by HPLC-DAD-TOF-MS. *(See table 1 for identification numbers).*

3.2. Chemometric approach

Because of the large amounts of data obtained, it was indispensable to adapt the appropriate statistical method so that it could facilitate discrimination between cultivars and/or sampling times. Hence, the data on the concentrations determined for the 28 compounds were grouped into five groups **(Table 1)**: secoiridoids, group 1 (oleuropein aglycon, demethyloleuropein, three isomers of oleuropein diglucoside, two isomers of 2″-methoxyoleuropein, two isomers of oleuropein, oleuropein/oleuroside, and ligstroside); flavonoids, group 2 (luteolin diglucoside, luteolin rutinoside, apigenin rutinoside, two isomers of luteolin

glucoside, luteolin, chrysoeriol-7-*O*-glucoside, and rutin); simple phenols, group 3 (hydroxytyrosol-hexose and isomer, and tyrosol glucoside); oleosides, group 4 (oleoside, secologanoside isomers a and b); and elenolic acids, group 5 (elenolic acid glucoside isomers a, b, and c). Subsequently, the statistical analysis was applied to a data set consisting of a matrix of 288 rows (one for each analysis) and 5 columns (one for each compound group).

Table 1. Phenolic compounds identified in olive leaves extract by HPLC-DAD-TOF-MS, including retention time, *m/z*, discrimination group, means of all cultivars and total (mg/g of dry olive leaves), and standard deviations (in parentheses)[a].

	Name	*m/z*	RT (min)	Group	June	August	October	December
1	**Oleoside**	389	4.27	4	0.023(0.012)	0.007(0.003)	0.026(0.005)	0.039(0.012)
2	**Hydroxytyrosol-hexose isomer a**	315	4.54	3	0.490(0.534)	0.126(0.070)	0.120(0.074)	0.518(0.260)
3	**Hydroxytyrosol-hexose isomer b**	315	4.75	3	1.121(0.423)	0.179(0.046)	0.362(0.185)	1.284(0.401)
4	**Secologanoside isomer a**	389	4.89	4	0.430(0.164)	0.153(0.051)	0.389(0.136)	0.574(0.191)
5	**Tyrosol glucoside**	299	6.54	3	0.461(0.473)	0.041(0.022)	0.138(0.106)	1.083(0.689)
6	**Elenolic acid glucoside isomer a**	403	8.65	5	0.838(1.303)	0.080(0.077)	0.124(0.126)	0.062(0.038)
7	**Secologanoside isomer b**	389	8.93	4	0.784(0.272)	0.163(0.064)	0.300(0.128)	0.478(0.138)
8	**Elenolic acid glucoside isomer b**	403	10.30	5	0.065(0.037)	0.045(0.030)	0.068(0.025)	0.097(0.059)
9	**Oleuropein aglycon**	377	11.14	1	0.183(0.176)	0.050(0.033)	0.080(0.053)	0.167(0.102)
10	**Luteolin-diglucoside**	609	11.73	2	0.170(0.163)	0.150(0.065)	0.194(0.086)	0.175(0.071)
11	**Elenolic acid glucoside isomer c**	403	12.33	5	0.110(0.098)	0.050(0.027)	0.152(0.099)	0.250(0.160)
12	**Demethyloleuropein**	525	13.83	1	1.230(0.866)	0.354(0.091)	0.282(0.170)	0.345(0.215)
13	**Rutin**	609	14.40	2	0.967(0.656)	0.205(0.171)	0.314(0.283)	0.267(0.157)
14	**Luteolin rutinoside**	593	14.62	2	0.458(0.384)	0.271(0.181)	0.298(0.189)	0.300(0.135)
15	**Luteolin glucoside isomer a**	447	15.32	2	0.872(0.567)	0.376(0.142)	0.729(0.259)	0.973(0.315)
16	**Apigenin rutinoside**	577	16.96	2	0.081(0.065)	0.074(0.059)	0.084(0.031)	0.111(0.087)
17	**Oleuropein diglucoside isomer a**	701	17.23	1	0.075(0.045)	0.090(0.031)	0.089(0.031)	0.132(0.056)
18	**Oleuropein diglucoside isomer b**	701	17.48	1	0.210(0.162)	0.156(0.078)	0.129(0.066)	0.232(0.092)
19	**Oleuropein diglucoside isomer c**	701	17.81	1	0.422(0.267)	0.294(0.101)	0.326(0.114)	0.521(0.140)
20	**Luteolin glucoside isomer b**	447	17.95	2	0.581(0.473)	0.291(0.158)	0.526(0.174)	0.678(0.168
21	**Chrysoeriol-7-*O*-glucoside**	461	18.36	2	0.090(0.078)	0.057(0.033)	0.108(0.060)	0.089(0.041)
22	**2"-Methoxyoleuropein isomer a**	569	19.02	1	0.391(0.285)	0.187(0.052)	0.244(0.120)	0.079(0.123)
23	**2"-Methoxyoleuropein isomer b**	569	19.17	1	0.615(0.479)	0.300(0.174)	0.524(0.181)	0.160(0.186)
24	**Oleuropein isomer a**	539	19.52	1	33.142(11.806)	9.868(2.373)	26.966(4.693)	42.493(15.897)
25	**Oleuropein isomer b**	539	20.12	1	0.594(0.246)	0.120(0.054)	0.316(0.150)	0.847(0.450)
26	**Oleuropein/Oleuroside**	539	20.51	1	1.340(0.751)	0.396(0.158)	0.884(0.340)	2.007(0.778)

	Name	*m/z*	RT (min)	Group	June	August	October	December
27	**Ligstroside**	523	21.37	1	0.599(0.243)	0.159(0.056)	0.432(0.142)	0.829(0.450)
28	**Luteolin**	285	21.86	2	0.026(0.015)	0.023(0.015)	0.020(0.008)	0.020(0.015)
	Total				53.73(8.16)	14.27(3.09)	34.23(5.37)	54.81(19.03)

[a]Group 1 (secoiridoid); group 2 (flavonoids); group 3 (simple phenols); group 4 (oleosides); group 5 (elenolic acids)

3.2.1. Comparison of total phenolic compounds among sampling times

The total concentration of phenolic compounds in the samples collected in June and December presented the highest mean concentrations, while those collected in August presented the minimum values. This general trend was also observed for most of the individual phenolic compounds **(Table 1)**. The total concentration means of phenolic compounds for the six cultivars are in agreement with those reported for every single cultivar, except for 'Koroneiki' and 'Arbosana' cultivar samples, in which the variation in phenolic compound content did not show any significant difference from October to December (**Tables a, c, d, e, f, g,** Supporting Information). Considering the standard deviation (SD), there was little variability between cultivars in August and October. To evaluate whether the differences between sampling times were statistically significant or not, a matched Student's t-test was performed between the total concentrations from each pair of seasons. Highly significant differences were obtained for all seasonal pair comparisons (p<0.001), except when comparing June and December (p=0.70). In fact, the phenolic compound content of olive leaves is in general greatly influenced by the time of collection[28]. Indeed, studies carried out on different cultivars and in different seasons by Hashemi et al.[25] reported that the concentration of oleuropein, the major component of olive leaves, was significantly higher in the cold season than in the warm season. The same results have been confirmed by other authors, such as Brahmi et al.[22] for total *O*-diphenols and total flavonoids. Mert et al.[24] studied the annual variation in the quantity of oleuropein in the leaves of the 'Gemlik' cultivar and demonstrated the same pattern observed in the present study. Changes in oleuropein levels in different flower and fruit developmental stages have also been reported previously. Thus, Malik and Bradford[29] found a sharp decrease in oleuropein level during the transition from vegetative to flower buds, followed by a rapid increase at the initial stages of fruit development and again a sharp decrease with fruit maturity.

3.2.2. Seasonal variability of phenolic compound groups

To analyze the seasonal variability in the phenolic content in olive leaves, total concentrations for each compound group were averaged for all samples (individuals and replicas) for each sampling time and cultivar. Considering the general mean of each phenolic compound group for all cultivars per sampling time (**Table 2**), an evident reduction in concentrations was observed in August with respect to June. From August on, the concentrations gradually increased until December. In fact, the mean secoiridoids concentration decreased from 46.82 mg/g dry olive leaves in June to 11.97 mg/g dry olive leaves in August, before starting to increase up to a maximum value in December, 47.81 mg/g dry olive leaves. Regarding flavonoids, mean values decreased from 3.18 mg/g dry olive leaves in June to 1.45 mg/g dry olive leaves in August, but returned to increasing in December, reaching 2.61 mg/g dry olive leaves.

Table 2. Mean values (mg/g of dry olive leaves) for the phenolic groups studied in the four sampling times[a]

	Cultivar	June	August	October	December
Group 1: Secoiridoids	'Arbequina'	44.22^{a,b}	09.86^{c}	24.14^{d}	37.20^{c}
	'Arbosana'	49.40^{a,b}	15.32^{a}	27.55^{d,c}	29.81^{c}
	'Changlot Real'	43.26^{b}	13.62^{b}	29.95^{b,c}	73.26^{a}
	'Koroneiki'	53.48^{a}	12.30^{b}	36.99^{a}	32.62^{d}
	'Picual'	45.93^{a,b}	12.59^{b}	32.78^{a,b}	70.69^{a}
	'Sikitita'	44.62^{a,b}	08.16^{d}	30.21^{b,c}	43.29^{b}
Group 2: Flavonoids	'Arbequina'	2.71^{c}	0.96^{c}	2.46^{c}	2.46^{c}
	'Arbosana'	5.30^{b}	2.00^{a}	3.10^{b}	3.20^{a,b}
	'Changlot Real'	0.65^{e}	1.68^{b}	1.82^{d}	2.95^{b}
	'Koroneiki'	6.11^{a}	1.74^{b}	3.57^{a}	1.72^{e}
	'Picual'	2.40^{c}	1.55^{b}	1.74^{d}	3.32^{a}
	'Sikitita'	1.90^{d}	0.76^{c}	0.96^{e}	2.03^{d}
Group 3: Simple phenols	'Arbequina'	1.27^{e}	0.43^{a}	0.96^{a}	2.13^{d}
	'Arbosana'	1.79^{c}	0.43^{a}	0.37^{e}	1.15^{e}
	'Changlot Real'	1.32^{e}	0.17^{d}	0.36^{e}	3.36^{b}
	'Koroneiki'	3.50^{a}	0.33^{c}	0.86^{b}	3.96^{a}
	'Picual'	1.53^{d}	0.35^{b,c}	0.70^{c}	2.87^{c}
	'Sikitita'	2.50^{b}	0.37^{b}	0.48^{d}	3.85^{a}
Group 4: Oleosides	'Arbequina'	1.42^{b}	0.22^{d}	0.72^{c,d}	0.93^{b}
	'Arbosana'	1.05^{d}	0.32^{b}	0.79^{b,c}	0.72^{c}
	'Changlot Real'	1.21^{c,d}	0.34^{b}	0.63^{d}	1.48^{a}
	'Koroneiki'	1.77^{a}	0.39^{a}	0.99^{a}	0.91^{b}
	'Picual'	1.33^{b,c}	0.40^{a}	0.87^{a,b}	1.50^{a}
	'Sikitita'	1.40^{b}	0.27^{c}	0.30^{e}	1.01^{b}
Group 5: Elenolic acids	'Arbequina'	0.30^{c}	0.13^{c}	0.29^{c}	0.17^{e}
	'Arbosana'	0.95^{a}	0.33^{a}	0.49^{a}	0.28^{d}
	'Changlot Real'	0.27^{c}	0.18^{b}	0.32^{c}	0.61^{b}
	'Koroneiki'	0.46^{b}	0.20^{b}	0.43^{b}	0.34^{c}
	'Picual'	0.22^{c,d}	0.20^{b}	0.40^{b}	0.68^{a}
	'Sikitita'	0.13^{d}	0.05^{d}	0.14^{d}	0.37^{c}

[a]Different letters indicate significant differences among cultivar by sampling time and phenolic group (p<0.001).

The mean concentrations of the simple phenols, oleosides, and elenolic acids groups decreased from 1.98, 1.36, and 0.39 mg/g dry olive leaves (June) to 0.35, 0.32, and 0.18 mg/g dry olive l leaves (August), respectively, while in December their concentrations reached 2.89, 1.09, and 0.41 mg/g dry olive leaves, respectively. The increase in elenolic acids content observed from August is

likely the result of some oleuropein degradation that is accentuated in autumn[30]. The same behavior was observed in all cultivars for the five groups, although some exceptions were noted, especially between concentrations in October and December **(Table 2)**. In fact, the concentration of the flavonoids group for 'Changlot Real' increased from June to December. The concentration of the flavonoids group for the 'Koroneiki' cultivar was decreased by one-half in December from its value in October. The concentration of the oleosides group showed a small decrease, by 8.2% and 8.9%, from October to December for the 'Koroneiki' and 'Arbosana' cultivars, respectively. Finally, the concentrations in the elenolic acids group decreased from October to December for the 'Koroneiki' and 'Arbequina' cultivars, displaying decreases of 20.8% and 42.6%, respectively. The large increase observed in winter in all phenolic groups probably occurred as a consequence of olive tree reaction to the low temperatures (**Figure a**, Supporting Information)

3.2.3. Comparison of phenolic compounds groups among cultivars

It is noteworthy that the concentration of each phenolic group was greatly dependent on the cultivar. In fact, the sum of squares (SS) distribution between sources of variation (cultivar and experimental error) showed a great influence of the cultivar on all sampling dates. Indeed, cultivar was responsible for 76%−99% of the variation in all groups. This result supports other studies reporting that genetic factors have a significant impact in the content of oleuropein in olive leaves[21,31]. Significant differences in phenolic compound composition have also been observed in the oils of different olive breeding selections[32]. In June, the 'Koroneiki' cultivar showed the highest concentrations of phenolic compounds for all cultivars and for all groups except the elenolic acid group, for which 'Arbosana' presented by far the highest concentration **(Table 2)**. In August, the 'Arbosana' cultivar presented the highest values for secoiridoids, flavonoids, simple phenols (with 'Arbequina'), and elenolic acids, while 'Koroneiki' and 'Picual' showed the highest concentrations for oleosides. In October, the highest

values for secoiridoids, oleosides, and flavonoids were registered for the 'Koroneiki' cultivar, whereas the highest value for simple phenols was registered for the 'Arbequina' cultivar. 'Arbosana' showed the highest concentration of elenolic acids. In contrast, the 'Sikitita' cultivar presented the lowest concentrations of flavonoids, oleosides, and elenolic acids in October. Except for simple phenols, the 'Picual' cultivar was the richest in terms of secoiridoids, oleosides, flavonoids, and elenolic acids among the studied samples in December. In addition, 'Changlot Real' also showed a high value of secoiridoids at that sampling time, with 73.25 mg/g dry olive leaves, insignificantly different from the 'Picual' cultivar with 70.69 mg/g dry olive leaves **(Table 2)**. Previous studies have reported that oleuropein concentration in the 'Picual' cultivar decreases significantly in fruits while increasing in leaves during olive ripening[33]. Many studies also stipulate that antioxidant capacity, assumed to be almost entirely due to phenolic compounds, is greatly dependent on the severity of the stress as well as the species and developmental stage[34]. Hence, differences observed are probably associated with each cultivar's resistance or tolerance to environmental conditions for each season and the plants' need for protection against external pathogenic agents, which are according to the genetic endowment.

3.2.4. Principal component analysis (PCA) of the phenolic content

Even though the seasonal tendency was similar for all compounds groups and cultivars, the plots in **Table 1** also show differences among cultivars or among compound groups. To study these differences, a principal component analysis (PCA) was applied to the data set containing the concentrations of the five previously described compound groups, including all 288 samples. Before applying the PCA, the data were preprocessed by applying a 10-base logarithm to the concentrations and mean and variance normalization. The logarithmic scaling puts the emphasis on relative variations in concentration (i.e., a change from 1 to 1.5 is more important than a change from 100 to 100.5), and the normalization

equalizes the different components of the data vector. The PCA analysis was performed using the MatLab function "princomp()". **Tables 3** and **4** show the variances of the principal components, the cumulative variance expressed as a percentage, and the coefficients providing the principal components as a function of the original components. As can be seen, the first and second principal components describe 88.4% of the data variability.

Table 3. Variances of principals' components.

Component	PC1	PC2	PC3	PC4	PC5
Variance	3.63	0.79	0.31	0.19	0.08
Cumulative variance (%)	72.61	88.44	94.63	89.41	100.00

Table 4. Coefficients of the principal components.

	PC1	PC2	PC3	PC4	PC5
Concentration Group 1	-0.4899	0.2266	-0.2894	0.3299	0.7184
Concentration Group 2	-0.3985	-0.5929	0.6784	0.0710	0.1560
Concentration Group 3	-0.4333	0.5101	0.2705	-0.6914	-0.0299
Concentration Group 4	-0.4874	0.2530	0.0276	0.5309	-0.6449
Concentration Group 5	-0.4194	-0.5224	-0.6181	-0.3554	-0.2070

According to the coefficients, principal components 1 and 2 can be computed from the normalized concentrations for different groups of compounds according to the following equations:

$$PC1 = -0.4899 * C_{G1} - 0.3985 * C_{G2} - 0.4333 * C_{G3} - 0.4874 * C_{G4} - 0.4194 * C_{G5}$$

$$PC2 = 0.2266 * C_{G1} - 0.5929 * C_{G2} + 0.5101 * C_{G3} + 0.2530 * C_{G4} - 0.5224 * C_{G5}$$

Under these equations, the first principal component is a weighted average of the normalized concentration for each group, where all of the original components contribute with a negative coefficient to the first PC (and, therefore, a decrease in this principal component represents a global increase in the phenolic content of

the sample). On the other hand, the second principal component is clearly dominated by the concentration of group 2 (although again with a negative coefficient; i.e., a decrease in PC2 can be roughly interpreted as an increase in the concentration of compounds in group 5).

Figures 2 and **3** represent scatter plots of the first and second principal components. To allow a detailed analysis of the PCA study, the scatter plots have been separated by seasons **(Figure 2)** and cultivars **(Figure 3)**. As can be seen, different cultivars can be separated in the scatter plots: the separation becomes difficult only between 'Picual' and 'Arbequina' in October, with 'Koroneiki' in August and December, and between 'Picual' and 'Changlot Real' in December. This separation reveals a strong dependence of the phenolic profile's seasonal pattern on the cultivar. This dependence has already been described in the paragraph above.

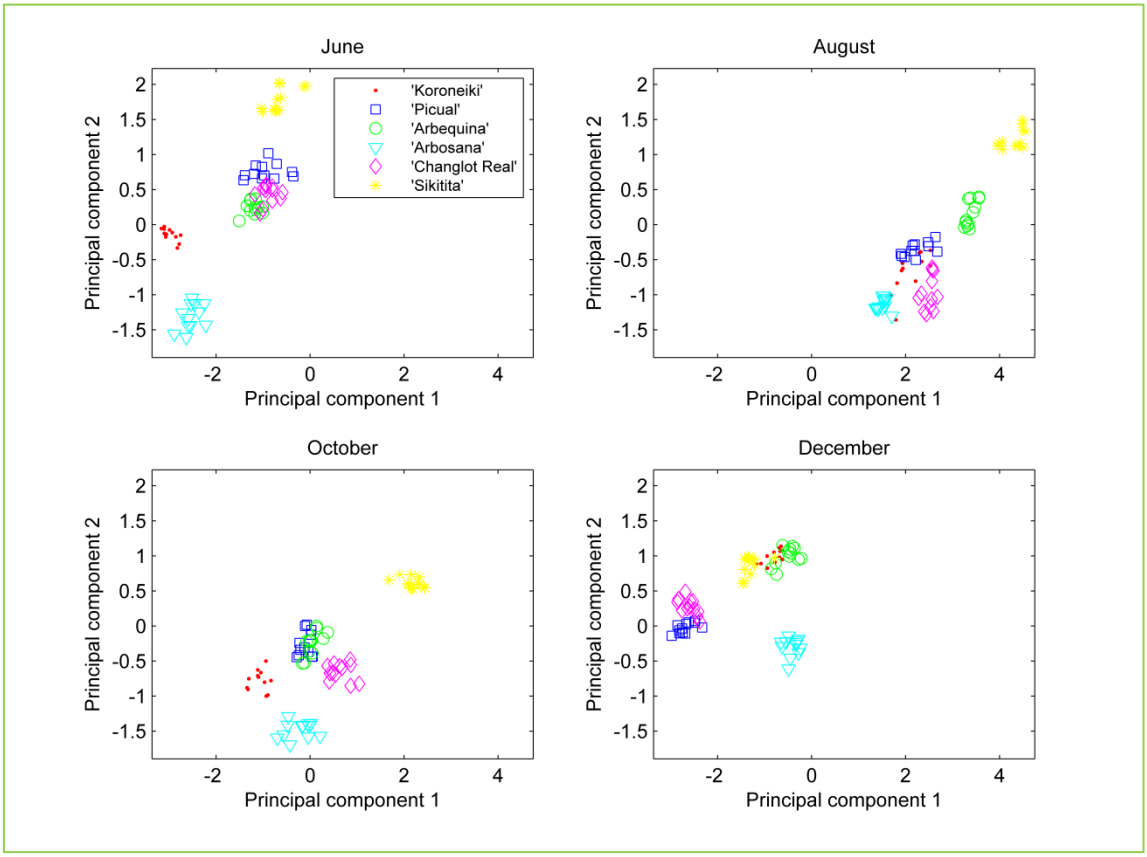

Figure 2. Scatter plots of the first and second principal components for sampling time.

Figure 3 reveals a strong dependence of the phenolic profile on the season, because the points corresponding to each season are clearly separable in each plot. In general, all of the cultivars showed global reductions in their phenolic contents in August (principal component 1 reaches its highest value at this sampling time for all cultivars). The largest increase in phenolic content (lowest value of PC1) was reached in June or December, depending on the cultivar. The plots in **Figure 3** evidence again the differences in the seasonal changes associated with each cultivar. According to these results, it can be concluded that the principal component analysis enables separation of the phenolic profile in olive leaves for different seasons and cultivars. This separation capacity is notable because PCA does not aim to discriminate among classes but to represent the maximum variability in the data with the minimum number of components, and reveal changes in the phenolic profile associated with the season (or seasonal changes in the environment) and with the cultivar.

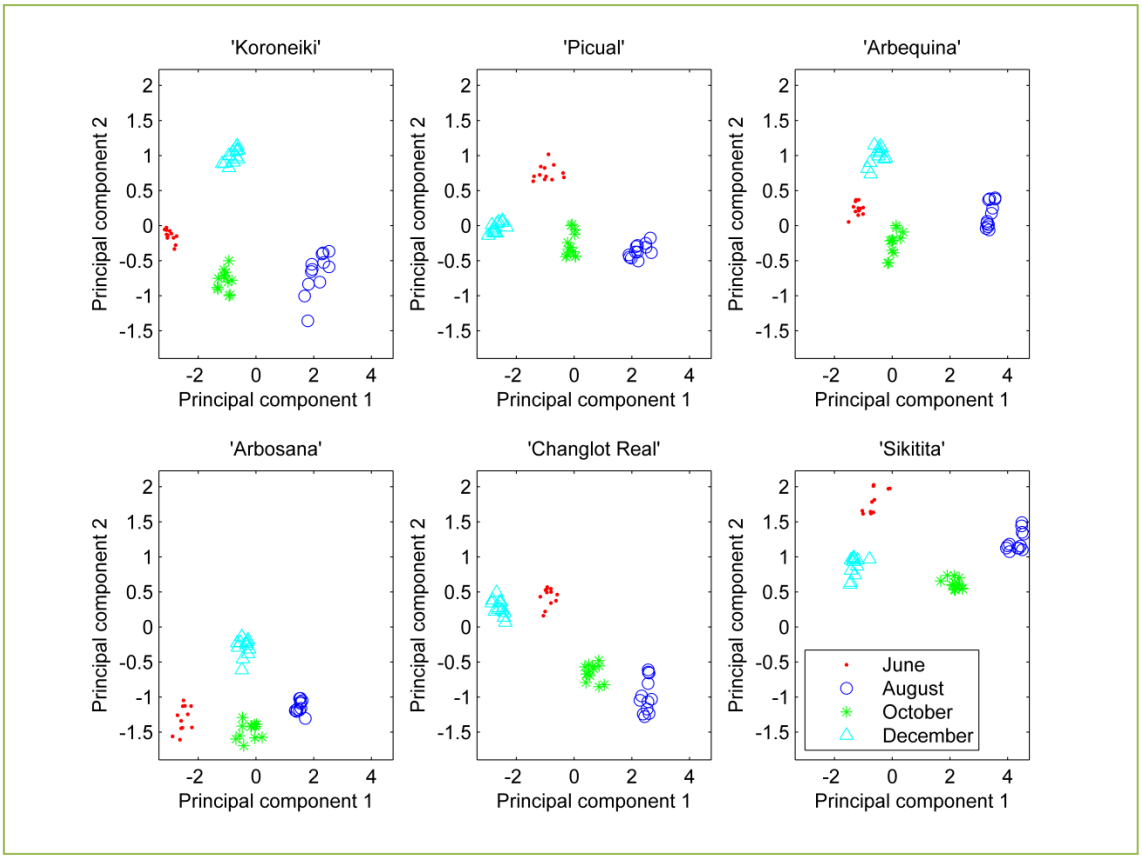

Figure 3. Scatter plots of the first and second principal components for sampling time.

4. Conclusions

In summary, olive leaves from different cultivars grown in the same orchard under the same environmental conditions and collected at different times showed marked differences in individual and total phenolic compound content. Despite these differences observed among cultivars, all of them showed a similar trend during ripening. The differences among seasons and cultivars should be taken into account for understanding the availability of phenolic compounds in each cultivar and the importance of seasonal changes in the context of the plants' biochemistry. On the other hand, knowledge of the phenolic profile for each season and cultivar is useful when the olive leaves are considered as a source of specific phenolic compounds, because the availability of each compound depends on both the season and the cultivar. To our knowledge, the present study

reports the seasonal variability of a large number of phenolic compounds for the first time. At the studied sampling times, June and December seem to be the best periods to use olive leaves as a source of phenolic compounds as both of them present similar concentrations of phenolic compounds. In practice, the results shown in December could be rewarding as it coincides with the fruit harvesting period in Andalusia (Spain), the region where the study was performed, during which 432 984 tons of olive leaves is produced annually[16]. In this period, olive leaves could be valorized efficiently as olive by-products.

References

(1) Latouche, G.; Bellow, S.; Poutaraud, A.; Meyer, S.; Cerovic, Z. G. Influence of constitutive phenolic compounds on the response of grapevine (*Vitis vinifera* L.) leaves to infection by *Plasmopara viticola*. *Planta* **2013**, 237, 351–61.

(2) Bennett, B.; Richard, N.; Wallsgrove, R. M. Secondary metabolites in plant defence mechanisms. *New Phytol*. **1994**, 127, 617–633.

(3) Gómez-Caravaca, A. M.; Verardo, V.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Caboni, M. F. Phenolic compounds and saponins in plants grown under different irrigation regimes. *In stress and polyphenols in plants*; 2013; Vol. Watson, pp. 37–48.

(4) Ben Abdallah, S.; Rabhi, M.; Harbaoui, F.; Zar-kalai, F.; Lachâal, M.; Karray-Bouraoui, N. Distribution of phenolic compounds and antioxidant activity between young and old leaves of *Carthamus tinctorius* L. and their induction by salt stress. *Acta Physiol. Plant*. **2012**, 35, 1161–1169.

(5) Yordanov, I.; Velikova, V.; Tsonev, T. Plant responses to drought and stress tolerance. *Bulg. J. Plant Physiol*. **2003**, 187–206.

(6) Palese, A. M.; Nuzzo, V.; Favati, F.; Pietrafesa, A.; Celano, G.; Xiloyannis, C. Effects of water deficit on the vegetative response, yield and oil quality of olive trees (*Olea europaea* L., cv. Coratina) grown under intensive cultivation. *Sci. Hortic.* (*Amsterdam, Neth.*). **2010**, 125, 222–229.

(7) Lattanzio, V. Bioactive Polyphenols : Their role in quality and storability of fruit and vegetables. *J. Appl. Bot*. **2003**, 77, 128–146.

(8) Kliebenstein, D. J. Plant defense compounds: Systems approaches to metabolic analysis. *Annu. Rev. Phytopathol*. **2012**, 50, 155–73.

(9) Seigler, D. S. *Plant Secondary Metabolism*; Kluwer Academic Publishers, Boston MA., 1998; p. 711.

(10) Ghanbari, R.; Anwar, F.; Alkharfy, K. M.; Gilani, A.-H.; Saari, N. Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.)-A review. *Int. J. Mol. Sci.* **2012**, 13, 3291–3340.

(11) Sofo, A.; Manfreda, S.; Fiorentino, M.; Dichio, B.; Xiloyannis, C. The olive tree : A paradigm for drought tolerance in Mediterranean climates. *Hydrol. Earth Syst. Sci.* **2008**, 12, 293–301.

(12) Bacelar, E. A.; Santos, D. L.; Moutinho-Pereira, J. M.; Lopes, J. I.; Gonçalves, B. C.; Ferreira, T. C.; Correia, C. M. Physiological behaviour, oxidative damage and antioxidative protection of olive trees grown under different irrigation regimes. *Plant Soil **2007***, 292, 1–12.

(13) Del Río, J. A.; Báidez, A. G.; Botía, J. M.; Ortuño, A. Enhancement of phenolic compounds in olive plants (*Olea europaea* L.) and their influence on resistance against *Phytophthora sp*. *Food Chem*. **2003**, 83, 75–78.

(14) El Aabidine, A. Z.; Baissac, Y.; Moukhli, A.; Jay-Allemand, C.; Khadari, B.; El Modafar, C. Resistance of olive tree to *Spilocaea oleagina* is mediated by the synthesis of phenolic compounds. *Int. J. Agric. Biol*. **2010**, 12, 61–67.

(15) Báidez, A. G.; Gómez, P.; Del Río, J. A.; Ortuño, A. Dysfunctionality of the xylem in *Olea europaea* L. Plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. *J. Agric. Food Chem.* **2007**, 55, 3373–3377.

(16) López, J. A. C.; Heras, T. P.; Gordillo, T. M. Potencial energético de los subproductos de la industria olivarera en Andalucía; *Secretaría General Del Medio Rural Y la Producción Ecolgóica Junta de Andalucia*, 2010; pp. 1–68.

(17) Molina-Alcaide, E.; Yáñez-Ruiz, D. R. Potential use of olive by-products in ruminant feeding: A review. *Anim. Feed Sci. Technol*. **2008**, 147, 247–264.

(18) D'Andria, R.; Di Salle, A.; Petillo, O.; Sorrentino, G.; Peluso, G. Nutraceutical, cosmetic, health products derived from olive: Present and future of the Mediterranean olive sector. *Options Méditerranéennes, A* **2013**, 106, 153–161.

(19) Susalit, E.; Agus, N.; Effendi, I.; Tjandrawinata, R. R.; Nofiarny, D.; Perrinjaquet-Moccetti, T.; Verbruggen, M. Olive (*Olea europaea*) leaf extract effective in patients with stage-1 hypertension: Comparison with Captopril. *Phytomedicine* **2011**, 18, 251–258.

(20) Sada, A. Olive Leaf. *Altern. Med. Rev*. Vol. **2009**, 14, 62–66.

(21) Ortega-García, F.; Peragón, J. Phenol metabolism in the leaves of the olive tree (*Olea europaea* L.) cv. Picual, Verdial, Arbequina, and Frantoio during ripening. *J. Agric. Food Chem*. **2010**, 58, 12440–12448.

(22) Brahmi, F.; Mechri, B.; Dhibi, M.; Hammami, M. Variations in phenolic compounds and antiradical scavenging activity of *Olea europaea* leaves and fruits extracts collected in two different seasons. *Ind. Crops Prod.* **2013**, 49, 256–264.

(23) Petridis, A.; Therios, I.; Samouris, G.; Koundouras, S.; Giannakoula, A. Effect of water deficit on leaf phenolic composition, gas exchange, oxidative damage and antioxidant activity of four Greek olive (*Olea europaea* L.) cultivars. *Plant Physiol. Biochem.* **2012**, 60, 1–11.

(24) Mert, C.; Barut, E.; Ipek, A. Quantitative seasonal changes in the leaf phenolic content related to the alternate-bearing patterns of olive (*Olea europaea* L. cv. Gemlik). *J. Agric. Sci. Technol*. **2013**, 15, 995–1006.

(25) Hashemi, P.; Delfan, B.; Ghiasvand, A. R.; Alborzi, M.; Raeisi, F. A study of the effects of cultivation variety, collection time, and climate on the amount of oleuropein in olive leaves. *Acta Chromatogr*. **2010**, 22, 133–140.

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

(27) Rallo, L.; Barranco, D.; De la Rosa, R.; León, L. "Chiquitita" Olive. *HortScience* **2008**, 43, 529–531.

(28) Papoti, V. T.; Tsimidou, M. Z. Impact of sampling parameters on the radical scavenging potential of olive (*Olea europaea* L.) leaves. *J. Agric. Food Chem*. **2009**, 57, 3470–3477.

(29) Malik, N. S. A.; Bradford, J. M. Changes in oleuropein levels during differentiation and development of floral buds in 'Arbequina' olives. *Sci. Hortic. (Amsterdam, Neth.)*. **2006**, 110, 274–278.

(30) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Importance and evolution of phenolic compounds in olive during growth and maturation. *J. Agric. Food Chem*. **1986**, 34, 823–826.

(31) Ranalli, A.; Contento, S.; Lucera, L.; Di Febo, M.; Marchegiani, D.; Di Fonzo, V. Factors affecting the contents of iridoid oleuropein in olive leaves (*Olea europaea* L.). *J. Agric. Food Chem*. **2006**, 54, 434–440.

(32) El Riachy, M.; Priego-Capote, F.; Rallo, L.; Luque-de Castro, M. D.; León, L. Phenolic composition of virgin olive oils from cross breeding segregating populations. *Eur. J. Lipid Sci. Technol*. **2012**, 114, 542–551.

(33) Ortega-García, F.; Blanco, S.; Peinado, M. A.; Peragón, J. Polyphenol oxidase and its relationship with oleuropein concentration in fruits and leaves of olive (*Olea europaea*) cv. Picual trees during fruit ripening. *Tree Physiol. **2008***, 28, 45–54.

(34) Chaves, M. M.; Maroco, J. P.; Pereira, J. S. Understanding plant responses to drought from genes to the whole plant. *Funct. Plant Biol*. **2003**, 30, 239–264.

Supporting information

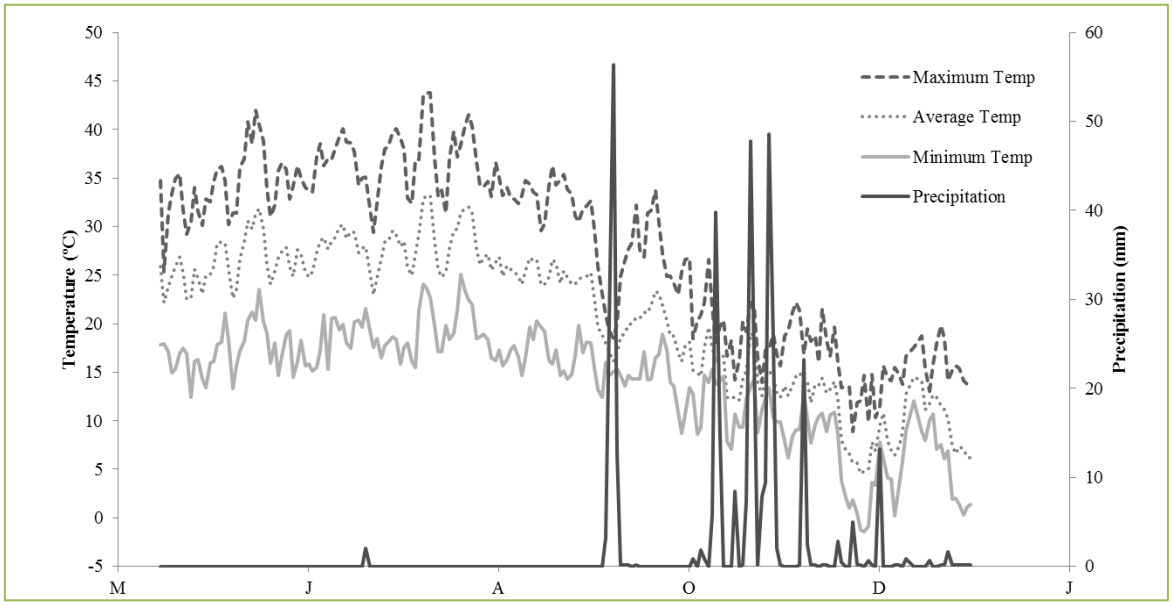

Figure a. Climatic data during the studied sampling times: J(June), A(August), O (October), D (December).

Table a. Quantification of the identified phenolic compounds in olive leaves of 'Arbosana' cultivar expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

Phenolic compounds 'Arbosana'	**Sampling time**			
	June	**August**	**October**	**December**
Oleoside	0.02^{c}	0.01^{d}	0.02^{b}	0.03^{a}
Hydroxytyrosol-hexose isomer a	1.05^{a}	0.22^{c}	0.12^{d}	0.40^{b}
Hydroxytyrosol-hexose isomer b	0.74^{a}	0.22^{c}	0.25^{c}	0.57^{b}
Secologanoside isomer a	0.57^{a}	0.23^{c}	0.42^{b}	0.43^{b}
Tyrosol glucoside	n.d	n.d	n.d	0.18
Elenolic acid glucoside isomer a	0.76^{a}	0.23^{c}	0.35^{b}	0.13^{d}
Secologanoside isomer b	0.46^{a}	0.07^{d}	0.35^{b}	0.26^{c}
Elenolic acid glucoside isomer b	0.11^{a}	0.03^{d}	0.07^{b}	0.05^{c}
Oleuropein aglycon	0.07^{a}	0.03^{d}	0.04^{c}	0.05^{b}
Luteolin-diglucoside	0.31^{a}	0.25^{c}	0.27^{bc}	0.29^{ab}
Elenolic acid glucoside isomer c	0.08^{b}	0.07^{b}	0.07^{b}	0.10^{a}
Demethyloleuropein	1.39^{a}	0.39^{c}	0.52^{b}	0.31^{c}
Rutin	1.49^{a}	0.54^{c}	0.71^{b}	0.57^{c}
Luteolin rutinoside	0.97^{a}	0.41^{c}	0.51^{b}	0.44^{c}
Luteolin glucoside isomer a	1.17^{a}	0.37^{c}	0.83^{b}	0.88^{b}
Apigenin rutinoside	0.19^{b}	0.07^{d}	0.10^{c}	0.24^{a}
Oleuropein diglucoside isomer a	0.13^{b}	0.15^{a}	0.09^{d}	0.10^{c}
Oleuropein diglucoside isomer b	0.36^{a}	0.13^{b}	0.12^{b}	0.14^{b}
Oleuropein diglucoside isomer c	0.58^{a}	0.37^{c}	0.41^{bc}	0.46^{b}
Luteolin glucoside isomer b	1.04^{a}	0.31^{d}	0.61^{c}	0.70^{b}
Chrysoeriol-7-*O*-glucoside	0.08^{a}	0.03^{d}	0.04^{c}	0.05^{b}
2"-Methoxyoleuropein isomer a	0.27^{a}	0.16^{b}	0.17^{b}	0.03^{c}
2"-Methoxyoleuropein isomer b	0.42^{a}	0.23^{b}	0.43^{a}	0.15^{c}
Oleuropein isomer a	43.07^{a}	12.83^{d}	24.17^{b}	26.50^{c}
Oleuropein isomer b	0.74^{a}	0.22^{d}	0.30^{c}	0.42^{b}
Oleuropein/Oleuroside	2.23^{a}	0.70^{d}	0.89^{c}	1.36^{b}
Ligstroside	0.16^{c}	0.11^{d}	0.42^{a}	0.29^{b}
Luteolin	0.04^{a}	0.02^{b}	0.02^{b}	0.02^{b}
Total	$\mathbf{58.48^{a}}$	$\mathbf{18.39^{c}}$	$\mathbf{32.30^{b}}$	$\mathbf{35.16^{b}}$

n.d (non determined).

Table b. Analytical parameters of the method proposed.

Analytes	RSD %	LOD (µg/L)	LOQ (µg/L)	Calibration range (10^3µg/L)	Calibration equations	r^2
Apigenin	0.37	0.53	1.76	LOQ 1.25-5	y = 365026x + 89376	r^2 = 0.9941
	1.50	1.83	6.10	LOQ 5-80	y = 105448x + 2E+06	r^2 = 0.9950
Hydroxytyrosol	0.54	5.38	17.95	LOQ 1.25-80	y = 35824x + 117962	r^2 = 0.9951
Luteolin	3.70	0.56	1.88	LOQ 1.25-5	y = 342636x - 9007.4	r^2 = 0.9975
	0.30	2.02	6.75	LOQ 5-80	y = 95266x + 1E+06	r^2 = 0.9934
Oleuropein	4.42	0.62	2.08	LOQ 1.25-5	y = 309291x + 37581	r^2 = 0.9936
	0.73	2.49	8.29	LOQ 5-80	y = 77570x + 1E+06	r^2 = 0.9956
	0.64	22.38	74.60	LOQ 500-1000	y = 8618.7x + 8E+06	r^2 = 0.9857
Tyrosol	1.44	27.63	92.10	LOQ 20-80	y = 6981.4x - 38670	r^2 = 0.9892

Table c. Quantification of the identified phenolic compounds in olive leaves of 'Changlot Real' cultivar expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

Phenolic compounds 'Changlot Real'	**Sampling time**			
	June	**August**	**October**	**December**
Oleoside	0.02^{c}	0.01^{d}	0.03^{b}	0.05^{a}
Hydroxytyrosol-hexose isomer a	0.05^{b}	0.03^{c}	0.02^{c}	0.13^{a}
Hydroxytyrosol-hexose isomer b	1.05^{b}	0.10^{c}	0.26^{c}	1.72^{a}
Secologanoside isomer a	0.50^{b}	0.18^{d}	0.43^{c}	0.75^{a}
Tyrosol glucoside	0.22^{b}	0.05^{c}	0.08^{c}	1.51^{a}
Elenolic acid glucoside isomer a	0.03^{b}	0.02^{b}	0.02^{b}	0.06^{a}
Secologanoside isomer b	0.69^{a}	0.14^{b}	0.16^{b}	0.68^{a}
Elenolic acid glucoside isomer b	0.06^{c}	0.09^{b}	0.10^{b}	0.22^{a}
Oleuropein aglycon	0.06^{b}	0.06^{b}	0.07^{b}	0.32^{a}
Luteolin-diglucoside	0.04^{c}	0.16^{a}	0.11^{b}	$0.15^{a,b}$
Elenolic acid glucoside isomer c	0.18^{b}	0.07^{c}	0.20^{b}	0.34^{a}
Demethyloleuropein	1.02^{a}	0.41^{c}	0.39^{c}	0.75^{b}
Rutin	0.03^{c}	0.09^{a}	0.05^{b}	0.08^{a}
Luteolin rutinoside	0.04^{c}	0.15^{a}	0.07^{b}	0.13^{a}
Luteolin glucoside isomer a	0.28^{d}	0.62^{c}	0.81^{b}	1.39^{a}
Apigenin rutinoside	0.06^{d}	0.18^{b}	0.12^{c}	0.22^{a}
Oleuropein diglucoside isomer a	0.04^{c}	0.07^{b}	0.04^{c}	0.13^{a}
Oleuropein diglucoside isomer b	0.07^{c}	0.13^{b}	0.04^{d}	0.24^{a}
Oleuropein diglucoside isomer c	0.48^{b}	0.21^{c}	0.24^{c}	0.74^{a}
Luteolin glucoside isomer b	0.15^{d}	0.36^{c}	0.49^{b}	0.81^{a}
Chrysoeriol-7-*O*-glucoside	0.04^{c}	0.09^{b}	0.13^{a}	0.12^{a}
2"-Methoxyoleuropein isomer a	0.36^{a}	0.26^{b}	0.20^{c}	0.35^{a}
2"-Methoxyoleuropein isomer b	0.58^{a}	0.44^{b}	$0.52^{a,b}$	0.55^{a}
Oleuropein isomer a	38.02^{b}	11.56^{d}	26.79^{c}	64.08^{a}
Oleuropein isomer b	0.64^{b}	0.09^{d}	0.34^{c}	1.41^{a}
Oleuropein/Oleuroside	1.20^{b}	0.27^{d}	0.87^{c}	2.98^{a}
Ligstroside	0.77^{b}	0.12^{d}	0.45^{c}	1.71^{a}
Luteolin	0.01^{c}	0.05^{a}	0.03^{b}	0.05^{a}
Total	**76.72^{b}**	**15.99^{d}**	**33.07^{c}**	**81.65^{a}**

Table d. Quantification of the identified phenolic compounds in olive leaves of 'Sikitita' cultivar expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

Phenolic compounds 'Sikitita'	**Sampling time**			
	June	**August**	**October**	**December**
Oleoside	0.02^{c}	0.004^{d}	0.03^{b}	0.03^{a}
Hydroxytyrosol-hexose isomer a	0.34^{b}	0.08^{c}	0.03^{c}	0.79^{a}
Hydroxytyrosol-hexose isomer b	1.73^{a}	0.21^{c}	0.10^{d}	1.62^{b}
Secologanoside isomer a	0.37^{b}	0.09^{d}	0.13^{c}	0.51^{a}
Tyrosol glucoside	0.44^{b}	0.07^{d}	0.34^{c}	1.44^{a}
Elenolic acid glucoside isomer a	0.01^{b}	0.01^{c}	0.02^{b}	0.02^{a}
Secologanoside isomer b	1.00^{a}	0.17^{c}	0.14^{c}	0.47^{b}
Elenolic acid glucoside isomer b	0.05^{b}	0.02^{c}	0.05^{b}	0.08^{a}
Oleuropein aglycon	0.07^{b}	0.02^{d}	0.05^{c}	0.10^{a}
Luteolin-diglucoside	0.04^{c}	0.08^{b}	0.08^{b}	0.15^{a}
Elenolic acid glucoside isomer c	0.07^{b}	0.02^{c}	0.07^{b}	0.28^{a}
Demethyloleuropein	2.00^{a}	0.28^{b}	0.15^{c}	0.21^{bc}
Rutin	0.23^{a}	0.07^{d}	0.13^{c}	0.19^{b}
Luteolin rutinoside	0.27^{a}	0.11^{c}	0.11^{c}	0.19^{b}
Luteolin glucoside isomer a	0.76^{a}	0.23^{b}	0.27^{b}	0.79^{a}
Apigenin rutinoside	0.06^{b}	0.02^{d}	0.07^{a}	0.03^{c}
Oleuropein diglucoside isomer a	0.07^{b}	0.07^{c}	0.06^{c}	0.13^{a}
Oleuropein diglucoside isomer b	0.25^{b}	0.15^{c}	0.09^{d}	0.26^{a}
Oleuropein diglucoside isomer c	0.49^{a}	0.28^{b}	0.16c	0.50^{a}
Luteolin glucoside isomer b	0.45^{b}	0.20^{c}	0.21^{c}	0.63^{a}
Chrysoeriol-7-*O*-glucoside	0.08^{a}	0.02^{d}	0.07^{b}	0.06^{c}
2"-Methoxyoleuropein isomer a	0.50^{a}	0.24^{b}	0.14^{c}	0.02^{d}
2"-Methoxyoleuropein isomer b	0.76^{a}	0.29^{b}	0.22^{c}	0.04^{d}
Oleuropein isomer a	37.83^{a}	6.37^{c}	28.71^{b}	38.78^{a}
Oleuropein isomer b	0.60^{b}	0.05^{d}	0.13^{c}	0.70^{a}
Oleuropein/Oleuroside	1.34^{b}	0.23^{c}	0.31^{c}	1.84^{a}
Ligstroside	0.80^{a}	0.18^{c}	0.19^{c}	0.71^{b}
Luteolin	0.02^{a}	0.01^{b}	0.01^{b}	0.01^{c}
Total	**50.65^{a}**	**9.60^{c}**	**32.08^{b}**	**50.56^{a}**

Table e. Quantification of the identified phenolic compounds in olive leaves of 'Picual' cultivar expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

Phenolic compounds 'Picual'	**Sampling time**			
	June	**August**	**October**	**December**
Oleoside	0.01^{c}	0.006^{d}	0.03^{b}	0.05^{a}
Hydroxytyrosol-hexose isomer a	0.38^{b}	0.09^{c}	0.16^{c}	0.85^{a}
Hydroxytyrosol-hexose isomer b	0.94^{b}	0.21^{d}	0.42^{c}	1.40^{a}
Secologanoside isomer a	0.43^{c}	0.17^{d}	0.51^{b}	0.90^{a}
Tyrosol glucoside	0.20^{b}	0.05^{d}	0.11^{c}	0.62^{a}
Elenolic acid glucoside isomer a	0.06^{a}	0.07^{b}	0.03^{c}	0.06^{a}
Secologanoside isomer b	0.89^{a}	0.23^{d}	0.33^{c}	0.54^{b}
Elenolic acid glucoside isomer b	0.07^{b}	0.06^{c}	0.06^{c}	0.10^{a}
Oleuropein aglycon	0.22^{b}	0.09^{d}	0.12^{c}	0.24^{a}
Luteolin-diglucoside	0.06^{c}	$0.16^{a,b}$	0.14^{b}	0.17^{a}
Elenolic acid glucoside isomer c	0.09^{c}	0.06^{c}	0.31^{b}	0.52^{a}
Demethyloleuropein	0.01^{c}	0.35^{a}	0.03^{c}	0.16^{b}
Rutin	0.09^{c}	0.12^{b}	0.07^{c}	0.27^{a}
Luteolin rutinoside	0.17^{c}	0.22^{b}	$0.19^{b,c}$	0.35^{a}
Luteolin glucoside isomer a	0.87^{b}	0.46^{d}	0.71^{c}	1.39^{a}
Apigenin rutinoside	0.09^{a}	0.11^{a}	0.05^{c}	0.08^{b}
Oleuropein diglucoside isomer a	0.05^{c}	0.10^{b}	0.10^{b}	0.25^{a}
Oleuropein diglucoside isomer b	0.09^{d}	0.18^{c}	0.23^{b}	0.40^{a}
Oleuropein diglucoside isomer c	0.20^{c}	0.36^{b}	0.33^{b}	0.56^{a}
Luteolin glucoside isomer b	0.99^{a}	0.35^{c}	0.52^{b}	0.90^{a}
Chrysoeriol-7-*O*-glucoside	0.09^{b}	0.10^{b}	0.05^{c}	0.15^{a}
2"-Methoxyoleuropein isomer a	0.17^{a}	0.13^{b}	0.17^{a}	0.04^{c}
2"-Methoxyoleuropein isomer b	0.25^{b}	0.25^{b}	0.60^{a}	0.05^{c}
Oleuropein isomer a	43.24^{b}	10.49^{d}	29.24^{c}	63.64^{a}
Oleuropein isomer b	0.70^{b}	0.15^{d}	0.39^{c}	1.51^{a}
Oleuropein/Oleuroside	0.37^{c}	0.40^{c}	1.17^{b}	3.08^{a}
Ligstroside	0.63^{b}	0.11^{d}	0.39^{c}	0.77^{a}
Luteolin	0.04^{a}	0.03^{b}	0.006^{d}	0.01^{b}
Total	**51.41^{b}**	**15.09^{d}**	**36.48^{c}**	**79.05^{a}**

Table e. Quantification of the identified phenolic compounds in olive leaves of 'Arbequina' cultivar expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

Phenolic compounds	**Sampling time**			
'Arbequina'	**June**	**August**	**October**	**December**
Oleoside	0.02^{b}	0.004^{d}	0.02^{c}	0.03^{a}
Hydroxytyrosol-hexose isomer a	0.11^{d}	0.20^{b}	0.16^{c}	0.35^{a}
Hydroxytyrosol-hexose isomer b	0.95^{b}	0.19^{d}	0.63^{c}	1.17^{a}
Secologanoside isomer a	0.44^{a}	0.10^{c}	0.33^{b}	0.45^{a}
Tyrosol glucoside	0.20^{b}	0.04^{c}	0.16^{b}	0.62^{a}
Elenolic acid glucoside isomer a	0.14^{b}	0.11^{c}	0.21^{a}	0.03^{d}
Secologanoside isomer b	0.95^{a}	0.12^{d}	0.37^{c}	0.45^{b}
Elenolic acid glucoside isomer b	0.06^{b}	0.02^{d}	0.04^{c}	0.10^{a}
Oleuropein aglycon	0.05^{b}	0.01^{d}	0.03^{c}	0.07^{a}
Luteolin-diglucoside	0.22^{b}	0.11^{c}	0.29^{a}	0.22^{b}
Elenolic acid glucoside isomer c	0.10^{a}	0.01^{c}	0.04^{b}	0.05^{b}
Demethyloleuropein	1.58^{a}	0.22^{c}	0.24^{c}	0.47^{b}
Rutin	0.22^{b}	0.11^{c}	0.25^{a}	0.21^{b}
Luteolin rutinoside	0.70^{a}	0.24^{c}	0.44^{b}	0.44^{b}
Luteolin glucoside isomer a	0.79^{a}	0.24^{c}	0.67^{b}	0.77^{a}
Apigenin rutinoside	0.06^{a}	0.03^{c}	0.05^{b}	0.06^{a}
Oleuropein diglucoside isomer a	0.10^{b}	0.07^{c}	0.12^{a}	0.09^{b}
Oleuropein diglucoside isomer b	0.15^{a}	0.12^{b}	0.10^{b}	0.17^{a}
Oleuropein diglucoside isomer c	0.67^{a}	0.19^{d}	0.48^{c}	0.54^{b}
Luteolin glucoside isomer b	0.57^{b}	0.20^{c}	0.56^{b}	0.63^{a}
Chrysoeriol-7-*O*-glucoside	0.11^{b}	0.03^{c}	0.17^{a}	0.10^{b}
2"-Methoxyoleuropein isomer a	0.67^{a}	0.17^{c}	0.48^{b}	0.03^{d}
2"-Methoxyoleuropein isomer b	1.02^{a}	0.20^{c}	0.69^{b}	0.15^{c}
Oleuropein isomer a	37.10^{a}	8.10^{d}	20.52^{c}	32.95^{b}
Oleuropein isomer b	0.57^{a}	0.09^{d}	0.18^{c}	0.48^{b}
Oleuropein/Oleuroside	1.48^{a}	0.43^{c}	0.75^{b}	1.55^{a}
Ligstroside	0.82^{a}	0.26^{d}	0.56^{c}	0.70^{b}
Luteolin	0.02^{b}	0.01^{c}	0.03^{a}	0.03^{ab}
Total	**49.92^{a}**	**11.60^{d}**	**28.57^{c}**	**42.89^{b}**

Table e. Quantification of the identified phenolic compounds in olive leaves of 'Koroneiki' cultivar expressed as mg/g of dry olive leaf. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

Phenolic compounds 'Koroneiki'	**Sampling time**			
	June	**August**	**October**	**December**
Oleoside	0.05^{a}	0.01^{c}	0.03^{b}	0.03^{b}
Hydroxytyrosol-hexose isomer a	1.36^{a}	0.14^{c}	0.22^{c}	0.60^{b}
Hydroxytyrosol-hexose isomer b	1.63^{a}	0.15^{d}	0.51^{c}	1.23^{b}
Secologanoside isomer a	0.67^{a}	0.14^{d}	0.51^{b}	0.40^{c}
Tyrosol glucoside	0.51^{b}	0.04^{c}	0.13^{c}	2.13^{a}
Elenolic acid glucoside isomer a	0.03^{c}	0.07^{b}	0.11^{a}	0.08^{b}
Secologanoside isomer b	1.04^{a}	0.25^{c}	0.45^{b}	0.48^{b}
Elenolic acid glucoside isomer b	0.11^{a}	0.06^{c}	0.10^{b}	0.04^{c}
Oleuropein aglycon	0.28^{a}	0.09^{c}	0.17^{d}	0.21^{b}
Luteolin-diglucoside	0.42^{a}	0.14^{c}	0.26^{b}	0.07^{d}
Elenolic acid glucoside isomer c	0.32^{a}	0.07^{c}	0.21^{b}	0.22^{b}
Demethyloleuropein	2.19^{a}	0.48^{b}	0.38^{b}	0.16^{c}
Rutin	1.26^{a}	0.30^{c}	0.68^{b}	0.29^{c}
Luteolin rutinoside	0.77^{a}	0.50^{b}	0.46^{b}	0.26^{c}
Luteolin glucoside isomer a	1.91^{a}	0.34^{d}	1.09^{b}	0.62^{c}
Apigenin rutinoside	0.14^{a}	0.04^{c}	0.12^{b}	0.04^{c}
Oleuropein diglucoside isomer a	0.11^{a}	0.09^{b}	0.12^{a}	0.09^{b}
Oleuropein diglucoside isomer b	0.44^{a}	0.23^{b}	0.19^{c}	0.17^{c}
Oleuropein diglucoside isomer c	0.65^{a}	0.35^{c}	0.32^{b,c}	0.33^{b,c}
Luteolin glucoside isomer b	1.31^{a}	0.34^{d}	0.76^{b}	0.40^{c}
Chrysoeriol-7-*O*-glucoside	0.24^{a}	0.08^{c}	0.19^{b}	0.05^{c}
2"-Methoxyoleuropein isomer a	0.78^{a}	0.16^{c}	0.30^{b}	0.01^{d}
2"-Methoxyoleuropein isomer b	1.34^{a}	0.40^{c}	0.69^{b}	0.02^{d}
Oleuropein isomer a	44.04^{a}	9.86^{d}	32.36^{b}	29.02^{c}
Oleuropein isomer b	0.97^{a}	0.12^{c}	0.56^{b}	0.56^{b}
Oleuropein/Oleuroside	2.07^{a}	0.34^{c}	1.31^{b}	1.24^{b}
Ligstroside	0.60^{b}	0.18^{c}	0.59^{b}	0.80^{a}
Luteolin	0.05^{a}	0.01^{c}	0.02^{b}	0.004^{d}
Total	**65.32^{a}**	**14.96^{c}**	**42.85^{b}**	**39.55^{b}**

CHAPTER 4. Phenolic compounds and *in vitro* immunomodulatory properties of three Andalusian olive leaf extracts

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Abstract

In the present study, it has been effected an analytical determinations of phenolic compounds in extracts of three Andalusian olive leaf cultivars using HPLC-DAD-TOF-MS. Then, the determination of the immunomodulatory properties of the whole phenolic extracts has been carried out on RAW 264 mouse macrophages as a preliminary *in vitro* study. Twenty-eight phenolic compounds were determined in the olive leaf extracts and high contents in total phenolic compounds were shown, particularly for 'Picual' cultivar. In addition, all olive leaf extracts inhibited the release of the pro-inflammatory mediator nitric oxide in LPS-stimulated RAW264.7 cells revealing their immunomodulatory properties. As a preliminary result, it could be deduced that the inhibition of NO by the olive leaf extract may depend on the type of phenolic compounds rather than on phenolic total contents. It is important to highlight that this is the first time that whole phenolic extracts of olive leaves have been used to *in vitro* study the anti-inflammatory properties.

Keywords: Olive leaf extracts, phenolic compounds, HPLC-DAD-TOF-MS, *in vitro* essays.

1. Introduction

Olive leaves (*Olea europaea*) have been used as folk medicine throughout the history of civilization in the Mediterranean area. However, they became important when olive leaf extract was reported to be effective in treating fever and malaria in 1854[1]. Current scientific researchers have reported that olive leaves contain phenolic compounds responsible for several biological activities, including antioxidant and anti-inflammatory, antimicrobial, antiviral, anti-carcinogenic, as well as beneficial cardiovascular effects[2–5]. The majority of studies attribute active biological activities of olive leaf extracts to total phenols or individual phenolic compounds such as secoiridoids and, particularly, oleuropein[6] and hydroxytyrosol[7], and to flavonoids such as diosmetin, quercetin, luteolin, apigenin and their derivatives[8,9].

The major classes of phenolic compounds in olive leaf extract are reported to be phenolic acids, phenolic alcohols, flavonoids and secoiridoids, mainly vanillic acid, caffeic acid, hydroxytyrosol, tyrosol, rutin, verbascoside, luteolin, quercetin, oleuropein, demethyloleuropein and ligstroside[10].

When considering the potential effects of these active compounds as anti-inflammatory agents, it is interesting to note that nitric oxide (NO) has been proposed to play a key role in the pathogenesis of the inflammatory response. NO is a free radical gas messenger molecule with both intra and extracellular regulatory functions for many cells. Endogenous NO is generated from L-arginine by oxidation of terminal nitrogen in the guanidine group in reaction catalyzed by the enzyme nitric oxide synthase (NOS)[11]. Different functional forms of NOS can be recognized: constitutive and inducible forms. NO synthesis by the constitutive isoforms, endothelial (eNOS) and neuronal (nNOS), generate low levels of NO under normal physiological conditions[11]. In the gastrointestinal tract (GIT) constitutive isoforms found in the endothelial cells (eNOS) and certain nerve terminals (nNOS) innervating the colon, regulate blood flow and

bowel motility by promoting muscle relaxation of the vessels and the bowel, respectively[12]. The inducible isoform, iNOS, is highly expressed in cells involved in the inflammatory response like macrophages and neutrophils, as well as in endothelial and smooth muscle cells, upon different stimuli, like endotoxine and/or cytokines, whose production is increased in the inflammatory environment[13]. After its induction, iNOS generates high, sustained levels of NO that may be toxic to the healthy tissue. Thus, tissue injury may result from the interaction of NO with superoxide anion, one of the reactive oxygen species, resulting in a formation of peroxynitrite that further contributes to tissue damage and up-regulation of the inflammatory response[12]. All the above support the important role ascribed to NO in chronic inflammatory conditions, including Crohn's disease (CD) and ulcerative colitis (UC), the major forms of inflammatory bowel diseases (IBD)[14]. Therefore, those compounds or product able to down regulate an exacerbated NO production could represent an important therapeutic tool in the management of inflammatory conditions, like IBD.

Thus, the aim of this study was, firstly, to determine the phenolic compounds in olive leaf extracts from three different olive cultivars grown in the same experimental orchard under the same agronomic and environmental conditions and collected in December, and, secondly, to evaluate for the first time the anti-inflammatory properties of the whole phenolic extracts of olive leaves by determining the inhibitory effect towards NO production in LPS-stimulated RAW 264.7 cells of the three olive leaf cultivars and compare the results among them. December is the period in which olive leaves of the cultivars studied has presented the highest phenolic contents among summer and winter periods[15]. This work is as a preliminary study to perform a subsequent bioguided isolation of the bioactive fractions of olive leaf extracts in the future.

2. Materials and Methods

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. HPLC-grade acetonitrile and acetic acid were purchased from Labscan (Dublin, Ireland) and Fluka (Switzerland), respectively. Standard compounds such as hydroxytyrosol, tyrosol, luteolin, and apigenin and all chemicals for MTT test and Griess assay were obtained from Sigma-Aldrich (St. Louis, MO, USA), and oleuropein from Extrasynthèse (Lyon, France). Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). The stock solutions containing these analytes were prepared in methanol (Panreac, Barcelona, Spain). All the solutions were stored in a dark flask at -20 °C until use.

2.2 Extraction of phenolic compounds from olive leaf extracts

Samples of olive leaves (*O. europaea* L.) of 'Arbequina', 'Picual' and 'Sikitita' cultivars, cultivated in the same orchards in IFAPA of Cordoba (Spain) under the same agronomic and environmental conditions were used in this study. The leaves were collected at the same time from three individuals of each cultivar and dried at room temperature. The extraction of phenolic compounds was performed as described previously by Talhaoui et al.[16]. Three replicates of each sample were processed. The extracts designed to the *in vitro* study were evaporated and concentrated in a SpeedVac concentrator (Savan SC250EXP (Thermo Sci.)).

2.3 HPLC-DAD-TOF-MS analysis

The analyses of the phenolic fraction of olive leaves were performed by a Poroshell 120 EC-C18 analytical column (4.6×100 mm, 2.7 μm) from Agilent Technologies, on an Agilent 1200 series Rapid Resolution Liquid

Chromatograph (Agilent Technologies, CA, USA). The method used was the previously described by Talhaoui et al.[16]. The gradient eluent, at flow rate of 0.8 mL/min, and the mobile phases used were water with acetic acid (1%) (phase A) and acetonitrile (phase B), and the solvent gradient changed according to the following conditions: 0 min, 5% B; 4 min, 9% B; 7 min,12% B; 8 min,15% B; 9min,16% B; 14 min, 20% B; 15 min, 22% B; 18 min, 28% B; 19 min, 30% B; 20 min, 31% B; 21.50 min, 32% B; 23 min, 34% B; 24 min, 35% B; 25.5 min, 40% B; 27 min, 50% B; 30 min, 100% B; 35 min, 100% B; 37 min, 5% B. The column temperature was maintained at 25 °C and the injection volume was 2.5µL.

The HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio=1:3). Analysis parameters were set using a negative-ion mode with spectra acquired over a mass range from *m/z* 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bars. The accurate mass data on the molecular ions was processed through the newest Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany). The quantification was carried out using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics, Bremen, Germany). Five standard calibration graphs were prepared for quantification of the phenolic compounds in fruits using five commercial standards (oleuropein, hydroxytyrosol, tyrosol, apigenin, luteolin).

2.4 *In vitro* effects on NO production in RAW 264.7 cells

Macrophages are considered the main source of the pro-inflammatory mediators in inflammatory conditions, including IBD[17]. The effects of the different olive extracts on NO production in LPS-stimulated RAW 264.7 cells were tested. The mouse macrophages RAW 264.7 cells were obtained from the Cell Culture Unit of the University of Granada (Granada, Spain) and cultured in RPMI Medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (2 mmol l^{-1}), penicillin (100 units ml^{-1}) and streptomycin (1 mg ml^{-1}) in a humidified 5% CO_2 atmosphere at 37%. Cells were seeded into 96 well plates at a density of $5x10^5$ cells/well, grown until the formation of a monolayer, pre-incubated with different concentrations of each olive leaf extract from the three cultivars ranging from 0.1 to 100 μg/mLand stimulated with the bacterial lipopolysaccharide LPS (100 ng/ml) for 24h. Untreated unstimulated cells and LPS-stimulated cells were used as negative and positive controls. Once the 24h period was finished, the supernatants were collected and nitrite levels measured by Griess Assay[18]. Cell viability was examined by the MTT-test described elsewhere[19].

2.5 Statistical analysis

All analytical assays were run in triplicate. Results were tested for statistical significance by one-way ANOVA, using Statistica 6.0 software (2001, StatSoft, Tulsa, OK, USA); Significant statistical differences among treatments (p<0.01) were assessed by Turkey's honest significant-difference multiple comparisons.

3. Results and discussion

3.1 Quantitative analysis of phenolic compounds of olive leaf extracts

Phenolic compounds of the olive leaf extracts under study were identified as reported in a previous work[16]. The compounds were identified by interpreting their mass spectra determined *via* TOF-MS and UV spectra provided by DAD supported by data reported in literature. Overall, twenty-eight compounds were detected in olive leaf extracts **(Table 1)**.

For quantitative purposes, standard calibration graphs were prepared from the injection of five standards (oleuropein, hydroxytyrosol, tyrosol, apigenin, luteolin) in the same condition of the samples analysis. The validation of the proposed method was performed with linearity, sensitivity, and precision parameters. The limits of detection (LOD) was ranging between 0.53 and 27.63 µg/L and the limit of quantification (LOQ) was ranging between was within 1.76 and 92.10µg/L. The LOD and LOQ for individual compounds in standard solutions were also calculated as S/N=3 and S/N=10, respectively, where S/N is the signal-to-noise ratio. The repeatability of the method was measured as the relative standard deviation (RSD %) in terms of concentration. The olive-leaf extract was injected several times (n=6) on the same day (intraday precision) and 3 times on 2 consecutive days (interday precision, n=18). The intraday repeatability of the developed method for all analytes ranged from 0.05 and 1.03%, whereas the interday repeatability ranged from 0.17 to 3.6%.

All calibration curves showed good linearity (r^2>0.99) between different concentrations depending on the analytes studied. The phenolic compound concentrations were determined by using the corresponded area for each individual compound and by interpolating in the corresponding calibration curve. The compounds that did not have their correspondent standard were tentatively quantified on the basis of calibration curves from other compounds with

structural similarities. Thus, oleuropein and other secoiridoids were quantified with the calibration curve of oleuropein; hydroxytyrosol-hexose isomers were quantified with the calibration curve of hydroxytyrosol; tyrosol glucoside was quantified with the calibration curve of tyrosol; apigenin rutinoside was quantified with the calibration curve of apigenin and luteolin, luteolin-diglucoside and the other flavonoids were quantified with the calibration curve of luteolin.

Table 1 summarizes all data about identification and quantification of phenolic compounds in olive leaf extracts of the three cultivars. A one-way analysis of variance was also performed and it indicates significant differences among the three cultivars for all the compounds and for total phenols (p<0.01). Studies performed on phenolic compounds in olive leaf extract demonstrate that genotypes are one of the most important factors which contribute to differences in quantitation of phenolic compounds[20]. Taking as reference data reported in green and black teas[21], which are known for their high content in phenolic compounds, total phenols obtained for the three cultivars, especially for 'Picual', could be considered relatively higher. In addition, olive leaves generally have higher total phenols and oleuropein contents than other tissues of the olive tree, notably fruits. In fact, a study carried out in 11 Greek cultivars showed concretively that both leaves and fruits are important sources of phenols. However, for all cultivars, the content of total phenols in olive leaves was 48-61% superior than total phenols content in fruits[22]. A study carried in 'Arbequina', 'Picual' and 'Sikitita' olive fruits at the same conditions than those described in the present study, reported insignificants values (1,3-9.3 mg/kg of fresh weight) of total phenols in fruits compared to olive leaves contents (**Table 1**)[23].

Oleuropein which has been reported to possess high antioxidant activity *in vitro* and anti-inflammatory effect[24] among others beneficial effects, was the major compound for the three cultivars, constituting 85.6%, 82.1% and 82.5% of total

phenols for ‘Picual’, ‘Arbequina’ and ‘Sikitita’, respectively. The sum of the other secoiridoids (oleoside, secologanoside, oleuropein aglycone, demethyloleuropein, oleuropein glucoside, 2"-methoxyoleuropein and ligstroside) were found at percentages ranging between 5.4 % and 6.8 % of total phenols, whereas simple phenols such as hydroxytyrosol-hexose and tyrosol glucoside represented 3.8%, 5.2% and 6.8 % of total phenols for ‘Picual’, ‘Arbequina’ and ‘Sikitita’, respectively. Elenolic acids contents were very low in all cultivars, between 0.3% and 0.8% of total phenols. The high content of oleuropein in all cultivars extracts and notably the low content of elenolic acid, which is a part of oleuropein moieties, could indicate that phenolic compounds in olive leaf extract samples did not suffer big degradations during leaves drying or during phenols extraction.

Flavonoids were represented by luteolin and their glycosides forms: luteolin rutinoside, rutin, apigenin rutinoside and chrysoeriol glucoside. In fact, free and/or bound forms of luteolin are considered the major flavonoids in olive leaf extracts[9,25]. Flavonoids contents ranged between 4.0% and 5.6% of total phenols. Interestingly, even though the contents of secoiridoids and flavonoids were different from cultivar leaf extract to another, their proportion in each cultivar olive extract was similar.

Table 1. The quantitative results for compounds identified in the dry olive leaves (mg/g dry leaves) (value=X±SD). Values with different letters are significantly different among cultivars at $p<0.01$.

Peak	Compounds Name	Rt (min)	*m/z* experimental	*m/z* calcultaed	Error (ppm)	mili Sigma	Molecular formula	'Picual'	'Arbequina'	'Sikitita'
1	**Oleoside**	4,08	389,1097	389,1089	1,8	7,1	$C_{16}H_{22}O_{11}$	0.051^{a}±0.004	0.031^{b}±0.004	0.035^{b}±0.002
2	**Hydroxytyrosol-hexose isomer a**	4,48	315,1096	315,1085	2,7	3,5	$C_{14}H_{20}O_{8}$	0.78^{a}±0.12	0.34^{b}±0.04	0.71^{a}±0.07
3	**Hydroxytyrosol-hexose isomer b**	4,67	315,1108	315,1085	5,7	16,9	$C_{14}H_{20}O_{8}$	1.59^{a}±0.15	1.27^{b}±0.08	1.52^{a}±0.12
4	**Secologanoside isomer a**	4,82	389,1107	389,1089	3,1	21,3	$C_{16}H_{22}O_{11}$	0.87^{a}±0.05	0.45^{c}±0.04	0.54^{b}±0.03
5	**Tyrosol glucoside**	6,46	299,1141	299,1102	1,7	14,3	$C_{14}H_{20}O_{7}$	0.76^{b}±0.07	0.79^{b}±0.11	1.44^{a}±0.13
6	**Elenolic acid glucoside isomer a**	8,61	403,1247	403,1246	0,4	12,7	$C_{17}H_{24}O_{11}$	0.066^{a}±0.009	0.031^{b}±0.003	0.017^{c}±0.002
7	**Secologanoside isomer b**	8,83	389,1115	389,1089	5,2	24,5	$C_{16}H_{22}O_{11}$	0.62^{a}±0.07	0.47^{b}±0.06	0.36^{b}±0.07
8	**Elenolic acid glucoside isomer b**	10,25	403,1256	403,1246	2,4	4,0	$C_{17}H_{24}O_{11}$	0.10^{a}±0.01	0.08^{a}±0.01	0.09^{a}±0.01
9	**Oleuropein aglycon**	11,09	377,1474	377,1453	5,5	14,9	$C_{16}H_{26}O_{10}$	0.27^{a}±0.04	0.09^{b}±0.01	0.11^{b}±0.02
10	**Luteolin-diglucoside**	11,67	609,1473	609,1461	2,1	25,2	$C_{27}H_{30}O_{16}$	0.19^{b}±0.03	0.25^{a}±0.02	0.15^{b}±0.01
11	**Elenolic acid glucoside isomer c**	12,26	403,1278	403,1246	7,9	9,3	$C_{17}H_{24}O_{11}$	0.47^{a}±0.05	0.04^{c}±0.01	0.33^{b}±0.03
12	**Demethyloleuropein**	13,75	525,1640	525,1614	1,7	11,3	$C_{24}H_{30}O_{13}$	0.18^{b}±0.02	0.40^{a}±0.03	0.22^{b}±0.03
13	**Rutin**	14,30	609,1513	609,1461	8,5	25,9	$C_{27}H_{30}O_{16}$	0.29^{a}±0.04	0.23^{b}±0.02	0.18^{c}±0.02
14	**Luteolin rutinoside**	14,52	593,1532	593,1512	3,4	6,2	$C_{27}H_{30}O_{15}$	0.38^{a}±0.03	0.41^{a}±0.02	0.19^{b}±0.02
15	**Luteolin glucoside isomer a**	15,22	447,0882	447,0933	5,2	2,2	$C_{21}H_{20}O_{11}$	1.54^{a}±0.09	0.86^{b}±0.11	0.87^{b}±0.07
16	**Apigenin rutinoside**	16,88	577,1599	577,1563	6,3	3,6	$C_{27}H_{30}O_{14}$	0.061^{a}±0.013	0.061^{a}±0.010	0.033^{b}±0.005
17	**Oleuropein glucoside isomer a**	17,16	701,2330	701,2298	3,4	19,7	$C_{31}H_{42}O_{18}$	0.28^{a}±0.02	0.08^{c}±0.01	0.14^{b}±0.01
18	**Oleuropein glucoside isomer b**	17,43	701,2322	701,2298	3,4	14,1	$C_{31}H_{42}O_{18}$	0.43^{a}±0.02	0.15^{c}±0.02	0.30^{b}±0.02
19	**Oleuropein glucoside isomer b**	17,76	701,2347	701,2298	8,5	23,7	$C_{31}H_{42}O_{18}$	0.77^{a}±0.10	0.49^{b}±0.05	0.54^{b}±0.04
20	**Luteolin glucoside isomer b**	17,86	447,0937	447,0933	0,8	1,5	$C_{21}H_{20}O_{11}$	1.08^{a}±0.09	0.67^{b}±0.06	0.67^{b}±0.04
21	**Chrysoeriol-7-*O*-glucoside**	18,28	461,1149	461,1089	12,9	7,7	$C_{22}H_{22}O_{11}$	0.173^{a}±0.011	0.098^{b}±0.019	0.058^{c}±0.003

Peak	Compounds Name	Rt (min)	*m/z* experimental	*m/z* calcultaed	Error (ppm)	mili Sigma	Molecular formula	'Picual'	'Arbequina'	'Sikitita'
22	**2"-Methoxyoleuropein isomer a**	18,98	569,1905	569,1876	5,1	15,7	$C_{26}H_{34}O_{14}$	0.034^{a}±0.005	0.025^{b}±0.004	0.016^{c}±0.002
23	**2"-Methoxyoleuropein isomer b**	19,13	569,1895	569,1876	3,4	14,3	$C_{26}H_{34}O_{14}$	0.061^{b}±0.006	0.161^{a}±0.025	0.035^{c}±0.004
24	**Oleuropein isomer a**	19,45	539,1915	539,177	1,4	16,4	$C_{25}H_{32}O_{13}$	66.44^{a}±2.81	35.66^{c}±1.61	41.68^{b}±2.64
25	**Oleuropein isomer b**	20,70	539,1844	539,177	3,7	8,3	$C_{25}H_{32}O_{13}$	1.55^{a}±0.05	0.52^{c}±0.05	0.77^{b}±0.04
26	**Oleuropein/Oleuroside**	20,45	539,1728	539,177	1,3	4,0	$C_{25}H_{32}O_{13}$	3.29^{a}±0.28	1.61^{b}±0.14	1.87^{b}±0.11
27	**Ligstroside**	21,34	523,1862	523,1282	10,1	9,4	$C_{25}H_{32}O_{12}$	0.99^{a}±0.11	0.78^{a}±0.08	0.81^{a}±0.07
28	**Luteolin**	21,78	285,0412	285,0405	2	6,7	$C_{15}H_{10}O_{6}$	0.021^{a}±0.003	0.025^{a}±0.002	0.006^{b}±0.001
	Total							**83.30^{a}±3.15**	**46.04^{c}±1.94**	**53.68^{b}±2.98**

3.2 Immunomodulatory properties of olive extracts on RAW 264 mouse macrophages

In order to initially characterize and compare the immunomodulatory properties of the olive extracts, their effects on nitrite production in LPS-stimulated macrophage cell line RAW.264 were assayed. Macrophages are considered as the main source of the pro-inflammatory mediators like NO in different inflammatory conditions, including IBD, actively contributing to their pathology[17], particularly in the initiation and perpetuation of inflammation and in the subsequent tissue damage[26].

The incubation of these cells with different concentrations of the olive leaf extracts in basal conditions did not result in an increased production of nitrites in comparison with un-treated cells (data not shown). After treatment with LPS (100 ng/mL) for 24 hours, nitrite concentration in the medium increased remarkably by about 10-fold in comparison with basal levels. However, the pre-treatment of the cells with olive leaf extracts inhibited dose-dependently the increased nitrite production induced by LPS, showing IC50 values of 129.9±23.5 μg/mL ('Sikitita'), 34.8±8.9 μg/mL ('Arbequina') and 23.3±7.3 μg/mL ('Picual'). The maximum inhibition exerted by all these extracts was greater than 75% at the concentration of 100 μg/mL and olive leaf extracts from 'Arbequina' cultivar showed the highest inhibitory effect **(Figure 1)**. These results are much more effective and higher than those presented before in bibliography for the individual phenol oleuropein from olive leaf on the same type of cell (NO production reduced by 42% at oleuropein concentration of 162 μg/mL)[27]. High quantities of oleuropein were required to obtain lesser effects than using lower quantities of the whole olive leaf phenolic extract. The difference between the effect of the whole olive leaf extract and oleuropein as individual compound is possibly due to the synergistic relationship among the phenolic compounds present in the whole olive leaf extract. Besides, these results are considered promoting for the olive leaf extract phenols when comparing to other sources of

olive phenolic compounds. For instance, the phenolic extracts of extra virgin olive oil tested in LPS-activated murine peritoneal macrophages cells have inhibited the NO production by 50% at a concentration of 50 μg/mL[28]. In addition, the immunomodulatory properties of olive oil waste has been tested on human macrophage cell line THP-1, and it has been found to be able to reduce LPS-induced TNF-α production (tumor necrosis factor TNF is the primary cytokine induced a consequence of NO production) by 50% at a concentration of 500 μg/mL[29].

Intriguingly, olive leaf extracts of 'Arbequina' cultivar was the one that presented the lowest content in total phenolic compounds (**Table 1**). Furthermore, this cultivar presented the lowest content in secoiridoids, flavonoids and simple phenols groups. i.e. about 40.9 mg/g of dry leaf content in total secoiridoids, in front of 75.8 and 47.4 mg/g of dry leaf shown in 'Picual' and 'Sikitita' respectively. This finding could reveal that the inhibition of NO by the olive leaf extract may depend on the type of phenolic compounds rather than on their total concentrations.

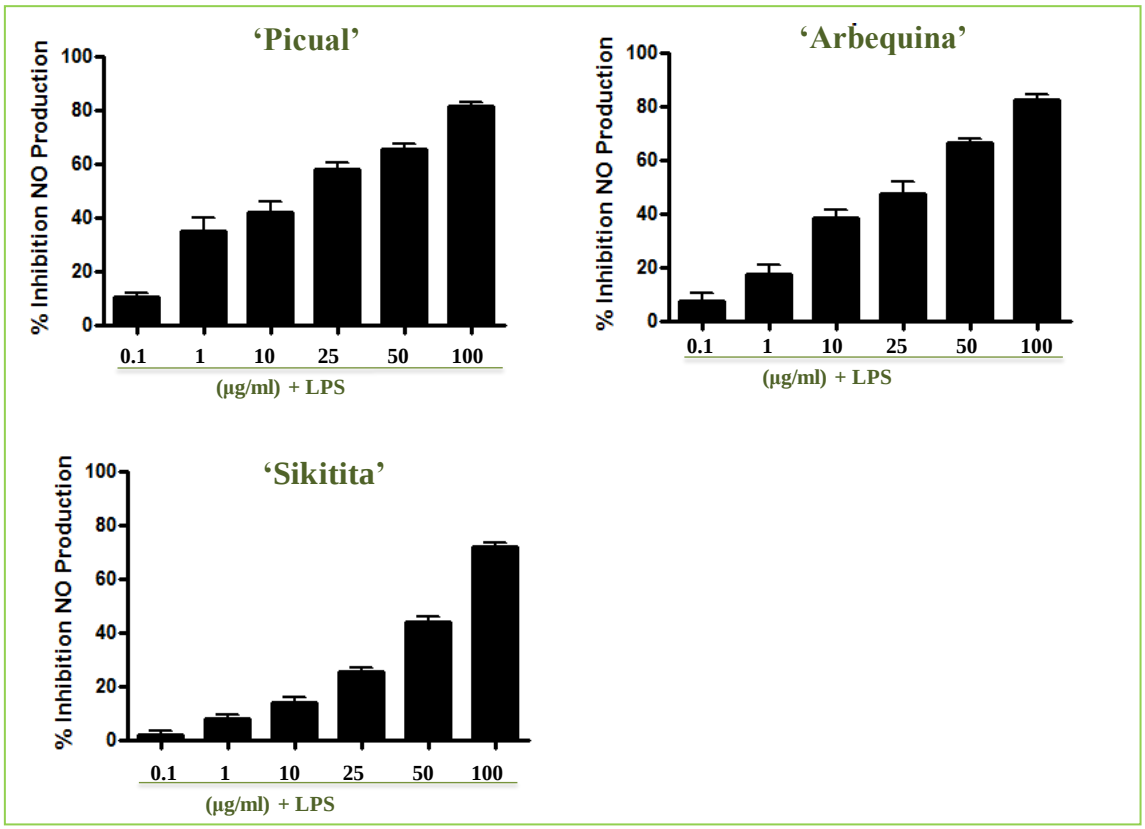

Figure 1. Effects of olive leaf extracts incubation in LPS-stimulated RAW 264.7 cells on nitrite oxide (NOx-) production evaluated by the Griess assay. The experiments were performed three times.

Of note, the viability of activated macrophages was not significantly altered by the different concentration of the olive leaf extracts as determined by MTT assays, thereby indicating that the inhibition of NO synthesis by these extracts was not simply due to cytotoxic effects (**Figure 2**).

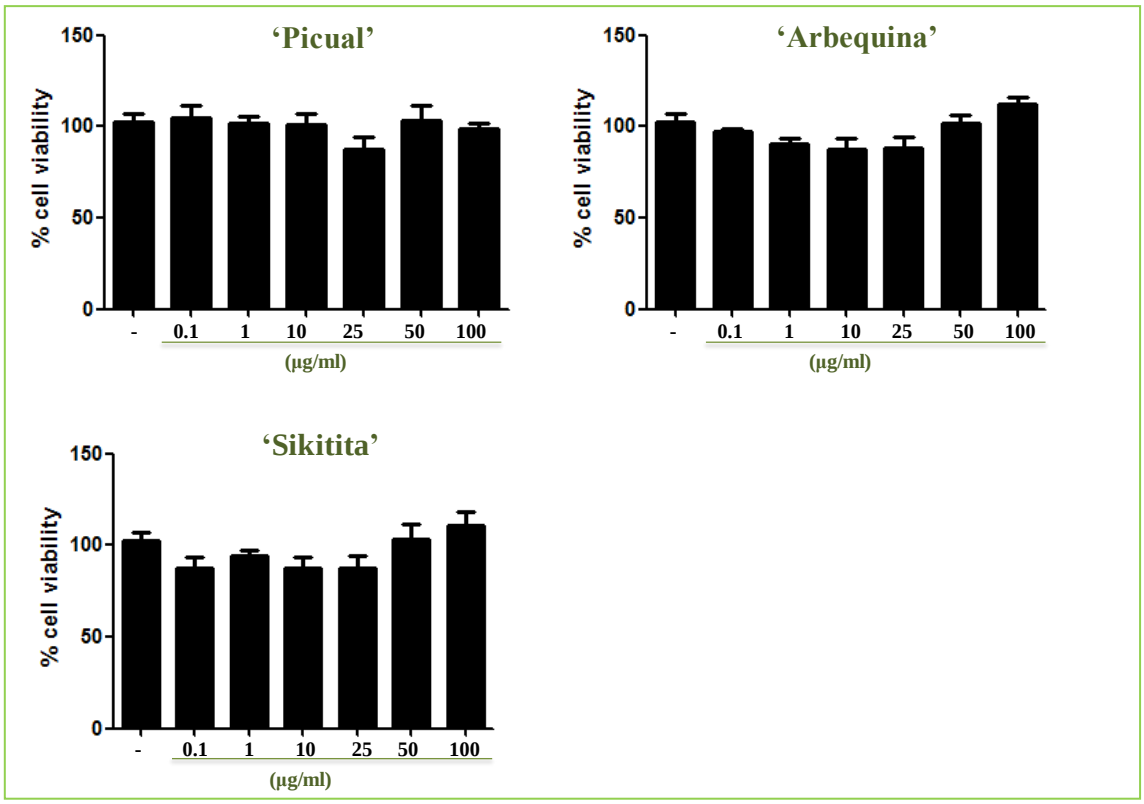

Figure 2. Effects of olive leaf extracts on cell viability in RAW264.7 cells. The experiments were performed three times.

All this data demonstrate inhibitory activities of the three olive leaf extracts against NO production in LPS-stimulated cells, suggesting their potential therapeutic use for the treatment of inflammatory diseases[30]. In fact, a number of studies have shown that olive leaf extracts are considered to be a good source of natural antioxidant molecules especially vitamins, terpenoids, polyphenols, alkaloids, coumarins, and other metabolites[31]; most of them with reported ability to exert anti-inflammatory properties[32]. Consequently, olive leaf extracts can be considered as promising candidates for alternative and/or complementary treatments of inflammatory conditions, including human IBD[33], a chronic disease of the digestive tract, typically characterized by chronic and spontaneously relapsing inflammation. Since the symptoms of the disease greatly diminish the quality of life for IBD patients and increase their risk for colorectal cancer, the prevention and treatment of IBD is of crucial importance[34]. At present, management of the disease involves different pharmacological therapies, mainly

anti-inflammatory drugs or immunosuppressive drugs, such as aminosalicylates, glucocorticoids, azathioprine or biologicals (anti-TNF antibodies like infliximab or adalimumab). Although these drugs are effective for temporary relief of symptoms, they are usually associated to the onset of severe side effects that may limit their use with time[35]; moreover, not all patients are responsive to therapy[35,36]. Therefore, it is crucial to identify new and safe therapeutic strategies for preventing or treating IBD. This can be the case of these olive leaf extracts, since they show immunomodulatory properties as it has been reported in the present study, thus supporting the future study in preclinical models of intestinal inflammation in rodents.

4. Conclusions

In this study, HPLC-DAD-ESI-TOF-MS allowed the identification and quantification of twenty-eight compounds in olive leaf extracts of the three cultivars studied. All olive leaf cultivars have shown high contents in total phenolic compounds, especially 'Picual' cultivar, being oleuropein the major compound for the three cultivars. Moreover, this is the first time that the whole phenolic extracts of olive leaves from olive trees grown in the same experimental orchard and under the same agronomic and environmental conditions have been used to *in vitro* study the immunomodulatory properties and it has been shown that they inhibited the release of the pro-inflammatory mediator NO, when evaluated *in vitro* in LPS-stimulated RAW264.7 cells, a macrophage cell type. Although significant differences have been seen among the three olive leaf cultivars for all the compounds and for total phenols, their respective *in vitro* activities were relatively similar. The findings of the present study clearly provide evidences that support the traditional use of medicinal plants in the treatment of inflammatory diseases. Further studies in relation to immunomodulatory and anti-inflammatory potentials of the extracts fractions will be performed in the future.

References

(1) Altinyay, Ç.; Altun, M. L. HPLC analysis of oleuropein in *Olea europaea* L. *J. Fac. Pharm. Ankara* **2006**, 35, 1–11.

(2) Lee-Huang, S.; Zhang, L.; Huang, P. L.; Chang, Y. T.; Huang, P. L. Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem. Biophys. Res. Commun*. **2003**, 307, 1029–1037.

(3) Lee, O.-H.; Lee, B.-Y. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. *Bioresour. Technol*. **2010**, 101, 3751–3754.

(4) Taamalli, A.; Arráez-Román, D.; Barrajón-Catalán, E.; Ruiz-Torres, V.; Pérez-Sánchez, A.; Herrero, M.; Ibañez, E.; Micol, V.; Zarrouk, M.; Segura-Carretero, A.; et al. Use of advanced techniques for the extraction of phenolic compounds from Tunisian olive leaves: Phenolic composition and cytotoxicity against human breast cancer cells. *Food Chem. Toxicol*. **2012**, 50, 1817–1825.

(5) Poudyal, H.; Campbell, F.; Brown, L. Olive leaf extract attenuates cardiac , hepatic , and metabolic changes in high carbohydrate, high fat fed fats 1-3. *J. Nutr*. **2010**, 140, 946–953.

(6) Al-Azzawie, H. F.; Alhamdani, M. S. S. Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits. *Life Sci*. **2006**, 78, 1371–1377.

(7) Bouallagui, Z.; Han, J.; Isoda, H.; Sayadi, S. Hydroxytyrosol rich extract from olive leaves modulates cell cycle progression in MCF-7 human breast cancer cells. *Food Chem. Toxicol*. **2011**, 49, 179–184.

(8) Poór, M.; Veres, B.; Jakus, P. B.; Antus, C.; Montskó, G.; Zrínyi, Z.; Vladimir-Knežević, S.; Petrik, J.; Kőszegi, T. Flavonoid diosmetin increases ATP levels in kidney cells and relieves ATP depleting effect of ochratoxin A. *J. Photochem. Photobiol. B*. **2014**, 132, 1–9.

(9) Goulas, V.; Papoti, V. T.; Exarchou, V.; Tsimidou, M. Z.; Gerothanassis, I.

P. Contribution of flavonoids to the overall radical scavenging activity of olive (*Olea europaea* L.) leaf polar extracts. *J. Agric. Food Chem*. **2010**, 58, 3303–3308.

(10) Talhaoui, N.; Taamalli, A.; Gómez-Caravaca, A. M.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Phenolic compounds in olive leaves: Analytical determination, biotic and abiotic influence, and health benefits. *Food Res. Int*. **2015**, 77, 92–108.

(11) Calcerrada, P.; Peluffo, G.; Radi, R. Nitric oxide-derived oxidants with a focus on peroxynitrite molecular targets, cellular responses and therapeutic implications. *Curr. Pharm. Des*. **2011**, 17, 3905–3932.

(12) Kolios, G.; Valatas, V.; Ward, S. G. Nitric oxide in inflammatory bowel disease: A universal messenger in an unsolved puzzle. *Immunology* **2004**, 113, 427–437.

(13) Palatka, K.; Serfőző, Z.; Veréb, Z.; Hargitay, Z.; Lontay, B.; Erdődi, F.; Bánfalvi, G.; Nemes, Z.; Udvardy, M.; Altorjay, I. Changes in the expression and distribution of the inducible and endothelial nitric oxide synthase in mucosal biopsy specimens of inflammatory bowel disease. *Scand. J. Gastroenterol*. **2005**, 40, 670–680.

(14) Roediger, W. E.; Lawson, M.; Nance, S.; Radcliffe, B. Detectable colonic nitrite levels in inflammatory bowel disease- Mucosal or bacterial malfunction. *Digestion*. **1986**, 35, 199–204.

(15) Talhaoui, N.; Gómez-Caravaca, A. M.; Roldán, C.; León, L.; De la Rosa, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. Chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages. *J. Agric. Food Chem.* **2015**, *63*, 1722–1729.

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

(17) Comalada, M.; Camuesco, D.; Sierra, S.; Ballester, I.; Xaus, J.; Gálvez, J.; Zarzuelo, A. *In vivo* quercitrin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF-kB pathway. *Eur. J. Immunol*. **2005**, 35, 584–592.

(18) Green, L. C.; Wagner, D. a; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem*. **1982**, 126, 131–138.

(19) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, 65, 55–63.

(20) Ben Salah, M.; Abdelmelek, H.; Abderraba, M. Medicinal chemistry study of phenolic composition and biological activities assessment of olive leaves from different varieties grown in Tunisia. *Med. Chem*. **2012**, 2, 107–111.

(21) Khokhar, S.; Magnusdottir, S. G. M. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *J. Agric. Food Chem*. **2002**, 50, 565–570.

(22) Petridis, A.; Therios, I.; Samouris, G. Genotypic variation of total phenol and oleuropein concentration and antioxidant activity of 11 Greek olive cultivars (*Olea europaea* L.). *HortScience* **2012**, *47*, 339–342.

(23) Talhaoui, N.; Gómez-Caravaca, A. M.; Leon, L.; de la Rosa, R.; Fernandez-Gutierrez, A.; Segura-Carretero, A. Pattern of variation of fruit traits and phenol content in olive fruits from six different cultivars. *J. Agric. Food Chem.* **2015**, *63*, 10466–10476.

(24) Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life Sci*. **1998**, 62, 541–546.

(25) Quirantes-Piné, R.; Zurek, G.; Barrajón-Catalán, E.; Bäßmann, C.; Micol, V.; Segura-Carretero, A.; Fernández-Gutiérrez, A. A metabolite-profiling approach to assess the uptake and metabolism of phenolic compounds from olive leaves in SKBR3 cells by HPLC–ESI-QTOF-MS. *J. Pharm. Biomed.*

Anal. **2013**, 72, 121–126.

(26) Oudkerk Pool, M.; Bouma, G.; Visser, J.; Kolkman, J.; Tran, D.; Meuwissen, S. G.; Peña, A. Serum nitrate levels in ulcerative colitis and Crohn's disease. *Saudi Med J.* **2012**, 33, 3–8.

(27) Ryu, S.J.; Choi, H.S.; Yoon, K.Y.; Lee, O.H.; Kim, K.J.; Lee, B.Y. Oleuropein suppresses LPS-induced inflammatory responses in RAW 264.7 cell and zebrafish. *J. Agric. Food Chem.* **2015**, *63*, 2098–2105.

(28) Cárdeno, A.; Sánchez-Hidalgo, M.; Aparicio-Soto, M.; Sánchez-Fidalgo, S.; Alarcón-de la Lastra, C. Extra virgin olive oil polyphenolic extracts downregulate inflammatory responses in LPS-activated murine peritoneal macrophages suppressing NFκB and MAPK signalling pathways. *Food Funct.* **2014**, *5*, 1270–1277.

(29) Bitler, C. M.; Viale, T. M.; Damaj, B.; Crea, R. Hydrolyzed olive vegetation water in mice has anti-inflammatory activity. *J. Nutr*. **2005**, *135*, 1475–1479.

(30) Pacher, P.; Beckman, J. S.; Liaudet, L. Nitric oxide and peroxynitrite in health and disaese. *Physiol. Rev*. **2007**, 87, 315–424.

(31) Vassiliki, G. K.; Gerothanassis, I. P. Phenolic compounds and antioxidant activity of olive leaf extracts. *Nat. Prod. Res*. **2012**, 26, 186–189.

(32) Cárdeno, A.; Sánchez-Hidalgo, M.; Alarcón-de-la-Lastra, C. An up-date of olive oil phenols in inflammation and cancer: Molecular mechanisms and clinical implications. *Curr. Med. Chem*. **2013**, 20, 4758–4776.

(33) Hur, S. J.; Kang, S. H.; Jung, H. S.; Kim, S. C.; Jeon, H. S.; Kim, I. H.; Lee, J. D. Review of natural products actions on cytokines in inflammatory bowel disease. *Nutr. Res*. **2012**, 32, 801–816.

(34) Srensen, G. V.; Erichsen, R.; Sværke, C.; Farkas, D. K.; Srensen, H. T. Risk of cancer in patients with inflammatory bowel disease and venous thromboembolism: A nationwide cohort study. *Inflamm. Bowel Dis*. **2012**, 18, 1859–1863.

(35) Triantafillidis, J.; Merikas, E.; Georgopoulos, F. Current and emerging drugs for the treatment of inflammatory bowel disease. *Drug Des. Devel.*

Ther. **2011**, 5, 185–210.

(36) Baumgart, D. C.; Sandborn, W. J. Inflammatory bowel disease: Clinical aspects and established and evolving therapies. *Lancet* **2007**, 369, 1641–1657.

CHAPTER 5. Pattern of variation of fruit traits and phenol content in olive fruits from six different cultivars.

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Pattern of Variation of Fruit Traits and Phenol Content in Olive Fruits from Six Different Cultivars

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Abstract

In the present study, olive fruits from six cultivars grown under similar agronomical and environmental conditions were collected at four different times during fruit ripening. Some agronomical traits were determined, and general increases in the size of the fruit and oil contents were recorded for all cultivars. The phenolic fraction in fruits was also identified and quantified during the same period using high performance liquid chromatography−diode array detection−time-of-flight-mass spectrometry. Thus, a total of 57 phenolic compounds were determined and qualitative and quantitative differences among cultivars and also among sampling times were observed. In contrast to the agronomical traits, a general decrease of total phenolic compounds was observed, characterized by a domination of secoiridoids at the beginning of ripening and by a domination of simple phenols and flavonoids in the end. This is the first time that four of the six cultivars have been studied regarding phenolic compounds evolution during ripening.

Keywords: Olive fruits, agronomical traits, phenolic compounds, six cultivars, ripening.

1. Introduction

Olive (*Olea europaea* L.) is one of the major crops in the Mediterranean region as the source of olive oil and table olives. Besides their economical contribution, they are important foods in terms of their nutritional value. Olives and olive oil may have a role in the prevention of coronary heart disease and certain cancers because of their high levels of monosaturated fatty acids and phenolic compounds[1]. These phenolic compounds may contribute to fruit quality in numerous ways; for example, by providing different sensory attributes, such as color and flavor[2], or making olive and olive oil both fairly stable against autoxidation and suitable for human health[3].

Phenolic compounds concentration in olive fruits could reach values ranging between 1 and 3% of the fresh pulp weight[4]. They, in particular, oleuropein, are responsible of the intense fruit bitterness[5]. Several studies have explored the presence of a high number of phenolic compounds in olive fruits, notably, hydroxytyrosol, tyrosol, luteolin glucoside, rutin, verbascoside, oleuropein, and salidroside[2,4]. Significant changes in the phenolic composition in fruits have been shown during fruit ripening[6,7] besides the physical changes that occur in fruits such as fruit size, moisture, and oil accumulation[8].

Many agronomic factors contribute to the variability in phenolic composition of olive fruits: cultivar, ripening, position on the tree, growing area, water availability, temperature, rootstock, and agronomic practices[2]. Studies of changes in the phenolic profile and content related to ripening have largely been focused on the olive fruit and particularly on variations in the oleuropein content[9]. Oleuropein, the major phenolic compound in immature olive fruit, amounts to up to 14% of the dry weight but, during ripening, undergoes hydrolysis and 50 yields several simple molecules such as hydroxytyrosol and oleuropein aglycon[9,10].

Because of its good separation of metabolites, efficiency, versatility, and speed of analysis, the most frequently used analytical technique for the separation of phenolic compounds in foods is reversed-phase high-performance liquid chromatography (RP-HPLC), with diode array detection (DAD) and mass spectrometry (MS) detection. Currently, this is the most widely used combination of techniques for the separation, identification, and quantification of phenolic compounds in olives[11].

Despite the numerous techniques applied and the large amount of published literature on the subject, olive cultivars are far from being fully characterized regarding phenolic compounds. The purpose of this study was the qualitative and quantitative evaluation of the main phenolic compounds in olive fruits from six different cultivars, using HPLC-DAD-MS, and the comparison of the phenolic profile and other agronomical traits changes during the ripening period. Some of these cultivars have been studied for the first time regarding phenolic compounds evolution during ripening.

2. Materials and methods

2.1. Chemicals and reagents

Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, and caffeic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and oleuropein was purchased from Extrasynthèse (Lyon, France). Methanol reagent was from Panreac (Barcelona, Spain). HPLC grade acetonitrile and acetic acid (assayed at >99.5%) used for preparing mobile phases were from Labscan (Dublin, Ireland) and Fluka (Switzerland), respectively. Ultrapure water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). The stock solutions containing these analytes were prepared in methanol. All chemicals were of analytical reagent grade and used as received. All of the solutions were stored in a dark flask at -20 °C until use

2.2. Samples

Samples from olive fruits (*O. europaea* L.) from six important cultivars were used for this study. These cultivars were selected as some of the most widely used in new orchards currently in Spain because of their high productivity and suitability for modern olive growing systems: 'Arbequina', 'Arbosana', 'Changlot Real', 'Koroneiki', 'Picual', and 'Sikitita'. All cultivars were grown under the same agronomic and environmental conditions in the same olive orchard in IFAPA, Centro Alameda del Obispo, Cordoba, Spain (37°51'36.5"N 4°47'53.7"W). The trees were 5 years old, planted at 7 × 5 m spacing, and trained as single-trunk open vase configuration. Standard cultural practices were followed, with minimal pruning to allow early bearing and irrigation by in-line drip with 2000 m3/ha per year to avoid water stress of plants. Samples were processed at four times: mid-June, mid-August, mid-October, and mid-December. About 250 g of fruits from all sides and orientations of each olive tree were collected. Sampling was done from three individuals of each cultivar, immediately transferred to the laboratory, and stored at -80°C until further analysis.

2.3. Determination of fruit traits

Fruit ripening index (RI) was calculated on the basis of color changes of peel and pulp according to the method of Frias et al.[12] Afterward, three subsamples of around 25g were randomly selected to measure fruit fresh weight (FrFW). Oil content was determined in the subsamples following the process proposed by Del Rio and Romero[13]. Briefly, the whole fruit subsamples were individually deposited in Petri dishes covered by a plastic film resistant to high temperatures and then dried in a forced-air oven at 105 °C (221 °F) until reaching constant mass. The dried subsamples were weighed to determine the fruit moisture (FrM) content they had lost and the fruit dry weight (FrDW). Oil content was determined in the subsamples using a NMR fat analyzer (Minispec MQone,

Bruker Optik GmbH, Ettlingen, Germany) according to Bruker manufacturer specifications for determination of oil content in olive fruits: 4 scans per sample, 9.95 MHz frequency, 56 db instrument gain, and 40 °C. The NMR used a linear calibration calculated from three reference standards. Oil content was expressed as percentage on both fresh (OCFrFW) and dry (OCFrDW) basis.

2.4 Extraction of phenolic compounds from olive fruits

The phenolic extracts of olive fruits were obtained using the method of Vinha et al.[14] with some modifications. Briefly, fresh and destined olive fruits (FFr) (2 g) were crushed and extracted *via* Ultra-Turrax IKA T18 basic with 30 mL of MeOH/H_2O (80:20). After that, the sample was placed in an ultrasonic bath (10 min) and centrifuged at 5000g for 15 min. Then, the supernatant was removed, and the extraction was repeated twice more. The supernatants were collected, and the extract was then evaporated. After that, the extract was reconstituted with 20 mL of acidified water (at pH 2.3) and washed twice with 40 mL of hexane to remove the possible oil. Then 40 mL of methanol was added to the solution (water and extract) and evaporated again. Finally, the extract was reconstituted with 2 mL of MeOH/H_2O (50:50) v/v. Samples were picked from 6 cultivars, 3 trees per cultivar and 3 replicates from each tree, which were 9 samples per cultivar and 54 samples in total in each sampling time. All cultivars showed similar high yields corresponding to an "on" year.

2.5. Determination of phenolic compounds by HPLC-DAD-TOF-MS

Separation of the phenolic fraction of olive leaves was performed by using a Poroshell 120 EC-C18 analytical column (4.6 × 100 mm, 2.7 μm) from Agilent Technologies, on an Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA). The gradient, at a flow rate of 0.8 mL/min, was the same as previously described by Talhaoui et al.[15] The column temperature was maintained at 25°C, and the injection volume was 2.5 μL.

The HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio=1:3). Analysis parameters were set using a negative-ion mode with spectra acquired over a mass range from *m/z* 50 to 1000. The optimum values of the ESI-MS parameters were as follows: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bars. The accurate mass data on the molecular ions were processed using Data Analysis 4.0 software (Bruker Daltonics), which provided a list of possible elemental formulas *via* the Smart Formula Editor. The Smart Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The quantification was carried out using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics). Six standard calibration graphs were prepared for quantification of the phenolic compounds in fruits using six commercial standards: oleuropein, hydroxytyrosol, tyrosol, apigenin, luteolin, and caffeic acid.

2.6 Statistical analysis

All assays were run in triplicate. Values of different results of phenolic compounds were expressed as the means in milligrams per kilogram fresh olive fruits (FrF). Results were tested for statistical significance by one-way ANOVA. Significant statistical differences among treatments ($p<0.001$) were assessed by Tukey's honest significant difference multiple comparisons. Statistica 8.0 software (2001, StatSoft, Tulsa, OK, USA) was used for statistical

determinations and for principal component analysis (PCA) for the phenol families grouped by cultivars and harvest dates.

3. Results and discussion

As expected, RI greatly increased from August to December, showing great differences among cultivars as previously reported[16]. 'Changlot Real' showed the highest RI along the sampling time, whereas 'Arbosana' and 'Koroneiki' alternated for the lowest RI **(Figure 1)**.

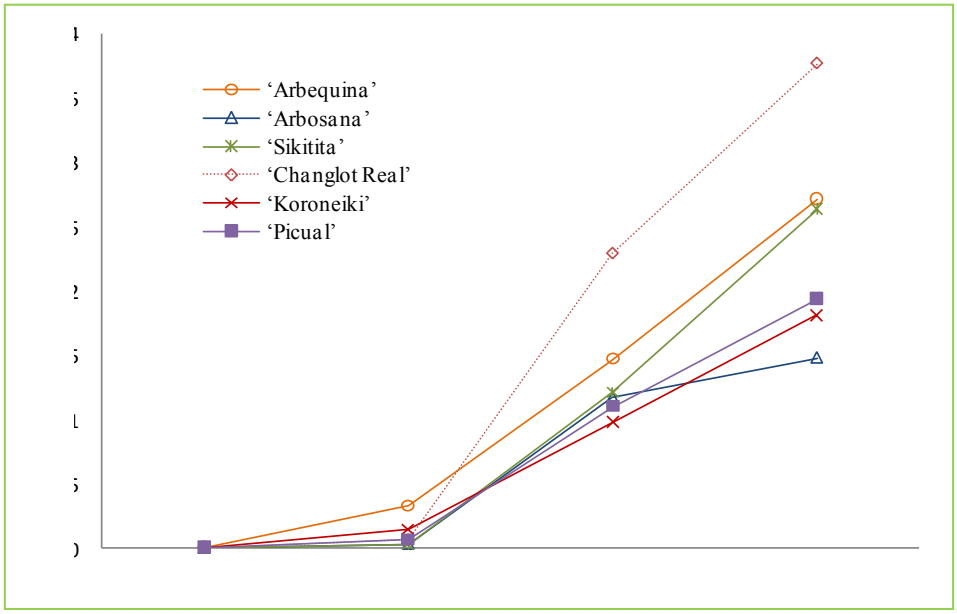

Figure 1. Evolution of the olive ripening index of the six cultivars among the sampling times.

With regard to FrFW, 'Changlot Real' and 'Picual' showed the highest values during all of the ripening periods and 'Koroneiki' the lowest ones **(Figure 2a)**. FrDW differed among cultivars with the same pattern as observed for the FrFW **(Figure 2b)**, except for 'Changlot Real', which showed a stable FrDW between October and December.

FrM ranged from 59 to 73% over all the ripening periods except in summer, where a notable decrease was recorded **(Figure 2e)**, likely due to the high

evapotranspiration because of the high temperatures (**Figure S1, Supporting Information**). The only exception was 'Changlot Real', for which the FrM decrease in August was very low. After October, the moisture tended to stabilize in all of the cultivars except for 'Arbequina'. Water is a major component in olive fruit, and its content can be influenced by numerous factors including rainfall, evaporation, irrigation events, soil type, and tree health[8].

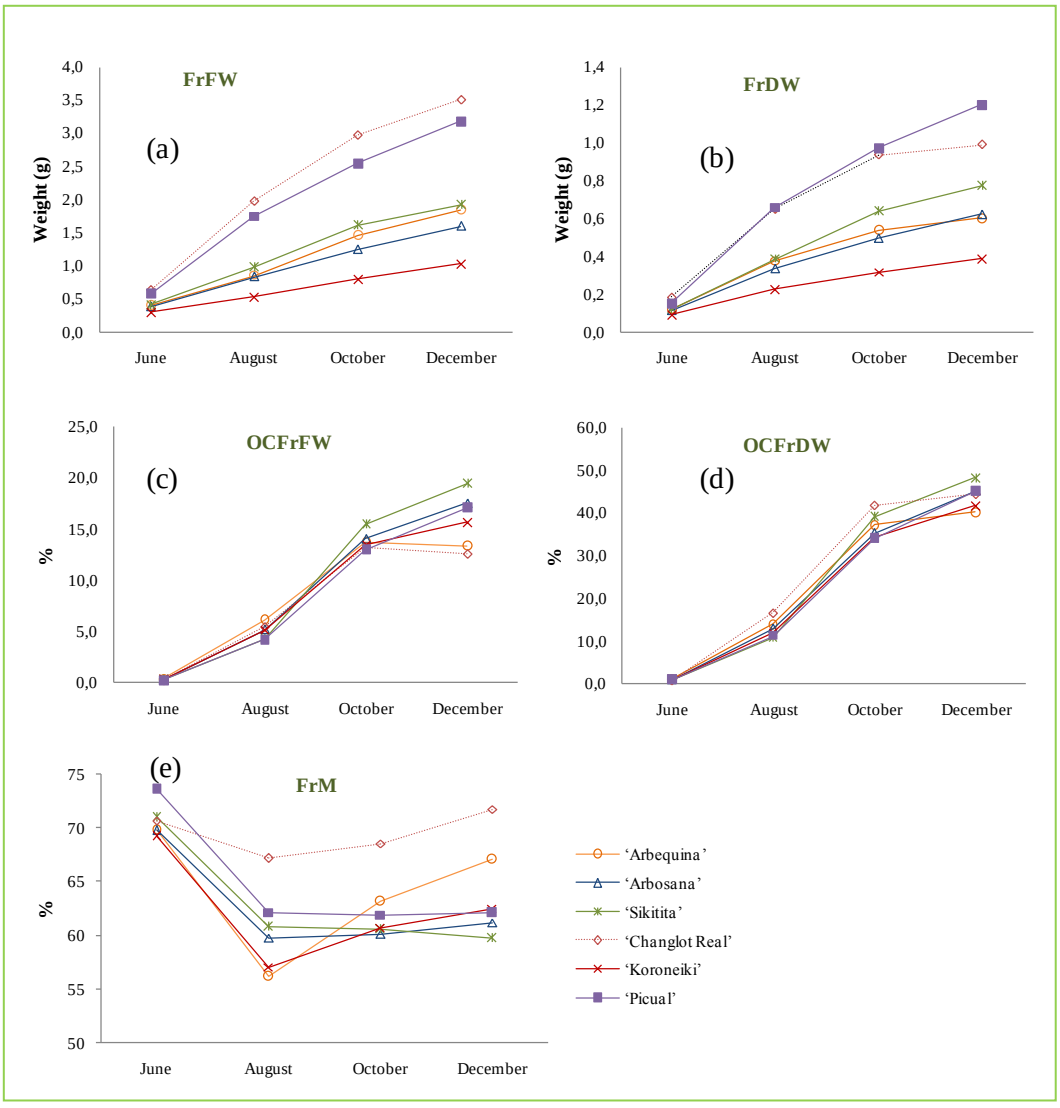

Figure 2. Evolution of the olive agronomical parameters of the six cultivars among sampling times. FrFW, fruit fresh weight; FrDW, fruit dry weight; OCFrFW, oil content in fruit fresh weight; OCFrDW, oil content in fruit dry weight; FrM, fruit moisture.

As ripening progressed, OCFrFW increased for all cultivars analyzed. The oil accumulation started from June, but from August it sharply increased. From

October the rate of oil accumulation was lower (14−24%), and it even seemed to decrease in cases of 'Changlot Real' and 'Arbequina'. This can be due to a stagnation of oil accumulation together with an increase in FrM **(Figure 2 c,e)**. At the end of the ripening period, the fruits of 'Sikitita' showed the highest OCFrFW (19.4%), whereas fruits of 'Changlot Real' showed the lowest OCFrFW (12.6%). Logically, the OCFrFW was heavily affected by climatic conditions, as varies with FrM. Because of that, the OCFrDW is more accurate for comparing oil content between cultivars[16]. OCFrDW increased during the ripening period except for 'Changlot Real' and 'Arbequina', which tend to stabilize from October to December **(Figure 2d)**. This tendency was in agreement with the results obtained by different authors[17]. Cultivars evaluated in this study showed values of OCFrDW between 40.3 and 48.2% for 'Arbequina' and 'Sikitita', respectively, at the end of the ripening period **(Figure 2d)**.

3.1. Identification and quantification of phenolic compounds

3.1.1 Identification of phenolic compounds in olive fruits

Phenolic extracts from olive fruits picked at different times were analyzed by HPLC coupled to UV-vis and MS detection. On the basis of Talhaoui et al.[15], TOF-MS analyses of the olive fruit extracts were performed in the negative mode because of its high sensitivity for the detection of phenolic compounds.

The compounds were identified by interpreting their mass spectra determined *via* TOF-MS and the fragments generated taking into account the data reported in the literature. All of these results were complemented with the UV spectra provided by the DAD, which gave additional information about the family of compounds, in terms of the absorbance bands. As result, a total of 57 different phenolic compounds were characterized taking into account the samples of all the cultivars and all sampling times. Phenolic compounds belonged to simple phenols, secoiridoids, flavonoids, and oleosides. The characterized phenolic

compounds are summarized in **Table 1** including retention times, experimental and calculated *m/z*, and molecular formula together with their proposed identities and the references on which identification is based. It also includes phenolic compounds concentration (mg/kg of FrF) of each cultivar at the four sampling times considered: June, August, October, and December.

Qualitative differences in phenolic compounds were detected among fruits from the same cultivar during the sampling times (**Table 1** and **Tables S1−S6**, **Supporting Information**). Some compounds identified at the initial sampling times were not detected later, whereas some others that could not be initially detected were observed in the later stages.

Table 1. Phenolic compounds identified in olive fruit extracts by HPLC-DAD-TOF-MS, including retention time, UV max absorption, *m/z*, and range means of individual and total phenolic compounds (mg/kg of FrFW) in all the cultivars by sampling time.

	Compounds[a]	Rt (min)	UV Max (nm)	*m/z* expe.	*m/z* calc.	Molecular Formula	June	August	October	December	Ref.
1	**Hydroxytyrosol-glucoside isomer a** [3]	2.31	237	315.1080	315.1085	$C_{14}H_{20}O_8$	n.d[b]-652.7	n.d.-139.7	n.d.-33.1	n.d.-91.7	[36]
2	**Hydroxytyrosol-diglucoside isomer a** [2]	3.00	226	477.1621	477.1614	$C_{20}H_{30}O_{13}$	n.d.-272.1	n.d.	n.d.	n.d.	[37]
3	**Hydroxytyrosol-glucoside isomer b**[3]	4.58	227,276	315.1091	315.1085	$C_{14}H_{20}O_8$	n.d.	n.d.	n.d.	n.d.-212.3	[36,38]
4	**Oleoside isomer a** [4]	4.22	227	389.1083	389.1089	$C_{16}H_{22}O_{11}$	n.d.-6.4	n.d.	n.d.	n.d.	[38]
5	**Hydroxytyrosol-glucoside isomer c** [3]	4.80	226,280	315.1096	315.1085	$C_{14}H_{20}O_8$	n.d.-622.1	n.d.-38.2	n.d.-186.3	n.d.-440.6	[36,38]
6	**Hydroxytyrosol-glucoside isomer d**[3]	4.82	226,280	315.1098	315.1085	$C_{14}H_{20}O_8$	n.d.-555.0	76.5-541.1	54.0-289.8	n.d.-433.3	[36,38]
7	**Oleoside isomer b**[4]	4.99	227,280	389.1101	389.1089	$C_{16}H_{22}O_{11}$	4.0-86.9	n.d.	n.d.	n.d.	[38]
8	**Hydroxytyrosol**[3]	5.14	226,280	153.0549	153.0577	$C_8H_{10}O_3$	n.d.	57.4-209.9	57.1-171.7	61.6-108.0	[39]
9	**Oleoside derivative isomer a**[4]	5.67	226,276	407.1546	407.1559	$C_{17}H_{28}O_{11}$	1.8-11.6	2.1-12.5	11.6-24.8	12.3-24.3	[38]
10	**Hydroxytyrosol-diglucoside isomer b**[2]	5.91	226	477.1605	477.1614	$C_{20}H_{30}O_{13}$	n.d.-4.5	n.d.	n.d.	n.d.	[37]
11	**Oleoside derivative isomer b**[4]	6.26	226	407.1579	407.1559	$C_{17}H_{28}O_{11}$	n.d.-8.5	34.3-123.3	62.4-213.2	52.5-231.2	[38]
12	**Tyrosol glucoside**[3]	6.59	226,276	299.1139	299.1136	$C_{14}H_{20}O_7$	44.5-1180.7	38.7-323	n.d.-102.9	n.d.-615.2	[38]
13	**Tyrosol**[3]	7.73	226,276	137.0602	137.0608	$C_8H_{10}O_2$	n.d.	19.5-141	28.5-34.7	n.d.	[36,38]
14	**Elenolic acid glucoside isomer a**[5]	8.70	226,276	403.1261	403.1246	$C_{17}H_{24}O_{11}$	n.d.-161.4	n.d.	n.d.	n.d.	[38]
15	***p*-coumaric acid**[3]	8.73	227	163.0396	163.0401	$C_8H_8O_3$	n.d.	37.1-133.4	19.2-86.2	21.1-79.0	[38]
16	**oleoside isomer c**[4]	9.00	228,280	389.1929	389.1089	$C_{16}H_{22}O_{11}$	30.8-336.4	n.d.-7.6	n.d.-1.8	n.d.	[36,38]

	Compounds[a]	Rt (min)	UV Max (nm)	*m/z* expe.	*m/z* calc.	Molecular Formula	June	August	October	December	Ref.
17	**6-ß-glucopyranosyl oleoside[4]**	9.37	226	551.1568	551.1618	$C_{22}H_{32}O_{16}$	n.d.	1.0-6.9	n.d.	n.d.	[38,39]
18	**Gallocatechin[2]**	10.19	226	305.0699	305.0667	$C_{15}H_{14}O_7$	n.d.	1.5-5.9	0.2-4.6	n.d.-3.1	[40]
19	**Elenolic acid glucoside isomer b[5]**	10.35	226,276	403.1252	403.1246	$C_{17}H_{24}O_{11}$	1.6-7.2	n.d.	n.d.	n.d.	[38]
20	**Elenolic acid glucoside isomer c[5]**	10.53	226,276	403.1245	403.1246	$C_{17}H_{24}O_{11}$	n.d.-14.4	n.d.	n.d.	n.d.	[38]
21	**Oleuropein aglycon derivative[1]**	11.44	226	377.1403	377.1453	$C_{16}H_{26}O_{10}$	38.9-281.1	27.9-273.1	15.7-146.3	11.7-106.2	[36,38,41]
22	**Luteolin diglucoside[2]**	11.76	227,268,337	609.1438	609.1461	$C_{27}H_{30}O_{16}$	0.6-4.3	n.d.	n.d.	n.d.	[4,15]
23	**ß-hydroxyverbascoside (ß-hydroxyacteoside) iso a[3]**	12.06	227	639.1946	639.1931	$C_{29}H_{36}O_{16}$	n.d.	n.d.-1.3	4.7-23.8	n.d.-17.0	[42,43]
24	**ß-hydroxyverbascoside (ß-hydroxyacteoside) iso b[3]**	12.21	227	639.1920	639.1931	$C_{29}H_{36}O_{16}$	n.d.	n.d.	4.8-41.1	n.d.-15.1	[42,43]
25	**Elenolic acid glucoside isomer d[5]**	12.39	226,276	403.1252	403.1246	$C_{17}H_{24}O_{11}$	0.5-69.4	n.d.	n.d.	n.d.	[38]
26	**Demethyloleuropein[1]**	13.95	228	525.1556	525.1614	$C_{24}H_{30}O_{13}$	n.d.	n.d.	n.d.-6.8	n.d.-23.7	[15,39]
27	**10-Hydroxy-oleuropein[1]**	14.27	227	555.1715	555.1719	$C_{25}H_{32}O_{14}$	2.9-106.9	n.d.	n.d.	n.d.	[36,44]
28	**Rutin[2]**	14.48	228,255,354	609.1477	609.1461	$C_{27}H_{30}O_{16}$	166.3-412.5	75.7-425.0	9.5-273.1	19.5-189.4	[4,38]
29	**Quercetin3-*O*-glucoside[2]**	15.27	228	463.0888	463.0882	$C_{21}H_{20}O_{12}$	2.4-15.4	n.d.	n.d.	n.d.	[45]
30	**Luteolin glucoside isomer a[2]**	15.40	228,253,348	447.0961	447.0933	$C_{21}H_{20}O_{11}$	101.3-206.2	14.0-179.3	4.8-70.4	14.9-149.3	[15,39]
31	**Verbascoside isomer a[2]**	15.61	228,286,331	623.1990	623.1981	$C_{29}H_{36}O_{15}$	n.d.-101.7	549.3-2125.4	482.5-1269.0	307.9-731.3	[36,41]
32	**Oleuropein hexose isomer a[1]**	16.22	227	701.2288	701.2298	$C_{31}H_{42}O_{18}$	1.8-17.4	n.d.	n.d.	n.d.	[39]
33	**Verbascoside b[3]**	16.96	228,286,330	623.1983	623.1981	$C_{29}H_{36}O_{15}$	n.d.	n.d.	71.7-205.7	37.6-103.9	[36,38,41]
34	**Oleuropein hexose isomer b[1]**	17.28	228	701.2269	701.2298	$C_{31}H_{42}O_{18}$	4.5-63.3	n.d.-13.3	n.d.	n.d.	[4,39]

	Compounds[a]	Rt (min)	UV Max (nm)	*m/z* expe.	*m/z* calc.	Molecular Formula	June	August	October	December	Ref.
35	**Luteolin glucoside isomer b[2]**	17.49	229,268,336	447.1748	447.0933	$C_{21}H_{20}O_{11}$	n.d.-13.9	n.d.	n.d.	n.d.	[39]
36	**Oleuropein hexose isomer c[1]**	17.75	226	701.2280	701.2298	$C_{31}H_{42}O_{18}$	3.8-17.1	12.8-39.1	n.d.	n.d.	[4,38]
37	**Apigen.d.n rutinoside[2]**	17.95	230-273-339	577.1545	577.1563	$C_{27}H_{30}O_{14}$	n.d.	n.d.	n.d.-6.7	n.d.-7.8	[39]
38	**Luteolin glucoside isomer c[2]**	18.03	229,268,336	447.0946	447.0933	$C_{21}H_{20}O_{11}$	12.1-38.8	n.d.-45.3	n.d.-11.2	n.d.	[39]
39	**Oleuropein hexose isomer d[1]**	18.05	226	701.2291	701.2298	$C_{31}H_{42}O_{18}$	n.d.-7.2	n.d.-27.3	n.d.	n.d.-3.5	[4,38]
40	**Caffeoyl-6-oleoside[4]**	18.48	226	551.1424	551.1406	$C_{25}H_{28}O_{14}$	n.d.	n.d.	n.d.-94.2	n.d.-87.8	[46,47]
41	**Oleuropein isomer a[1]**	18.87	235,282	539.1767	539.1770	$C_{25}H_{32}O_{13}$	n.d.	n.d.	n.d.-6.7	n.d.-7.8	[38,39]
42	**2″-Methoxyoleuropein isomer a [1]**	19.05	230,280	569.1891	569.1876	$C_{26}H_{34}O_{14}$	n.d.-45.2	n.d.	n.d.	n.d.	[15,44]
43	**Oleuropein isomer b[1]**	19.07	235,282	539.1759	539.1770	$C_{25}H_{32}O_{13}$	n.d.	n.d.	n.d.-9.4	n.d.-5.5	[38,39]
44	**2″-Methoxyoleuropein isomer b[1]**	19.20	235,282	569.1885	569.1876	$C_{26}H_{34}O_{14}$	n.d.-72.5	n.d.	n.d.	n.d.	[15,44]
45	**Oleuropein isomer c[1]**	19.53	235,282	539.1767	539.1770	$C_{25}H_{32}O_{13}$	289.0-5422.3	5.1-299.7	n.d.-11.0	n.d.-29.0	[38,39]
46	**6-*p*-Coumaroyl secologanoside isomer[4]**	19.80	232,310	535.1457	535.1457	$C_{25}H_{28}O_{13}$	n.d.	n.d.-96.3	19.9-184.0	9.9-176.4	[36,46]
47	**Oleuropein isomer d[1]**	20.15	235	539.1795	539.1770	$C_{25}H_{32}O_{13}$	n.d.-91.2	n.d.	n.d.-5.9	n.d.-2.3	[38,39]
48	**Oleuropein isomer e[1]**	20.47	235	539.1789	539.1770	$C_{25}H_{32}O_{13}$	n.d.-5.4	n.d.	n.d.-6.6	n.d.-4.2	[38,39]
49	**Oleuropein aglycon isomer a[1]**	20.59	232,280	377.1236	377.1242	$C_{19}H_{22}O_8$	23.3-146.6	n.d.-198.7	n.d.-9.5	n.d.-9.5	[38,39,48]
50	**6-*p*-Coumaroyl secologanoside isomer b[4]**	20.72	233	535.1454	535.1457	$C_{25}H_{28}O_{13}$	n.d.	n.d.	n.d.	n.d.-2.6	[36,46]
51	**Oleuropein aglycon isomer b[1]**	20.86	232,280	377.1242	377.1242	$C_{19}H_{22}O_8$	n.d.	n.d.-184.0	n.d.-19.9	n.d.-9.9	[38,39,48]
52	**Oleuropein isomer f[1]**	21.22	235	539.1756	539.1770	$C_{25}H_{32}O_{13}$	n.d.	n.d.	n.d.-5.4	n.d.-3.9	[38,39]

	Compounds[a]	Rt (min)	UV Max (nm)	*m/z* expe.	*m/z* calc.	Molecular Formula	June	August	October	December	Ref.
53	**Elenolic acid derivative isomer e**[5]	21.29	233	601.2182	601.2138	$C_{27}H_{38}O_{15}$	n.d.-135.9	n.d.	n.d.	n.d.	[49,50]
54	**Ligstroside**[1]	21.41	233,280	523.1905	523.1821	$C_{25}H_{32}O_{12}$	17.8-437.4	n.d.	n.d.	n.d.	[4]
55	**Luteolin**[2]	21.94	236	285.0411	285.0399	$C_{15}H_{10}O_6$	1.8-18.9	15.1-279.6	25.3-223.6	18.4-68.5	[38,41,39]
56	**Apigenin**[2]	24.62	236	269.0461	269.0455	$C_{15}H_{10}O_5$	n.d.	n.d.	n.d.-6.4	n.d.-2.6	[38,39]
57	**Diosmetin**[2]	25.54	236	299.0561	299.0551	$C_{16}H_{12}O_6$	n.d.	n.d.	1.5-12.7	0.5-8.2	[44]
	Total						**1911.3-9343.7**	**2102.7-3883.9**	**1368.3-2388.6**	**1066.8-2699.9**	

[a] Superscript numbers indicate phenolic groups: 1, secoiridoid; 2, flavonoids; 3, simple phenols; 4, oleosides; 5, elenolic acids glucosides. [b]n.d, not detected.

Specifically, many glycosylated phenolic compounds were probably hydrolyzed during the ripening, which was observed by the disappearance of hydroxytyrosol glucoside isomer a, hydroxytyrosol diglucoside isomer a, all oleoside isomers, tyrosol glucoside, elenolic acid glucoside, luteolin diglucoside, 10-hydroxy-oleuropein, quercetin 3-*O*-glucoside, oleuropein hexose, methoxyoleuropein isomers, and ligstroside.

In this sense, many studies demonstrated the presence of a high *β*-glucosidase activity in the pulp tissue and its hydrolyzing effect on glycoside phenolic compounds during ripening[18,19]. As a result of the hydrolysis produced in the olive fruits, other phenolics appeared. Oleuropein aglycon isomer a was detected only in June and August. Other compounds also appeared during the ripening, such as hydroxytyrosol, tyrosol, *p*-coumaric acid, *β*-hydroxyverbascoside, verbascoside, apigenin rutinoside, 6-*p* coumaryl secologanoside, and apigenin. It has been suggested a metabolic relationship between oleuropein and verbascoside during ripening, based on the partial degradation of the oleuropein molecule that could be responsible for the formation of verbascoside[20,21].

It is worth noting that other compounds such as isomers c and d of hydroxytyrosol glucoside, oleoside derivative isomer a, oleuropein aglycon derivative, rutin, isomer a of luteolin glucoside, oleuropein isomer c, and luteolin were detected at all sampling times during the fruit ripening. Dağdelen et al.[22] reported the presence of those phenolic compounds among the ripening stages in ‘Ayvalik’ cultivar, too. However, the rest of the compounds (mainly isomers) were detected indifferently among sampling times and/or cultivars. With regard to cultivars, some qualitative differences among phenolic compounds were also noted; that is, demethyloleuropein was detected only in ‘Arbequina’ and ‘Arbosana’ in October and December, whereas diosmetin was identified only in ‘Sikitita’ in the same months (**Tables S1−S6, Supporting Information**). Gómez-Rico et al.[21] reported the presence of demethyloleuropein only in

'Arbequina' fruits, whereas it was absent in the rest of the six Spanish cultivars compared in their study.

3.1.2 Quantification of phenolic compounds in olive fruits

Oleuropein and other secoiridoids were quantified with the calibration curve of oleuropein; hydroxytyrosol-hexose isomers were quantified with the calibration curve of hydroxytyrosol; tyrosol and tyrosol glucoside was quantified with the calibration curve of tyrosol; gallocatechin, rutin, quercetin glucoside, and apigenin were quantified with the calibration curve of apigenin; luteolin, luteolin glucoside, and luteolin diglucoside were quantified with the calibration curve of luteolin; and *p*-coumaric acid and hydroxyverbascoside isomers were quantified with the calibration curve of caffeic acid. All phenolic compounds contents for all cultivars and sampling times are reported as milligrams of compound per kilogram of FFr (**Tables S1−S6, Supporting Information**).

3.2 Pattern of phenolic compounds in olive fruit Cultivars among sampling times

3.2.1 Total phenolic compounds

For all cultivars, total concentrations of phenols decreased significantly during ripening (**Figure 3a**), being more pronounced from June to August for the cultivars with major phenolic contents, that is, 'Picual' and 'Changlot Real'. The decrease of total phenols is mainly the result of the oleuropein hydrolysis during ripening[5,23]. In addition, an increase in phenolic compounds was observed in 'Changlot Real' in December. With regard to 'Koroneiki' cultivar, total phenols content sharply decreased between June and October. With regard to the phenolic concentration of the other cultivars, 'Arbequina' and 'Sikitita' showed a slow diminution pattern between June and October, accentuated after October with 26% decrease for 'Arbequina' and 43% for 'Sikitita'. However, 'Arbosana'

showed an increase from June to August (28%) and decreased as well after this sampling time (**Figure 3**). This behavior registered for 'Arbosana' between June and August could be due to the ripening stage experienced by this cultivar. In fact, in that period 'Arbosana' had not started the process of ripening yet (RI 0.03, **Figure 1**) and, consequently, it was still accumulating phenols, specifically oleuropein.

Intriguingly, 'Changlot Real' had significantly the highest content of total phenols through all of the sampling times, whereas the ranking of other cultivars in regard to total phenols contents fluctuated between sampling times (**Figure 3a** and **Table S7, Supporting Information**).

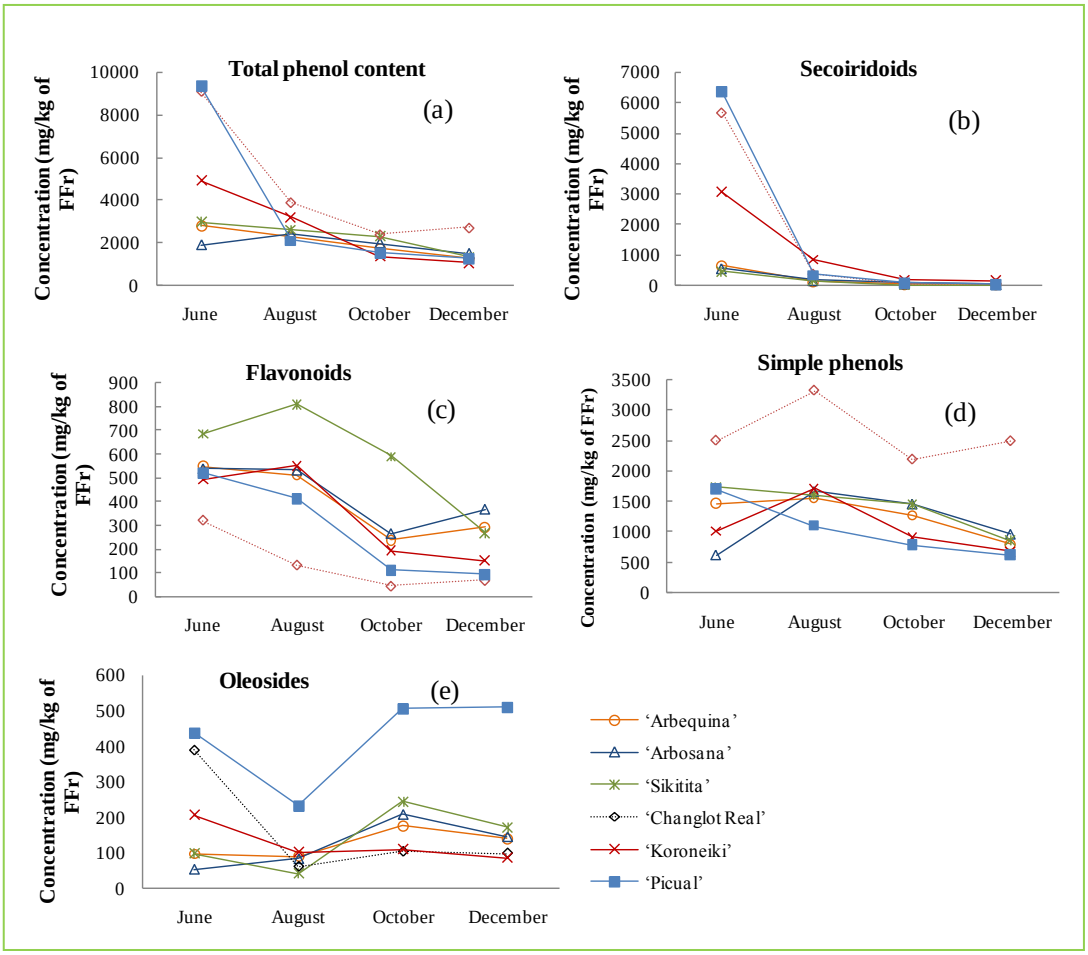

Figure 3. Evolution of the olive phenolic compounds groups of the six cultivars among sampling times.

3.2.2 Seasonal variability of phenolic compound groups

Data about the phenolic compounds concentrations were grouped into five groups **(Table 1)**: secoiridoids, group 1 (oleuropein aglycon derivative, oleuropein aglycon and isomer, demethyloleuropein, 10-hydroxy-oleuropein, oleuropein hexose and isomers, 2″-methoxyoleuropein and isomer, oleuropein and isomers, and ligstroside); flavonoids, group 2 (luteolin, luteolin diglucoside, gallocatechin, rutin, quercetin-3-*O*-glucoside, luteolin glucoside and isomers, verbascoside, apigenin, apigenin rutinoside, and diosmetin); simple phenols, group 3 (hydroxytyrosol, hydroxytyrosol-glucoside, hydroxytyrosol diglucoside and isomers, tyrosol, tyrosol glucoside, *p*-coumaric acid, and *β*-hydroxyverbascoside and isomer); oleosides, group 4 (oleoside and isomers, 6-*p*-coumaroyl secologanoside and isomer, 6-*β*-glucopyranosyl oleoside, caffeoyl-6-oleoside); and elenolic acid glucosides, group 5 (elenolic acid glucoside and isomers). Elenolic acid glucosides group was represented only for June.

Secoiridoids were the major components in fruits at the first sampling time **(Figure 3)**. Consequently, their pattern of variation during the ripening period in some cultivars was relatively the same as described before for total phenols. In fact, secoiridoids concentrations decreased sharply between June and October for all cultivars; the rate of decrease was between 89% for ‘Arbosana’ and 99% for ‘Changlot Real’ **(Figure 3b)**.There is a characteristic accumulation of oleuropein (the main secoiridoid in olive) content during the early stages of fruit development, which is attributed to a growth phase that occurs prior to ripening[5], and as the ripening progresses, oleuropein decreases significantly[5,2].

Flavonoids also decreased during fruit ripening, showing different rates of decrease for each cultivar **(Figure 3c)**. In ‘Changlot Real’ the decrease was relatively accentuated, likely due to its fast ripening **(Figure 1)**. In the other cultivars (‘Arbequina’, ‘Arbosana’, and ‘Picual’) the content of flavonoids

decreased to a lesser extent from June to August. On the contrary, 'Koroneiki' and 'Sikitita' registered a relative increase in this period. This increase was the result of the appearance of some major flavonoids and/or the increase of their contents in that sampling time, especially verbascoside, luteolin, and rutin (**Tables S4, S6, Supporting Information**). The increase of those three flavonoids was previously reported by other authors[21]. Also, Ryan et al.[24] reported a similar behavior in verbascoside contents for two cultivars ('Manzanillo' and 'Cucco'). Verbascoside gradually decreased with ripening in 'Manzanillo' olive fruits, whereas in 'Cucco' olive fruits verbascoside was not initially detected, but it started to accumulate after day 21 of ripening and then decreased once again to an undetectable level at black ripening. These results, as well as the identification described, lead to the conclusion that flavonoids are mainly synthesized at the beginning of ripening (August), but after the synthesis of secoiridoids. In December, an increase of flavonoids contents was noted in 'Changlot Real', 'Arbequina', and 'Arbosana', especially compounds such as luteolin glucoside, apigenin rutinoside, hydroxyverbascoside, and rutin. Bouaziz et al.[25] reported an increase in total flavonoids for four Tunisian cultivars during the period of fruit ripening.

Simple phenols contents showed a big increase between June and August (for example, 170 and 68% for 'Arbosana' and 'Koroneiki', respectively), except for 'Picual', for which the content gradually decreased during the ripening periods (**Figure 3d**). The content of simple phenols decreased slowly after August for all cultivars except 'Changlot Real', in which a small increase (13%) was observed after October. The increase of simple phenols at the beginning of ripening is the obvious result of the hydrolysis of glucoside phenolic compounds[18,21,26]. The general decrease in simple phenols is in accordance with observations for other olive cultivars[25,27,28].

Oleosides, considered derivatives of elenolic acids[29], showed a decrease from the beginning of fruit growth until August. The degree of decrease varied widely

from one cultivar to another, for example, 7% for ‘Arbequina’ and 84% for ‘Changlot Real’. Since August, the contents in oleosides increased tremendously in some cultivars, for example, 540% in ‘Sikitita’; however, they barely increased in ‘Koroneiki’ (8%) **(Figure 3e)**. The elenolic acid glucosides group was identified and quantified only in June (**Table S7, Supporting Information**). The increase of oleosides in August could probably due to the metabolism of transformation of elenolic acids glucosides into oleosides in fruits.

Metabolites isolated from natural sources are not necessarily the metabolites that are present in the living tissue. In addition, olive fruit is in a dynamic state, and the level of metabolites at any given time represents a composite of both catabolic and anabolic processes[30].

3.2.3 Tentative profiling on the basis of overall phenolic composition

PCA contributed to a further profiling of the accessions considered, and it was applied to the data set containing the concentrations of the five previously described compound groups. The first (PC1) and second principal components (PC2) described >73.9% of the data variability for all cases of the analysis. PC1 was clearly linked to elenolic acids, secoiridoids, and total phenols groups. In fact, it has the highest correlations with all of the components of those groups (r=-0.92, r=-0.98, and r=-0.97, respectively). PC2 was correlated to the flavonoids group (r=-0.59), oleosides group (r=0.78), and simple phenols (r=-0.57) (**Table S8, Supporting Information**). To allow a detailed analysis of the PCA study, the scatter plots have been separated by sampling time **(Figure 4)** and cultivar **(Figure 5)**.

The different cultivars were clearly separated in the scatter plots **(Figure 4)**, except between ‘Arbequina’ and ‘Arbosana’ in August and October and between both cultivars and ‘Sikitita’ in December. This confirms the high genetic variability on fruit phenol profile on olive[9,25]. ‘Sikitita’, which comes from a

cross between ‘Picual’ and ‘Arbequina’[31], was located close to ‘Arbequina’ in the scores plot. This suggests that, in contrast to the result found for leaf phenols[15], ‘Sikitita’ fruit phenols profile is more similar to ‘Arbequina’ than to ‘Picual’. However, a higher degree of similarity between ‘Sikitita’ and ‘Arbequina’ was also found in olive oil phenols profile[32].

Furthermore, a strong dependence of the phenolic profile on the sampling time was revealed, as the points corresponding to each sampling time were clearly separable in each plot (**Figure 5**). Nevertheless, the separation was difficult between October and December for almost all cultivars. This is due to the tendency of the phenolic group’s contents to stabilize at the end of the ripening stage.

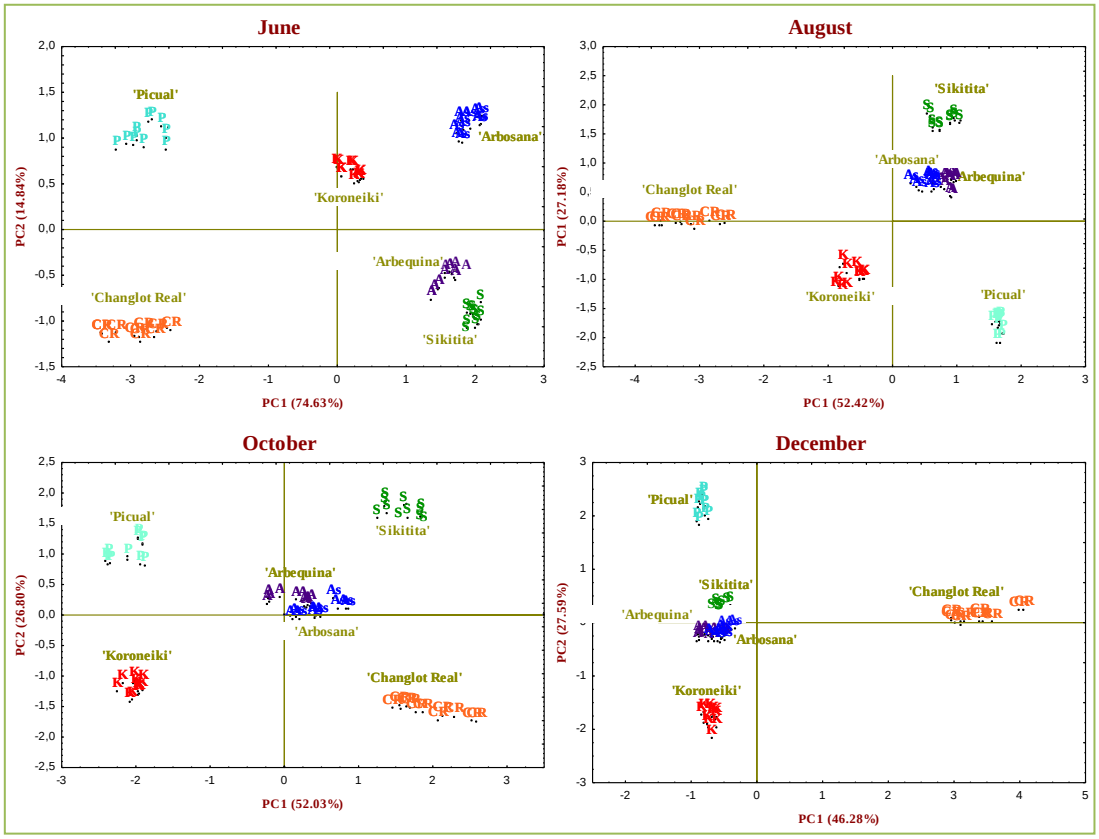

Figure 4. Scatter plots of the first and second principal components for sampling time.

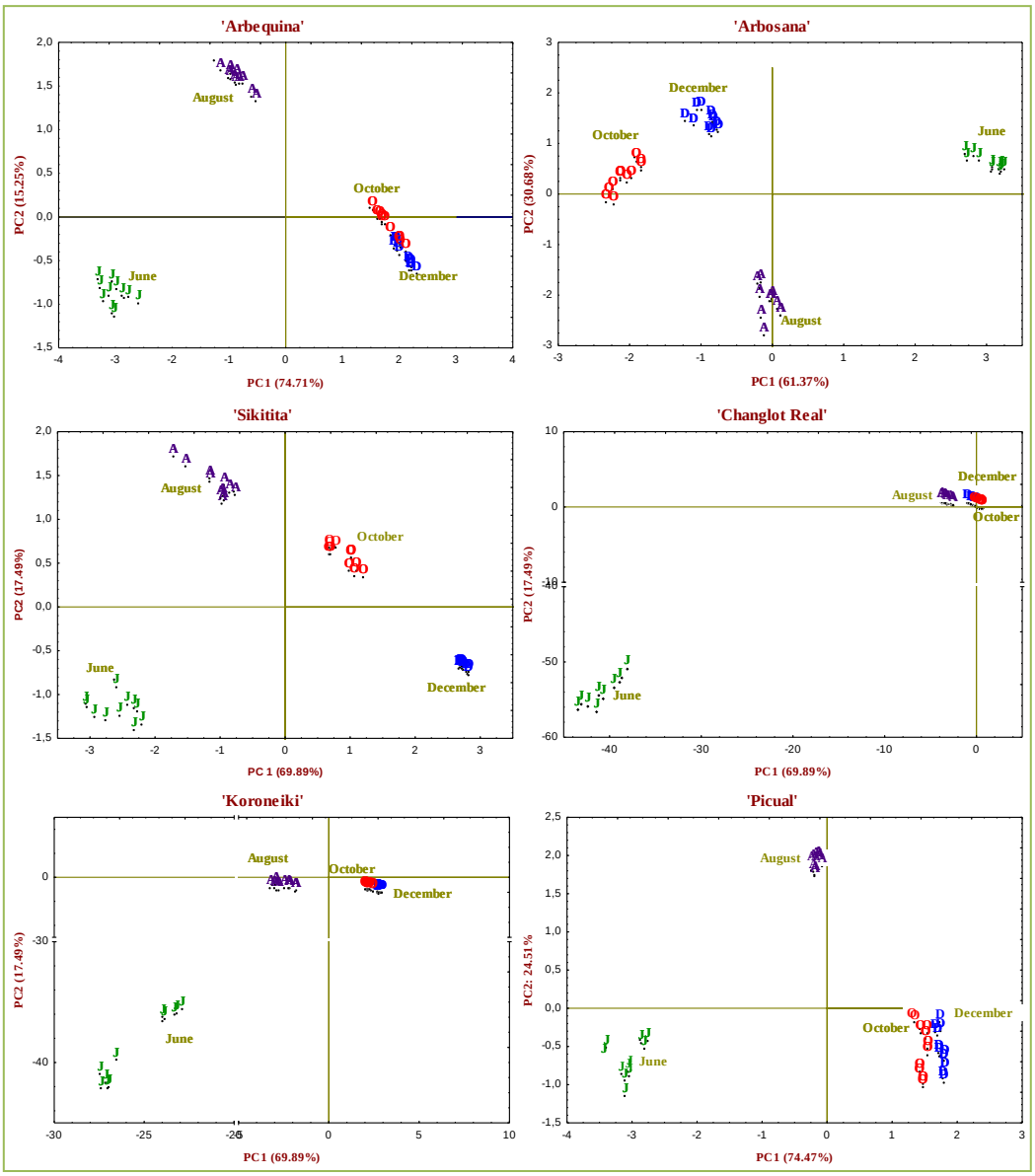

Figure 5. Scatter plots of the first and second principal components for cultivar.

3.3 Relationship between fruits traits and phenolic profile

As expected, the general tendency of fruit characteristics was an increase in the size of the fruits and oil content and, at the same time, a decrease of total phenolic compounds was observed. Despite the big differences observed in the evolution pattern of RI for the different cultivars, especially between October and December, the evolution patterns of the rest of almost all the parameters did not show big differences among cultivars (**Figure 2**).

On the one hand, the cultivar ‘Changlot Real’, which presented the highest levels of total phenols, secoiridoids, and simple phenols and the lowest level of flavonoids, showed a high FrDW and the major FrFW, medium OCFrDW, and minor OCFrFW and, finally, the major RI and FrM. On the other hand, ‘Sikitita’ cultivar, which presented the opposite profiles (the lowest total phenols and secoiridoids and the highest level of flavonoids), revealed medium FrDW, FrFW, and RI, but showed the highest OCFrFW and OCFrDW, and the lowest FrM. It is noteworthy that the fruit moisture and, thus, water content are key factors in the extraction of phenolic compounds from fruits. In fact, phenolic compounds are more soluble in water than in oil[33]. Studies on olive oil phenolic fraction have demonstrated that a reduction in oil moisture content facilitates its extraction, notably secoiridoids[34,35]. The results obtained in the study can be explained by considering that the increase of the moisture content in fruits permits a higher availability of phenolic compounds and, thus, they are more efficiently extracted with a methanol-water mixture.

It is noteworthy to mention that this study has been performed in the “on” year regarding olive alternate bearing. Thus, the study has been managed in one year as preliminary study. Despite this limitation, the results were consistent with those reported in the literature. Two more consecutive years will be studied in the future.

4. Conclusions

In summary, despite the high number of olive cultivars conserved in different cultivar collections, there are few reports on the genetic variability of fruit phenolic compounds. This study reports the evolution of agronomical fruit traits and the phenolic compounds of six of the most widely cultivated olive cultivars in Spain. In this sense, the evolution of phenolic compounds of ‘Koroneiki’, ‘Arbosana’, ‘Changlot Real’, and ‘Sikitita’ cultivars during ripening has been reported for the first time. Major changes occurred between June and October,

whereas the period between October and December was characterized by a stability of almost all studied parameters. With regard to phenolic compounds, qualitative and quantitative differences among cultivars were highlighted. Interestingly, a common pattern was observed: The first part of the ripening period was dominated by secoiridoids (mainly oleuropein), but as the ripening progressed simple phenols and flavonoids became the major components. PCA provided a separation of the phenolic profile in olive fruits for different sampling times and cultivars. However, at the opposite of the agronomical traits, the ranking of cultivars in regard to phenolic compounds was not stable during ripening. The content of the different phenol groups was very variable among cultivars and sampling times. However, it can be observed that 'Koroneiki' stands out for its flavonoids content, whereas 'Changlot Real' for simple phenols and 'Picual' for oleosides.

References

(1) Bulotta, S.; Celano, M.; Lepore, S. M.; Montalcini, T.; Pujia, A.; Russo, D. Beneficial effects of the olive oil phenolic components oleuropein and hydroxytyrosol: Focus on protection against cardiovascular and metabolic diseases. *J. Transl. Med*. **2014**, 12, 219.

(2) Ryan, D.; Robards, K. Critical review. Phenolic compounds in olives. *Analyst* 1**998**, 123, 31R–44R.

(3) Uylaşer, V.; Yildiz, G. The historical development and nutritional importance of olive and olive oil constituted an important part of the Mediterranean diet. *Crit. Rev. Food Sci. Nutr*. **2014**, 54, 1092–1101.

(4) Silva, S.; Gomes, L.; Leitao, F.; Coelho, A. V.; Boas, L. V. Phenolic compounds and antioxidant activity of *Olea europaea* L. fruits and leaves. *Food Sci. Technol. Int*. **2006**, 12, 385–396.

(5) Amiot, M. J.; Fleuriet, A.; Macheix, J. J. Accumulation of oleuropein derivatives maturation. *Phytochemistry* **1989**, 28, 67–69.

(6) Conde, C.; Delrot, S.; Gerós, H. Physiological, biochemical and molecular changes occurring during olive development and ripening. *J. Plant Physiol*. **2008**, 165, 1545–1562.

(7) Briante, R.; Patumi, M.; Limongelli, S.; Febbraio, F.; Vaccaro, C.; Di Salle, A.; La Cara, F.; Nucci, R. Changes in phenolic and enzymatic activities content during fruit ripening in two Italian cultivars of *Olea europaea* L. *Plant Sci*. **2002**, 162, 791–798.

(8) Mailer, R. J.; Ayton, J.; Conlan, D. Influence of harvest timing on olive (*Olea europaea*) oil accumulation and fruit characteristics under Australian conditions. *J. Food, Agric. Environ*. **2007**, 5, 58–63.

(9) Morelló, J.-R.; Romero, M.-P.; Motilva, M.-J. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from 'Arbequina', 'Farga', and 'Morrut' cultivars. *J. Agric. Food Chem*. **2004**, 52, 6002–6009.

(10) Tan, H.; Tuck, K. L.; Stupans, I.; Hayball, P. J. Simultaneous determination of oleuropein and hydroxytyrosol in rat plasma using liquid chromatography with fluorescence detection. *J. Chromatogr. B*. **2003**, 785, 187–191.

(11) El Riachy, M.; Priego-Capote, F.; León, L.; Rallo, L.; Luque de Castro, M. D. Hydrophilic antioxidants of virgin olive oil. Part 2: Biosynthesis and biotransformation of phenolic compounds in virgin olive oil as affected by agronomic and processing factors. *Eur. J. Lipid Sci. Technol*. **2011**, 113, 692–707.

(12) Frias, L.; Garcia-Ortiz, A.; Hermoso, M.; Jimenez, A.; Llavero Del Pozo, M. P.; Morales, J.; Ruano, T.; Uceda, M. *Analista de laboratorio de almazara*; Junta de Andalucía. Consejería de Aglricultura y Pesca, Ed.; Viceconsejería. Servicio de Publicaciones y Divulgación, 1991.

(13) Del Río, C.; Romero, A. M. Whole, unmilled olives can be used to determine their oil content by nuclear magnetic resonance. *HorTechnology* **1999**, 9, 675–680.

(14) Vinha, A. F.; Silva, B. M.; Andradc, P. B.; Rosa, M.; Pcrcira, J. A.; Beatriz,

M. Development and evaluation of an HPLC/DAD method for the analysis of phenolic compounds from olive fruits. *J. Liq. Chromatogr. Relat. Technol*. **2002**, 25, 151–160.

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

(16) Beltrán, G.; del Río, C.; Sánchez, S.; Martínez, L. Seasonal changes in olive fruit characteristics and oil accumulation during ripening process. *J. Sci. Food Agric*. **2004**, 84, 1783–1790.

(17) Baccouri, B.; Ben Temime, S.; Taamalli, W.; Daoud, D.; M'Sallem, M.; Zarrouk, M. Analytical charachteristics of virgin olive oils from two new varieties obtained by controlled crossing on 'Meski' variety. *J. Food Lipids* **2007**, 14, 19–34.

(18) Romero-Segura, C.; García-Rodríguez, R.; Sánchez-Ortiz, A.; Sanz, C.; Pérez, A. G. The role of olive *β*-glucosidase in shaping the phenolic profile of virgin olive oil. *Food Res. Int*. **2012**, 45, 191–196.

(19) Japón Luján, R.; Priego Capote, F.; Luque de Castro, M. D. Temporal metabolomic analysis of *O*-glucoside phenolic compounds and their aglycone forms in olive tree and derived materials. *Phytochem. Anal*. **2009**, 20, 221–230.

(20) Servili, M.; Baldioli, M.; Selvaggini, R.; Macchioni, A.; Montedoro, G. Phenolic compounds of olive fruit: One- and two-dimensional nuclear magnetic resonance characterization of nuzhenide and its distribution in the constitutive parts of fruit. *J. Agric. Food Chem*. **1999**, 47, 12–18.

(21) Gómez-Rico, A.; Fregapane, G.; Salvador, M. D. Effect of cultivar and ripening on minor components in Spanish olive fruits and their corresponding virgin olive oils. *Food Res. Int*. **2008**, 41, 433–440.

(22) Dağdelen, A.; Tümen, G.; Özcan, M. M.; Dündar, E. Phenolics profiles of olive fruits (*Olea europaea* L.) and oils from 'Ayvalık', 'Domat' and

'Gemlik' varieties at different ripening stages. *Food Chem*. **2013**, 136, 41–45.

(23) Muzzalupo, I.; Stefanizzi, F.; Perri, E.; Chiappetta, A. Variation of the antioxidant compounds in Italian olive (*Olea europaea*) drupes during ripening stage. *Acta Hortic*. **2012**, 949, 209–212.

(24) Ryan, D.; Robards, K.; Lavee, S. Changes in phenolic content of olive during maturation. *Int. J. Food Sci. Technol*. **1999**, 34, 265–274.

(25) Bouaziz, M.; Jemai, H.; Khabou, W.; Sayadi, S. Oil content, phenolic profiling and antioxidant potential of Tunisian olive drupes. *J. Sci. Food Agric*. **2010**, 90, 1750–1758.

(26) Mazzuca, S.; Spadafora, A.; Innocenti, A. M. Cell and tissue localization of beta-glucosidase during the ripening of olive fruit (*Olea europaea*) by *in situ* activity assay. *Plant Sci*. **2006**, 171, 726–733.

(27) Machado, M.; Felizardo, C.; Fernandes-Silva, A. A.; Nunes, F. M.; Barros, A. Polyphenolic compounds, antioxidant activity and l-phenylalanine ammonia-lyase activity during ripening of olive cv. Cobrançosa under different irrigation regimes. *Food Res. Int*. **2013**, 51, 412–421.

(28) Vinha, A. F.; Ferreres, F.; Silva, B. M.; Valentão, P.; Gonçalves, A.; Pereira, J. A.; Oliveira, M. B.; Seabra, R. M.; Andrade, P. B. Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): Influences of cultivar and geographical origin. *Food Chem*. **2005**, 89, 561–568.

(29) Silva, S.; Gomes, L.; Leitão, F.; Bronze, M.; Coelho, A. V.; Boas, L. V Secoiridoids in olive seed: Characterization of nüzhenide and 11-methyl oleosides by liquid chromatography with diode array and mass spectrometry. *Grasas y Aceites* **2010**, 61, 157–164.

(30) Ryan, D.; Antolovich, M.; Prenzler, P.; Robards, K.; Lavee, S. Biotransformations of phenolic compounds in *Olea europaea* L. *Sci. Hortic. (Amsterdam)*. **2002**, 92, 147–176.

(31) Rallo, L.; Barranco, D.; De la Rosa, R.; León, L. "Chiquitita" Olive. *HortScience* **2008**, 43, 529–531.

(32) Garcia-Gonzalez, D.; Tena, N.; Aparicio, R. Quality characterization of the

new virgin olive oil Var. 'Sikitita' by phenols and volatile compounds. *J. Agric. Food Chem*. **2010**, 58, 8357–8364.

(33) Benito, M.; Lasa, J. M.; Gracia, P.; Oria, R.; Abenoza, M.; Varona, L.; Sánchez-Gimeno, A. C. Olive oil quality and ripening in super-high-density 'Arbequina' orchard. *J. Sci. Food Agric*. **2013**, 93, 2207–2220.

(34) Bakhouche, A.; Lozano-Sánchez, J.; Ballus, C. A.; Bendini, A.; Gallina-Toschi, T.; Fernández-Gutiérrez, A.; Segura-Carretero, A. A new extraction approach to correct the effect of apparent increase in the secoiridoid content after filtration of virgin olive oil. *Talanta* **2014**, 127, 18–25.

(35) Gómez-Caravaca, A. M.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Lercker, G. Effect of filtration systems on the phenolic content in virgin olive oil by HPLC-DAD-MSD. *Am. J. Food Technol*. **2007**, 2, 671–678.

(36) Rubio-Senent, F.; Lama-muñoz, A.; Rodríguez-Gutiérrez, G.; Fernanández-Bolaños, J. Isolation and identification of phenolic glucosides from thermally treated olive oil by-products. *J. Agric. Food Chem*. **2013**, 187, 166-173.

(37) Schieber, A.; Keller, P.; Streker, P.; Klaiber, I.; Carle, R. Detection of isorhamnetin glycosides in extracts of apples (*Malus domestica* cv. Brettacher) by HPLC-PDA and HPLC-APCI-MS/MS. *Phytochem. Anal*. **2002**, 13, 87–94.

(38) Cardoso, S. M.; Guyot, S.; Marnet, N.; Lopes-da-Silva, J. A.; Renard, C. M.; Coimbra, M. A. Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J. Sci. Food Agric*. **2005**, 85, 21–32.

(39) Savarese, M.; Demarco, E.; Sacchi, R. Characterization of phenolic extracts from olives (*Olea europaea* cv. Pisciottana) by electrospray ionization mass spectrometry. *Food Chem*. **2007**, 105, 761–770.

(40) Rodríguez-pérez, C.; Quirantes-Piné, R.; Amessis-ouchemoukh, N.; Madani, K.; Segura-Carretero, A.; Fernández-Gutierrez, A. A metabolite-profiling approach allows the identification of new compounds from

Pistacia lentiscus leaves. *J. Pharm. Biomed. Anal*. **2013**, 77, 167–174.

(41) Peralbo-Molina, A.; Priego-capote, F.; Luque de Castro, M. D. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography tandem mass spectrometry with a quadrupole−quadrupole-time-of-flight mass detector. *J. Agric. Food Chem*. **2012**, 60, 11542−11550.

(42) Jerman Klen, T.; Golc Wondra, A.; Vrhovšek, U.; Mozetič Vodopivec, B. Phenolic profiling of olives and olive oil process-derived matrices using UPLC-DAD-ESI-QTOF-HRMS analysis. *J. Agric. Food Chem*. **2015**, 63, 3859–3872.

(43) Lama-Muñoz, A.; Rodríguez-Gutiérrez, G.; Rubio-Senent, F.; Palacios-Díaz, R.; Fernández-Bolaños, J. A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste. *Food Chem*. **2013**, 140, 154–60.

(44) Taamalli, A.; Gómez-Caravaca, A. M.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area. *Eur. Food Res. Technol*. **2010**, 231, 965–975.

(45) Barros, A. I.; Freire, I.; Gonçalves, B.; Bacelar, E.; Gomes, S.; Lopes, J.; Guedes-Pinto, H.; Martins-Lopes, P. Evaluation of chemical and phenotypic changes in 'Blanqueta', 'Cobrançosa', and 'Galega' during olive fruits ripening. *CyTA - J. Food.* **2013**, 11, 136–141.

(46) Obied, H. K.; Bedgood, D. R.; Prenzler, P. D.; Robards, K. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Anal. Chim. Acta.* **2007**, 603, 176–189.

(47) Kanaki, P.; Termentzi, A.; Michel, T.; Gikas, E.; Halabalaki, M.; Skaltsounis, A.-L. From olive drupes to olive oil. An HPLC-orbitrap-based qualitative and quantitative exploration of olive key metabolites. *Planta Med*. **2013**, 79, 1576–1587.

(48) Aranda, E.; García-Romera, I.; Ocampo, J. A.; Carbone, V.; Mari, A.;

Malorni, A.; Sannino, F.; De Martino, A.; Capasso, R. Chemical characterization and effects on *Lepidium sativum* of the native and bioremediated components of dry olive mill residue. *Chemosphere.* **2007**, 69, 229–239.

(49) Fu, S.; Arráez-Roman, D.; Segura-Carretero, A.; Menéndez, J. A.; Menéndez-Gutiérrez, M. P.; Micol, V.; Fernández-Gutiérrez, A. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal. Bioanal. Chem*. **2010**, 397, 643–654.

(50) Herrero, M.; Temirzoda, T. N.; Segura-Carretero, A.; Quirantes, R.; Plaza, M.; Ibañez, E. New possibilities for the valorization of olive oil by-products. *J. Chromatogr. A*. **2011**, 1218, 7511–7520.

Supporting information

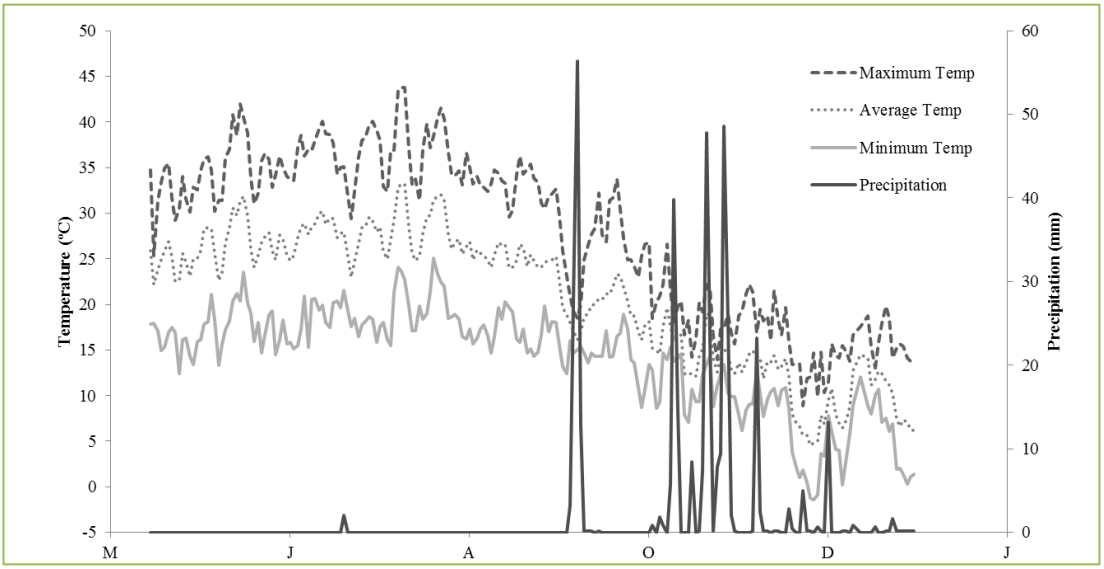

Figure S1. Climatic data during the studied sampling times: J(June), A(August), O (October), D (December).

Table S1. Quantification of the identified phenolic compounds in olive fruits of 'Arbequina' cultivar expressed as mg/kg of FrFW. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Phenolic compounds	Sampling time			
	'Arbequina'	June	August	October	December
1	**Hydroxytyrosol-glucoside isomer a**	358.9	NI	NI	NI
2	**Hydroxytyrosol-diglucoside isomer a**	55.3	NI	NI	NI
3	**Hydroxytyrosol-glucoside isomer b**	NI	NI	NI	NI
4	**Oleoside isomer a**	1.7	NI	NI	NI
5	**Hydroxytyrosol-glucoside isomer c**	NI	NI	NI	NI
6	**Hydroxytyrosol-glucoside isomer d**	555.0^{a}	522.2^{a}	257.5^{b}	257.4^{b}
7	**Oleoside isomer b**	4.0^{a}	NI	NI	NI
8	**Hydroxytyrosol**	NI	86.5^{c}	72.8^{a}	62.9^{c}
9	**Oleoside derivative isomer a**	1.8^{d}	7.9^{c}	21.9^{a}	20.2^{b}
10	**Hydroxytyrosol-diglucoside isomer b**	NI	NI	NI	NI
11	**Oleoside derivative isomer b**	8.5^{d}	38.9^{c}	63.5^{a}	57.9^{b}
12	**Tyrosol glucoside**	504.8^{a}	139.2^{b}	NI	NI
13	**Tyrosol**	NI	19.5^{b}	31.6^{a}	NI
14	**Elenolic acid glucoside isomer a**	27.4^{a}	NI	NI	NI
15	***p*-coumaric acid**	NI	77.0^{a}	35.0^{b}	27.9^{c}
16	**oleoside isomer c**	79.8^{a}	5.7^{b}	1.0^{b}	NI
17	**6- ß -glucopyranosyl oleoside**	NI	2.5^{a}	NI	NI
18	**Gallocatechin**	NI	4.5^{a}	2.4^{b}	1.4^{c}
19	**Elenolic acid glucoside isomer b**	2	NI	NI	NI
20	**Elenolic acid glucoside isomer c**	8.8	NI	NI	NI
21	**Oleuropein aglycon derivative**	134.6^{a}	28.8^{b}	16.6^{c}	12.7^{c}
22	**Luteolin diglucoside**	1.1	NI	NI	NI
23	**ß -Hydroxyverbascoside isomer a**	NI	NI	10.3	NI
24	**ß -Hydroxyverbascoside isomer b**	NI	NI	14	NI
25	**Elenolic acid glucoside isomer d**	5.8	NI	NI	NI
26	**Demethyloleuropein**	NI	NI	6.8^{b}	23.7^{a}
27	**10-Hydroxy-oleuropein**	8.9	NI	NI	NI
28	**Rutin**	297.3^{a}	295.2^{a}	159.3^{b}	110.2^{c}
29	**Quercetin3-*O*-glucoside**	2.9	NI	NI	NI
30	**Luteolin glucoside isomer a**	201.0^{a}	179.3^{b}	43.5^{d}	149.3^{c}
31	**Verbascoside isomer a**	NI	717.7^{a}	674.7^{a}	340.7^{b}
32	**Oleuropein hexose isomer a**	7.7	NI	NI	NI
33	**Verbascoside isomer b**	NI	NI	174.6^{a}	103.9^{b}
34	**Oleuropein hexose isomer b**	63.3	NI	NI	NI
35	**Luteolin glucoside isomer b**	NI	NI	NI	NI

	Phenolic compounds	Sampling time			
	'Arbequina'	June	August	October	December
36	**Oleuropein hexose isomer c**	6.4[b]	12.9[a]	NI	NI
37	**Apigenin rutinoside**	NI	NI	2.1[b]	5.9[a]
38	**Luteolin glucoside isomer c**	32.0[a]	NI	NI	NI
39	**Oleuropein hexose isomer d**	7.2[b]	25.1[a]	NI	NI
40	**Caffeoyl-6-oleoside**	NI	NI	NI	NI
41	**Oleuropein isomer a**	NI	NI	NI	NI
42	**2″-Methoxyoleuropein isomer a**	.9[a]	NI	NI	NI
43	**Oleuropein isomer b**	NI	NI	6.5[a]	3.9[b]
44	**2″-Methoxyoleuropein isomer b**	6.5[a]	NI	NI	NI
45	**Oleuropein isomer c**	316.0[a]	18.4[b]	NI	1.8[b]
46	**6-*p*-coumaroyl secologanoside isomer a**	NI	33.3[c]	89.5[a]	58.9[b]
47	**Oleuropein isomer d**	10	NI	NI	NI
48	**Oleuropein isomer e**	5.4	NI	NI	NI
49	**Oleuropein aglycon isomer a**	23.3[a]	15.8[b]	NI	NI
50	**6-*p*-Coumaroyl secologanoside isomer b**	NI	NI	NI	1.4
51	**Oleuropein aglycon isomer b**	NI	NI	NI	NI
52	**Oleuropein isomer f**	NI	NI	NI	NI
53	**Elenolic acid derivative isomer e**	NI	NI	NI	NI
54	**Ligstroside**	45.2	NI	NI	NI
55	**Luteolin**	13.8[d]	34.2[a]	28.4[b]	25.4[c]
56	**Diosmetin**	NI	NI	NI	0.5
57	**Apigenin**	NI	NI	2.1[a]	0.5[b]
	Total	**2801.2[a]**	**2264.6[b]**	**1714.0[c]**	**1266.7[d]**

NI (Non Identified)

Table S2. Quantification of the identified phenolic compounds in olive fruits of 'Arbosana' cultivar expressed as mg/kg of FrFW. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Phenolic compounds	Sampling time			
	'Arbosana'	June	August	October	December
1	**Hydroxytyrosol-glucoside isomer a**	316.3[a]	139.7[b]	NI	NI
2	**Hydroxytyrosol-diglucoside isomer a**	59.3	NI	NI	NI
3	**Hydroxytyrosol-glucoside isomer b**	NI	NI	NI	NI
4	**Oleoside isomer a**	1.6[a]	NI	NI	NI
5	**Hydroxytyrosol-glucoside isomer c**	NI	NI	NI	NI
6	**Hydroxytyrosol-glucoside isomer d**	96.9[c]	76.5[d]	158.0[b]	276.7[a]
7	**Oleoside isomer b**	7.5	NI	NI	NI
8	**Hydroxytyrosol**	NI	84.6[b]	106.3[a]	73.4[c]
9	**Oleoside derivative isomer a**	11.6[b]	12.5[b]	24.8[a]	24.3[a]
10	**Hydroxytyrosol-diglucoside isomer b**	NI	NI	NI	NI
11	**Oleoside derivative isomer b**	NI	53.9[c]	81.9[a]	71.3[b]
12	**Tyrosol glucoside**	44.5[a]	46.2[a]	NI	NI
13	**Tyrosol**	NI	46.8[a]	31.9[b]	NI
14	**Elenolic acid glucoside isomer a**	124.4	NI	NI	NI
15	***p*-coumaric acid**	NI	133.4[a]	86.2[b]	79.0[b]
16	**oleoside isomer c**	30.8[a]	5.9[b]	0.7[c]	NI
17	**6-ß-Glucopyranosyl oleoside**	NI	NI	NI	NI
18	**Gallocatechin**	NI	1.5[a]	0.2[b]	NI
19	**Elenolic acid glucoside isomer b**	2.7	NI	NI	NI
20	**Elenolic acid glucoside isomer c**	14.4[a]	NI	NI	NI
21	**Oleuropein aglycon derivative**	172.7[a]	101.5[b]	44.4[c]	21.0[d]
22	**Luteolin diglucoside**	1.5	NI	NI	NI
23	**ß -Hydroxyverbascoside isomer a**	NI	NI	23.8[a]	11.0[b]
24	**ß -Hydroxyverbascoside isomer b**	NI	NI	41.1[a]	14.0[b]
25	**Elenolic acid glucoside isomer d**	4.2	NI	NI	NI
26	**Demethyloleuropein**	NI	NI	0.4[b]	9.7[a]
27	**10-Hydroxy-oleuropein**	2.9	NI	NI	NI
28	**Rutin**	357.7[a]	351.2[a]	118.4[c]	189.4[b]
29	**Quercetin3-*O*-glucoside**	7.9	NI	NI	NI
30	**Luteolin glucoside isomer a**	151.8[a]	114.6[c]	70.4[d]	129.8[b]
31	**Verbascoside isomer a**	101.7[d]	1145.1[a]	834.6[b]	406.4[c]
32	**Oleuropein hexose isomer a**	1.8	NI	NI	NI
33	**Verbascoside isomer b**	NI	NI	182.2[a]	101.8[b]
34	**Oleuropein hexose isomer b**	12.2	NI	NI	NI
35	**Luteolin glucoside isomer b**	NI	NI	NI	NI

	Phenolic compounds	Sampling time			
	'Arbosana'	June	August	October	December
36	**Oleuropein hexose isomer c**	3.8[b]	12.8[a]	NI	NI
37	**Apigenin rutinoside**	NI	NI	6.7[b]	7.8[a]
38	**Luteolin glucoside isomer c**	13.6	NI	NI	NI
39	**Oleuropein hexose isomer d**	4.1[b]	27.3[a]	NI	NI
40	**Caffeoyl-6-oleoside**	NI	NI	NI	NI
41	**Oleuropein isomer a**	NI	NI	NI	NI
42	**2″-Methoxyoleuropein isomer a**	3.2[a]	NI	NI	NI
43	**Oleuropein isomer b**	NI	NI	9.4	NI
44	**2″-Methoxyoleuropein isomer b**	4.2	NI	NI	NI
45	**Oleuropein isomer c**	289.0[a]	17.7[b]	NI	NI
46	**6-*p*-coumaroyl secologanoside isomer a**	NI	9.0[c]	98.9[a]	45.1[b]
47	**Oleuropein isomer d**	8.3	NI	NI	NI
48	**Oleuropein isomer e**	NI	NI	6.6[a]	4.2[b]
49	**Oleuropein aglycon isomer a**	35.0[a]	7.8[b]	NI	NI
50	**6-*p*-Coumaroyl secologanoside isomer b**	NI	NI	NI	2.6
51	**Oleuropein aglycon isomer b**	NI	NI	NI	NI
52	**Oleuropein isomer f**	NI	NI	NI	NI
53	**Elenolic acid derivative isomer e**	NI	NI	NI	NI
54	**Ligstroside**	18.4	NI	NI	NI
55	**Luteolin**	7.4[d]	65.9[a]	57.1[b]	33.0[c]
56	**Diosmetin**	NI	NI	NI	NI
57	**Apigenin**	NI	NI	12.7[a]	8.2[b]
	Total	**1911.3[b]**	**2453.8[a]**	**1996.8[b]**	**1509.0[c]**

NI (Non Identified)

Table S3. Quantification of the identified phenolic compounds in olive fruits of 'Changlot Real' cultivar expressed as mg/kg of FrFW. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Phenolic compounds	Sampling time			
	'Changlot Real'	June	August	October	December
1	**Hydroxytyrosol-glucoside isomer a**	652.7[a]	78.4[c]	33.1[d]	91.7[b]
2	**Hydroxytyrosol-diglucoside isomer a**	272.1	NI	NI	NI
3	**Hydroxytyrosol-glucoside isomer b**	NI	NI	NI	NI
4	**Oleoside isomer a**	5.8	NI	NI	NI
5	**Hydroxytyrosol-glucoside isomer c**	NI	38.2[c]	186.3[b]	440.6[a]
6	**Hydroxytyrosol-glucoside isomer d**	401.2[b]	492.8[a]	289.8[c]	433.3[b]
7	**Oleoside isomer b**	36	NI	NI	NI
8	**Hydroxytyrosol**	NI	168.9[a]	147.8[b]	73.5[c]
9	**Oleoside derivative isomer a**	9.4[c]	8.9[c]	17.5[b]	23.4[a]
10	**Hydroxytyrosol-diglucoside isomer b**	NI	NI	NI	NI
11	**Oleoside derivative isomer b**	NI	34.8[c]	62.4[a]	63.8[a]
12	**Tyrosol glucoside**	1180.7[a]	265.3[c]	102.9[d]	615.2[b]
13	**Tyrosol**	NI	113.7[a]	30.6[b]	NI
14	**Elenolic acid glucoside isomer a**	50.0[a]	NI	NI	NI
15	***p*-Coumaric acid**	NI	37.1[a]	19.2[b]	21.1[b]
16	**oleoside isomer c**	336.4[a]	3.0[b]	1.8[b]	NI
17	**6-ß-Glucopyranosyl oleoside**	NI	1.0[a]	NI	NI
18	**Gallocatechin**	NI	2.7[a]	1.8[b]	1.3[c]
19	**Elenolic acid glucoside isomer b**	7.2[a]	NI	NI	NI
20	**Elenolic acid glucoside isomer c**	12.9[a]	NI	NI	NI
21	**Oleuropein aglycon derivative**	281.1[a]	146.9[b]	37.5[c]	24.6[c]
22	**Luteolin diglucoside**	0.8	NI	NI	NI
23	**ß -Hydroxyverbascoside isomer a**	NI	NI	11.1[b]	17.0[a]
24	**ß -Hydroxyverbascoside isomer b**	NI	NI	15.5[a]	15.1[a]
25	**Elenolic acid glucoside isomer d**	69.4	NI	NI	NI
26	**Demethyloleuropein**	NI	NI	NI	NI
27	**10-Hydroxy-oleuropein**	9.8	NI	NI	NI
28	**Rutin**	166.3[a]	75.7[b]	9.5[d]	20.3[c]
29	**Quercetin3-O-glucoside**	2.4	NI	NI	NI
30	**Luteolin glucoside isomer a**	114.6[a]	14.0[c]	4.8[d]	24.9[b]
31	**Verbascoside isomer a**	NI	2125.4[a]	1269.0[b]	731.3[c]
32	**Oleuropein hexose isomer a**	17.4	NI	NI	NI
33	**Verbascoside isomer b**	NI	NI	93.2[a]	58.2[b]
34	**Oleuropein hexose isomer b**	5.1	NI	NI	NI
35	**Luteolin glucoside isomer b**	13.9	NI	NI	NI

	Phenolic compounds	Sampling time			
	'Changlot Real'	June	August	October	December
36	**Oleuropein hexose isomer c**	13.4[b]	19.2[a]	NI	NI
37	**Apigenin rutinoside**	NI	NI	NI	NI
38	**Luteolin glucoside isomer c**	19	NI	NI	NI
39	**Oleuropein hexose isomer d**	NI	NI	NI	3.5
40	**Caffeoyl-6-oleoside**	NI	NI	NI	NI
41	**Oleuropein isomer a**	NI	NI	NI	NI
42	**2″-Methoxyoleuropein isomer a**	NI	NI	NI	NI
43	**Oleuropein isomer b**	NI	NI	NI	5.5
44	**2″-Methoxyoleuropein isomer b**	NI	NI	NI	NI
45	**Oleuropein isomer c**	4792.9[a]	6.1[b]	6.9[b]	4.4[b]
46	**6-*p*-Coumaroyl secologanoside isomer a**	NI	11.4[b]	19.9[a]	9.9[c]
47	**Oleuropein isomer d**	NI	NI	NI	NI
48	**Oleuropein isomer e**	NI	NI	NI	NI
49	**Oleuropein aglycon isomer a**	125.2[b]	185.5[a]	9.5[c]	9.5[c]
50	**6-*p*-Coumaroyl secologanoside isomer b**	NI	NI	NI	NI
51	**Oleuropein aglycon isomer b**	NI	NI	NI	NI
52	**Oleuropein isomer f**	NI	NI	NI	NI
53	**Elenolic acid derivative isomer e**	39.8	NI	NI	NI
54	**Ligstroside**	437.4	NI	NI	NI
55	**Luteolin**	1.8[d]	38.7[a]	25.3[b]	18.6[c]
56	**Diosmetin**	NI	NI	NI	NI
57	**Apigenin**	NI	NI	1.5[a]	1.4[b]
	Total	**9074.8[a]**	**3883.9[b]**	**2388.6[c]**	**2699.9[c]**

NI (Non Identified)

Table S4. Quantification of the identified phenolic compounds in olive fruits of 'Koroneiki' cultivar expressed as mg/kg of FrFW. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Phenolic compounds 'Koroneiki'	**Sampling time**			
		June	**August**	**October**	**December**
1	**Hydroxytyrosol-glucoside isomer a**	NI	NI	NI	NI
2	**Hydroxytyrosol-diglucoside isomer a**	NI	NI	NI	NI
3	**Hydroxytyrosol-glucoside isomer b**	NI	NI	NI	NI
4	**Oleoside isomer a**	NI	NI	NI	NI
5	**Hydroxytyrosol-glucoside isomer c**	NI	25.4^{c}	38.5^{b}	96.8^{a}
6	**Hydroxytyrosol-glucoside isomer d**	471.8^{b}	541.1^{a}	97.0^{c}	114.7^{c}
7	**Oleoside isomer b**	11.7	NI	NI	NI
8	**Hydroxytyrosol**	NI	108.8^{a}	61.2^{b}	61.6^{b}
9	**Oleoside derivative isomer a**	6.4^{d}	10.7^{c}	14.7^{b}	17.7^{a}
10	**Hydroxytyrosol-diglucoside isomer b**	NI	NI	NI	NI
11	**Oleoside derivative isomer b**	NI	40.3^{b}	55.1^{a}	52.5^{a}
12	**Tyrosol glucoside**	547.4^{a}	323.0^{b}	NI	NI
13	**Tyrosol**	NI	64.8^{a}	28.5^{b}	NI
14	**Elenolic acid glucoside isomer a**	21.5	NI	NI	NI
15	***p*-Coumaric acid**	NI	108.1^{a}	50.4^{b}	53.5^{b}
16	**oleoside isomer c**	187.0^{a}	7.6^{b}	NI	NI
17	**6-ß-Glucopyranosyl oleoside**	NI	6.9	NI	NI
18	**Gallocatechin**	NI	1.8^{a}	1.0^{b}	0.3^{c}
19	**Elenolic acid glucoside isomer b**	6	NI	NI	NI
20	**Elenolic acid glucoside isomer c**	NI	NI	NI	NI
21	**Oleuropein aglycon derivative**	274.9^{a}	273.1^{a}	146.3^{b}	106.2^{c}
22	**Luteolin diglucoside**	1.7	NI	NI	NI
23	**ß -Hydroxyverbascoside isomer a**	NI	NI	8.1^{a}	6.0^{b}
24	**ß -Hydroxyverbascoside isomer b**	NI	NI	11.7^{a}	4.0^{b}
25	**Elenolic acid glucoside isomer d**	6	NI	NI	NI
26	**Demethyloleuropein**	NI	NI	NI	NI
27	**10-Hydroxy-oleuropein**	106.9	NI	NI	NI
28	**Rutin**	370.2^{b}	425.0^{a}	139.5^{c}	113.5^{c}
29	**Quercetin3-*O*-glucoside**	4.4	NI	NI	NI
30	**Luteolin glucoside isomer a**	101.3^{b}	111.3^{a}	20.7^{c}	14.9^{c}
31	**Verbascoside isomer a**	NI	549.3^{a}	530.3^{a}	292.9^{b}
32	**Oleuropein hexose isomer a**	12.9	NI	NI	NI
33	**Verbascoside isomer b**	NI	NI	85.3^{a}	46.3^{b}
34	**Oleuropein hexose isomer b**	10.9^{b}	13.3^{a}	NI	NI
35	**Luteolin glucoside isomer b**	NI	NI	NI	NI

	Phenolic compounds	Sampling time			
	'Koroneiki'	June	August	October	December
36	**Oleuropein hexose isomer c**	9.3[b]	39.1[a]	NI	NI
37	**Apigenin rutinoside**	NI	NI	0.9[b]	2.1[a]
38	**Luteolin glucoside isomer c**	12.1	NI	NI	NI
39	**Oleuropein hexose isomer d**	NI	NI	NI	NI
40	**Caffeoyl-6-oleoside**	NI	NI	NI	NI
41	**Oleuropein isomer a**	NI	NI	NI	NI
42	**2″-Methoxyoleuropein isomer a**	45.2	NI	NI	NI
43	**Oleuropein isomer b**	NI	NI	NI	NI
44	**2″-Methoxyoleuropein isomer b**	72.5	NI	NI	NI
45	**Oleuropein isomer c**	2364.4[a]	299.7[b]	11.0[c]	29.0[c]
46	**6-*p*-Coumaroyl secologanoside isomer a**	NI	34.2[b]	37.9[a]	14.3[c]
47	**Oleuropein isomer d**	NI	NI	5.9[a]	2.3[b]
48	**Oleuropein isomer e**	NI	NI	NI	NI
49	**Oleuropein aglycon isomer a**	27.5[b]	198.7[a]	NI	NI
50	**6-*p*-Coumaroyl secologanoside isomer b**	NI	NI	NI	NI
51	**Oleuropein aglycon isomer b**	NI	NI	NI	NI
52	**Oleuropein isomer f**	NI	NI	NI	NI
53	**Elenolic acid derivative isomer e**	49.8	NI	NI	NI
54	**Ligstroside**	151.8	NI	NI	NI
55	**Luteolin**	3.6[d]	15.1[c]	29.6[a]	18.4[b]
56	**Diosmetin**	NI	NI	NI	NI
57	**Apigenin**	NI	NI	2.2[a]	1.1[b]
	Total	**4873.9[a]**	**3206.7[b]**	**1368.3[c]**	**1066.8[d]**

NI (Non Identified)

Table S5. Quantification of the identified phenolic compounds in olive fruits of 'Picual' cultivar expressed as mg/kg of FrFW. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Phenolic compounds 'Picual'	Sampling time			
		June	August	October	December
1	**Hydroxytyrosol-glucoside isomer a**	422.2	NI	NI	NI
2	**Hydroxytyrosol-diglucoside isomer a**	147.7	NI	NI	NI
3	**Hydroxytyrosol-glucoside isomer b**	NI	NI	NI	NI
4	**Oleoside isomer a**	6.4	NI	NI	NI
5	**Hydroxytyrosol-glucoside isomer c**	363.0^{a}	28.8^{d}	54.3^{c}	79.7^{b}
6	**Hydroxytyrosol-glucoside isomer d**	NI	275.3^{b}	54.0^{a}	58.3^{a}
7	**Oleoside isomer b**	86.9	NI	NI	NI
8	**Hydroxytyrosol**	NI	57.4^{b}	57.1^{b}	108.0^{a}
9	**Oleoside derivative isomer a**	7.6^{b}	8.1^{b}	11.6^{a}	12.3^{a}
10	**Hydroxytyrosol-diglucoside isomer b**	3.8	NI	NI	NI
11	**Oleoside derivative isomer b**	NI	123.3^{b}	213.2^{a}	231.2^{a}
12	**Tyrosol glucoside**	736.5^{a}	60.0^{b}	NI	NI
13	**Tyrosol**	NI	73.9^{a}	30.7^{b}	NI
14	**Elenolic acid glucoside isomer a**	161.4	NI	NI	NI
15	***p*-Coumaric acid**	NI	52.8^{a}	31.0^{b}	27.5^{b}
16	**oleoside isomer c**	335.6	NI	NI	NI
17	**6-ß-Glucopyranosyl oleoside**	NI	2.9	NI	NI
18	**Gallocatechin**	NI	5.9^{a}	3.8^{b}	1.1^{c}
19	**Elenolic acid glucoside isomer b**	9	NI	NI	NI
20	**Elenolic acid glucoside isomer c**	10.5	NI	NI	NI
21	**Oleuropein aglycon derivative**	216.8^{a}	132.0^{b}	53.6^{c}	18.5^{d}
22	**Luteolin diglucoside**	4.3	NI	NI	NI
23	**ß -Hydroxyverbascoside isomer a**	NI	NI	4.7^{b}	5.2^{a}
24	**ß -Hydroxyverbascoside isomer b**	NI	NI	4.8^{a}	2.4^{b}
25	**Elenolic acid glucoside isomer d**	15.4^{a}	NI	NI	NI
26	**Demethyloleuropein**	NI	NI	NI	NI
27	**10-Hydroxy-oleuropein**	87.3	NI	NI	NI
28	**Rutin**	323.2^{a}	244.4^{b}	21.9^{c}	19.5^{c}
29	**Quercetin3-*O*-glucoside**	15.4	NI	NI	NI
30	**Luteolin glucoside isomer a**	153.4^{a}	31.9^{b}	19.3^{c}	18.9^{c}
31	**Verbascoside isomer a**	NI	556.2^{a}	482.5^{b}	307.9^{c}
32	**Oleuropein hexose isomer a**	13.9	NI	NI	NI
33	**Verbascoside isomer b**	NI	NI	71.7^{a}	37.6^{b}
34	**Oleuropein hexose isomer b**	4.5^{b}	7.0^{a}	NI	NI
35	**Luteolin glucoside isomer b**	7.8	NI	NI	NI

	Phenolic compounds 'Picual'	Sampling time			
		June	August	October	December
36	**Oleuropein hexose isomer c**	11.8[b]	21.1[a]	NI	NI
37	**Apigenin rutinoside**	NI	NI	NI	4.3
38	**Luteolin glucoside isomer c**	16	NI	NI	NI
39	**Oleuropein hexose isomer d**	NI	NI	NI	NI
40	**Caffeoyl-6-oleoside**	NI	NI	94.2[a]	87.8[a]
41	**Oleuropein isomer a**	NI	NI	NI	NI
42	**2″-Methoxyoleuropein isomer a**	15.4	NI	NI	NI
43	**Oleuropein isomer b**	NI	NI	NI	NI
44	**2″-Methoxyoleuropein isomer b**	22.8	NI	NI	NI
45	**Oleuropein isomer c**	5422.3[a]	5.2 [b]	6.7[b]	1.5[b]
46	**6-*p*-Coumaroyl secologanoside isomer a**	NI	96.3 [b]	184.0[a]	176.4[a]
47	**Oleuropein isomer d**	91.2	NI	NI	NI
48	**Oleuropein isomer e**	NI	NI	NI	NI
49	**Oleuropein aglycon isomer a**	146.6	NI	NI	NI
50	**6-*p*-Coumaroyl secologanoside isomer b**	NI	NI	NI	NI
51	**Oleuropein aglycon isomer b**	NI	184.0[a]	19.9[b]	9.9[b]
52	**Oleuropein isomer f**	NI	NI	5.4[a]	3.9[b]
53	**Elenolic acid derivative isomer e**	135.9	NI	NI	NI
54	**Ligstroside**	316.4	NI	NI	NI
55	**Luteolin**	3.0[d]	130.0[a]	64.0[b]	47.5[c]
56	**Diosmetin**	NI	NI	NI	NI
57	**Apigenin**	NI	NI	1.9[a]	1.5[b]
	Total	**9343.7[a]**	**2102.7[b]**	**1504.6[c]**	**1249.4[c]**

NI (Non Identified)

Table S6. Quantification of the identified phenolic compounds in olive fruits of 'Sikitita' cultivar expressed as mg/kg of FrFW. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Phenolic compounds 'Sikitita'	**Sampling time**			
		June	**August**	**October**	**December**
1	**Hydroxytyrosol-glucoside isomer a**	380.8[a]	7.23[b]	NI	NI
2	**Hydroxytyrosol-diglucoside isomer a**	66.8	NI	NI	NI
3	**Hydroxytyrosol-glucoside isomer b**	NI	NI	NI	212.3
4	**Oleoside isomer a**	1.2	NI	NI	NI
5	**Hydroxytyrosol-glucoside isomer c**	622.1[a]	21.8[d]	95.2[b]	56.4[c]
6	**Hydroxytyrosol-glucoside isomer d**	NI	257.8[a]	86.9[b]	NI
7	**Oleoside isomer b**	5.6	NI	NI	NI
8	**Hydroxytyrosol**	NI	290.9[a]	171.7[b]	85.2[c]
9	**Oleoside derivative isomer a**	7.7[c]	2.1[d]	14.6[b]	19.8[a]
10	**Hydroxytyrosol-diglucoside isomer b**	4.5	NI	NI	NI
11	**Oleoside derivative isomer b**	NI	34.3[b]	71.6[a]	72.1[a]
12	**Tyrosol glucoside**	667.0[a]	38.7[b]	NI	46.3[b]
13	**Tyrosol**	NI	141.0[a]	34.7[b]	NI
14	**Elenolic acid glucoside isomer a**	NI	NI	NI	NI
15	***p*-Coumaric acid**	NI	51.9[b]	64.9[a]	61.7[a]
16	**oleoside isomer c**	81.8[a]	1.0[b]	NQ	NQ
17	**6-ß-Glucopyranosyl oleoside**	NI	1.9	NI	NI
18	**Gallocatechin**	NI	5.5[a]	4.6[b]	3.1[c]
19	**Elenolic acid glucoside isomer b**	1.6	NI	NI	NI
20	**Elenolic acid glucoside isomer c**	NI	NI	NI	NI
21	**Oleuropein aglycon derivative**	38.9[a]	27.9[b]	15.7[c]	11.7[d]
22	**Luteolin diglucoside**	0.6	NI	NI	NI
23	**ß -Hydroxyverbascoside isomer a**	NI	1.3[c]	16.7[a]	11.4[b]
24	**ß -Hydroxyverbascoside isomer b**	NI	0.8[b]	10.2[a]	10.2[a]
25	**Elenolic acid glucoside isomer d**	0.5	NI	NI	NI
26	**Demethyloleuropein**	NI	NI	NI	NI
27	**10-Hydroxy-oleuropein**	10.5	NI	NI	NI
28	**Rutin**	412.5[a]	400.2[a]	273.1[b]	114.3[c]
29	**Quercetin3-*O*-glucoside**	8.8	NI	NI	NI
30	**Luteolin glucoside isomer a**	206.2[a]	80.6[b]	63.4[c]	69.8[c]
31	**Verbascoside isomer a**	NI	799.2[a]	772.3[a]	308.5[b]
32	**Oleuropein hexose isomer a**	3.8	NI	NI	NI
33	**Verbascoside isomer b**	NI	NI	205.7[a]	69.3[b]
34	**Oleuropein hexose isomer b**	15.5	NI	NI	NI
35	**Luteolin glucoside isomer b**	NI	NI	NI	NI

	Phenolic compounds ‘Sikitita’	Sampling time			
		June	August	October	December
36	**Oleuropein hexose isomer c**	17.1[a]	16.9[a]	NI	NI
37	**Apigenin rutinoside**	NI	NI	3.6[b]	6.1[a]
38	**Luteolin glucoside isomer c**	38.8[b]	45.3[a]	11.2[c]	NI
39	**Oleuropein hexose isomer d**	NI	NI	NI	NI
40	**Caffeoyl-6-oleoside**	NI	NI	56.9[a]	25.0[b]
41	**Oleuropein isomer a**	NI	NI	6.7[b]	7.8[a]
42	**2′′-Methoxyoleuropein isomer a**	5.1	NI	NI	NI
43	**Oleuropein isomer b**	NI	NI	NI	4.4
44	**2′′-Methoxyoleuropein isomer b**	7	NI	NI	NI
45	**Oleuropein isomer c**	281.8[a]	5.1[b]	NI	NI
46	**6-*p*-Coumaroyl secologanoside isomer a**	NI	NI	100.3[a]	50.4[b]
47	**Oleuropein isomer d**	NI	NI	NI	NI
48	**Oleuropein isomer e**	NI	NI	NI	NI
49	**Oleuropein aglycon isomer a**	54.7[b]	97.9[a]	NI	NI
50	**6-*p*-Coumaroyl secologanoside isomer b**	NI	NI	NI	2.1
51	**Oleuropein aglycon isomer b**	NI	NI	NI	NI
52	**Oleuropein isomer f**	NI	NI	NI	NI
53	**Elenolic acid derivative isomer e**	4.2	NI	NI	NI
54	**Ligstroside**	17.8	NI	NI	NI
55	**Luteolin**	18.9[d]	279.6[a]	223.6[b]	68.5[c]
56	**Diosmetin**	NI	NI	6.4[a]	2.6[b]
57	**Apigenin**	NI	NI	4.3[a]	1.7[b]
	Total	**2981.6[a]**	**2618.1[b]**	**2318.9[c]**	**1318.0[d]**

NI (Non Identified)

Table S7. Mean values (mg/kg of FrFW) of total phenols and phenolic groups for cultivars studied at the four sampling times. Values with different letters are significantly different among cultivars at $p \leq 0.001$.

	Cultivar	June	August	October	December
Total phenols	**'Arbequina'**	2801.18^{c}	2264.65^{c}	1714.03^{c}	1266.74^{c}
	'Arbosana'	1911.33^{d}	2453.78^{b}	1996.79^{b}	1508.95^{b}
	'Sikitita'	2981.57^{d}	2618.08^{d}	2318.92^{d}	1318.03^{d}
	'Changlot Real'	9074.79^{a}	3883.90^{a}	2388.64^{a}	2699.89^{a}
	'Koroneiki'	4927.00^{b}	3206.74^{b}	1368.34^{d}	1066.84^{e}
	'Picual'	9343.67^{a}	2102.74^{e}	1504.60^{e}	1249.35^{d}
Group 1: Secoiridoids	**'Arbequina'**	639.37^{d}	100.94^{d}	29.89^{c}	42.11^{c}
	'Arbosana'	555.59^{d}	167.01^{c}	60.82^{d}	34.94^{d}
	'Sikitita'	452.05^{d}	147.75^{c}	22.44^{e}	23.92^{e}
	'Changlot Real'	5682.37^{b}	357.76^{b}	53.91^{b}	49.00^{b}
	'Koroneiki'	3076.33^{c}	831.18^{a}	163.17^{a}	137.62^{a}
	'Picual'	6349.12^{a}	349.20^{b}	85.70^{d}	33.68^{d}
Group 2: Flavonoids	**'Arbequina'**	548.10^{b}	513.10^{c}	237.78^{c}	293.25^{b}
	'Arbosana'	539.88^{b}	533.24^{b,c}	266.60^{b}	368.28^{a}
	'Sikitita'	685.75^{a}	811.12^{a}	590.25^{a}	266.23^{c}
	'Changlot Real'	318.74^{d}	131.10^{e}	42.83^{f}	66.47^{f}
	'Koroneiki'	493.35^{c}	553.16^{b}	193.94^{d}	150.40^{d}
	'Picual'	523.12^{b}	412.34^{d}	111.01^{e}	92.77^{e}
Group 3: Simple phenols	**'Arbequina'**	1474.08^{d}	1562.03^{d}	1270.50^{e}	792.86^{d}
	'Arbosana'	618.71^{f}	1672.29^{b,c}	1464.01^{c}	962.33^{b}
	'Sikitita'	1741.26^{b}	1610.67^{d,c}	1458.44^{f}	861.32^{c}
	'Changlot Real'	2506.68^{a}	3319.82^{a}	2198.54^{d}	2496.91^{a}
	'Koroneiki'	1019.24^{e}	1720.52^{b}	911.06^{a}	675.94^{e}
	'Picual'	1702.79^{c}	1104.51^{e}	790.66^{b}	626.56^{e}
Group 4: Oleosides	**'Arbequina'**	95.66^{d}	88.44^{c}	175.85^{d}	138.52^{c}
	'Arbosana'	51.45^{e}	81.24^{d}	206.36^{c}	143.41^{c}
	'Sikitita'	96.27^{d}	39.20^{f}	243.35^{b}	169.30^{b}
	'Changlot Real'	387.65^{b}	59.09^{e}	101.53^{e}	97.05^{d}
	'Koroneiki'	205.06^{c}	99.65^{b}	107.71^{e}	84.43^{e}
	'Picual'	436.49^{a}	230.62^{a}	502.96^{a}	507.65^{a}

	Cultivar	June	August	October	December
Group 5: Elenolic acids glucosides	**'Arbequina'**	43.98[e]	0.00	0.00	0.00
	'Arbosana'	145.70[c]	0.00	0.00	0.00
	'Sikitita'	6.24[f]	0.00	0.00	0.00
	'Changlot Real'	179.35[b]	0.00	0.00	0.00
	'Koroneiki'	133.02[d]	0.00	0.00	0.00
	'Picual'	332.15[a]	0.00	0.00	0.00

Table S8. Correlations between phenolic groups and the principal components

	PC 1	PC 2	PC 3	PC 4	PC5
Elenolic acid	-0.920024	0.059128	-0.203724	-0.272538	-0.185149
Flavonoids	-0.223601	-0.592280	-0.716034	0.293547	-0.018230
Oleosides	-0.483577	0.780032	0.029415	0.393133	-0.047797
Secoiriodis	-0.983281	0.040716	-0.029267	-0.095874	0.146469
Simple phenols	-0.328525	-0.573110	0.722431	0.189293	-0.076669
Total phenol	-0.975947	-0.154644	0.127827	0.027745	0.080638

CHAPTER 6. From olive fruits to olive oil: Phenolic compounds transfer in six different olive cultivars grown under the same agronomical conditions.

This work is submitted to International Journal of Molecular Sciences.

Talhaoui, N.; Gómez-Caravaca, A. M.; León, L.; De la Rosa, R.; Fernández-Gutiérrez; Segura Carretero, A. From olive fruits to olive oil: Phenolic compounds transfer in six different olive cultivars grown under the same agronomical conditions.

Abstract

Phenolic compounds are responsible of the nutritional and sensory quality of virgin olive oil (VOO). The composition of phenolic compounds in VOO is related to the initial content of phenolic compounds in the olive fruit tissues and the activity of enzymes acting on these compounds during the industrial process to obtain the oil. In this work, the phenolic composition was studied in six important cultivars grown at the same orchard under the same agronomical and environmental conditions to test the effects of cultivars on phenolic composition in both fruits and oils and transfer between matrices. The phenolic fractions were identified and quantified using high performance liquid chromatography-diode array detector-time-of-flight-mass spectrometry. A total of 33 phenolic compounds were determined in the fruits samples whereas a total of 20 compounds were determined in their correspondent oils. Qualitative and quantitative differences in phenolic composition were found among cultivars in both matrices, as well as regarding the transfer rate of phenolic compounds from fruits to oil. Transfer rates also varied according to the different phenolic groups evaluated, with secoiridoids showing the highest transfer rate from fruits to oils. Principal Component Analysis confirmed a strong genetic effect on the basis of the phenolic profile in both olive fruits and oils.

Keywords: Phenolic compounds, EVOO, olive fruit, six cultivars, transfer rates.

1. Introduction

In the Mediterranean area, healthy, nutritional and sensorial properties of olive oil have been known for a many centuries. Olive oil is considered the main fat source of the Mediterranean diet and it is appreciable for its distinguishable characteristics such as: aroma, taste, color and nutritive properties than other vegetable oils. Besides to the monounsaturated/saturated fatty acid ratio and tocopherols, the positive effects of virgin olive oil (VOO) are also due to polyphenols. Indeed, many scientific studies have demonstrated the healthy benefits of these antioxidant compounds, including the reduction of the risk of factors of coronary heart disease, the prevention of several chronic diseases (atherosclerosis), cancer, strokes and other degenerative diseases[1]. Moreover, polyphenols are found to be responsible for the typical bitter and pungent taste of VOO, which strongly affect its sensory properties[2,3], and contribute to the stability of the oil to autoxidation[4].

The amount of polyphenols in VOO are variable, depending on several factors such as geographical zone[5–8], agro-climatic conditions[9–11], degree of fruit ripeness[12] and oil extraction processing[2,3,13]. Additionally, the phenolic fraction of olive oil can greatly vary among cultivars[6,14], although this aspect has been scarcely studied.

In the olive fruit, the main phenols are secoiridoids such as oleuropein, demethyloleuropein, phenolic glycosides as ligstroside, and hydroxycinnamic acid derivatives as verbascoside[15]. During crushing and malaxing processes, oleuropein and demethyloleuropein are hydrolyzed by endogenous β-glycosidases to 3,4-DHPEA-EDA and 3,4-DHPEA-EA. These newly formed substances are the most abundant secoiridoids in VOO[16]. Jerman Klen and co-workers[17] studied four cultivars with the same ripening index (RI) and demonstrated that during crushing and malaxation in industrial-scale extraction systems, only 0.3-1.5% of available phenols in fruits were transferred to olive oil,

whereas the rest ended up in wastes. Another study was also achieved with one cultivar by a laboratory-scale in which only 0.53% of phenolic compounds ended-up in olive oil[18].

The purpose of the current work was to study the transfer of single phenolic compounds from fruits to oil at laboratory-scale, using six different cultivars grown at the same orchard under the same agronomical and environmental conditions. The obtained results have the goal of strengthening the previous works in relation to cultivars effects on phenolic compounds transfer.

2. Materials and Methods

2.1 Chemicals and reagents

Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin and pinoresinol were purchased from Sigma-Aldrich (St. Louis, MO, USA), and oleuropein from Extrasynthèse (Lyon, France). Methanol reagent was from Panreac (Barcelona, Spain). HPLC-grade acetonitrile and acetic acid (assayed at >99.5%) used for preparing mobile phases were from Labscan (Dublin, Ireland) and Fluka (Switzerland) respectively. Distilled water with a resistance of 18.2 MΩ was deionized in a Milli-Q system (Millipore, Bedford, MA, USA). The stock solutions containing these analytes were prepared in methanol. All chemicals were of analytical reagent grade and used as received. All the solutions were stored in a dark flask at -20 °C until use.

2.2 Samples

Olives from the cultivars ‘Arbosana’, ‘Koroneiki’, ‘Picual’, ‘Sikitita’, ‘Arbequina’, and ‘Changlot Real’ were harvested at the same time in mid-December from the same olive orchards in “IFAPA, Centro Alameda del Obispo” in Córdoba, Spain (37°51'36.5"N 4°47'53.7"). Only healthy fruits

without any kind of infection or physical damage were processed. Olive oil samples were prepared at laboratory scale using the Abencor system (Comercial Abengoa, S.A., Seville, Spain) equipped with a hammer crusher, malaxer, and centrifuge that simulates the industrial process of VOO production. Malaxation was carried out at 25 ºC for 30 min and centrifugation of the kneaded paste was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oils were decanted, paper filtered and transferred into dark glass bottles until analysis.

2.3 Extraction of phenolic compounds from olive fruits and oils

2g of fresh olive fruits (FrF) were crushed and extracted *via* Ultra-Turrax IKA T18 basic with 30 mL of MeOH/H_2O (80/20). After that, the sample was placed in an ultrasonic bath (10 min) and centrifuged at 4500 rpm for 15 min. Then, the supernatant was removed, and the extraction was repeated twice more. The supernatants were collected and the extract was then evaporated. After that, the extract was reconstituted with 20 ml of acidified water (at pH 2.3) and washed up twice with 40 ml of hexane to remove the possible oil. Then 40 ml of methanol was added to the aqueous solution and evaporated again. Finally, the extract was reconstituted with 2 mL of MeOH/H_2O (50/50).

The polar fraction of olive oil was extracted according to Taamalli et al.[7] with some modifications. Briefly, 2 g of oil sample was weighted and washed with 3 mL of hexane. After, 2 mL of methanol: water (60/40) was added, the mixture was vortexed and then centrifuged at 3000 rpm during 5 min. Then, the supernatant was removed, and the extraction was repeated twice more. The polar extract was evaporated in a rotary evaporator. The residue was dissolved in 0.25 mL of MeOH/H_2O (50/50).

2.4 Determination of phenolic compounds by HPLC-DAD-TOF-MS

HPLC analyses were carried out using an Agilent 1200 series Rapid Resolution Liquid Chromatograph (Agilent Technologies, CA, USA). The separation of the phenolic fractions was performed by a Poroshell 120 EC-C18 analytical column (4.6 × 100 mm, 2.7 μm). The gradient eluent used was at flow rate of 0.8 mL/min, following the method previously described by Talhaoui et al.[19]. The column temperature was maintained at 25 °C and the injection volume was 2.5 μL.

Besides, the HPLC system was coupled to a micrOTOF (Bruker Daltonics, Bremen, Germany), an orthogonal-accelerated TOF mass spectrometer, using an electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA). The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio=1:3). Analysis parameters were set using a negative-ion mode with spectra acquired over a mass range from *m/z* 50 to 1000. The optimum values of the ESI-MS parameters were: capillary voltage, +4.5 kV; drying gas temperature, 190 °C; drying gas flow, 9.0 L/min; and nebulizing gas pressure, 2 bars. The accurate mass data on the molecular ions was processed through Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulae via the Smart Formula Editor. The Smart Formula Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration and ring-plus double-bond equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (Sigma Value) for increased confidence in the suggested molecular formula. The quantification was carried out using Bruker Compass Target Analysis 1.2 software for compound screening (Bruker Daltonics, Bremen, Germany).

Quantification was made according to the linear calibration curves of standard compounds. Different calibration curves were prepared using the following standards: oleuropein, hydroxytyrosol, tyrosol, apigenin, luteolin and pinoresinol. All calibration curves showed good linearity among different concentrations. The calibration plots revealed good correlation between peak areas and analyte concentrations, and the regression coefficients were always higher than 0.995. Limit of detection (LOD) was found to be within the range 0.053–0.233 µg/mL whereas limit of quantification (LOQ) was within 0.175–0.679 µg/mL.

2.5 Statistical analysis

All the statistical analyses (ANOVA and principal component analysis) were performed by Statistica 8.0 software (2001, StatSoft, Tulsa, OK, USA). Samples were collected in three trees per cultivar and all assays were run in triplicate. Significant statistical differences among treatments (p<0.001) were assessed by Tukey's honest significant-difference multiple comparisons. Values of different results of phenolic compounds were expressed as the means mg/kg fresh fruits weight (FrFW), and as the means mg/kg olive oil. Principal components analysis (PCA) was performed to detect structure in the relationships between variables, allowing the classification and the separation of each cultivar.

2. Results and discussion

2.1 Quantitative characterization of phenolic compounds

The identification of phenolic compounds in olive fruit and oil was carried out by the interpretation of their UV-Vis and mass spectra provided by HPLC-DAD-TOF-MS and the information previously reported in the literature. The base peak chromatograms (BPCs) of two representative phenol extracts of both matrices of the cultivar 'Arbosana', in negative ionization mode, are shown in **Figure 1**. The tentatively identified phenolic compounds are summarized in **Table 1**, including

retention times, *m/z* and molecular formula together with their proposed identities. A total of 33 phenolic compounds have been determined in the fruits samples, whereas a total of 20 compounds were determined in their correspondent oils. Only five compounds (hydroxytyrosol, diosmetin, apigenin, luteolin, and oleuropein aglycone isomer b) have been found both in fruits and oil.

Qualitative differences have also been detected among cultivars for the phenolic profile of fruits. For example, tyrosol glucoside have been detected only in 'Sikitita' and 'Changlot Real', demethyloleuropein only in 'Arbequina' and 'Picual', and oleuropein glucoside only in 'Arbosana' and 'Changlot Real'. Otherwise, hydroxyverbascoside and apigenin rutinoside have been detected in all cultivars except for 'Arbequina' and 'Changlot Real' fruits, respectively. Qualitative differences has also been observed among oils, as, hydroxytyrosol acetate, pinoresinol and diosmetin have only been detected in 'Arbequina', 'Sikitita' and 'Arbosana', whereas elenolic acid methyl ester has been detected in all cultivars as exception of 'Changlot Real' and 'Koroneiki' and tyrosol was only absent in 'Arbequina' cultivar.

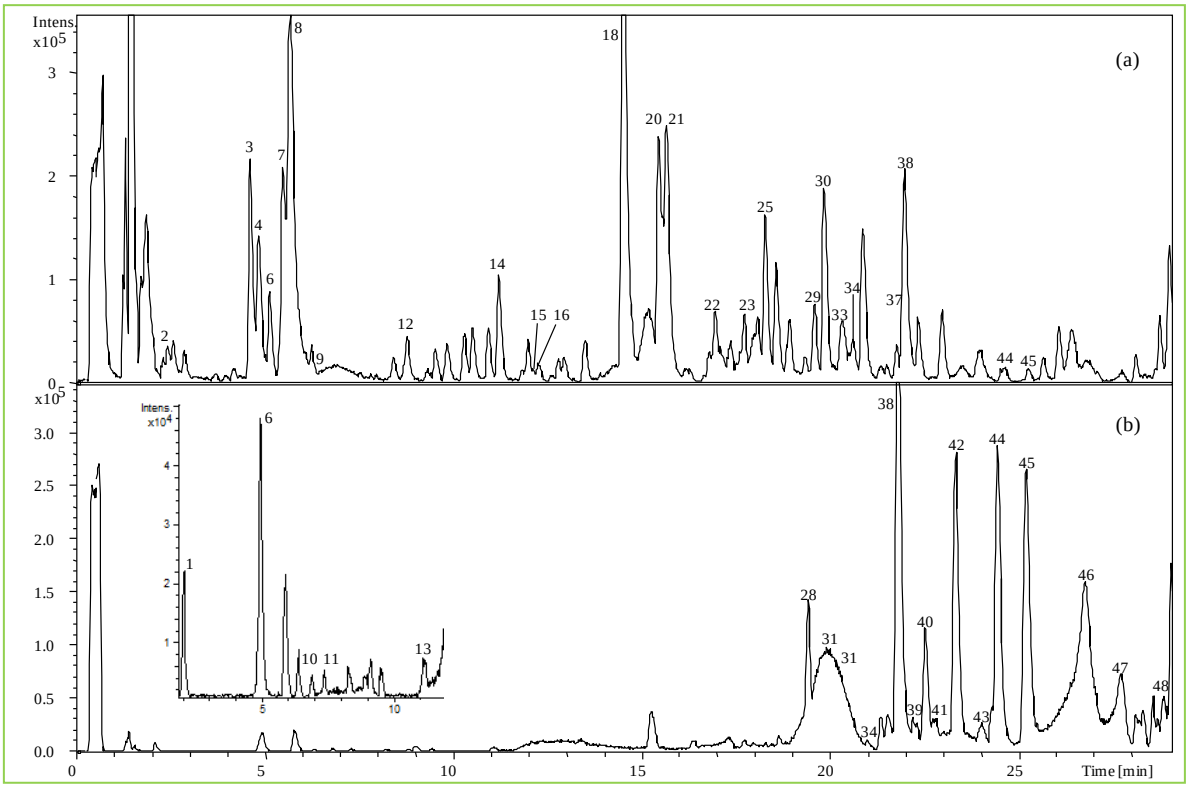

Figure 1. Base peak chromatogram (BPC) of 'Arbosana' phenolic compounds of olive fruits (a) and olive oil (b), using HPLC-DAD-TOF-MS. Proposed phenolic compounds have been numbered by elution order (See table 1 for peak numbers).

Quantification data of olive fruit and oil phenolic compounds for the six cultivars have also been reported in **Table 1**. As expected, for all cultivars, hydroxytyrosol glucoside and verbascoside were the major phenolic compounds determined in ripe fruits. Oleuropein aglycone and deacetoxyoleuropein aglycone isomers were the major compounds in olive oils. However, significant differences between cultivars for both fruits and oil phenolic compounds contents were observed. In fact, several papers have reported the genetic effect of the cultivar on the content of phenolic compounds in fruit as well as in oil[6,20–22]. For all cultivars, hydroxytyrosol glucoside and verbascoside were the major phenolic compounds determined in ripe fruits. However, oleuropein aglycone and deacetoxyoleuropein aglycone isomers were the major compounds in olive oils. Overall, phenols contents have shown low values in fruits as well as in oils, likely due to the late fruits sampling time, as reported in previous study[23].

'Changlot Real' olive fruits showed a total phenol content much higher than the rest of cultivars. In the case of oil 'Picual', 'Koroneiki' and 'Changlot Real' showed the highest phenol content oil. This fact highlights the big effect of the extraction process on olive oil phenolic content[24].

Table 1. Phenolic compounds determined in olive fruits and oils extract by HPLC-DAD-TOF-MS, including retention time, *m/z*, formula, means of compounds by cultivar, and total (mg/kg of FrFW or oil).Standard deviations (in parentheses). n.q (not quantified) and n.i (not identified)

					Phenolic Content (mg/kg FrFW or mg/kg oil)											
					'Arbequina'		'Picual'		'Sikitita'		'Arbosana'		'Changlot Real'		'Koroneiki'	
	Compounds[a]	**Rt min**	***m/z***	**Formula**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**
1	Vanillin isomer a[3]	2.09	151	$C_8H_8O_3$	n.i	0.10 (0.01)	n.i	0.18 (0.01)	n.i	0.21 (0.02)	n.i	0.40 (0.01)	n.i	0.11 (0.01)	*n.i*	0.12 (0.01)
2	Hydroxytyrosol glucoside isomer a[3]	2.31	315	$C_{14}H_{20}O_8$	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	91.74 (7.53)	n.i	n.i	n.i
3	Hydroxytyrosol glucoside isomer b[3]	4.58	315	$C_{14}H_{20}O_8$	n.i	n.i	n.i	n.i	212.34 (23.07)	n.i	n.i	n.i	n.i	n.i	n.i	n.i
4	Hydroxytyrosol glucoside isomer c[3]	4.8	315	$C_{14}H_{20}O_8$	n.i	n.i	79.68 (7.89)	n.i	56.37 (2.04)	n.i	n.i	n.i	440.58 (40.67)	n.i	96.80 (7.09)	n.i
5	Hydroxytyrosol glucoside isomer d[3]	4.82	315	$C_{14}H_{20}O_8$	257.39 (22.06)	n.i	58.32 (5.01)	n.i	n.i	n.i	276.71 (16.71)	n.i	433.30 (35.44)	n.i	114.74 (7.39)	n.i
6	Hydroxytyrosol [3]	5.14	153	$C_8H_{10}O_3$	62.91 (4.93)	0.29 (0.01)	107.97 (7.40)	1.12 (0.04)	85.16 (8.26)	0.83 (0.07)	73.41 (6.08)	1.39 (0.07)	73.53 (7.59)	1.63 (0.71)	61.61 (5.43)	1.57 (0.11)
7	Oleoside derivative isomer a[4]	5.67	407	$C_{17}H_{28}O_{11}$	20.22 (1.17)	n.i	12.26 (0.98)	n.i	19.80 (0.76)	n.i	24.33 (1.18)	n.i	23.36 (1.95)	n.i	17.67 (1.87)	n.i
8	Oleoside derivative isomer b[4]	6.26	407	$C_{17}H_{28}O_{11}$	57.93 (3.00)	n.i	231.21 (22.43)	n.i	72.07 (3.41)	n.i	71.31 (6.25)	n.i	63.80 (4.94)	n.i	52.48 (4.32)	n.i
9	Tyrosol glucoside[3]	6.59	299	$C_{14}H_{20}O_7$	n.i	n.i	n.i	n.i	46.27 (3.88)	n.i	n.i	n.i	615.18 (49.57)	n.i	n.i	n.i
10	Vanillin isomer b[3]	6.81	151	$C_8H_8O_3$	n.i	0.022 (0.001)	n.i	n.i	n.i	0.040 (0.004)	n.i	0.028 (0.002)	n.i	n.i	n.i	n.i
11	Tyrosol[3]	7.28	137	$C_8H_{10}O_2$	n.i	n.i	n.i	1.99 (0.12)	n.i	2.28 (0.24)	n.i	2.06 (0.15)	n.i	10.25 (0.96)	n.i	3.46 (0.12)
12	*p*-coumaric	8.73	163	$C_8H_8O_3$	27.88	n.i	27.51	n.i	61.71		79.01	n.i	21.09	n.i	53.50	n.i

					Phenolic Content (mg/kg FrFW or mg/kg oil)											
					'Arbequina'		'Picual'		'Sikitita'		'Arbosana'		'Changlot Real'		'Koroneiki'	
	Compounds[a]	**Rt min**	***m/z***	**Formula**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**
	acid[3]				(2.35)		(2.40)		(4.57)		(5.59)		(2.03)		(3.19)	
13	Vanillin isomer c[3]	11.06	151	$C_8H_8O_3$	n.i	0.27 (0.03)	n.i	n.i	n.i	0.18 (0.02)	n.i	0.28 (0.02)	n.i	n.i	n.i	n.i
14	Oleuropein aglycone derivative[1]	11.44	377	$C_{16}H_{26}O_{10}$	12.70 (0.42)	n.i	18.47 (1.12)	n.i	11.72 (0.87)		21.01 (1.80)		24.55 (2.38)	n.i	106.25 (9.67)	n.i
15	ß-hydroxy-verbascoside isomer a[3]	12.06	639	$C_{29}H_{36}O_{16}$	n.i	n.i	5.22 (0.08)	n.i	11.40 (0.72)		10.99 (0.54)		16.96 (1.65)	n.i	5.99 (0.42)	n.i
16	ß-hydroxy - verbascoside isomer b[3]	12.21	639	$C_{29}H_{36}O_{16}$	n.i	n.i	2.36 (0.07)	n.i	10.19 (0.79)		14.02 (1.32)		15.07 (0.77)	n.i	4.02 (0.40)	n.i
17	Demethyl oleuropein[1]	13.95	525	$C_{24}H_{30}O_{13}$	9.75 (0.94)	n.i	23.68 (2.15)	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i
18	Rutin[2]	14.48	609	$C_{27}H_{30}O_{16}$	110.16 (6.09)	n.i	19.48 (1.79)	n.i	114.34 (10.88)		189.44 (18.01)		20.27 (0.45)	n.i	113.53 (10.67)	n.i
19	Hydroxytyrosol acetate/3,4-DHPEA-AC[3]	15.22	195	$C_{10}H_{12}O_4$	n.i	2.67 (0.20)	n.i	n.i	n.i	2.06 (0.14)	n.i	2.37 (0.08)	n.i	n.i	n.i	n.i
20	Luteolin glucoside isomer [2]	15.4	447	$C_{21}H_{20}O_{11}$	149.29 (10.75)	n.i	18.87 (0.70)	n.i	69.84 (3.29)	n.i	129.79 (12.63)	n.i	24.94 (2.42)	n.i	14.92 (0.74)	n.i
21	Verbascoside isomer a[3]	15.61	623	$C_{29}H_{36}O_{15}$	340.74 (33.43)	n.i	307.91 (25.61)	n.i	308.55 (13.75)	n.i	406.40 (28.24)	n.i	731.26 (59.36)	n.i	292.94 (27.97)	n.i
22	Verbascoside isomer b[3]	16.96	623	$C_{29}H_{36}O_{15}$	103.94 (8.40)	n.i	37.58 (3.75)	n.i	69.32 (4.01)	n.i	101.78 (8.34)	n.i	58.20 (4.96)	n.i	46.35 (4.59)	n.i
23	Apigenin rutinoside[2]	17.95	577	$C_{27}H_{30}O_{14}$	5.94 (0.39)	n.i	4.32 (0.41)	n.i	6.14 (0.50)	n.i	7.81 (0.70)	n.i	n.i	n.i	2.12 (0.23)	n.i
24	Oleuropein glucoside[1]	18.05	701	$C_{31}H_{42}O_{18}$	n.i	n.i	n.i	n.i	n.i	n.i	24.98 (2.43)	n.i	3.50 (0.30)	n.i	n.i	n.i
25	Caffeoyl-6-oleoside[4]	18.48	551	$C_{25}H_{28}O_{14}$	n.i	n.i	87.76 (8.34)	n.i	24.98 (2.42)	n.i	n.i	n.i	n.i	n.i	n.i	n.i
26	Oleuropein isomer a[1]	18.87	539	$C_{25}H_{32}O_{13}$	n.i	n.i	n.i	n.i	n.i	n.i	7.82 (0.77)	n.i	n.i	n.i	n.i	n.i

					Phenolic Content (mg/kg FrFW or mg/kg oil)											
					'Arbequina'		'Picual'		'Sikitita'		'Arbosana'		'Changlot Real'		'Koroneiki'	
	Compounds[a]	**Rt min**	***m/z***	**Formula**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**
27	Oleuropein isomer b[1]	19.07	539	$C_{25}H_{32}O_{13}$	n.i	n.i	3.93 (0.28)	n.i	n.i	n.i	4.38 (0.23)	n.i	5.47 (0.59)	n.i	n.i	n.i
28	10-Hydroxy oleuropein aglycone[1]	19.38	335	$C_{17}H_{20}O_7$	n.i	0.71 (0.05)		0.62 (0.04)	n.i	7.91 (0.86)	n.i	3.20 (0.27)	n.i	0.23 (0.02)	n.i	0.22 (0.02)
29	Oleuropein isomer c[1]	19.53	539	$C_{25}H_{32}O_{13}$	1.80 (0.09)	n.i	1.46 (0.14)	n.i	n.i	n.i	n.i	n.i	5.37 (0.18)	n.i	29.05 (2.21)	n.i
30	6-p-Coumaroyl secologanoside isomer a[4]	19.80	535	$C_{25}H_{28}O_{13}$	58.93 (1.82)	n.i	176.42 (13.78)	n.i	50.38 (4.10)	n.i	45.13 (3.61)	n.i	9.89 (0.84)	n.i	14.29 (1.39)	n.i
31	Deacetoxy oleuropein aglycone isomer a[1]	19.87	319	$C_{17}H_{20}O_6$	n.i	7.77 (0.62)	n.i	1.26 (0.09)	n.i	12.14 (1.20)	n.i	29.86 (2.29)	n.i	2.14 (0.22)	n.i	2.90 (0.09)
32	Oleuropein isomer d[1]	20.15	539	$C_{25}H_{32}O_{13}$	n.i	n.i	n.i	n.i	2.32 (0.17)	n.i	n.i	n.i	n.i	n.i	n.i	n.i
33	Oleuropein isomer e[1]	20.47	539	$C_{25}H_{32}O_{13}$	4.18 (0.17)	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i
34	Oleuropein Aglycone isomer a[1]	20.59	377	$C_{19}H_{22}O_8$	n.i	n.i	4.29 (0.22)	n.i	n.i	n.i	n.i	n.i	9.52 (0.87)	n.i	n.i	12.22 (1.01)
35	6-p-Coumaroyl secologanoside isomer b[4]	20.72	535	$C_{25}H_{28}O_{13}$	2.63 (0.26)	n.i	1.43 (0.11)	n.i	n.i	n.i	2.07 (0.19)	n.i	n.i	n.i	n.i	n.i
36	Oleuropein aglycone isomer b[1]	20.86	377	$C_{19}H_{22}O_8$	n.i	n.i	9.89 (0.82)	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i
37	Oleuropein isomer f[1]	21.22	539	$C_{25}H_{32}O_{13}$	n.i	n.i	3.86 (0.29)	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i	n.i
38	Luteolin[2]	21.94	285	$C_{15}H_{10}O_6$	25.42 (1.93)	3.51 (0.31)	47.50 (2.81)	1.93 (0.08)	68.53 (6.57)	3.19 (0.20)	33.01 (3.24)	3.65 (0.38)	18.60 (1.34)	2.20 (0.04)	18.44 (1.01)	1.31 (0.08)
39	Deacetoxy oleuropein aglycone	22.29	319	$C_{17}H_{20}O_6$	n.i	n.q	n.i	1.09 (0.09)	n.i	0.11 (0.01)	n.i	0.71 (0.03)	n.i	0.05 (0.01)	n.i	n.q

					Phenolic Content (mg/kg FrFW or mg/kg oil)											
					'Arbequina'		'Picual'		'Sikitita'		'Arbosana'		'Changlot Real'		'Koroneiki'	
	Compounds[a]	**Rt min**	***m/z***	**Formula**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**	**Fruit**	**oil**
	isomer b[1]															
40	Oleuropein aglycone c[1]	22.48	377	$C_{19}H_{22}O_8$	n.i	0.68 (0.06)	n.i	1.76 (0.10)	n.i	1.02 (0.07)	n.i	0.32 (0.03)	n.i	0.94 (0.08)	n.i	1.98 (0.15)
41	Elenolic acid methyl ester[5]	22.61	255	$C_{12}H_{16}O_6$	n.i	0.18 (0.01)	n.i	0.21 (0.02)	n.i	0.12 (0.01)	n.i	1.55 (0.15)	n.i	n.i	n.i	
42	Acetoxy pinoresinol[6]	23.3	415	$C_{22}H_{24}O_8$	n.i	13.04 (1.39)	n.i	0.13 (0.01)	n.i	8.27 (0.80)	n.i	11.70 (0.76)	n.i	7.00 (0.47)	n.i	5.88 (0.55)
43	Pinoresinol[6]	23.93	357	$C_{20}H_{22}O_6$	n.i	0.46 (0.04)	n.i	n.i	n.i	0.42 (0.03)	n.i	0.81 (0.08)	n.i	n.i	n.i	n.i
44	Apigenin[2]	24.62	269	$C_{15}H_{10}O_5$	0.49 (0.03)	1.42 (0.04)	1.52 (0.09)	0.73 (0.02)	1.69 (0.17)	1.06 (0.06)	8.22 (0.92)	3.73 (0.21)	1.38 (0.04)	1.15 (0.02)	1.09 (0.07)	0.72 (0.05)
45	Diosmetin[2]	25.54	299	$C_{16}H_{12}O_6$	0.53 (0.05)	0.55 (0.03)	n.i	n.q	2.64 (0.26)	1.91 (0.08)	n.i	0.12 (0.01)	n.i	n.q	n.i	n.q
46	Oleuropein aglycone d[1]	26.73	377	$C_{19}H_{22}O_8$	n.i	2.74 (0.19)	n.i	89.63(7.49)	n.i	17.49 (1.76)	n.i	21.46 (2.92)	n.i	57.21 (4.83)	n.i	118.39 (16.09)
47	Oleuropein aglycone e[1]	27.79	377	$C_{19}H_{22}O_8$	n.i	0.96 (0.07)	n.i	36.13 (3.41)	n.i	6.62 (0.35)	n.i	14.28 (1.11)	n.i	4.49 (0.22)	n.i	3.43 (0.30)
48	Ligstroside aglycone[1]	28.76	361	$C_{19}H_{22}O_7$	n.i	0.54 (0.05)	n.i	32.08 (2.22)	n.i	0.45 (0.05)	n.i	1.20 (0.08)	n.i	3.72 (0.17)	n.i	3.56 (0.20)
	Total				**1265.33 (55.34)**	**35.92 (1.68)**	**1249.35 (53.14)**	**173.13 (8.66)**	**1318.03 (48.95)**	**66.33 (2.16)**	**1508.95 (43.54)**	**99.20 (4.55)**	**2699.89 (200.56)**	**155.76 (16.69)**	**1066.84 (47.69)**	**169.56 (7.65)**

[a] Superscript numbers indicate phenolic groups: 1, secoiridoid; 2, flavonoids; 3, simple phenols; 4, oleosides; 5, elenolic acids, 6, lignans.

2.2 Transfer of phenolic compounds from fruits to oil

Big changes occur to olive oil phenolic compounds comparing to those of fruits during oil extraction. These changes are qualitative and quantitative changes and they are due to different reasons.

2.2.1 Qualitative changes.

In the present study **(Table 1)** it has been seen that all glycoside phenols were transformed to their aglycone forms, i.e. hydroxytyrosol glucoside, tyrosol glucoside, luteolin glucoside and apigenin rutinoside. Besides, others complex phenols were totally hydrolyzed to simple phenols, i.e. oleuropein, demethyloleuropein, oleoside and verbascoside. The complete transformation of those phenols has been previously reported[17]. Such phenol transformation is the result of the activity of many enzymes that are released during pressing and malaxation steps. In particular, polyphenol oxidase could be responsible for an indirect oxidation of secoiridoids, and β-glucosidase could play a role in the production of phenol-aglycones such as the deacetoxyoleuropein aglycone, oleuropein aglycone and their isomers (the principal compounds in olive oil) by hydrolysis of oleuropein, dimethyloleuropein, etc.[24-27]. However, some compounds such as ligstroside aglycone and lignans (acetoxypinoresinol and pinoresinol) have ambiguously been determined in olive oil and not in olive fruit. Ligstroside aglycone is logically the result of ligstroside degradation. It is worth noted that ligstroside has been detected previously[23] at early stages of fruit ripening, and then its concentration decreased during ripening until not detected levels. This could be due to the fact that ligstroside is completely oxidized into other products when fruits ripe, whereas its respected aglycones found in olive oil are the hydrolysis products of others compounds structurally related to ligstroside[18]. In the case of acetoxypinoresinol and pinoresinol, few references reported the presence of such lignans in fruits, most mentioned the presence of higher amounts of lignans in virgin olive oils compared to olive fruit[28,29]. Brenes

et al.[30] speculated that lignans could be originated from hydrolysis of compounds similar to lignan linked to secoiridoid glucoside. Artajo et al.[24] explained the presence of lignans only in oil by their lipidic character and by the fact that these compounds could be releasable from the vegetable sources after hydrolysis treatments. However, their detection at certain levels in olive stones suggests that lignans in olive oil could proceed from stones after crushing and malaxation of the whole olive fruits[31].

2.2.2 Quantitative changes.

For both matrices (fruit and olive oil), the total phenolic contents were obtained summing up the individual phenolic contents determined by HPLC-DAD-TOF-MS. To better understand the transfer of the individual phenolic compounds from olive fruits to oils, they were grouped into six classes: secoiridoids (oleuropein and isomers, oleuropein glucoside, demethyloleuropein, oleuropein aglycone derivative, oleuropein aglycone and isomers, 10-hydroxyoleuropein aglycone, deacetoxyoleuropein aglycone and isomers, and ligstroside aglycone), flavonoids (luteolin glucoside and isomers, luteolin, apigenin rutinoside, apigenin, rutin and diosmetin), simple phenols (hydroxytyrosol glucoside and isomers, hydroxytyrosol acetate, hydroxytyrosol, tyrosol glucoside, tyrosol, verbascoside and isomers, *β*-hydroxyverbascoside and isomers, *p*-coumaric acid, and vanillin and isomers), oleosides (6-*p*-coumaroylsecologanoside and isomers, caffeoyl-6-oleoside, oleoside derivative and isomers), elenolic acid (elenolic acid methyl ester)and lignans (pinoresinol, acetoxypinoresinol).

Figure 2a and **2b** show groups and total phenolic contents expressed with the same unit mg/kg of fruit fresh weight (FrFW) for both matrices (fruits and olive oil). **Figure 2b** also presents the phenols transfer rates between fruits and oil of the six different cultivars. Those rates have been calculated and expressed as percentage of initial phenolic content of fresh olive fruits taking into account the percentage of olive oil obtained with one kilogram of olive fruits. In general

terms, a very few percentage of total phenols were transferred from fruits to oils for all cultivars (0.38%-1.95%). The result is in agreement with a previous report where only 0.3%-1.5% of available phenols were transferred to olive oil, whereas the rest ended up in wastes (>40%), depending on the extraction process[17]. In the present study, a big variability in the total phenol transfer rate among cultivars has been noticed, although the same abencor system extraction has been used to obtain oil for all cultivars. Thus, the hypothesis of the effect of the extraction process could be discarded. Interestingly, cultivars with less phenol transfer rates have coincided with those who presented a high percentage of humidity in fruit and *viceversa* (data of humidity not shown); *i.e.* 'Arbequina' and 'Changlot Real' presented the lowest transfer rates (0.38% and 0.45%) simultaneously with the highest fruit humidity (67%-72%), whereas 'Picual' and 'Koroneiki' showed the highest transfer rates (1.85% and 1.95%) and the lowest fruit humidity (62% for both). Olive oil phenolic compounds are amphiphilic in nature and are more soluble in water than in oil phase[32]. Besides, during oil extraction from olives, phenolic compounds are distributed between the oil and aqueous phases[33]. Because of those affirmations and the fact that all samples analyzed received the same irrigation and precipitations, it can be speculated that the humidity contained in fruits of each cultivar affects negatively the transfer of phenolic compounds to oil. Due to the fact that water uptake is cultivar dependent[34]; this result could highlight the influence of the genetic factor in the transfer of phenolic compounds from olive fruit to oil.

Among all phenolic groups, secoiridoids were the compounds with higher transfer rate from fruits to oil, followed by flavonoids and simple phenols (**Figure 2b**). In fact, secoiridoids which are the most lipophilic compounds may have suffered semi-degradation during crashing and malaxation, but this big phenolic group was still presented in oil as their aglycone forms. The dominance of secoiridoid derivatives, followed by flavonoids and phenolic alcohols in oil have also been reported by Artajo et al.[24] and Jerman Klen et al.[18]. In contrast, the low transfer of flavonoids could probably be due to the fact that rutin, the

major flavonoid found in olive fruits, is completely wasted in water without many alterations, such as hydrolysis and/or other degradation reactions, during the oil process[17,24,35]. The oleoside groups have been totally disappeared in oil which could be due to their abovementioned degradation pathways to simple phenols. On the contrary, a new group of lignans appeared in oil, the apparition of such group has been explained above for pinoresinol and acetoxypinoresinol.

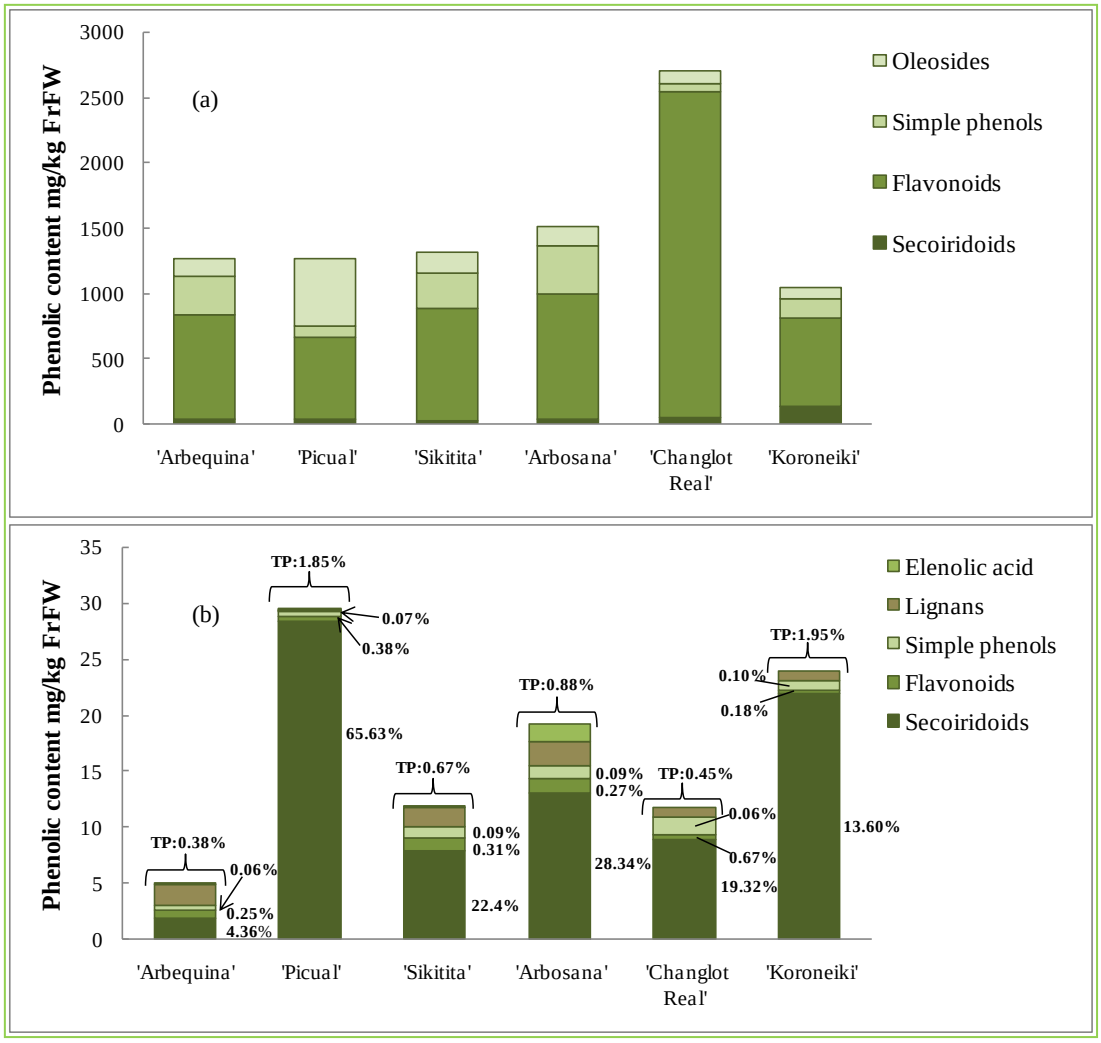

Figure 2. (a) Total and groups phenolic contents of the different olive fruits cultivars (b) total and groups phenolic contents of the different olive oil cultivars including the transfer rates. Rates are expressed in % of initial fresh olive fruits phenolic contents (mg/kg fresh fruits weight (FrFW)). Graphic (a) represents the 100%. TP (total phenol).

Furthermore, no clear differences have been detected in the transfer rates of simple phenols among cultivars (0.06%-0.10%). However, wide differences have

been registered for secoiridoids (4.36%-65.63%) and for flavonoids (0.18%-0.67%) transfer rates. In fact, secoiridoids have been greatly transferred in 'Picual' cultivar, whereas flavonoids have greatly been transferred in 'Changlot Real' cultivar. This behavior was not correlated with the original contents of secoiridoids and flavonoids in fruits of those cultivars, which discards the statement that more phenols in fruits could lead automatically to more phenols in oil. The result obtained could probably be due to the character amphiphilic of those phenolic groups in interaction with the humidity of each cultivar.

2.3 Chemometric analysis

PCA was applied to the different phenolic groups' contents and to total phenols of both olive fruits and oils at the same time. The first (PC1) and second (PC2) principal components described more than 77.60% of the data variability for all cases of the analysis. PC1 was clearly linked to fruits and oil secoiridoids, fruits and oil flavonoids, oil lignans and oil total phenol, whereas PC2 was correlated to fruit and oil simple phenols, fruits oleosides and fruits total phenols (**Figure 3**).

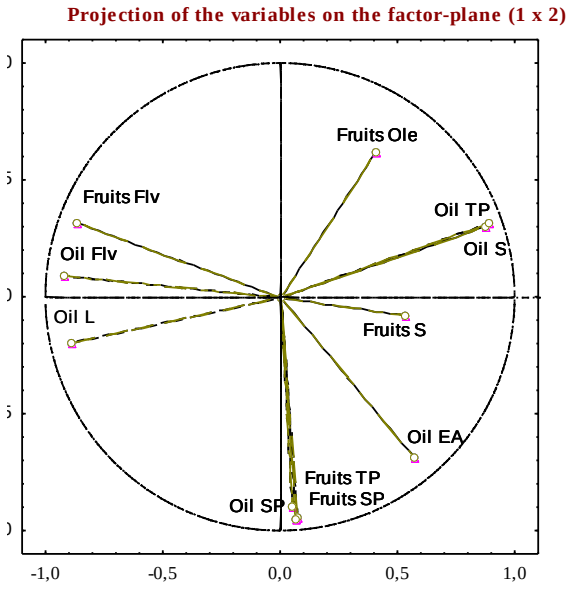

Figure 3. Projection on the factorial plane of olive fruits and oils variables. Ole (oleosides), TP (total phenols), S (secoiridoids), EA (elenolic acid), SP (simple phenol), Flv (flavonoid) and L (lignans)

Interestingly, the chemometric analysis showed that phenolic groups and total phenolic contents of fruits and oils were responsible of the discrimination of almost all cultivars (**Figure 4**). In fact, the different cultivars were greatly separated, as exception of ‘Arbequina’, ‘Sikitita’ and ‘Arbosana’. This result confirms once more the high genetic variability on the phenolic compounds profiles of olive fruit[36,37] as well as on oil[38,39]. The difficulty of separation among ‘Arbequina’, ‘Sikitita’ and ‘Arbosana’ cultivars is probably due to the proximity of their phenolic profiles. In fact, ‘Arbequina’ and ‘Sikitita’ are genetically related ('Sikitita' comes from a cross between 'Picual' x 'Arbequina'[40], and a higher degree of similarity of 'Sikitita' oil phenol composition with the 'Arbequina' than 'Picual' parent has previously been reported[41]. ‘Arbequina’ and ‘Arbosana’ are originated from the same geographical area (Catalonia, Spain) and, probably, could also be genetically related[42].

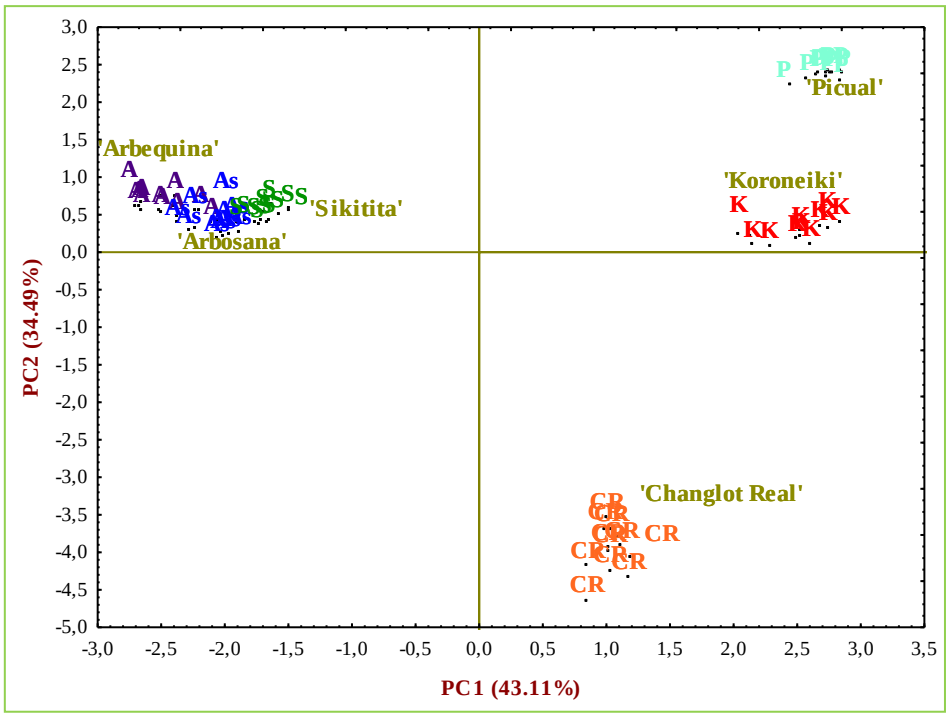

Figure 4. Scatter Plots of the first and second principal components for the different cultivars. P ('Picual'), K ('Koroneiki'), CR ('Changlot Real'), S ('Sikitita'), As ('Arbosana') and A ('Arbequina').

3. Conclusions

To sum up, phenolic compounds displayed high qualitative and quantitative differences among the cultivars considered in the present study and among olive fruit and olive oil. Concretely, after fruit processing, new compounds appeared in oil, notably aglycone forms because of the partial or total degradation during oil process of some original compounds detected in fruits, or totally new structures such as lignans. The phenolic transfer rate did not overcome 2% in all cultivars; however, big differences in transfer rate were detected in total phenol and individual phenolic groups rate transfers among cultivars. These results clearly revealed the genetic contribution on olive phenolic content and composition and their transfer between olive fruits and oil.

References

(1) Servili, M.; Sordini, B.; Esposto, S.; Urbani, S.; Veneziani, G.; Di Maio, I.; Selvaggini, R.; Taticchi, A. Biological activities of phenolic compounds of extra virgin olive oil. *Antioxidants* **2014**, 3, 1–23.

(2) Boskou, D.; Blekas, G.; Tsimidou, M. Phenolic compounds in olive oil and olives. *Curr. Top. Nutraceutical Res*. **2005**, 3, 125–136.

(3) Clodoveo, M. L.; Durante, V.; La Notte, D. Working towards the development of innovative ultrasound equipment for the extraction of virgin olive oil. *Ultrason. Sonochem*. **2013**, 20, 1261–1270.

(4) García, A.; Brenes, M.; García, P.; Romero, C.; Garrido, A. Phenolic content of commercial olive oils. *Eur. Food Res. Technol*. **2003**, 216, 520–525.

(5) Bakhouche, A.; Lozano-Sánchez, J.; Beltrán-Debón, R.; Joven, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia. *Food Res. Int*. **2013**, 50, 401–408.

(6) Dabbou, S.; Rjiba, I.; Nakbi, A.; Gazzah, N.; Issaoui, M.; Hammami, M. Compositional quality of virgin olive oils from cultivars introduced in Tunisian arid zones in comparison to Chemlali cultivars. *Sci. Hortic. (Amsterdam)*. **2010**, 124, 122–127.

(7) Taamalli, A.; Arráez Román, D.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Classification of ‘Chemlali’ accessions according to the geographical area using chemometric methods of phenolic profiles analysed by HPLC-ESI-TOF-MS. *Food Chem*. **2012**, 132, 561–566.

(8) Taamalli, A.; Gomez-Caravaca, A. M.; Zarrouk, M.; Segura-Carretero, A.; Fernandez-Gutierrez, A. Determination of apolar and minor polar compounds and other chemical parameters for the discrimination of six different varieties of Tunisian extra-virgin olive oil cultivated in their traditional growing area. *Eur. Food Res. Technol*. **2010**, 231, 965–975.

(9) Caruso, G.; Gucci, R.; Urbani, S.; Esposto, S.; Taticchi, A.; Di Maio, I.;

Selvaggini, R.; Servili, M. Effect of different irrigation volumes during fruit development on quality of virgin olive oil of cv. Frantoio. *Agric. Water Manag*. **2014**, 134, 94–103.

(10) Farinelli, D.; Tombesi, S. Performance and oil quality of 'Arbequina' and four Italian olive cultivars under super high density hedgerow planting system cultivated in central Italy. *Sci. Hortic. (Amsterda*m). **2015**, 192, 97–107.

(11) García, J. M.; Cuevas, M. V.; Fernández, J. E. Production and oil quality in 'Arbequina' olive (*Olea europaea*, L.) trees under two deficit irrigation strategies. *Irrig. Sci*. **2013**, 31, 359–370.

(12) Baccouri, B.; Zarrouk, W.; Baccouri, O.; Guerfel, M.; Nouairi, I.; Krichene, D.; Daoud, D.; Zarrouk, M. Composition, quality and oxidative stability of virgin olive oils from some selected wild olives (*Olea europaea* L. *subsp. oleaster*). *Grasas y Aceites* **2008**, 59, 346–351.

(13) Servili, M.; Esposto, S.; Fabiani, R.; Urbani, S.; Taticchi, A.; Mariucci, F.; Selvaggini, R.; Montedoro, G. F. Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology* **2009**, 17, 76–84.

(14) Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Cerretani, L.; Bendini, A.; Fernández-Gutiérrez, A. Use of capillary electrophoresis with UV detection to compare the phenolic profiles of extra-virgin olive oils belonging to Spanish and Italian PDOs and their relation to sensorial properties. *J. Sci. Food Agric*. **2009**, 89, 2144–2155.

(15) Alagna, F.; Mariotti, R.; Panara, F.; Caporali, S.; Urbani, S.; Veneziani, G.; Esposto, S.; Taticchi, A.; Rosati, A.; Rao, R.; et al. Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. *BMC Plant Biol*. **2012**, 12, 162.

(16) Bendini, A.; Cerretani, L.; Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Lercker, G. Phenolic molecules in virgin olive oils: A survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the

last decade. *Molecules* **2007**, 12, 1679–1719.

(17) Jerman Klen, T.; Mozetič Vodopivec, B. The fate of olive fruit phenols during commercial olive oil processing: Traditional press versus continuous two- and three-phase centrifuge. *LWT-Food Sci. Technol*. **2012**, 49, 267–274.

(18) Jerman Klen, T.; Wondra, A. G.; Vrhovšek, U.; Sivilotti, P.; Mozetič Vodopivec, B. Olive fruit phenols transfer, transformation, and partition trail during laboratory-scale olive oil processing. *J. Agric. Food Chem*. **2015**, 63, 4570–4579.

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

(20) Tura, D.; Gigliotti, C.; Pedò, S.; Failla, O.; Bassi, D.; Serraiocco, A. Influence of cultivar and site of cultivation on levels of lipophilic and hydrophilic antioxidants in virgin olive oils (*Olea Europaea* L.) and correlations with oxidative stability. *Sci. Hortic. (Amsterdam)*. **2007**, 112, 108–119.

(21) Dağdelen, A.; Tümen, G.; Ozcan, M. M.; Dündar, E. Phenolics profiles of olive fruits (*Olea europaea* L.) and oils from Ayvalık, Domat and Gemlik varieties at different ripening stages. *Food Chem*. **2013**, 136, 41–45.

(22) Ryan, D.; Robards, K. Critical Review. Phenolic compounds in olives. *Analyst.* **1998**, 123, 31R – 44R.

(23) Talhaoui, N.; Gómez-Caravaca, A. M.; Leon, L.; de la Rosa, R.; Fernandez-Gutierrez, A.; Segura-Carretero, A. Pattern of variation of fruit traits and phenol content in olive fruits from six different cultivars. *J. Agric. Food Chem*. **2015**, 63, 10466–10476.

(24) Artajo, L.-S.; Romero, M.-P.; Suárez, M.; Motilva, M.-J. Partition of phenolic compounds during the virgin olive oil industrial extraction process. *Eur. Food Res. Technol*. **2007**, 225, 617–625.

(25) Frankel, E.; Bakhouche, A.; Lozano-Sánchez, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. Literature review on production process to obtain extra virgin olive oil enriched in bioactive compounds. Potential use of by-products as alternative sources of polyphenols. *J. Agric. Food Chem*. **2013**, 61, 5179–5188.

(26) Sánchez de Medina, V.; Priego-Capote, F.; de Castro, M. D. L. Characterization of monovarietal virgin olive oils by phenols profiling. *Talanta*. **2015**, 132, 424–432.

(27) Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. Health and sensory properties of virgin olive oil hydrophilic phenols: agronomic and technological aspects of production that affect their occurrence in the oil. *J. Chromatogr. A.* **2004**, 1054, 113–127.

(28) Goulas, V.; Charisiadis, P.; Gerothanassis, I.; Manganaris, G. Classification, biotransformation and antioxidant activity of olive fruit biophenols: A review. *Curr. Bioact. Compd*. **2012**, 8, 232–239.

(29) Obied, H. K.; Karuso, P.; Prenzler, P. D.; Robards, K. Novel secoiridoids with antioxidant activity from Australian olive mill waste. *J. Agric. Food Chem*. **2007**, 55, 2848–2853.

(30) Brenes, M.; Hidalgo, F. J.; García, A.; Rios, J. J.; García, P.; Zamora, R.; Garrido, A. Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J. Am. Oil Chem. Soc*. **2000**, 77, 715–720.

(31) Oliveras López, M. J.; Innocenti, M.; Ieri, F.; Giaccherini, C.; Romani, A.; Mulinacci, N. HPLC/DAD/ESI/MS detection of lignans from Spanish and Italian *Olea europaea* L. fruits. *J. Food Compos. Anal*. **2008**, 21, 62–70.

(32) Benito, M.; Lasa, J. M.; Gracia, P.; Oria, R.; Abenoza, M.; Varona, L.; Sánchez-Gimeno, A. C. Olive oil quality and ripening in super-high-density Arbequina orchard. *J. Sci. Food Agric*. **2013**, 93, 2207–2220.

(33) Rodis, P. S.; Karathanos, V. T.; Mantzavinou, A. Partitioning of olive oil antioxidants between oil and water phases. *J. Agric. Food Chem*. **2002**, 50, 596–601.

(34) Kirkham, M. B. *Water use in crop production*; Kirkham, M. B., Ed.; The Haworth Press, 1999.

(35) Artajo, L.-S.; Romero, M. P.; Motilva, M. J. Transfer of phenolic compounds during olive oil extraction in relation to ripening stage of the fruit. *J. Sci. Food Agric*. **2006**, 86, 518–527.

(36) Bouaziz, M.; Jemai, H.; Khabou, W.; Sayadi, S. Oil content, phenolic profiling and antioxidant potential of Tunisian olive drupes. *J. Sci. Food Agric*. **2010**, 90, 1750–1758.

(37) Morelló, J.R.; Romero, M.P.; Motilva, M.J. Effect of the maturation process of the olive fruit on the phenolic fraction of drupes and oils from 'Arbequina' , 'Farga' , and 'Morrut' Cultivars. *J. Agric. Food Chem*. **2004**, 52, 6002–6009.

(38) Rivas, A.; Sanchez-Ortiz, A.; Jimenez, B.; García-Moyano, J.; Lorenzo, M. L. Phenolic acid content and sensory properties of two Spanish monovarietal virgin olive oils. *Eur. J. Lipid Sci. Technol*. **2013**, 115, 621–630.

(39) Rotondi, A.; Alfei, B.; Magli, M.; Pannelli, G. Influence of genetic matrix and crop year on chemical and sensory profiles of Italian monovarietal extra-virgin olive oils. *J. Sci. Food Agric*. **2010**, 90, 2641–2648.

(40) Rallo, L.; Barranco, D.; De la Rosa, R.; León, L. "Chiquitita" Olive. *HortScience* **2008**, 43, 529–531.

(41) Garcia-Gonzalez, D.; Tena, N.; Aparicio, R. Quality characterization of the new virgin olive oil Var . 'Sikitita' by phenols and volatile compounds. *J. Agric. Food Chem*. **2010**, 58, 8357–8364.

(42) Rallo, L.; Barranco, D.; Caballero, J. M.; Del Río, C.; Martín, A.; Tous, J.; Trujillo, I. *Variedades de olivo en España*, Mundi-Pren.; Rallo, L., Barranco, D., Caballero, J. M., Del Río, C., Martín, A., Tous, J., Trujillo, I., Eds.; Junta de Andalucía, MAPA: Madrid, 2005.

GENERAL CONCLUSIONS

1. The review concerning the importance of olives leaves as a source of phenolic compounds, the analytical determination of those compounds, biotic and abiotic influence on their presence and their health benefits indicates that olive growth is an object of a great importance in Mediterranean countries as well as in the new olive world. In this sense, the general improvement in extraction methods and analytical techniques for the determination of phenolic compounds has been useful for analyzing phenolic compounds in olive leaves. This review investigates the abiotic and biotic factors that could affect phenolic contents in olive leaves for the first time. This knowledge is of key importance, since it could predict which family of compounds is more available in foliage during leaf sampling for therapeutic and medicinal uses. Finally, the huge number of studies related to the valuable effect of olive-leaf phenolic compounds on health in last decade should encourage the industry to assess these leaves as a source of antioxidants to produce medicines, cosmetics, nutraceuticals and functional foods.

2. The study of the phenolic compounds profile in 'Sikitita', 'Picual' and 'Arbequina' cultivars showed that HPLC-DAD-ESI-TOF-MS is a powerful technique for determining olive-leaf phenolic compounds in the new cultivar 'Sikitita' and its parent cultivars 'Picual' and 'Arbequina'. The optimized methodology enabled the identification of a total of 30 compounds in the olive-leaf samples. Moreover, it was also possible to tentatively identify, for the first time in Spanish cultivars, two isomers of 2"-methoxyoleuropein. Furthermore, the comparison among cultivars on the basis of the 30 compounds showed that 'Arbequina' was the cultivar that presented the highest concentration of total phenols and almost of all single phenols in olive leaves. Also, the phenolic profile of the new cultivar 'Sikitita' was more similar to that of the 'Picual' parent than to that of 'Arbequina' parent.

3. The chemometric analysis for the evaluation of phenolic patterns in olive leaves from six cultivars at different growth stages revealed that foliage from

different cultivars grown in the same orchard under the same environmental and agronomic conditions and sampled at different times showed noticeable differences in individual and total phenolic compounds contents. However, all cultivars showed similar trends in the evaluation of phenolic contents during ripening. Since the olive leaves are considered one of the important sources of specific phenolic compounds, the knowledge of these differences between cultivars and between seasons is paramount for predicting the availability of phenolic compounds. Furthermore, this is the first time that the seasonal variability of such a high number of phenolic compounds has been reported. Finally, June and December appeared to be the best periods to use olive leaves as a source of phenolic compounds, both months presenting similar phenolic concentrations. Nevertheless, December could be advantageous, as it coincides with the harvest period in Andalusia (Spain). Thus, in this period, the leaves could be evaluated efficiently as olive by-products.

4. In ‘Sikitita’, ‘Picual’, and ‘Arbequina’, olive-leaf phenolic compounds were determined by HPLC-DAD-ESI-TOF-MS and afterwards assessed for the potential anti-inflammatory properties of those phenolic extracts. All olive cultivars showed high foliar contents in total phenolic compounds, especially ‘Picual’, with oleuropein as the major compound for the three cultivars. Moreover, olive-leaf extracts of the three cultivars presented great immunomodulatory properties since they all inhibited the release of the pro-inflammatory mediator NO, when evaluated *in vitro* in LPS-stimulated RAW264.7 cells, a macrophage cell type. Although significant differences were detected among the three cultivars olive-leaf extracts for all the compounds and for total phenols, their respective *in vitro* activities were found not to significantly differ. In any case, the findings of the present study provide clear evidence supporting the traditional use of medicinal plants in treating inflammatory diseases. Further studies in relation to immunomodulatory and anti-inflammatory potentials of the extracts fractions will be performed in the future.

5. The study of the variation pattern of fruit traits and phenolic contents in olive fruits from six different cultivars during ripening represents a solid contribution to research on olive fruits. Despite the high number of olive cultivars preserved in different cultivar collections, there are few reports on the genetic variability of phenolic compounds in the fruit. The time course of phenolic compounds in 'Koroneiki', 'Arbosana', 'Changlot Real', and 'Sikitita' olive fruit during ripening has been reported for the first time. Overall, the major agronomic changes occurred were registered between June and October, whereas the period between October and December was characterized by the stability of almost all the parameters studied. Regarding phenolic compounds, qualitative and quantitative differences among cultivars were highlighted, whereas a common pattern was observed among sampling time, characterized by a dominance of secoiridoids (mainly oleuropein) during the first part of the ripening period, but, as the ripening progressed, simple phenols and flavonoids became the major components. It bears noting that PCA provided a separation of the phenolic profile in olive fruits for different sampling times and cultivars. However, in contrast to the results for the agronomical traits, the ranking of cultivars with regard to phenolic compounds was not stable during ripening. Finally, it was observed that 'Koroneiki' stands out for its flavonoid content, 'Changlot Real' for simple phenols, and 'Picual' for oleosides.

6. The study of the transfer of phenolic compounds from olive fruits to VOO in six different cultivars grown under the same agronomical conditions showed that phenolic compounds displayed high qualitative and quantitative differences among cultivars for fruit and oil. Concretely, after fruit processing, new compounds appeared in the oil, notably aglycone forms because of the partial or total degradation during oil processing of some original compounds detected in fruits. Also totally new structures such as lignans were found. The phenolic transfer rate from olive fruit to the oil did not exceed 2% in any cultivar; however, substantial differences were detected among cultivars in the transfer rates of total phenolics and individual phenolic groups. These results clearly

reveal the genetic contribution to olive phenolic content and composition and the transfer of these compounds between olive fruits and oils.

CONCLUSIONES GENERALES

1. Se ha llevado a cabo una revisión bibliográfica acerca de la importancia de la hoja del olivo como fuente de compuestos fenólicos, su determinación analítica, la influencia de factores bióticos y abióticos, y los beneficios para la salud. La mejora de los métodos de extracción y de las técnicas analíticas existentes para la determinación de los compuestos fenólicos han sido muy útiles para el análisis de los compuestos fenólicos de la hoja de olivo. Así, este trabajo de revisión resume por primera vez los factores abióticos y bióticos que afectan a los compuestos fenólicos de la hoja de olivo. El conocimiento de la influencia de todos estos factores podría ayudar a predecir la familia de compuestos o los compuestos individuales que van a estar disponibles en el momento de la recogida de la hoja y que posteriormente serán usadas con fines terapéuticos y medicinales. Por último, el gran número de trabajos relacionados con los efectos beneficiosos de los compuestos fenólicos de la hoja de olivo realizados en la última década deberían servir para animar a la industria a revalorizar la hoja de olivo como una fuente de antioxidantes que puede ser empleada para la producción de medicinas, cosméticos, nutracéuticos y para el desarrollo de alimentos funcionales.

2. El estudio del perfil fenólico de la hoja de olivo de las variedades ‘Sikitita’, ‘Picual’ y ‘Arbequina’ mediante HPLC-DAD-ESI-TOF-MS ha demostrado que esta es una potente técnica para la determinación de los compuestos fenólicos de la hoja de olivo de la nueva variedad ‘Sikitita’ y en sus progenitores ‘Picual’ y ‘Arbequina’. El método optimizado hizo posible la identificación de un total de 30 compuestos en las muestras de hoja de olivo. Además, se consiguieron identificar por primera vez dos isómeros de 2”-metoxioleuropeina en variedades de hoja de olivo españolas. Por otra parte, la comparación entre las diferentes variedades mostró que la variedad ‘Arbequina’ fue la que presentó la mayor concentración de compuestos fenólicos totales y de casi todos los compuestos fenólicos a nivel individual. El perfil fenólico de la hoja de olivo de la nueva variedad ‘Sikitita’ resulta ser más parecido al perfil de la variedad ‘Picual’ que al perfil de la variedad ‘Arbequina’.

3. El análisis quimiométrico empleado para la evaluación del perfil fenólico de la hoja de olivo recogida en diferentes épocas del año reveló que la hoja de olivo de diferentes variedades cultivadas en el mismo campo experimental bajo las mismas condiciones ambientales y agronómicas muestran diferencias notables tanto en el contenido de compuestos fenólicos totales como individuales. A pesar de ello, las hojas de todas las variedades presentaron un comportamiento similar en cuanto a la evolución de los compuestos fenólicos durante la maduración. El conocimiento de las diferencias entre las muestras de hoja de diferentes variedades y entre los diferentes periodos de la recogida puede resultar de gran importancia para el pronóstico de la disponibilidad de los compuestos fenólicos. Cabe destacar que esta es la primera vez que se ha realizado un estudio acerca de la variabilidad estacional de un número tan elevado de compuestos fenólicos en la hoja de olivo. Junio y diciembre resultaron las mejores épocas para recoger la hoja con el fin de usarlas como fuente de compuestos fenólicos, ambos meses presentaron concentraciones altas y similares de estos compuestos. Sin embargo, diciembre podría ser la mejor época de recogida ya que coincide con la época de recogida de la aceituna en Andalucía (España). Por ello, en este periodo del año la hoja de olivo podría ser eficazmente revalorizada como subproducto del olivo.

4. Se han determinado los compuestos fenólicos de las hojas de olivo de las variedades Sikitita', 'Picual' y 'Arbequina', y a continuación, se ha llevado a cabo la evaluación del potencial antiinflamatorio de los extractos fenólicos. Todas las hojas de olivo de las diferentes variedades presentaron altas concentraciones de compuestos fenólicos totales, especialmente la variedad 'Picual', siendo la oleuropeína el compuesto mayoritario en las tres variedades estudiadas. Además, los extractos de hoja de olivo de las tres variedades mostraron tener excelentes propiedades inmunomodulatorias, ya que todos ellos inhibían la liberación del mediador pro-inflamatorio NO, al ser evaluados *in vitro* con células RAW264.7 estimuladas con LPS. Aunque se observaron diferencias significativas entre el contenido en compuestos fenólicos totales e individuales de las tres variedades de hoja, sus respectivas actividades *in vitro* fueron

relativamente similares. Estos resultados aportan claras evidencias que apoyan el uso tradicional de plantas medicinales para el tratamiento de enfermedades inflamatorias. Por ello, en un futuro próximo se llevarán a cabo estudios más profundos relacionados con el potencial inmunomodulatorio y antiinflamatorio de los extractos fenólicos de la hoja de olivo.

5. El estudio del patrón de variación de diferentes parámetros agronómicos de las aceitunas y del contenido en compuestos fenólicos de aceitunas pertenecientes a seis variedades diferentes y estudiadas a lo largo de su proceso de maduración, representa una gran contribución a la investigación de este campo. A pesar del gran número de variedades de olivo que se conservan en distintas colecciones en todo el mundo, hay muy pocos estudios acerca de la influencia de variabilidad genética sobre los compuestos fenólicos de la aceituna. Además, este estudio presenta por primera la evolución de los compuestos fenólicos de aceitunas de las variedades 'Koroneiki', 'Arbosana', 'Changlot Real' y 'Sikitita' durante su maduración. Se observó que los mayores cambios agronómicos ocurrían entre los meses de junio y octubre, mientras que el periodo entre octubre y diciembre se caracterizaba por la estabilidad de casi todos los parámetros estudiados. En cuanto a los compuestos fenólicos, se observaron diferencias cualitativas y cuantitativas entre las aceitunas de las distintas variedades estudiadas, aunque la tendencia de estos parámetros era común para todas ellas a lo largo de los diferentes periodos de toma de muestra. Esta tendencia se caracterizaba por un predominio de la familia de los secoiridoides (principalmente oleuropeína) al comienzo del periodo de maduración y, a medida que la maduración avanzaba, flavonoides y fenoles simples pasaban a ser los compuestos mayoritarios. El análisis PCA dio lugar a una separación de las diferentes variedades y los diferentes tiempos de toma de muestra usando los contenidos en compuestos fenólicos como parámetros de discriminación. Por otra parte, al contrario de lo que ocurría con los parámetros agronómicos, la clasificación de las variedades en cuanto a compuestos fenólicos no era estable durante la maduración. Por último, se pudo observar que 'Koroneiki' destacaba por su contenido en flavonoides,

mientras que ‘Changlot Real’ por los fenoles simples y ‘Picual’ por la familia de los oleósidos.

6. Se ha realizado un estudio de la transferencia de los compuestos fenólicos de la aceituna al aceite virgen empleando para ello seis variedades de olivo diferentes cultivados bajo las mismas condiciones agronómicas y ambientales. En primer lugar, se observó que había grandes diferencias cualitativas y cuantitativas entre los compuestos fenólicos de aceituna y aceite de las diferentes variedades. Después del procesado de la aceituna aparecían compuestos en el aceite que anteriormente no se encontraban en la aceituna, principalmente formas aglicona, debido a la degradación parcial o total de algunos compuestos de la aceituna. Además, en el aceite se identificaban nuevos compuestos anteriormente no presentes en la aceituna como son los lignanos. La transferencia de compuestos fenólicos entre la aceituna y el aceite no sobrepasaba el 2% en ninguna de las variedades; sin embargo, se detectaron grandes diferencias en la transferencia de compuestos fenólicos totales y de familias de compuestos fenólicos entre las distintas variedades estudiadas. Estos resultados revelan la contribución genética en el perfil y contenido de compuestos fenólicos y en su transferencia entre aceituna y aceite.

CONCLUSIONS GENERALES

1. Une révision bibliographique a été menée concernant notamment l'importance de la feuille d'olivier comme source de composés phénoliques, la détermination analytique de ces dits composés, l'influence des facteurs biotiques et abiotiques sur leur synthèse, ainsi que les bénéfices qu'ont ces facteurs sur la santé humaine. L'amélioration survenue au niveau des méthodes d'extraction et des techniques analytiques pour la détermination des composés phénoliques a aussi été d'une utilité considérable dans l'analyse des composés phénoliques des feuilles d'olivier. Il est à signaler que la présente révision résume pour la première fois les facteurs abiotiques et biotiques influençant sur le contenu des composés phénoliques au niveau des feuilles d'olivier. La prise de conscience de ce fait pourrait aider à prévoir la disponibilité de certaines familles et/ou composés phénoliques dans les feuilles à un moment donné, ce qui augmenterait le profit de l'usage des feuilles à des fins thérapeutiques et médicinales. Finalement, le grand nombre de recherches scientifiques menées au cours de la dernière décennie concernant les bienfaits des composés phénoliques des feuilles d'olivier sur la santé humaine devrait encourager le secteur de l'industrie à valoriser les feuilles d'olivier comme source d'antioxydant, dans le but de produire des médicaments, des cosmétiques et nutraceutiques, ainsi que pour développer des aliments fonctionnels.

2. L'étude du profil phénolique des feuilles appartenant aux variétés d'olivier dites 'Sikitita', 'Picual' et 'Arbequina' par l'HPLC-DAD-ESI-TOF-MS a démontré que cette dernière était une technique puissante pour la détermination des composés phénoliques au niveau des feuilles d'olivier de la nouvelle variété 'Sikitita', ainsi que de ses progéniteurs 'Picual' et 'Arbequina'. L'optimisation de la méthode de séparation au niveau de l'HPLC a permis d'identifier un total de 30 composés phénoliques présents au sein des échantillons de feuilles. En outre, deux composés ont été identifiés pour la première fois dans des variétés espagnoles. Il s'agit des deux isomères du 2"-methoxyoleuropéine. Par ailleurs, la comparaison faite entre les différentes variétés sur la base de ces composés phénoliques a montré que la variété 'Arbequina' a été celle dotée de la

concentration la plus élevée, tant au niveau de la concentration totale qu'individuelle, et ce dans presque tous les composés identifiés dans la feuille. Enfin, le profil phénolique des feuilles d'olivier de la nouvelle variété 'Sikitita' s'est avéré être d'avantage similaire à celui du progéniteur 'Picual' qu'à celui du progéniteur 'Arbequina'.

3. L'analyse chimiométrique a été utilisée afin d'évaluer le profil phénolique des feuilles d'olivier de six variétés collectées à différentes période de l'année (maturation du fruit). L'étude a révélé des différences significatives entre les échantillons, aussi bien en teneur totale qu'en teneur individuelle des composés phénoliques, au niveau des feuilles issues des différentes variétés cultivées dans le même verger expérimental et dans les mêmes conditions environnementales et agronomiques. Cependant, tous les échantillons ont présenté un comportement similaire en terme d'évolution de composés phénoliques au cours de la période de maturation. Connaître les différences entre les variétés et entre les périodes d'échantillonnage au cours de la maturation peut s'avérer être d'une grande importance, afin de prévoir la disponibilité des composés phénoliques au niveau des feuilles. Plus précisément, c'est la première fois qu'une étude de la variabilité saisonnière a été menée sur un aussi grand nombre de composés phénoliques présents dans la feuille d'olivier. Les mois de juin et décembre ont présenté les périodes les plus propices pour collecter les feuilles d'olivier afin d'en bénéficier comme source de composés phénoliques; au cours de ces deux périodes, de hautes et similaires concentrations en ces composés ont été enregistrées. Cependant, décembre pourrait être qualifié de meilleure période pour collecter les feuilles d'olivier, car celle-ci coïncide avec celle de la récolte des olives en Andalousie (Espagne). Par conséquent, à cette époque de l'année les feuilles d'olivier pourraient être efficacement valorisées comme un sous-produit de la culture d'olivier.

4. Les composés phénoliques des feuilles d'olivier des variétés 'Sikitita', 'Picual' et 'Arbequina' ont été déterminés. Par la suite, l'étude a été achevée par une

évaluation du potentiel anti-inflammatoire des extraits phénoliques des feuilles concernées. Toutes les feuilles des différentes variétés ont présenté des concentrations élevées en composés phénoliques totaux, en particulier la variété 'Picual', étant l'oleuropéine le composé majoritaire dans les trois variétés étudiées. Par ailleurs, les extraits de feuilles d'olivier des trois variétés ont montré d'excellentes propriétés immuno-modulatrices, en inhibant efficacement la libération des médiateurs pro-inflammatoires NO au niveau de l'étude *in vitro* sur des cellules macrophages type RAW264.7 stimulées par l'LPS. D'autre part, bien que des différences significatives entre les teneurs en composés phénoliques totaux et individuels eussent été observées au niveau des feuilles des trois variétés, leurs activités respectives dans l'étude *in vitro* étaient relativement similaires. Ces résultats fournissent des preuves positives en faveur de l'utilisation traditionnelle des plantes médicinales pour le traitement des maladies inflammatoires. Par conséquent, des études plus approfondies en relation avec les activités immuno-modulatrices et anti-inflammatoires des extraits phénoliques des feuilles d'olivier seront menées dans le futur.

5. Le suivi du modèle de variation des différents paramètres agronomiques des olives ainsi que de leur teneur en composés phénoliques dans six différentes variétés au cours de la maturation (juin, août, octobre et décembre), est considéré comme une contribution importante à la recherche dans ce domaine. Malgré le grand nombre de variétés d'olives conservé dans les différentes collections à travers le monde, il n'y a eu que très peu d'études traitant de l'influence de la variabilité génétique sur les composés phénoliques au niveau des olives. En outre, cette étude montre pour la première fois l'évolution des composés phénoliques au cours de la maturation à partir d'olives des variétés 'Koroneiki', 'Arbosana', 'Changlot Real' et 'Sikitita'. Alors que de grands changements au niveau des paramètres agronomiques ont été observés entre les mois de juin et d'octobre, on a remarqué que celle d'entre octobre et décembre a été caractérisée par la stabilité de presque tous les paramètres étudiés. En ce qui concerne les composés phénoliques, des différences qualitatives et quantitatives entre les

variétés étudiées ont été observées, bien que la tendance de ces paramètres eût été commune tout au long des périodes de collecte des olives. Cette tendance s'est caractérisée par une prédominance de la famille des secoiridoïdes (principalement l'oleuropéine) au début de la période de maturation, et au fur et à mesure que la maturation progressait, d'autres familles comme les flavonoïdes et les phénols simples commençaient à prendre de l'ampleur. En prenant les composés phénoliques comme paramètres de discrimination, l'analyse en composantes principales (ACP) a donné lieu à une séparation entre les différentes variétés d'une part et entre les périodes d'échantillonnage des olives d'autre part. Par ailleurs, contrairement à ce qui était observé au niveau des paramètres agronomiques, le classement des variétés en ce qui concerne les composés phénoliques n'était pas stable pendant la maturation. Enfin, la variété 'Koroneiki' s'est avérée avoir la plus haute concentration en flavonoïdes dans les olives, alors que la variété 'Changlot Real' a présenté la plus haute concentration en phénols simples. Quant à elle, la variété 'Picual' s'est distinguée par sa haute concentration en oléosidos.

6. Une étude portant sur le transfert des composés phénoliques des olives à l'huile d'olive vierge a été réalisée sur six variétés d'oliviers cultivées dans les mêmes conditions agronomiques et environnementales. Dans un premier temps, de grandes différences qualitatives et quantitatives ont été observées entre les composés phénoliques tant pour les olives que pour l'huile d'olive des différentes variétés. Dans un second temps, après le processus d'extraction de l'huile d'olive, de nouveaux composés sont apparus dans l'huile, principalement des composés en forme aglycone générés par la dégradation partielle ou totale de certains composés phénoliques existant dans les olives. Néanmoins, d'autres composés comme les lignanes ont été identifiés uniquement dans l'huile d'olive. Le transfert des composés phénoliques à l'huile d'olive n'a pas dépassé les 2% dans toutes les variétés; cependant, de grandes différences au niveau du transfert des contenus totaux et des contenus par familles de composés phénoliques entre les différentes variétés étudiées ont été détectées. Ces résultats mettent en

évidence la contribution génétique de la variété dans le profil et le contenu phénolique ainsi que leur transfert des olives à l'huile d'olive.