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NEW CHALLENGES IN ANALYTICAL DETERMINATION OF OLIVE OIL POLYPHENOLS. POTENTIAL USE AS MARKERS LINKED TO PEDOCLIMATIC, AGRONOMIC AND TECHNOLOGICAL CONDITIONS

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Submitted for a Doctoral degree in Chemistry

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by

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GRANADA, October 29, 2015

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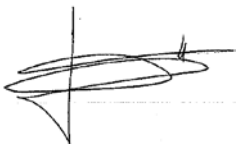

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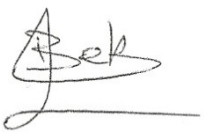

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Certify:

That the work presented in this doctoral thesis entitled "**NEW CHALLENGES IN ANALYTICAL DETERMINATION OF OLIVE OIL POLYPHENOLS. POTENTIAL USE AS MARKERS LINKED TO PEDOCLIMATIC, AGRONOMIC AND TECHNOLOGICAL CONDITIONS**" 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 Doctoral Degree in Chemistry from the University of Granada.

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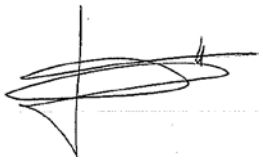

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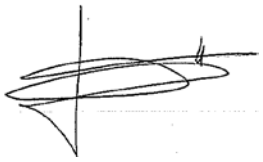

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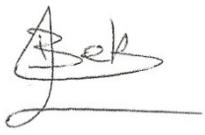

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List of Abbreviations

BP: before the Present

BCE: before the Common Era

HDL: high density lipoprotein

LDL: low density lipoprotein

3, 4-DHPEA: (3, 4-dihydroxyphenyl) ethanol or hydroxytyrosol

p-HPEA: (*p*-hydroxyphenyl) ethanol or tyrosol

3, 4-DHPEA-AC: hydroxytyrosol acetate

3,4-DHPEA-EA: oleuropein aglycone

p-HPEA-EA: ligstroside aglycone

3,4-DHPEA-EDA: decarboxymethyl oleuropein aglycone

p-HPEA-EDA: decarboxymethyl ligstroside aglycone

HPLC: high performance liquid chromatography

MS: mass spectrometry

TC: total cholesterol

CVD: cardiovascular diseases

TXB2: thromboxane B2

COX-1: cyclooxygenase-1

COX-2: cyclooxygenase-2

IL-1*β:* interleukin 1*β*

TNF-*α:* tumor necrosis factor-alpha

LTB4: leukotriene B4

DNA: deoxyribonucleic acid

PBMCs: peripheral blood mononuclear cells

SO: styrene oxide

ER*β:* estrogen receptor *β*

ERK: extracellular signal-regulated kinase

HER2: human epidermal growth factor receptor 2

PAL: phenylalanine ammonia lyase

PLS-DA: partial least square-discriminant analysis

ETc: crop evapotranspiration

RDI: regulated deficit irrigation

Mg: milligrams

Kg: kilogramme

$N_{2:}$ nitrogen

VOO: virgin olive oil

EVOO: extra-virgin olive oil

PE: polyethylene

LLE: liquid-liquid extraction

SPE: solid phase extraction

MeOH: methanol

H_2O: water

Ml: milliliter

LC: liquid chromatography

GC: gas chromatography

CE: capillary electrophoresis

RRLC: rapid-resolution liquid chromatography

UPLC: ultra-performance liquid chromatography

IT-MS: ion trap mass spectrometry

TOF-MS: time-of-flight mass spectrometry

Q-TOF-MS: quadrupole-time-of-flight mass spectrometry

Q-orbitrap-MS: hybrid quadrupole-orbitrap mass spectrometery

UV-visible: ultraviolet–visible detector

m/*z:* mass-to-charge ratio

ESI: electrospray ionization

API: atmospheric-pressure ionization

APCI: atmospheric-pressure chemical ionization

MALDI: matrix-assisted laser-desorption ionization

BPC: base-peak chromatogram

EIC: extracted ion chromatogram

DA: discriminant analysis

OMWW: olive mill wastewater

NO: nitric oxide

DAD: diode array detector

EFSA: European food safety authority

NMR: nuclear magnetic resonance spectroscopy

LLME: liquid–liquid microextraction

DLLME: dispersive liquid–liquid microextraction

RP-DLLME: reversed phase dispersive liquid–liquid microextraction

NACE: non-aqueous capillary electrophoresis

FID: flame ionization detector

HRMS: high resolution mass spectrometry

SUMMARY

Virgin olive oil (VOO) is the main source of fat in the Mediterranean region. Its consumption was found to be associated with low incidence of chronic diseases such as atherosclerosis, cancer, obesity and diabetes. The healthy properties of this oil were originally attributed to its high content of monounsaturated fatty acids. However, recent studies have demonstrated that the minor fractions, mainly polyphenols, also make a major contribution to healthy VOO properties. In addition, phenolic compounds are also associated with the oxidative stability and organoleptic quality of VOO.

The present doctoral thesis entitled "***New challenges in analytical determination of olive oil polyphenols. Potential use as markers linked to pedoclimatic, agronomic and technological conditions***", deals with the analysis of phenolic compounds in VOOs and the effect of different factors on their concentration, such as pedoclimatic, agronomic and technological conditions of production. For this purpose, the thesis is divided into two sections: introduction and experimental. The **INTRODUCTION** includes important information about VOO composition, oxidative stability, organoleptic and health properties of VOO polyphenols, factors affecting their content in VOO, and analytical procedures for the qualitative and quantitative characterization of this important fraction.

The EXPERIMENTAL SECTION, RESULTS AND DISCUSSIONS are presented in three sections according to the different topics under study:

SECTION I focuses on the study of the effect of geographical area of cultivation and agronomic practices on VOO phenolic composition. This section is divided into two chapters:

Chapter 1 concerns the phenolic characterization and geographical classification of commercial Arbequina VOOs produced in southern Catalonia. The aim of the study was to explore the phenolic profile of Arbequina VOO as one of the main Spanish olive varieties, and to look for possible differences in the phenolic composition among the geographical area under study. For this purpose, VOO samples were classified into three groups (group 1, group 2 and group 3) according to their geographical origin, which were demarcated

taking into account the edaphological characteristics and orography. A total of 32 olive oil samples were obtained from different mills. Then, phenolic compounds were extracted from the samples using solid-phase extraction, and the resulting extracts were analysed by high-performance liquid chromatography coupled to mass spectrometry (HPLC-ESI-TOF/MS). This study was carried out in collaboration with la Unitat de Recerca Biomèdica (URB-CRB) de la Universitat Rovira i Virgili (Reus).

In **chapter 2,** the changes in chemical composition, principally phenolic compounds, of Algerian Chemlal VOO with regard to irrigation and harvest time were studied. First, the olive grove was divided into two parts: one was under rain-fed conditions (non-irrigated) and the other was irrigated with 100% of crop evapotranspiration. Olive oil samples were obtained from irrigated and non-irrigated olives on three harvest dates for posterior analysis. Phenolic compounds were extracted using liquid-liquid extraction, and analysed by HPLC-ESI-TOF/MS in an effort to obtain detailed information about the phenolic behaviour under the effect of the studied factors. The work included in this chapter was carried out in collaboration with the Department of Agronomic Sciences of Tizi-Ouzou University in Algeria.

SECTION II is divided into three chapters and examines the VOO production process, and the best conditions for obtaining VOO with high phenolic content.

In **chapter 3,** the first part provides an overview of the different steps involved in the VOO elaboration process including olive harvesting time, crushing, malaxation, centrifugation, storage and filtration, and their effect on its phenolic composition. The objective was to establish the best conditions for obtaining VOO rich in phenolic compounds taking into account the reported data in the literature. The second part provides an overview of the different phenolic families characterized in olive oil by-products, and the possible use of these by-products as a potential alternative source of bioactive compounds. The work included in this chapter was the result of a collaboration with the Olive Center of California University, United States.

In **chapter 4**, the effect of harvest dates corresponding to different olive ripening stages on Algerian Azeradj olive oil quality was studied. To carry out the study, olive fruits from the Azeradj variety were manually collected on different dates (D1, 03 November 2013; D2, 27 November 2013; and D3, 21 December 2013) from trees cultivated in the same area (Haizar), in north-central Algeria. After that, olive oil samples were made on a laboratory scale using the Abencor system for their posterior analysis. This study is the first one available in which polyphenols of the Azeradj VOO variety have been characterized by HPLC-ESI-TOF/MS. The work included in this chapter was carried out in collaboration with the Department of Agronomic Sciences of Tizi-Ouzou University in Algeria.

Chapter 5 includes the monitoring of VOO moisture and phenolic compound content during the industrial filtration process. To achieve this, a conventional filtration process was performed in duplicate using two lots (lot 1 and lot 2), for a total amount of 45,000 kg of VOO each. The VOOs were from the main Spanish olive varieties (Hojiblanca, Manzanilla, Picual and Arbequina). Cloudy VOOs were filtered using Vitacel L-90 and Filtracel EFC-950 as filter aids together with a filtration tank. The moisture content was determined in unfiltered and filtered VOOs. In addition, the individual phenolic compounds were qualitatively and quantitatively characterized by HPLC-ESI-TOF/MS. This work was carried out in collaboration with Oleoestepa Company S.C.A, with the University of Bologna (Italy) and with University of Campinas (Brazil).

SECTION III is divided into two chapters and focuses on the analytical procedures for the determination of VOO phenolic composition, and the different limitations observed in the methods proposed until now.

In **chapter 6**, the first part provides an overview of the current extraction and analytical approaches for the qualitative and quantitative characterization of phenolic compounds in VOO as well as the advantages and disadvantages of each approach. Liquid-liquid extraction, solid-phase extraction, liquid chromatography, gas chromatography, capillary electrophoresis, UV-Vis and

mass spectrometry detectors were the analytical techniques reviewed in this first part. In the second part, the main current problems in the analysis of VOO phenolic compounds were discussed in order to take them into account in future studies aimed at olive oil phenolic characterization.

In **chapter 7,** a new approach has been developed for correcting the effect that moisture reduction after VOO filtration exerts on the apparent increase in the secoiridoid content by using an internal standard during extraction. The objective of the study was to resolve one of the major problems faced in the analysis of VOO phenolic compounds. Firstly, two main Spanish varieties (Picual and Hojiblanca) were submitted to industrial filtration of VOOs. Afterwards, the moisture content was determined in unfiltered and filtered VOOs, and liquid–liquid extraction of phenolic compounds was performed using different internal standards. The resulting extracts were analysed by HPLC-ESI-TOF/MS, in order to gain maximum information concerning the phenolic profiles of the samples under study. This research was carried out in collaboration with the University of Bologna (Italy), University of Campinas (Brazil) and Aceites Maeva Company S.L.

RESUMEN

El aceite de oliva virgen (AOV) es la fuente principal de grasa en la dieta mediterránea. Su consumo se ha asociado con una baja incidencia de enfermedades crónicas tales como aterosclerosis, cáncer, obesidad y diabetes. Estas propiedades saludables lo convierten en un alimento funcional, siendo su bioactividad tradicionalmente atribuida a su alto contenido en ácidos grasos monoinsaurados. Sin embargo, estudios recientes han demostrado que compuestos presentes en la fracción minoritaria pueden contribuir de forma significativa a las propiedades saludables de AOV. Dentro de estos compuestos se puede resaltar el papel de los polifenoles que son el objetivo de estudio en la presente memoria. Estos compuestos fenólicos además de poseer propiedades bioactivas demostradas científicamente contribuyen a la estabilidad oxidativa y la calidad organoléptica del AOV.

Dadas las múltiples funciones que presentan los polifenoles la presente tesis doctoral titulada " ***Estudio del efecto de los parámetros pedoclimáticos, agronómicos y tecnológicos en la composición fenólica del aceite de oliva virgen-extra "*** pretende profundizar en el conocimiento de esta familia de compuestos así como determinar el efecto que las diferentes variables mencionadas pueden tener sobre su composición final. El trabajo realizado se divide en dos bloques: introducción y parte experimental. La **INTRODUCCIÓN** incluye una revisión bibliográfica acerca de la composición fenólica del AOV, su importancia en la establidad oxidativa, propiedades organolépticas y saludables de este alimento, así como los factores que afectan a su contenido y técnicas analíticas utilizadas en la caracterización cualitativa y cuantitativa de esta fracción.

LA PARTE EXPERIMENTAL, RESULTADOS Y DISCUSIONES se divide en tres secciones cada una de las cuales agrupa los capítulos en los que se han llevado a cabo investigaciones dentro de una misma temática de trabajo:

La **SECCIÓN I** se centra en el estudio del efecto de la zona geográfica y las prácticas agronómicas en la composición fenólica de AOV. Esta sección se divide en dos capítulos.

En el **capítulo 1** se incluye el estudio de la fracción fenólica de aceites obtenidos a partir de la misma variedad de aceituna pero con distinto origen geográfico. El objetivo de este trabajo ha sido determinar el efecto de la zona geográfica en esta familia de compuestos. Para ello se han utilizado 32 muestras de AOVs comerciales de la variedad Arbequina producidos en el sur de Cataluña y se han clasificado en tres grupos (grupo 1, grupo 2, y el grupo 3) en base a su origen geográfico. Estas zonas han sido delimitadas teniendo en cuenta las características edafológicas y orografía. La caracterización cualitativa y cuantitativa de los polifenoles se ha llevado a cabo mediante cromatografía líquida de alta resolución acoplada a un espectrómetro de masas con analizador de tiempo de vuelo mediante el uso de interfase tipo electrospray (HPLC-ESI-TOF/MS). Este trabajo de investigación se llevó a cabo en colaboración con la Unitat de Recerca Biomèdica (URB-CRB) de la Universitat Rovira i Virgili de Reus.

En el **capítulo 2** se ha estudiado el efecto de la irrigación y el periodo de recolección en la composición química, principalmente en compuestos fenólicos, del AOV de la variedad Chemal cultivada en Argelia. Para ello se ha realizado un estudio en un cultivo experimental sometido a distintas condiciones de estrés hídrico. La recolección del fruto se ha llevado a cabo en distintos periodos. Los compuestos fenólicos se han analizado mediante HPLC-ESI-TOF/MS con el fin de establecer el efecto que estos factores presentan de forma individual en las distintas familias de polifenoles identificados en las muestras bajo estudio. El trabajo incluido en este capítulo fue llevado a cabo en colaboración con el Departamento de Ciencias Agronómicas de la Universidad de Tizi-Ouzou de Argelia.

La **SECCIÓN II** dividida en 3 capítulos, analiza el proceso de producción del AOV, con el objetivo de proponer las condiciones óptimas para la obtención de AOVs con alto contenido fenólico.

En el **capítulo 3** se ha incluido una revisión científica del estado del arte en la producción de aceite de oliva en relación con el contenido final en polifenoles. Esta revisión ha proporcionado una visión general sobre las

diferentes etapas del proceso de elaboración del AOV (periodo de recolección, la molienda, batido, centrifugación, almacenamiento y filtración) así como su efecto sobre la composición fenólica del AOV. Los cambios producidos a lo largo del diagrama de flujo del proceso se traducen en pérdidas de polifenoles que pasan a formar parte de los subproductos generados. En este sentido la revisión llevada a cabo ha incluido una descripción de la composición de estos subproductos así como sus posibles usos como fuentes alternativas de estos compuestos bioactivos. El trabajo incluido en este capítulo fue el resultado de una colaboración con Olive Center of California University, Estados Unidos.

En el **capítulo 4** se ha determinado el efecto del grado de maduración del fruto sobre la calidad de aceite de oliva. Para llevar a cabo este estudio se ha seleccionado una parcela de olivos experimentales de la variedad Azeradj en Haizar (norte-centro de Argelia). Se han recolectado muestras de frutos en tres fechas distintas y se ha obtenido el aceite de oliva empleando el sistema Abencor. La determinación del perfil fenólico de los distintos aceites se ha llevado a cabo empleando la misma metodología analítica que en capítulos anteriores. El trabajo incluido en este capítulo fue llevado a cabo en colaboración con el Departamento de Ciencias Agronómicas de la Universidad de Tizi-Ouzou de Argelia.

El **capítulo 5** trata del proceso industrial de filtración del aceite de oliva y su efecto en el contenido en humedad y polifenoles del aceite de oliva. Para establecer el efecto de esta etapa del procesado del aceite de oliva sobre ambos componentes (agua y polifenoles) se ha procedido a filtrar aceite obtenido a partir de las principales variedades españolas Hojiblanca, Manzanilla, Picual y Arbequina. Se han empleado como agentes filtrantes coadyuvantes orgánicos derivados de la celulosa (Vitacel L-90 y Filtracel EFC-950). La monitorización de los polifenoles a lo largo del proceso de filtración se ha llevado a cabo mediante HPLC-ESI-TOF/MS. Este trabajo se llevó a cabo en colaboración con la empresa Oleoestepa S.C.A, con la Universidad de Bolonia (Italia) y con la Universidad de Campinas (Brasil).

La **SECCIÓN III** comprende dos capítulos centrados en el estudio de los procedimientos analíticos para la determinación de la composición fenólica del AOV.

El **capítulo 6** incluye una revisión científica sobre los procedimientos de extracción y análisis de los compuestos fenólicos del AOV con información relativa a las ventajas e inconvenientes que cada autor ha establecido para cada metodología analítica: extracción líquido-líquido, extracción en fase sólida y técnicas separativas acopladas a distintos detectores.

En el **capítulo 7** se ha desarrollado un trabajo de investigación orientado a la puesta a punto de un nuevo procedimiento para determinar el efecto real de la filtración del aceite de oliva sobre la fracción fenólica. Este planteamiento se ha llevado a cabo como consecuencia del incremento aparente en el contenido en polifenoles por efecto de la reducción de la humedad del aceite. Para ello se han seleccionado aceites de las variedades españolas Picual y Hojiblanca y se ha procedido a su filtración a escala industrial. La extracción de los compuestos fenólicos se ha realizado empleando distintos procedimientos con la finalidad de obtener una solución analítica al problema. Este trabajo de investigación se realizó en colaboración con la Universidad de Bolonia (Italia), con la Universidad de Campinas (Brasil) y la empresa Aceites Maeva S. L.

OBJECTIVES

Virgin olive oil (VOO) is becoming increasingly popular around the world, not only because of its unique sensory characteristics but also because of the beneficial health effects associated with its consumption, particularly as part of the Mediterranean diet. The health-promoting effects of VOO have been attributed to its fatty acid profile, as well as to the presence of many bioactive components such as phenolic compounds. Unfortunately, the concentration of these analytes in VOO is not constant, and it can be affected by several factors related to agronomic conditions of cultivation and the extraction process of VOO. Therefore, studying the effect of these factors on VOO phenolic fraction and looking for the best conditions for producing VOO with high phenolic content are of great importance. However, developing efficient and accurate analytical methods for their correct qualitative and quantitative characterization in the produced oils is also vital. Therefore, the main **objectives** of this thesis are:

- To explore the phenolic profile of VOO and to evaluate the effect of the geographical area of cultivation on its phenolic composition. For this purpose, the phenolic extracts obtained from the Arbequina olive variety will be characterized using high-performance liquid chromatography coupled to electrospray time-of-flight mass spectrometry (HPLC-ESI-TOF/MS).

- To investigate the changes produced in VOO phenolic composition obtained under different irrigation treatments on tree harvest dates, in an effort to understand how the agronomic practices can alter its phenolic profile. To this end, the characterization of the phenolic extracts of Chemlal VOO will be done by HPLC-ESI-TOF/MS.

- To review the different steps involved in the VOO elaboration process, and their effect on VOO phenolic composition. Taking into account the data reported in the literature, the best conditions for the VOO production process will be summarized. Because of the importance of

phenolic compounds, the different phenolic families identified in VOO by-products and the different methods used for their recovery will also be reviewed.

- To study the variation in phenolic composition during olive maturation. To do this, Algerian Azeradj VOOs will be obtained at different olive fruit ripening stages, and their chemical composition will be determined in an effort to establish the best harvest period for the Azeradj variety. Notably, a detailed characterization of individual phenolic compounds in VOOs from this variety will be carried out using HPLC-ESI-TOF/MS.

- To evaluate the effect of the industrial filtration process on VOO moisture and phenolic compound content, in order to establish the relationship between making VOO brilliant and the richness of the filtered VOO on phenolic content. The phenolic profile of filtered and unfiltered VOOs will be analysed by HPLC-ESI-TOF/MS.

- To review the main methods used for extracting phenolic compounds from VOO as well as the separation techniques and the detectors used in their characterization. The methods reported in the literature will be evaluated and the drawbacks of each of them will be discussed in order to take them into account in VOO phenolic studies.

- To resolve analytical problems causing the apparent increase in secoiridoid content in filtered VOO, and then to evaluate what really happens to the phenolic compounds during VOO filtration. The characterization of phenolic compounds in this study will be done by HPLC-ESI-TOF/MS.

OBJETIVOS

El aceite de oliva virgen (AOV) es cada vez más popular en todo el mundo, no sólo por sus características sensoriales sino por los efectos saludables asociados a su consumo, en particular como parte de la dieta Mediterránea. Cada vez son más las publicaciones científicas que atribuyen estos efectos beneficiosos no sólo al perfil de ácidos grasos sino a sus componentes minoritarios, en concreto a los polifenoles. Estos componentes constituyen una fracción muy compleja cuya composición completa aún está por determinar. En esta labor es muy importante el desarrollo de métodos analíticos eficaces y precisos para su correcta caracterización cualitativa y cuantitativa.

Por otro lado, existe una dificultad añadida y es que tanto el perfil cualitativo como su concentración se pueden ver afectados por numerosos factores tales como pedoclimáticos, genéticos, agronómicos y tecnológicos. Por lo tanto, el estudio del efecto que estos factores ejercen en la fracción fenólica del AOV y la búsqueda de las mejores condiciones para producir AOV con alto contenido fenólico es de gran importancia. Es por ello que el objetivo principal de la presente tesis doctoral se ha centrado tanto en la determinación analítica como en la evaluación del efecto que los distintos factores ejercen en los polifenoles del aceite de oliva. Este doble objetivo se puede desglosar en:

- Explorar el perfil fenólico del AOV y evaluar el efecto de la zona geográfica en su composición fenólica. Para este fin, los extractos fenólicos obtenidos de la variedad Arbequina se caracterizarán mediante la plataforma analítica HPLC-ESI-TOF/ MS. De los resultados de caracterización se comprobará si el perfil fenólico permite establecer a esta fracción como huella dactilar del origen geográfico de los aceites bajo estudio.

- Investigar los cambios producidos en la composición fenólica del AOV obtenidos a partir de cultivos de olivo sometidos a distintas condiciones de estrés hídrico y monitorizar la evolución de estos compuestos. Para este fin, la caracterización de los extractos

fenólicos de AOV de la variedad Chemlal se hará mediante HPLC- ESI-TOF/MS.

- Determinar el efecto del proceso de elaboración de AOV en la composición fenólica. Para ello se llevará a cabo una evaluación sobe el estado de la técnica con un doble objetivo: establecer las mejores condiciones de elaboración para obtener un aceite enriquecido en polifenoles y evaluar los subproductos como fuente potencial de este tipo de compuestos bioactivos.

- Establecer la variación de la composición fenólica de aceites obtenidos a partir de aceitunas con distinto índice de madurez. Para este objetivo se utilizarán aceites obtenidos a partir de la variedad Azeradj y se llevará a cabo una caracterización detallada de los compuestos fenólicos individuales mediante HPLC-ESI-TOF/MS.

- Monitorizar la evolución de los polifenoles durante la filtración industrial para establecer el equilibrio entre la reducción de humedad y la pérdida de polifenoles durante esta etapa del proceso de elaboración del aceite.

- Establecer mediante revisión bibliográfica los principales métodos de extracción de los compuestos fenólicos del AOV, así como las técnicas separativas empleadas y los detectores utilizados en su caracterización. Resumir las principales ventajas e inconvenientes que los distintos autores han atribuido a cada una de las metodologías analíticas desarrolladas.

- Establecer posibles soluciones analíticas a los problemas en la determinación de polifenoles que imposibilitan evaluar el efecto de determinados factores en la composición final del aceite, como es el caso del efecto del proceso de producción, en concreto de la filtración del aceite de oliva.

INTRODUCTION

1. Olive and olive oil history

Plants and fruits mentioned in the Hebrew and Christian Bibles and in the Koran have long been of historic interest as they represent a broad picture of Middle Eastern people's interactions with their environment, and beliefs, for a period of more than two millennia. In these sacred writings, the olive (*Olea europaea* L.) is the most frequently mentioned fruit, reaching a total of 66 references (48 in the Hebrew Bible, 12 in the Christian Bible and six in the Koran)[1]. The olive tree is one of the most emblematic trees in the world. The geographic origin and timing of its domestication, the history of its early use and its symbolic significance are still openly debated.

Ancient wild olive trees flourished in the Holy Land woodlands of the Carmel hills, of Samaria, of Lower Galilee and of Gilead, but only isolated remains of olive branches and stones have been found in settlements from the Palaeolithic and Neolithic periods[2]. The earliest evidence of the use of wild olives dates to the Palaeolithic, at Ohalo II, Sea of Galilee, at *c.* 19000 BP[3]. However, olive oil production, with thousands of crushed olive stones and olive pulp, was discovered in the south of Haifa. This later includes olive oil extraction from wild forms started on the Carmel coast at *c.* 6500 BP. Olive cultivation, based on the domesticated form, is considered to have begun during the Chalcolithic cultural period when the development of techniques for oil extraction and the presence of olives are recorded at different sites in the Jurdan Valley and the rest of the Levant[4]. Since the early Bronze Age, the olive has become one of the most important crops grown in the dry farming regions of the Middle East, and one of the main economic plants of the Mediterranean basin.

At the beginning of the Bronze Age, the increase in the density of olive trees was clearly reflected in the pollen sequences from the northern Levant in the

[1] Kaniewski, D.; Van Campo, E.; Boiy, T.; Terral, J.-F.; Khadari, B.; Besnard, G. *Biol. Rev. Camb. Philos. Soc.* **2012**, *87*, 885–899.
[2] Galili, E.; Weinstein-Evron, M.; Hershkovitz, I.; Gopher, A.; Kislev, M.; Lernau, O.; Kolska Horwitz, L.; Lernau, H. *Journal F. Archaeol.* **1993**, *20*, 133–157.
[3] Kislev, M. E.; Nadel, D.; Carmi, I. *Rev. Palaeobot. Palynol.* **1992**, *73*, 161–166.
[4] Liphschitz, N.; Gophna, R.; Hartman, M.; Biger, G. *J. Archaeol. Sci*. **1991**, *18*, 441–453.

Ghab valley (Syria)[5]. In addition, it was indicated that fragments from olive trees increased from 20-30 % during the Chalcolithic to 40–60 % during the Early Bronze Age (*c.* 5300-4100 BP) at 47 archaeological sites in Israel. Nevertheless, the cultivation of O. *europea* seems to have been relatively restricted to an area close to the Mediterranean coast during the Early Bronze Age, and became even more restricted in the Middle Bronze Age[6]. As a consequence, olive oil was destined for an elite. It was considered a luxurious product; documents from Syria indicate that the value of olive oil was five times higher than that of wine and two and a half times more than that of seed oils. With regard to the Iron Age, controversial data have been published about the olive oil situation during this period. While archaeobotanical and olive press remains indicate the existence of a major olive oil production in Palestine (701-630/623 BCE), the pollen data from the southern Levant indicate a decrease in olive tree density during the Iron Age[1].

The spread of the olive tree probably coincided with the vegetative propagation. However, commerce played an important role in its expansion. The primary movements to the west were well documented. Records indicate the introduction of olives into Greece, Egypt and western Turkey. In those areas, there are many archaeological sites with olive-related findings, such as milling stones, decantation basins, storage vessels, frescos and ancient writings[7]. The great ancient writer and philosopher Homer stated in his writings that Greek courts sentenced people to death if they destroyed an olive tree. At the site of the ancient Olympic Stadium in Olympia, Greece (775 BCE), the winners were triumphantly acclaimed and crowned with wreaths made of olive twigs. Ancient gold coins that were minted in Athens depicted the face of the Goddess Athena wearing an olive leaf wreath on her helmet and holding a clay vessel of olive oil. At the palace of Knossos (1700 BCE) on the island of Crete, clay tables record the trade of olive oil. In Urla, a district of Izmir, Turkey, there is an ancient olive oil processing facility dating to 600 BCE. Many clay vessels, called amphorae, which were used to store and

[5] Yasuda, Y.; Kitagawa, H.; Nakagawa, T. *Quat. Int*. **2000**, *73*-74, 127–136.
[6] Riehl, S. *Veg. Hist. Archaeobot*. **2008**, *17*, 43–51.
[7] Vossen, P. *HortScience.* **2007**, *42*, 1093–1100.

transport olive oil, can be found in the ruins throughout this area[8]. Later, the Romans discovered olive trees through their contact with Greek colonies in Italy. Although they were not admirers of olives and olive oil, the Romans were responsible for spreading the tree through their huge empire. The value of the tree led the Roman agronomist Collumela to call the olive *the queen of trees*[9].

The rise of the Roman Empire and the conquest of Greece led to the introduction of olive oil processing facilities in the entire Mediterranean basin. The Iberian Peninsula (Spain and Portugal) and the north coast of Africa became the largest production areas of olive oil, which was shipped in large amphorae to England, Germany, France and Italy. Olive oil in these times had many documented uses; all cultures used olive oil primarily as lamp fuel, which was its greatest value. Many rituals involved the use of olive oil, including the anointing of royalty, warriors and the general public for religious purposes. The term "Messiah" means "the anointed one". Olive oil was used to make offerings to the gods, as pharmaceutical ointments to cure diseases and to make the skin and hair appear healthier. They were also used to make soap and to consecrate the dead. There is very little record of olive oil being used for human consumption[7,8].

During the middle Ages, olive oil continued to increase in production and importance primarily in Spain, Italy and Greece. It declined in North Africa and other areas taken over by Turks, but was revived later in Arab-controlled countries. In the late 19th and 20th centuries, the demand for olive oil decreased after the development of low-cost solvent extraction techniques for seed oils and the use of other sources of light (gas and electricity)[7]. Recently, it has been proven that olive oil provides valuable nutrients for humans, and these play important roles in the diets of the people in the areas of cultivation, which explains the appearance of new producer countries such as the United State of America, Argentina, Chile, Mexico, Peru and

[8] Uylaşer, V.; Yildiz, G. *Crit. Rev. Food Sci. Nutr*. **2014**, *54*, 1092–1101.
[9] Kapellakis, I. E.; Tsagarakis, K. P.; Crowther, J. C. *Rev. Environ. Sci. Bio/Technology*. **2007**, *7*, 1–26.

Australia[10]. The **Figure 1** shows the expansion of olive tree cultivation in the Mediterranean basin.

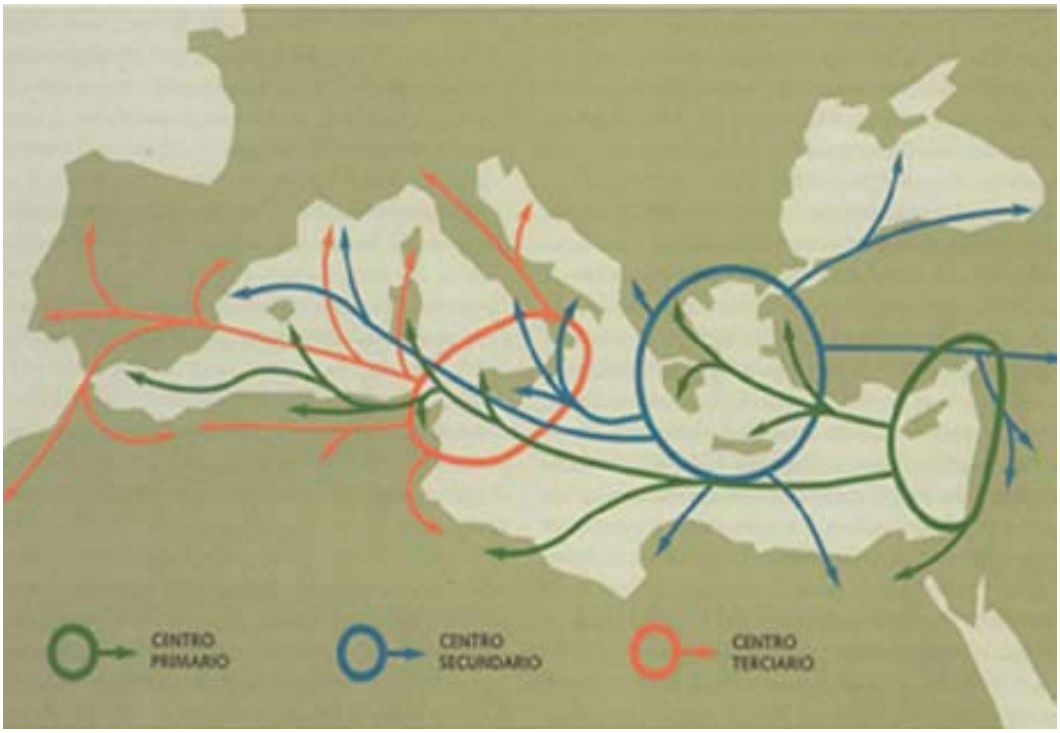

Figure 1. The expansion of olive tree cultivation in the Mediterranean basin through different civilizations**.**

2. Olive oil composition

Overall, olive oil can be divided, from a chemical composition point of view, into major and minor fractions. The major components, which include triacylglycerols, partial glycerides and free fatty acids, represent more than 98% of the total oil weight. Minor components, which are present in very low amounts of about 2% of oil weight, include compounds such as hydrocarbons, triterpenes, pigments, tocopherols and phenolic compounds.

2.1 Major fraction

Triacylglycerols are the main components of olive oil and are derived from the esterification of glycerol with three fatty acid molecules. Fatty acids may combine with any of the three hydroxyl groups of glycerol to create a wide

[10] García-González, D. L.; Aparicio, R. *J. Agric. Food Chem*. **2010**, *58*, 12569–12577.

diversity of compounds. If all three fatty acids are identical, it is a simple triacylglycerol. However, the more common forms are the "mixed" triacylglycerols in which two or three kinds of fatty acids are present in the molecule. The triacylglycerols found in significant proportions in olive oil are OOO (40-59 %), POO (12-20 %), OOL (12.5-20 %), POL (5.5-7 %) and SOO (3-7 %)[11,12]. POP, POS, OLnL, LOL, OLnO, PLL, PLnO and LLL, where O=oleic acid, L=linoleic acid, P=palmitic acid, Ln=linolenic acid and S=stearic acid, were reported in smaller amounts[13]. Fully saturated moieties have not been reported and the same applies for the tri-unsaturated ones containing linolenic acid. The presence of partial glycerides in olive oil is due either to incomplete triacylglycerol biosynthesis or hydrolytic reactions. While the concentration of diacylglycerols ranges from 1 to 2.8 % in virgin olive oil, the concentration of monoacylglycerols is present in much smaller quantities (less than 0.25%). During olive oil storage it was reported that 2-diacylglycerols tend to isomerize to the more stable 1,3-diacylglycerols. This rearrangement provides information about the age of olive oil and storage conditions, and a ratio of 1.3/1.2 diacylglycerols can be used as a criterion to monitor olive oil quality[14]. Furthermore, due to the information provided by the olive oil triacylglycerol profile, it was possible to discriminate olive oils according to the cultivar and geographical origin[15].

With regard to olive oil **fatty acid** composition, the main compounds detected were palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids. The amount of each fatty acid in olive oil was found to be dependent on many factors such as the area of production, the latitude, the climate, the variety and the stage of maturity of the fruit. Myristic (C14:0), heptadecanoic (C17:0), heptadecenoic (C17:1), arachidic (C20:0) and adoleic (C20:1) acids are found in trace amounts[15].

[11] Gökçebağ, M.; Dıraman, H.; Özdemir, D. *J. Am. Oil Chem. Soc*. **2013**, *90*, 1661–1671.
[12] Fuentes de Mendoza, M.; De Miguel Gordillo, C.; Marín Expóxito, J.; Sánchez Casas, J.; Martínez Cano, M.; Martín Vertedor, D.; Franco Baltasar, M. N. *Food Chem*. **2013**, *141*, 2575–2581.
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[14] García-González, D. L.; Aparicio-Ruiz, R.; Aparicio, R. *Eur. J. Lipid Sci. Technol*. **2008**, *110*, 602–607.
[15] Yorulmaz, A.; Yavuz, H.; Tekin, A. *J. Am. Oil Chem. Soc*. **2014**, *91*, 2077–2090.

However, the principal component is always oleic acid, contributing about 55–83 % of the total fatty acids[16]. As mentioned above, most of the fatty acids in olive oil are present as triacylglycerols. Nevertheless, in free forms, their content can be used as a criterion for olive oil freshness (acidity index). It was reported that a good olive oil quality is obtained when the acidity index is low[17].

The nutritional and healthy properties of olive oil have been attributed to its composition in monounsaturated fatty acids, mainly oleic acid. It is claimed to increase the plasma high-density lipoprotein (HDL) cholesterol and decrease the low-density lipoprotein (LDL) cholesterol. For this reason, oleic acid can prevent cardiovascular diseases, which are the major cause of mortality in industrialized countries[16]. Other healthy effects of oleic acid were reported, such as inhibition of coagulation, strengthening of cell-membrane integrity and helping to repair cells and damaged tissues, and protection from breast cancer and diabetes type 2[18,19]. From a nutritional point of view, the polyunsaturated fatty acids with 18 carbon atoms (linoleic and linolenic acids) present in olive oil are known as essential fatty acids. They cannot be synthesized by the body and therefore must be part of our diet. However, it seems important that they have to be present in a correct ratio in the diet, because their high reactivity and susceptibility to oxidation may represent a health risk[16].

2.2 Minor fraction

Hydrocarbons are present in considerable amounts in olive oil. Among the characterized hydrocarbons, squalene is reported as the main constituent of the unsaponifiable matter and makes up more than 90% of the hydrocarbon fraction. Its level in olive oil may range from 200 to 7500 mg/kg, although

[16] Ghanbari, R.; Anwar, F.; Alkharfy, K. M.; Gilani, A.-H.; Saari, N. *Int. J. Mol. Sci*. **2012**, *13*, 3291–3340.
[17] Bengana, M.; Bakhouche, A.; Lozano-Sánchez, J.; Amir, Y.; Youyou, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Food Res. Int*. **2013**, *54*, 1868–1875.
[18] Lopez, S.; Bermudez, B.; Pacheco, Y. M.; Ortega, A.; Varela, L. M.; Abia, R.; Muriana, F. J. G. *Olives and Olive Oil in Health and Disease Prevention*; Elsevier, 2010; pp. 1385–1393.
[19] Menendez, J. a; Vellon, L.; Colomer, R.; Lupu, R. *Ann. Oncol*. **2005**, *16*, 359–371.

much higher levels (up to 12000 mg/kg) were also found[20]. Squalene has been considered to be an important component due to its chemopreventative potential against cancer[16]. Moreover, it was reported that squalene administration at a dose of 1 g/kg decreased reactive oxygen species in lipoprotein fractions and caused a specific increase in high-density lipoprotein (HDL) cholesterol levels in animals[21]. Besides its biological properties, squalene can take part in olive oil oxidative stability as well as olive oil aroma and flavour saving due to its antioxidant activity[22]. Other hydrocarbons detected in olive oil included sesquiterpenes[23].

Triterpenes also represent a considerable part of olive oil minor fractions. In fact, two classes of triterpenic compounds are present, dialcohols (uvaol and erythrodiol) and triterpenic acids (oleanolic, ursolic and maslinic). Studying the triterpene composition of 40 Spanish olive cultivars, it was found that triterpenic dialcohol content ranges between 5.89 and 73.78 mg/kg, whereas triterpenic acid concentration ranges between 8.90 and 112.36 mg/kg[24]. The variation in the concentration of these analytes was attributed to genetic factors. However, variations in their content were also attributed to olive oil quality. It was reported that olive oil with an acidity index > 1% is richer in these compounds than olive oil with an acidity index < 1%[25]. Several studies have shown that these compounds possess healthy properties such as anti-inflammatory and vasodilatory properties[26,27].

Pigments are responsible for the colour of olive oil, ranging from yellow-green to greenish gold. Chlorophyll and carotenoids are the main olive oil

[20] Fernández-Cuesta, A.; León, L.; Velasco, L.; De la Rosa, R. *Food Res. Int*. **2013**, *54*, 1885–1889.
[21] Gabás-Rivera, C.; Barranquero, C.; Martínez-Beamonte, R.; Navarro, M. A.; Surra, J. C.; Osada, J. *PLoS One*. **2014**, *9*, e104224.
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[27] Juan, M. E.; Wenzel, U.; Daniel, H.; Planas, J. M. *Mol. Nutr. Food Res*. **2008**, *52*, 595–599.

pigments[28]. From a qualitative point of view, the chlorophyll profile of olive oil is determined by the pigments that are initially found in the fruits and the derivatives formed during olive oil processing. The chlorophylls "a" and "b", originally found in the fruit, are irreversibly converted into the more stable pheophytins due to the release of acids after the mechanical breakdown of the fruit tissue. Pheophytins are chlorophyll molecules where the central Mg2+ is replaced by two hydrogen ions. Among them, pheophytin "a" was reported as the major component; its concentration in olive oil was found to range from 3.3 to 40 mg/kg, while pheophytin "b" and chlorophyll "b" were present in trace amounts, and chlorophyll "a" has not been detected[29]. During olive oil storage a slowly developing decarbomethoxylation takes place on C_{13} of the pheophytin molecule, which originates pyropheophytin[30]. The olive oil carotenoid profile consists of lutein, ß-carotene, violaxanthine and neoxanthine[31]. When studying the composition of carotenoids in various Spanish olive oils, it was found that their concentration ranges from 3.1 to 9.2 mg/kg, with lutein being the main one[32]. In addition to the colour, chlorophylls and carotenoids play an important role in the oxidative stability of virgin olive oil due to their antioxidant nature in the dark and pro-oxidant activity in the light[33].

The **tocopherols** reported in olive oil are α-, ß-, γ- and δ-tocopherol. In fact, α tocopherol is the predominant form accounting for 90-95 % of total tocopherols. Research studies concerning the occurrence and levels of α-tocopherol in various sets of olive oils from all over the world have increased in the last two decades. Data indicated > 250 mg of α-tocopherol per kg of high-quality virgin olive oils analysed just after production, and even higher

[28] Motilva, M.; Romero, M. *The Effect of the Ripening Process of the Olive Fruit on the Chlorophyll and Carotenoid Fractions of Drupes and Virgin Oils*; Elsevier, 2010; pp. 59–68
[29] Psomiadou, E.; Tsimidou, M. *J. Agric. Food Chem.* **1998**, *46*, 5132–5138.
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[31] Aparicio-Ruiz, R.; Gandul-Rojas, B.; Roca, M. *J. Agric. Food Chem*. **2009**, *57*, 10831–10836.
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levels were found (>350 mg/ kg) in certain monovarietal products[34]. However, the concentration of ß-, γ- and δ-tocopherols ranges from traces to 25 mg/kg[16]. Olive oil tocopherol content is strongly variable according to pedoclimatic factors, agronomic conditions, fruit ripening and cultivars[35]. These compounds are known to contribute to olive oil oxidative stability[36]. In addition, they seem to have preventive properties against colon cancer, and defend the body against free radical attacks. However, the nature of this contribution is not yet fully understood. Some researchers have demonstrated a synergistic relationship between the antioxidant actions of tocopherols and some phenolic compounds, other minor components present in olive oil[16].

The **phenolic fraction** of olive oil has recently generated much interest regarding its health-promoting properties. Subsequent studies (*in vivo* and *in vitro*) have demonstrated that olive oil phenolics have a positive effect on certain physiological parameters, possibly reducing the risk of the development of chronic diseases[37]. Furthermore, phenolic compounds play an essential role in slowing down the oxidative phenomena of olive oil and strongly influence the organoleptic properties of the product[38]. The contribution of phenolic compounds to pungency and bitterness has to be taken into account to guarantee the best palatability of olive oil. Due to the importance of these analytes, and due to the fact that phenolic compounds are the subject of study in this thesis, they will be discussed in more detail in the following section.

[34]Tsimidou, M. Z. *Squalene and Tocopherols in Olive Oil: Importance and Methods of Analysis*; Elsevier, 2010; pp. 561–567.
[35] El Riachy, M.; Priego-Capote, F.; León, L.; Rallo, L.; Luque de Castro, M. D. *Eur. J. Lipid Sci. Technol*. **2011**, *113*, 678–691.
[36] Franco, M. N.; Galeano-Díaz, T.; Sánchez, J.; Miguel, C. De; Martín-Vertedor, D. *J. Oleo Sci.* **2014**, *63*, 115–125.
[37] Lozano-Sánchez, J.; Segura-Carretero, A.; Menendez, J. A.; Oliveras-Ferraros, C.; Cerretani, L.; Fernandez-Gutierrez, A. *J. Agric. Food Chem*. **2010**, *58*, 9942–9955.
[38] Servili, M.; Sordini, B.; Esposto, S.; Urbani, S.; Veneziani, G.; Di Maio, I.; Selvaggini, R.; Taticchi, A. *Antioxidants*. **2013**, *3*, 1–23.

3. Olive oil phenolic compounds

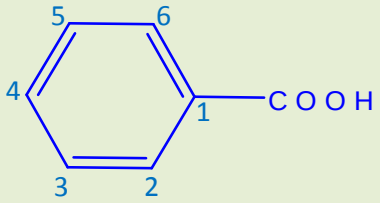
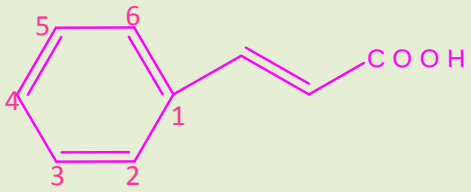
3.1 Classification

Phenolic compounds, known for many years as "polyphenols", are natural substances that possess a benzene ring bearing one or more hydroxy groups. In olive oil, the phenolic fraction consists of a heterogeneous mixture of compounds belonging to several families with different chemical structures. At least 36 structurally distinct phenolic compounds have been identified in this matrix. These compounds belong to five main classes: phenolic acids, phenolic alcohols, flavonoids, secoiridoids and lignans[39]. The synthesis of phenolic compounds in olive fruit occurs through the shikimate pathway, phenylpropanoid metabolism and mevalonic acid pathway. The latter is responsible for secoiridoid synthesis, and is typical of the *Oleaceae* family, which explains the presence of secoiridoids only in this family of plants[40,41].

Phenolic acids: Phenolic acids are secondary aromatic plant metabolites spread across a wide range of plants. They were the first group of phenolic compounds described in virgin olive oil. These compounds can be divided into two subgroups: benzoic acid derivatives with a basic chemical structure of C6–C1 and cinnamic acid derivatives with a basic chemical structure of C6–C3 (**Table 1**). While the benzoic acids include 3,4-dihydroxybenzoic acid, gentisic acid, gallic acid, vanillic acid and syringic acid, the cinnamic acids include *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid[42]. These substances are present in small amounts; their quantification in olive oil samples showed concentrations lower than 1 mg/kg[43].

[39] Bajoub, A.; Carrasco-Pancorbo, A.; Ajal, E. A.; Ouazzani, N.; Fernández-Gutiérrez, A. *Food Chem*. **2015**, *166*, 292–300.
[40] Gutierrez-Rosales, F.; Romero, M. P.; Casanovas, M.; Motilva, M. J.; Mínguez-Mosquera, M. I. *J. Agric. Food Chem*. **2010**, *58*, 12924–12933.
[41] El Riachy, M.; Priego-Capote, F.; León, L.; Rallo, L.; Luque de Castro, M. D. *Eur. J. Lipid Sci. Technol*. **2011**, *113*, 692–707.
[42] Tripoli, E.; Giammanco, M.; Tabacchi, G.; Di Majo, D.; Giammanco, S.; La Guardia, M. *Nutr. Res. Rev*. **2005**, *18*, 98–112.
[43] Bendini, A.; Cerretani, L.; Carrasco-Pancorbo, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Lercker, G. *Molecules*. **2007**, *12*, 1679–1719.

Table 1. The main phenolic acids identified in olive oil

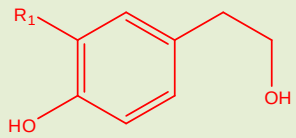
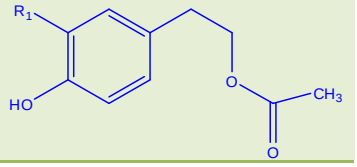
Compounds	Substituent	Structure
Benzoic acids and derivatives		
3,4-Dihydroxybenzoic acid	3,4-OH	
Gentisic acid	2,5-OH	
Vanillic acid	3-OCH_3, 4-OH	
Gallic acid	3,4,5-OH	
Syringic acid	3,5-OCH_3, 4-OH	
Cinnamic acids and derivatives		
p-Coumaric acid	4-OH	
Caffeic acid	3,4-OH	
Ferulic acid	3-OCH_3, 4-OH	
Sinapinic acid	3,5-OCH_3, 4-OH	

Phenolic alcohols: The main phenolic alcohols identified in olive oil are (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA) or hydroxytyrosol and (*p*-hydroxyphenyl)ethanol (*p*-HPEA) or tyrosol. Hydroxytyrosol and tyrosol are structurally identical except that hydroxytyrosol possesses an extra hydroxy group in the *meta* position. The concentration of these two phenolic alcohols is generally low in fresh oils but increases during olive oil storage as a consequence of hydrolysis of major phenolic compounds belonging to the secoiridoids group. In fact, when studying the phenolic composition of 18 virgin olive oil samples, it was found that the mean concentration of hydroxytyrosol and tyrosol was 14.4 and 27.45 mg/kg, respectively[44]. In another study where the extraction of the phenolic fraction from 210 olive oil

[44] Owen, R.; Giacosa, A.; Hull, W.; Haubner, R.; Spiegelhalder, B.; Bartsch, H. *Eur. J. Cancer* **2000**, *36*, 1235–1247.

samples from different areas of Mediterranean countries was carried out, the reported median values were 1.8 (lower quintile 1; upper quintile 3.6) and 1.9 (lower quintile 0.6; upper quintile 5.0) mg/kg of hydroxytyrosol and tyrosol, respectively[45]. Hydroxytyrosol acetate (3,4-DHPEA-AC), a derivative of hydroxytyrosol, was also found in virgin olive oil[46]. **Table 2** presents the main phenolic alcohols reported in olive oil.

Table 2. Different phenolic alcohols reported in olive oil

Compounds	R1	Structure
Hydroxytyrosol	OH	
Tyrosol	H	
Hydroxytyrosol acetate	OH	

Secoiridoids are compounds produced from the secondary metabolism of terpenes**.** These compounds are found only in plants belonging to the family of *Oleaceae*, which includes *Olea europaea* L. They are characterized by the presence of elenolic acid in their glucosidic or aglyconic form in their molecular structure. In particular, they are formed from a phenyl ethyl alcohol (hydroxytyrosol or tyrosol), elenolic acid and, eventually, a glucosidic residue[43]. In olive fruit, the main secoiridoids identified are oleuropein and ligstroside. While oleuropein was identified as an ester of hydroxytyrosol and elenolic acid glycoside (an oleosidic skeleton common to the secoiridoid glucosides of *Oleaceae*), ligstroside was identified as an ester of tyrosol and elenolic acid glucoside[47]. Secoiridoids of olive oil in aglyconic forms arise from glycosides in olive fruits by hydrolysis of endogenous *β*-glucosidase during

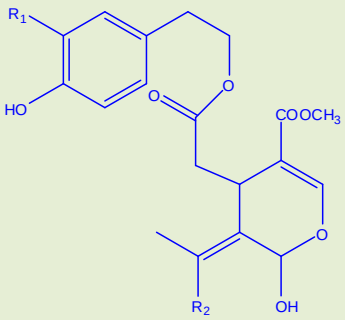
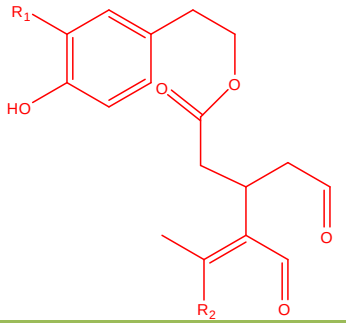
[45] Servili, M.; Selvaggini, R.; Esposto, S.; Taticchi, A.; Montedoro, G.; Morozzi, G. *J. Chromatogr. A*. **2004**, *1054*, 113–127.
[46] Carrasco-Pancorbo, A.; Arráez-Román, D.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Electrophoresis*. **2006**, *27*, 2182–2196.
[47] Klen, T. J.; Wondra, A. G.; Vrhovšek, U.; Vodopivec, B. M. *J. Agric. Food Chem*. **2015**, *63*, 3859–3872.

crushing and malaxation. In fact, in a recent study the transformation of oleuropein and ligstroside to their aglycone forms was monitored step by step during olive oil processing on a laboratory scale[48]. The results showed that oleuropein decreased after olive crushing by 94% from its initial content. However, ligstroside aglycone was not detected in the obtained olive paste. The authors explained this behaviour by their transformation primarily to the aglycone forms and further to their decarboxymethylated forms. Taking into account the obtained result, the authors suggested that crushing accelerated the enzymatic degradation of both glucosides, which had already begun in the fruit. This degradation process continued in the malaxation step; nevertheless, it was slower than that observed during crushing. These newly formed substances, which have amphiphilic characteristics, are partitioned between the oily layer and the vegetation water, and they are more concentrated in the latter fraction because of their polar functional groups[43].

The main secoiridoids present in olive oil are oleuropein aglycone, ligstroside aglycone and their decarboxymethylated forms. The presence of a series of degradation products, mainly in stored olive oils, due to different reactions such as methylation, hydroxylation and hydrolysis, was also reported[49]. Furthermore, isomers of oleuropein algycone, ligstroside algycone and elenolic acid were found in olive oil[50]. The concentration of secoiridoids varies depending on many factors. However, the sum of individual secoiridoids exceeds 100 mg/kg of olive oil in most analysed samples[39]. The **Table 3** includes the main secoiridoids identified in olive oil.

[48] Jerman Klen, T.; Golc Wondra, A.; Vrhovšek, U.; Sivilotti, P.; Vodopivec, B. M. *J. Agric. Food Chem*. **2015**, *63*, 4570–4579.
[49] Lozano-Sánchez, J.; Bendini, A.; Quirantes-Piné, R.; Cerretani, L.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Food Control.* **2013**, *30*, 606–615.
[50] Vichi, S.; Cortés-Francisco, N.; Caixach, J. *J. Chromatogr. A.* **2013**, *1301*, 48–59.

Table 3. The main secoiridoids identified in olive oil

Compounds	R1	R2	Structures
Oleuropein aglycone (3,4-DHPEA-EA)	OH	H	
Methyl oleuropein aglycone	OH	CH_3	
Hydroxy oleuropein aglycone	OH	OH	
Ligstroside aglycone (*p*-HPEA-EA)	H	H	
Decarboxymethyl oleuropein aglycone (3,4-DHPEA-EDA)	OH	H	
Hydroxy decarboxymethyl oleuropein aglycone	OH	OH	
Decarboxymethyl ligstroside aglycone (*p*-HPEA-EDA)	H	H	

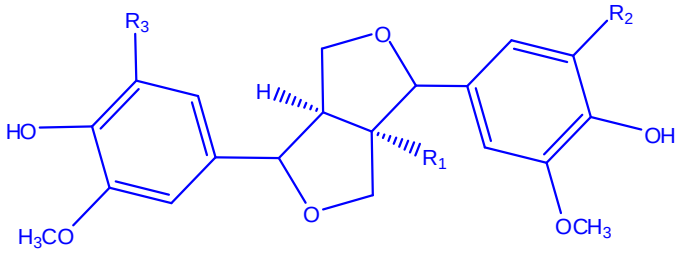
Lignans are present in the olive pulp and in the woody portion of the seed; they are released in olive oil during the mechanical extraction process without biochemical modification during the extraction[51]. The main lignans identified in olive oil are acetoxypinoresinol, pinoresinol hydroxypinoresinol and syringaresinol (**Table 4**). Their concentration may be up to 100 mg/kg, but considerable inter-oil variation exists[43]. The amount of lignans in olive oil may be used as a varietal marker. In fact, an interesting method was proposed to authenticate the Picual virgin olive oil variety[52]. In the proposed method, phenolic extraction from the Picual, Arbequina, Empeltre, Hojiblanca and Cornicabra varieties of virgin olive oil was carried out. The extracts were analysed using high-performance liquid chromatography with a fluorescence detector. As a result, the authors found that the very low concentration of acetoxypinoresinol in Picual olive oil can discriminate it from

[51] Servili, M.; Esposto, S.; Fabiani, R.; Urbani, S.; Taticchi, A.; Mariucci, F.; Selvaggini, R.; Montedoro, G. F. *Inflammopharmacology.* **2009**, *17*, 76–84.
[52] Brenes, M.; García, A.; Rios, J.; García, P.; Garrido, A. *Int. J. Food Sci. Technol.* **2002**, *37*, 615–625.

the rest of the varieties under study, which showed a high concentration of this analyte. In addition, it was assumed that lignan content doesn't change during olive oil storage for one year[53], which means that the differences observed between the monovarietal oils remain throughout the storage. The use of lignans as a varietal marker was reported in a more recent study[54].

Table 4. The main lignans identified in olive oil

Compounds	R1	R2	R3
Pinoresinol	H	H	H
Hydroxypinoresinol	OH	H	H
Acetoxypinoresinol	$COOCH_3$	H	H
Syringaresinol	H	OCH_3	OCH_3
Structure			

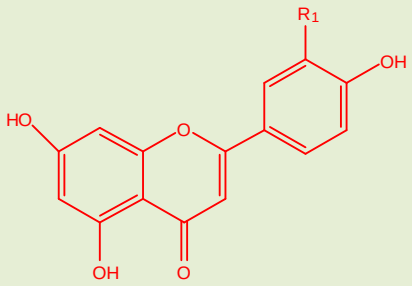


Flavonoids are largely planar molecules and their structural variation comes in part from the pattern of modification by hydroxylation, methoxylation, prenylation or glycosylation. Flavonoid aglycones are subdivided into flavones, flavonols, flavanones and flavanols depending upon the presence of a carbonyl carbon at C-4, a hydroxy group at C-3, a saturated single bond between C-2 and C-3, and a combination of no carbonyl at C-4 and a hydroxy group at C-3, respectively[43]. In olive oil, the main flavonoids identified belong to the flavones class such as luteolin and apigenin, which originate from glucosidic

[53] Brenes, M.; García, A.; García, P.; Garrido, A. *J. Agric. Food Chem.* **2001**, *49*, 5609–5614.
[54] Ballus, C. A.; Quirantes-Piné, R.; Bakhouche, A.; da Silva, L. F. D. O.; de Oliveira, A. F.; Coutinho, E. F.; da Croce, D. M.; Segura-Carretero, A.; Godoy, H. T. *Food Chem.* **2015**, *170*, 366–377.

forms present in the fruit. Indeed, luteolin may originate from rutin or luteolin-7-glucoside, and apigenin from apigenin glucosides. Their concentration was found to vary between 0.3 and 9 mg of analyte/kg of olive oil obtained from Spanish olive varieties[37]. A derivative of luteolin named methoxyluteolin was also reported in olive oil samples[55]. In **Table 5** the main flavones found in olive oil are shown.

Table 5. The main flavonoids identified in olive oil

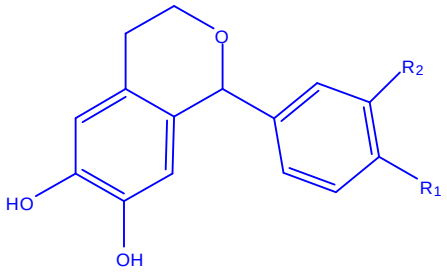
Compounds	R1	Structure
Luteolin	OH	
Apigenin	H	

Besides the different phenolic families mentioned above, another group of phenolic compounds was also identified in olive oil. This group is called **hydroxy-isochromans**. In fact, during the malaxation step of olive oil extraction, hydrolytic processes through the activity of glycosidases and esterases increase the quantity of hydroxytyrosol and carbonylic compounds, thereby favouring the presence of all compounds necessary for the formation of isochroman derivatives. Two hydroxy-isochromans, formed by the reaction between hydroxytyrosol and benzaldehyde or vanillin, have been identified by HPLC-MS/MS technique and quantified in commercial olive oils (**Table 6**)[56].

[55] De la Torre-Carbot, K.; Jauregui, O.; Gimeno, E.; Castellote, A. I.; Lamuela-Raventós, R. M.; López-Sabater, M. C. *J. Agric. Food Chem.* **2005**, *53*, 4331–4340.
[56] Bianco, A.; Coccioli, F.; Guiso, M.; Marra, C. *Food Chem.* **2001**, *77*, 405–411.

Table 6. Two hydroxy-isochromans identified in olive oil

Compounds	R1	R2
1-Phenyl-6,7-dihydroxyisochroman	H	H
1-(3'-Methoxy-4'-hydroxy)phenyl-6,7dihydroxyisochroman	OH	OCH_3
Structure		



3.2 Importance of olive oil phenolic compounds

3.2.1 Sensory properties and oxidative stability

Phenolic compounds were found to contribute to the organoleptic properties of olive oil, and some of olive oil positive attributes were reported to be related to the presence of secoiridoids[51]. Therefore, many researchers have been interested in studying the relationship between the content of secoiridoids in olive oil and the attributes of bitterness and pungency. Indeed, the major peaks found in the phenolic profile of olive oil were isolated using preparative liquid chromatography; after dissolving these purified molecules in water, they were tasted to evaluate the intensity of the bitterness[57]. It was concluded that the peaks corresponding to oleuropein algycone and its decarboxymethylated form were those mainly responsible for the bitter taste of olive oil. Furthermore, in another study evaluating the relationship between polyphenols and olive oil pungency, it was found that ligstroside

[57] Gutiérrez-Rosales, F.; Ríos, J.; Gómez-Rey, M. L. *J. Agric. Food Chem.* **2003**, *51*, 6021–6025.

aglycone is the key source of the burning sensation found in many olive oils[58]. Phenolic acids have also been associated with sensory qualities of olive oil[59]. In fact, several authors associated the off-flavour note of "fusty" with the presence of phenolic acids in olive oil.

As well as their contribution to olive oil organoleptic properties, polyphenols were reported as being excellent antioxidant compounds and playing an important role in olive oil oxidative stability[60]. In fact, the antioxidant activity of specific hydrophilic phenols of olive oil such as hydroxytyrosol, tyrosol and phenolic acids has been studied and the high antioxidant power of hydroxytyrosol has been clearly shown[45]. Moreover, when studying this property among a large number of phenolic compounds from olive oil using the Rancimat test, *o*-diphenols, such as hydroxytyrosol, showed a much higher antioxidant activity than tyrosol[61]. It was assumed that the highest antioxidant effects are observed with a 3,4-dihydroxy structure linked to an aromatic ring. In a more recent study, the antioxidant activity of several single phenolic compounds of olive oil was evaluated using different chemical approaches[62]. The authors verified that, on the one hand, the presence of a single hydroxyl group on the benzenic ring conferred only limited antioxidant activity. On the other hand, the presence of a catechol moiety enhances the ability of the phenolic compounds to act as antioxidants. The results obtained in all tests carried out in this experiment showed that hydroxytyrosol was one of the strongest compounds in terms of antioxidant power. The high

[58] Andrewes, P.; Bush, J. L. H. C.; De Joode, T.; Groenewegen, A.; Alexandre, H. *J. Agric. Food Chem.* **2003**, *51*, 1415–1420.
[59] Segura-Carretero, A.; Menéndez-Menéndez, J.; Fernández-Gutiérrez, A. *Polyphenols in Olive Oil: The Importance of Phenolic Compounds in the Chemical Composition of Olive Oil*; Elsevier, 2010; pp. 167–175.
[60] Rotondi, A.; Bendini, A.; Cerretani, L.; Mari, M.; Lercker, G.; Toschi, T. G. *J. Agric. Food Chem.* **2004**, *52*, 3649–3654.
[61] Baldioli, M.; Servili, M.; Perretti, G.; Montedoro, G. *J. Am. Oil Chem. Soc.* **1996**, *73*, 1589–1593.
[62] Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Del Carlo, M.; Gallina-Toschi, T.; Lercker, G.; Compagnone, D.; Fernández-Gutiérrez, A. *J. Agric. Food Chem.* **2005**, *53*, 8918–8925.

antioxidant activity of hydroxytyrosol acetate and secoiridoids such as oleuropein aglycone and its derivatives was also reported[63].

3.2.2 Bioactivity

Scientific research has shown that olive oil phenolic compounds possess important biological activities that may exert a preventative effect in regard to the development of chronic degenerative diseases.

As is well known, high levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) are recognized risk markers for atherosclerosis. This is considered to be the primary cause of cardiovascular diseases (CVDs), while high-density lipoprotein cholesterol (HDL-C) is assumed to be protective. The relationship between blood lipid profile and olive oil phenolic compounds has been evaluated by several authors. In fact, three different diets were given to male rats[64]. The first group of rats was fed a standard diet, the second group was fed a cholesterol-rich diet, and the third group was fed a cholesterol-rich diet supplemented with phenolic compounds (hydroxytyrosol and hydroxytyrosol acetate). The authors observed that supplementation of the diet with phenolic compounds reduced the low-density lipoprotein cholesterol level in blood compared to the diet based only on cholesterol. In addition, the effect of olive oil phenolic compounds on blood lipid profile was supported by another *in vivo* study[65]. In fact, 200 European participants were chosen for the intake of olive oil over three weeks. The participants were randomly assigned to three groups of olive oil differing in their phenolic content (low, medium and high). The obtained results showed that HDL-C linearly increased with the phenolic content, whereas the TC/HDL-C ratio linearly decreased. The LDL-C/HDL-C ratio and triglycerides decreased in those consuming medium and high-phenolic olive oils.

[63] Artajo, L. S.; Romero, M. P.; Morelló, J. R.; Motilva, M. J. *J. Agric. Food Chem.* **2006**, *54*, 6079–6088.

[64] Largo, C.; Mart, S.; Espartero, L.; Bravo, L.; Mateos, R. *Food Funct.* **2014**, *5*, 1556–1563.
[65] Amiot, M. J. *OCL.* **2014**, *21*, 1–8.

Another aspect reported about the relationship between olive oil phenolic compounds and blood lipid profile is their possible protection of LDL from oxidation. The oxidation of LDL causes damage to the vascular wall, stimulating macrophage uptake and the formation of foam cells, which in turn result in the formation of plaque within the arterial wall. In an effort to investigate the effect of phenolic compounds on the oxidation of LDL, an experiment was carried out *in vivo* where 12 healthy male volunteers ingested 40 ml of similar olive oils, but with high (366 mg/kg), moderate (164 mg/kg) and low (2.7 mg/kg) phenolic content[66]. The results of the experiment showed that LDL oxidation was lower as the phenolic content administered increased. In a more recent study, in order to understand how phenolic compounds can reduce the oxidation of LDL, the authors compared the concentration of phenolic compounds in the LDL of 30 healthy volunteers after consumption of three olive oils containing different concentrations of phenolic compounds[67]. The authors observed that the concentration of phenolic compounds in LDL directly correlated with the phenolic concentration in the olive oils ingested. Indeed, olive oils rich in phenolic compounds led to an increase in phenolic compounds in LDL. Consequently, the phenolic compounds that can bind LDL are likely to exert their peroxyl scavenging activity, avoiding the oxidation of LDL.

Platelet aggregability is a proxy of thrombogenic potential and an important marker of cardiovascular risk. Some researchers have interested in studying the effect of olive oil phenolic compounds on platelet aggregability. In this way, the anti-platelet activities of hydroxytyrosol and hydroxytyrosol acetate were explored *in vitro*[68]. The obtained results showed the positive effect of the phenolic extract on the inhibition of platelet aggregation, being higher in the case of hydroxytyrosol acetate, up to 38%, and for the first time its synergist effect with hydroxytyrosol has been proved, obtaining more than

[66] Covas, M.-I.; de la Torre, K.; Farré-Albaladejo, M.; Kaikkonen, J.; Fitó, M.; López-Sabater, C.; Pujadas-Bastardes, M. a; Joglar, J.; Weinbrenner, T.; Lamuela-Raventós, R. M.; de la Torre, R. *Free Radic. Biol. Med.* **2006**, *40*, 608–616.
[67] Gimeno, E.; de la Torre-Carbot, K.; Lamuela-Raventós, R. M.; Castellote, A. I.; Fitó, M.; de la Torre, R.; Covas, M.-I.; López-Sabater, M. C. *Br. J. Nutr.* **2007**, *98*, 1243–1250.
[68] Rubio-Senent, F.; de Roos, B.; Duthie, G.; Fernández-Bolaños, J.; Rodríguez-Gutiérrez, G. *Eur. J. Nutr.* **2014**. DOI 10.1007/s00394-014-0807-8.

double the inhibition. Few human studies have been performed to assess the *in vivo* antithrombotic potential of olive oil phenolic compounds. Indeed, the administration of pure hydroxytyrosol to human volunteers lowered thromboxane B2 (TXB2) production in a time-dependent manner[69]. Another published study on individuals with enhanced oxidative stress supports the *in vivo* antithrombotic activity of olive oil phenolic compounds in humans[70]. The administration of extra-virgin olive oil providing 6.6 mg/day of hydroxytyrosol for seven weeks to mildly hyperlipidemic individuals decreased serum TXB2 production compared with refined olive oil administration. These studies confirm the effect of olive oil phenolic compounds on the inhibition of platelet aggregation, and consequently the reduction of the incidence of cardiovascular diseases.

Many authors established the relation between olive oil phenolic compounds and inflammatory process. In fact, a phenolic compound called oleocanthal belonging to the secoiridoids group was shown to inhibit cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) activity (both involved in the inflammatory process) in the same way as the anti-inflammatory drug ibuprofen does[71]. In addition, hydroxytyrosol, known as an olive oil phenolic alcohol, was reported to have significant anti-inflammatory properties decreasing pro-inflammatory cytokines, interleukin 1*β* (IL-1*β*) and tumour necrosis factor-alpha (TNF-*α*) in rats[72]. In an *in vivo* study, it was found that consuming extra-virgin olive oil rich in phenolic compounds reduced the production of Leukotriene B4 (LTB4) in blood by 31.6% after 2 hours[73]. Moreover, the effect of two similar olive oils, but with differences in their phenolic content, on inflammatory markers was evaluated in stable coronary

[69]Ruiz-Gutie, V.; Torre, R. de; Kafatos, A.; Lamuela-Ravento, R. M.; Owen, R. W.; Visioli, F. *Nutr. Rev.* **2006**, *64*, S20–S30.
[70] Visioli, F.; Caruso, D.; Grande, S.; Bosisio, R.; Villa, M.; Galli, G.; Sirtori, C.; Galli, C. *Eur. J. Nutr.* **2005**, *44*, 121–127.
[71] Beauchamp, G. K.; Keast, R. S. J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C.-H.; Smith, A. B.; Breslin, P. A. S. *Nature.* **2005**, *437*, 45–46
[72] Gong, D.; Geng, C.; Jiang, L.; Cao, J.; Yoshimura, H.; Zhong, L. *Phyther. Res.* **2009**, *23*, 646–650.
[73] Bogani, P.; Galli, C.; Villa, M.; Visioli, F. *Atherosclerosis.* **2007**, *190*, 181–186.

heart disease patients[74]. The pro-inflammatory agents evaluated in the study were interleukin-6 and C-reactive protein. The obtained results again showed a decrease in both markers after taking virgin olive oil rich in phenolic compounds.

In recent years, several studies have been focused on the antimicrobial activity of olive oil phenolic compounds. *Helicobacter pylori* was reported as responsible for most peptic ulcers and some gastric cancer, and infections. It is currently eradicated with antibiotics, although this therapy fails in 10–30 % of patients[75]. The *in vitro* activity of olive oil phenolic compounds against *H. pylori* has been studied[76]. The researchers discovered a strong anti-*H. pylori* activity exerted by olive oil extracts rich in phenolic compounds. This activity was even effective against some antibiotic-resistant strains and, more importantly, a very low concentration of the olive oil extract was necessary. The compounds found to be responsible for this strong anti-*H. pylori* activity were decarboxymethyl elenolic acid linked to hydroxytyrosol and decarboxymethyl elenolic acid linked to tyrosol. In addition, the antimicrobial activity of hydroxytyrosol and tyrosol extracts against a variety of microorganisms of the gut microbiota was tested *in vitro*[77]. On the one hand, the inhibitory effect of hydroxytyrosol and tyrosol against the tested strains, in particular *Clostridium clostridiiforme* and *Enterococcus faecalis* (normally considered not beneficial for health), was observed, with the synergetic action of both compounds being more effective. On the other hand, the addition of olive oil phenolic extracts (hydroxytyrosol and tyrosol) promoted the growth of *Bifidobacterium adolescentis* by two or three orders of magnitude, and revealed a property of virgin olive oil as a bifidogenic substance. Several important health benefits may be associated with bifidogenesis. At the same time as this bifidobacterial predominance, a stable

[74] Fitó, M.; Cladellas, M.; de la Torre, R.; Martí, J.; Muñoz, D.; Schröder, H.; Alcántara, M.; Pujadas-Bastardes, M.; Marrugat, J.; López-Sabater, M. C.; Bruguera, J.; Covas, M. I. *Eur. J. Clin. Nutr.* **2008**, *62*, 570–574.
[75] Cavallaro, L. G.; Egan, B.; Morain, C. O.; Mario, F. Di. *Helicobacter.* **2006**, *11*, 36–39.
[76] Romero, C.; Medina, E.; Vargas, J.; Brenes, M.; De Castro, A. *J. Agric. Food Chem.* **2007**, *55*, 680–686.
[77] Zampa, A.; Silvi, S.; Servili, M.; Montedoro, G.; Orpianesi, C.; Cresci, A. *Microb. Ecol. Health Dis.* **2006**, *18*, 147–153.

number of *Lactobacillus salivarius* was present that did not seem to be affected by the olive oil phenolic compounds. Taking into account the obtained results, the authors believed that olive oil phenolic compound consumption can modulate the growth of intestinal microbiota. In another study, the antimicrobial activities of olive oil polyphenols were tested *in vitro* against three foodborne pathogenic bacteria: *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enteritidis*[78]. A synergistic interaction was noted amongst various olive oil phenolic compounds and this synergism appeared to increase antimicrobial capacity compared to that of individual compounds. The authors concluded that the use of extra-virgin olive oil in foods may help to prevent foodborne diseases.

With regard to the carcinogenic process, which is usually divided into three different steps (initiation, promotion and progression), it is possible to identify different points of control in which the phenolic compounds were reported to play an important role in protecting against cancer incidence. They were found to have an anti-initiation capacity. In fact, olive oil phenolic extract was used to prevent oxidative DNA damage on human peripheral blood mononuclear cells (PBMCs) caused by styrene oxide (SO)[79]. In the experiment it was observed that the addition of 1 µg/ml of olive oil phenolic extract to PBMC suspensions and their further exposition to 25 µM of SO reduced the DNA oxidation by 51% after 2 hours of incubation at 37 ºC, in comparison to the control in which no phenolic extract was added. These results are of great importance given that the prevention of DNA damage in lymphocytes can be considered a phenomenon predictive of a lower cancer risk. Moreover, an *in vivo* study showed a decrease in the amount of 8-oxo-7,8-dihydro-2'deoxyguanosine (a marker of DNA oxidation) in the mitochondrial DNA of mononuclear cells and in urine, after short-term consumption of olive oil with a linear trend significantly correlated to the content of phenols[80]. Similarly, it

[78] Karaosmanoglu, H.; Soyer, F.; Ozen, B.; Tokatli, F. *J. Agric. Food Chem.* **2010**, *58*, 8238–8245.
[79] Fuccelli, R.; Sepporta, M. V.; Rosignoli, P.; Morozzi, G.; Servili, M.; Fabiani, R. *Nutr. Cancer.* **2014**, *66*, 1322–1330.
[80] Weinbrenner, T.; Torre, R. De; Saez, G. T.; Rijken, P.; Tormos, C.; Coolen, S. *J. Nutr.* **2004**, *134*, 2314–2321.

showed a 30% reduction of oxidative DNA damage in peripheral blood lymphocytes during intervention on postmenopausal women with virgin olive oil containing high amounts of phenolic compounds[81].

Olive oil phenolic compounds were also reported to have anti-promotion and anti-progression capacity as they were able to inhibit the proliferation and induce apoptosis in cancer cells. In fact, it was demonstrated *in vitro* that olive oil phenolic extracts have an antiproliferative effect on colon cancer cells through the interaction with estrogen-dependent signals involved in tumour cell growth[82]. Specifically, the ability of olive oil extracts to inhibit cell proliferation was superimposable to the activation of estrogen receptor *β (*ER*β)*. In addition, hydroxytyrosol was found to exert strong antiproliferative effects against human colon adenocarcinoma cells via its ability to induce a cell cycle block in G2/M[83]. These antiproliferative effects were preceded by a strong inhibition of extracellular signal-regulated kinase (ERK)1/2 phosphorylation and a downstream reduction of cyclin D1 expression. Furthermore, in order to assess the anticancer properties of olive oil phenolic extracts using *in vitro* models, it was reported as results of the study that olive oil extract containing a high amount of pinoresinol (62%) possesses an evident antiproliferative and proapoptotic effect, which was more pronounced in *p53*-proficient cells (colon cell line)[84].

The effect of olive oil phenolic compounds has also been evaluated in breast cancer cells. Indeed, an MTT-based cell viability protocol was employed to assess the effects of crude extra-virgin olive oil phenolic extracts on the metabolic status of cultured SKBR3 human breast cancer cells[37]. MTT-based cell viability assays revealed a wide range of breast cancer cytotoxic potencies among individual crude phenolic extracts obtained from Spanish

[81] Salvini, S.; Sera, F.; Caruso, D.; Giovannelli, L.; Visioli, F.; Saieva, C.; Masala, G.; Ceroti, M.; Giovacchini, V.; Pitozzi, V.; Galli, C.; Romani, A.; Mulinacci, N.; Bortolomeazzi, R.; Dolara, P.; Palli, D. *Br. J. Nutr.* **2007**, *95*, 742–751.
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[83] Corona, G.; Deiana, M.; Incani, A.; Vauzour, D.; Dessì, M. A.; Spencer, J. P. E. *Mol. Nutr. Food Res.* **2009**, *53*, 897–903.
[84] Fini, L.; Hotchkiss, E.; Fogliano, V.; Graziani, G.; Romano, M.; De Vol, E. B.; Qin, H.; Selgrad, M.; Boland, C. R.; Ricciardiello, L. *Carcinogenesis.* **2008**, *29*, 139–146.

extra-virgin olive oil monovarietals. Remarkably, breast cancer cell sensitivity to crude extra-virgin olive oil phenolic extracts was higher when the extract was rich in secoiridoids. Again, hydroxytyrosol was reported to have an antiproliferative effect on MCF-7 breast cancer cells[85]. This effect was attributed to its ability to inhibit estrogen-dependent rapid signals involved in uncontrolled tumour cell growth. Oleuropein aglycone, one of the main phenolic compounds identified in olive oil, through its specific inhibition of human epidermal growth factor receptor 2 (HER2) oncogene, may exert a protective effect not only on the promotion but further on the progression (invasion and metastasis) of human breast cancer[86]. **Figure 2** shows a schematic presentation of the main biological activities attributed to olive oil phenolic compounds.

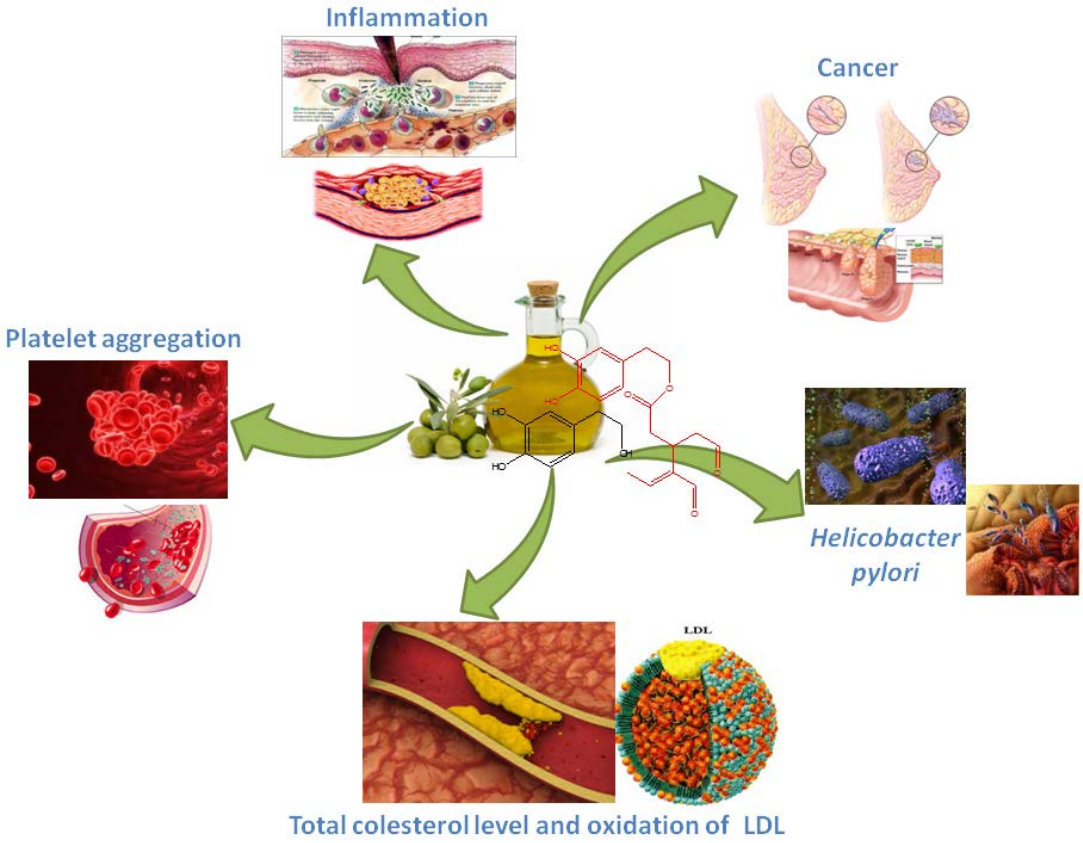

Figure 2. Main biological activities attributed to olive oil phenolic compounds.

[85] Sirianni, R.; Chimento, A.; De Luca, A.; Casaburi, I.; Rizza, P.; Onofrio, A.; Iacopetta, D.; Puoci, F.; Andò, S.; Maggiolini, M.; Pezzi, V. *Mol. Nutr. Food Res.* **2010**, *54*, 833–840.
[86] Menendez, J. A.; Vazquez-Martin, A.; Colomer, R.; Brunet, J.; Carrasco-Pancorbo, A.; Garcia-Villalba, R.; Fernandez-Gutierrez, A.; Segura-Carretero, A. *BMC Cancer* **2007**, *7*, 1–19.

4. Factors affecting olive oil phenolic composition

Olive oil polyphenols vary depending on several factors such as genetic, pedoclimatic and agronomic conditions of cultivation. Overall, the interaction of the mentioned factors combined with technological factors related to the olive oil production process determines the final olive oil phenolic composition.

4.1 Genetic, pedoclimatic and agronomic factors

With regard to **olive cultivar**, it was reported that the differences observed among olive varieties are related to genetic factors that regulate the expression of phenolic compounds, causing the variability found in olive pulps which consequently are reproduced in olive oil[87]. Comparing the chemical composition of two cultivars named Arbequina and Chemlali, cultivated within the same area[88], it was found that chemlali is richer than Arbequina in term of total phenolic content. In addition, differences in the individual concentration of phenolic compounds between the two varieties were also reported. In another study, the phenolic profile of six Tunisian varieties from the same location was determined[89]. From the results, it could be seen that the distribution of phenolic compounds varied significantly in the different cultivars. Among the major secoiridoids, oleuropein aglycone, ligstroside aglycone and decarboxymethyl oleuropein aglycone were found in higher concentrations in Chetoui/El Hor, Chemlali and Chemchali, respectively. Meanwhile, Oueslati and Jarboui presented the lowest content in oleuropein aglycone and decarboxymethyl oleuropein aglycone, respectively. Moreover, comparing Spanish olives in terms of phenolic content, the main differences were found in oleuropein aglycone, ligstroside aglycone and their decarboxymethylated forms[90]. Among these compounds, decarboxymethyl oleuropein aglycone showed the highest value in Cornicabra olive oil followed

[87] Charoenprasert, S.; Mitchell, A. *J. Agric. Food Chem.* **2012**, *60*, 7081–7095.
[88] Chtourou, M.; Gargouri, B.; Jaber, H.; Abdelhedi, R.; Bouaziz, M. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 631–640.
[89] Taamalli, A.; Gómez-Caravaca, A. M.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Eur. Food Res. Technol.* **2010**, *231*, 965–975.
[90] Gómez-Rico, A.; Fregapane, G.; Salvador, M. D. *Food Res. Int.* **2008**, *41*, 433–440.

by Picual, Arbequina, Picolimon and Morisca, respectively. The total phenolic content and phenolic profile of four Italian varieties (Coratina, Nocellara, Ogliarola and Peranzana) showed a great variability among the cultivars grown in the same location[91], which confirms the effect of genetic matrix on olive oil phenolic composition.

Olive oils from different cultivars have also been found to possess characteristic phenolic profiles, which made it possible to classify olive oils according to the cultivar. In fact, a good separation was achieved among Picual, Arbequina, Hojiblanca, Manzanilla and Cornezuelo varieties using their phenolic composition[37].

Pedoclimatic conditions such as soil characteristics, precipitation, temperature and relative humidity also modulate the genetic expression of phenolic compounds in olive cultivars. It is well known that these conditions vary depending on the geographical area of cultivation. Therefore, many studies were carried out in an effort to monitor the variability of olive oil phenolic composition among the geographical origins for the same cultivar. An in-depth phenolic characterization of the Chemlali olive variety from the north, centre and south of Tunisia was carried out[92]. The analysis of the profiles of the different oils showed significant differences among the sites of cultivation under study. Secoiridoids were the most concentrated phenolic family in Chemlali olive oil and reached 98% of total phenolic content. Indeed, the highest concentrations of ligstroside aglycone, oleuropein aglycone and its decarboxymethylated form were found in olive oils from the Siliana, Oueslatia and Sidi Bou Zid regions, respectively. A variation in the rest of the phenolic families such as phenolic alcohols and flavones was observed among the sites of cultivation. It is of great importance to know that all the samples were obtained from olives with the same maturity index, and belong to the same variety. Therefore, the observed differences in the Chemlali phenolic profile may be the result of differences in pedoclimatic conditions characteristic of

[91] Baiano, A.; Terracone, C.; Viggiani, I.; Nobile, M. A. Del. *J. Am. Oil Chem. Soc.* **2012**, *90*, 103–111.
[92] Taamalli, A.; Arráez Román, D.; Zarrouk, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Food Chem.* **2012**, *132*, 561–566.

each geographical area. Furthermore, the phenolic composition of Arbequina commercial olive oils obtained from Jaén, Tarragona and Lleida was determined[93]. The results showed no qualitative differences among olive oils from different growing regions. However, significant quantitative differences were observed in a large number of phenolic compounds. The hydroxytyrosol and tyrosol contents were significantly higher in olive oils from Jaén. However, secoiridoids and total phenol contents were higher in olive oils from Tarragona. Olive oils from Lleida were characterized by their higher ratio of decarboxymethyl ligstroside aglycone/lignans. The authors attributed this distribution to the differences in altitude and climatic conditions among the three regions.

In order to study further the effect of pedoclimatic conditions on the phenolic composition of olive oil, some authors were interested in understanding the mechanism through which these conditions can change the olive oil phenolic profile. Therefore, the relationship between soil composition and olive oil phenolic content was studied[94]. In fact, it was reported that nitrogen nutrition is related to phenylalanine ammonia-lyase (PAL) activity. The high availability of nitrogen probably induces protein synthesis rather than the synthesis of phenylpropanoids via PAL, thereby decreasing the phenolic contents in olive oil. In addition, high concentrations of nitrogen and potassium enhance the polyphenol oxidase activity, which catalyses the oxidation of phenolic compounds. Therefore, soils rich in these nutrients can negatively affect the phenolic composition of olive oils produced from these areas. In another study, in an effort to evaluate the effect of climatological conditions on the olive oil phenolic fraction, samples of Arbequina olive oils were obtained in four successive crop seasons, corresponding to 2000/01, 2001/02, 2002/03 and 2003/04[95]. From the results, it was shown that olive oils produced from frost-damaged olive fruit (temperatures below −5 °C in December 2001) had a lower content of secoiridoids than normal drupes due

[93] Criado, M. N.; Morelló, J. R.; Motilva, M. J.; Romero, M. P. *J. Am. Oil Chem. Soc.* **2004**, *81*, 633–640.
[94] Romero, N.; Saavedra, J.; Tapia, F.; Sepúlveda, B.; Aparicio, R. *J. Sci. Food Agric.* **2015.** DOI 10.1002/jsfa.7127.
[95] Morelló, J. R.; Romero, M. P.; Motilva, M. J. *J. Am. Oil Chem. Soc.* **2006**, *83*, 683–690.

to freeze-fracturing of the cell walls in the drupes, leading to oxidation of phenolic compounds. With regard to the precipitation, geographical areas with high annual precipitation led to the increase of water availability in the fruit, which can affect the solubilization of phenolic compounds and alters the release of polysaccharide-linked phenolic compounds in the cell wall during the milling and malaxation steps of the olive oil production process[94].

The differences observed in the olive oil phenolic profile among the cultivation sites raised the idea of using this fraction as a geographical marker. Therefore, many studies have been reported on the classification of olive oils according to geographical origins by means of various chemometric methods applied to phenolic composition. The application of principal component analyses enabled classification of the Oueslati olive oils produced in different geographical areas of Tunisia into three groups using their phenolic profile[96]. The first group was composed by Oueslati oils from Ala, the second group was characterized by the Oueslati oils from Jebel Rihan, and the third group was composed of Oueslati oils from the Haffouz, Ain Jloula Khit el Oued, Menzel Raiss and Sfax areas. In another study, the phenolic compounds of olive oil samples from different locations in the Aegean coastal area of Turkey were characterized[97]. After that, the application of partial least squares discriminant analysis (PLS-DA) revealed that oils from the north Aegean and south Aegean areas had different phenolic profiles. The phenolic compounds, which played significant roles in the discrimination of the olive oils, were tyrosol, oleuropein aglycone, cinnamic acid, apigenin and hydroxytyrosol to tyrosol ratio. The study showed that the olive oils from different parts of the region have their own defining characteristics that can be used in the authentication studies and geographical labelling of Turkish olive oils.

Some agronomic practices also have a considerable effect on olive oil phenolic composition. Among them, irrigation is an essential parameter, even when

[96] Ouni, Y.; Taamalli, A.; Gómez-Caravaca, A. M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Zarrouk, M. *Food Chem.* **2011**, *127*, 1263–1267.
[97] Alkan, D.; Tokatli, F.; Ozen, B. *J. Am. Oil Chem. Soc.* **2011**, *89*, 261–268.

water is unrestricted, to achieve better production and productivity. However, the balance between production and olive oil quality needs to be taken into account by olive oil producers. Due to the well-known role played by phenolic compounds in the quality of olive oil, it is of great importance to have maximum information about the behaviour of this fraction in olive oils obtained under olive stress or irrigation conditions. Controversial data were reported about the effect of irrigation on olive oil phenolic profile. Several studies support the fact that increased amounts of water produce oils with lower phenolic content. Nevertheless, some authors reported no effect, or even an increase in phenolic compounds. An experiment carried out in a high-density olive orchard (Frantoio cultivar) to determine the effect of different irrigation regimes (full, deficit, complementary) on olive oil quality over three consecutive years was reported[98]. It was observed that irrigation had a strong effect on phenolic content. The concentrations of secoiridoids, such as decarboxymethyl oleuropein aglycone, oleuropein aglycone or isomer, and decarboxymethyl ligstroside aglycone, were lower in olive oils from fully irrigated trees than olive oils from trees under deficit irrigation or those that received complementary irrigation only. The same results for these compounds were obtained comparing Koroneiki olive oils from irrigated and rain-fed orchards[99]. Moreover, the variation in the composition of Cornicabra olive oils obtained using different irrigation strategies was reported[100]. The applied irrigation treatments were based on regulated deficit irrigation (RDI), 100% of crop evapotranspiration (ETc), 125% of ETc, and rain-fed as control. The obtained data showed that the total phenol content, which affects olive oil's sensory characteristics, decreased significantly as the amount of supplied water increased. However, this effect was considered by the authors to be an advantage due to the high bitterness and pungency of Cornicabra olive oils, and therefore the right level of irrigation could enhance its organoleptic quality. The reduction effects observed in olive oils from irrigated orchards

[98] Caruso, G.; Gucci, R.; Urbani, S.; Esposto, S.; Taticchi, A.; Di Maio, I.; Selvaggini, R.; Servili, M. *Agric. Water Manag.* **2014**, *134*, 94–103.
[99] Stefanoudaki, E.; Williams, M.; Chartzoulakis, K.; Harwood, J. *J. Agric. Food Chem.* **2009**, *57*, 7048–7055.
[100] Gómez-Rico, A.; Salvador, M. D.; Moriana, A.; Pérez, D.; Olmedilla, N.; Ribas, F.; Fregapane, G. *Food Chem.* **2007**, *100*, 568–578.

were attributed, firstly, to the role played by the irrigation in the synthesis of phenolic compounds in the fruit. It was reported that irrigation influences phenolic compounds' metabolic pathways, mainly the phenylpropanoid one. L-Phenylalanine ammonia-lyase is a key enzyme in the metabolic pathways of phenolic compounds[101]. It was observed that its activity is positively correlated with total phenol and orthodiphenol content, and negatively correlated with the amount of water applied to the trees[102]. Secondly, during olive oil extraction the quantity of water involved (added water plus water from irrigated olives) causes a higher loss of hydrosoluble phenolic compounds in the obtained olive oil[103]. Nevertheless, these explanations may partly, but not fully, explain the mechanisms through which irrigation affects the olive oil phenolic fraction, because some authors reported that phenolic compound content increased after the application of irrigation to olive trees. In fact, it was observed that in olive oils from the Arbequina cultivar grown under linear irrigation strategies, the concentration of vanillin and lignans such as pinoresinol and acetoxypinoresinol increased with higher irrigation treatments[104].

4.2 Olive oil processing

4.2.1 Olive harvesting

There are many different methods of olive harvesting depending on the tree size and shape, and orchard terrain[105]. Traditional olive orchards are usually established in dry-farmed areas characterized by low planting densities, and low olive production per unit of ground area[106]. Trees grown in the traditional orchards are almost always harvested manually by hand. This method is used extensively. Unfortunately, it is considered inefficient, slow and very

[101] Morelló, J.-R.; Romero, M.-P.; Ramo, T.; Motilva, M.-J. *Plant Sci.* **2005**, *168*, 65–72.
[102] Tovar, M. J.; Romero, M. P.; Girona, J.; Motilva, M. J. *J. Sci. Food Agric.* **2002**, *82*, 892–898
[103] Artajo, L.-S.; Romero, M.-P.; Tovar, M.-J.; Motilva, M.-J. *Eur. J. Lipid Sci. Technol.* **2006**, *108*, 19–27.
[104] Tovar, M. J.; Motilva, M. J.; Romero, M. P. *J. Agric. Food Chem.* **2001**, *49*, 5502–5508.
[105] Guardia Rubio, M.; Ruiz Medina, A.; Molina Díaz, A.; Ayora Cañada, M. J. *J. Agric. Food Chem.* **2006**, *54*, 8538–8544.
[106] Sola-Guirado, R. R.; Castro-García, S.; Blanco-Roldán, G. L.; Jiménez-Jiménez, F.; Castillo-Ruiz, F. J.; Gil-Ribes, J. A. *Biosyst. Eng.* **2014**, *118*, 186–193.

expensive[7,105,107]. Because of this, changing plantation systems and mechanization of olive harvesting were extremely necessary. Among the technologies innovated in order to facilitate this step of production process, the most frequently reported ones are: limb-shaking devices, trunk shakers, double- or single-sided picking head mechanisms and straddle-type harvesters, which can achieve 90% of removal efficiency[107-109]. The main olive harvest systems are shown in **Figure 3**.

Different factors related to olive harvesting affect olive oil phenolic compounds. In this sense, it was found that the damaged fruits obtained by different harvesting methods produce olive oil with low phenolic content[110]. Moreover, the olive ripening stage is a key factor to take into account before harvesting. It was reported to have a strong effect on olive oil phenolic composition[17]. In fact, as ripening advances, the phenolic content in the obtained olive oil tends to decrease. Therefore, it is recommended that an optimal maturity index be determined for each cultivar.

Figure 3. Different olive harvest systems.

4.2.2 Leaf removal and washing

After their harvesting from the orchards, olives are put into a large feeding hopper attached to a moving belt. Generally, harvested olives are contaminated with vegetal impurities, such as leaves or twigs, and with mineral impurities, such as soil, dust and stone fragments. Extraneous matter, even if its origin is natural, mingled with olives must be removed to avoid

[107] Ferguson, L. *Grasas y Aceites.* **2006**, *57*, 9–15.
[108] Ravetti, L.; Robb, S. *Adv. Hortic. Sci.* **2010**, *24*, 71–77.
[109] Deboli, R.; Calvo, A.; Preti, C.; Inserillo, M. *Int. J. Ind. Ergon.* **2014**, *44*, 581–589.
[110] Yousfi, K.; Weiland, C. M.; García, J. M. *J. Agric. Food Chem.* **2012**, *60*, 4743–4750.

negative influences on the quality of olive oil and on the mechanical safety of the equipment utilized for olive oil extraction[7,111]. However, the water used in olive washing was reported to affect olive oil phenolic composition. In a recent study, the phenolic profiles of olive oils obtained from unwashed and washed olives were compared[112]. A decrease in phenolic acids, phenolic alcohols, secoiridoids and flavones was observed in olive oils obtained from washed olives in comparison to the unwashed ones. The **Figure 4** shows the washing machine used in olive oil factories.

Figure 4. Olive washing machine.

4.2.3 Crushing

Different technologies were applied to carry out this step, and the effects that are exerted on the phenolic fraction were various. Crushing is normally carried out using a traditional stone mill or by means of a disc or hammer crusher **(Figure 5)**. The stone mill consists of three stone rollers or wheels, which roll in circles on a slab of granite to grind the olives into a paste[113,114]. The slow movement of the stone crushers does not heat the paste and results in less emulsification, so the oil is easier to extract. The disadvantages of this method are the bulky machinery and its slowness, its high cost and its

[111] Di Giovacchino, L.; Sestili, S.; Di Vincenzo, D. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 587–601

[112] Vichi, S.; Boynuegri, P.; Caixach, J.; Romero, A. *Eur. J. Lipid Sci. Technol.* **2015**, *117*, 0000–0000.

[113] Servili, M.; Piacquadio, P.; Stefano, G. De; Taticchi, A.; Sciancalepore, V. *Eur. J. Lipid Sci. Technol.* **2002**, *104*, 483–489.

[114] Amirante, P.; Clodoveo, M. L.; Tamborrino, A.; Leone, A.; Paice, A. G. *Influence of the Crushing System : Phenol Content in Virgin Olive Oil Produced from Whole and De-stoned Pastes*; Elsevier Inc., 2010; pp. 69–76.

inability to be continuously operated[7,115]. In hammer crushing machines, a three- or four-lobe rotor with wear-resistant metal plates crushes the olives against a stationary grid. The diameter of the grid holes determines the thickness of the paste. Disc crushing machines, on the other hand, crush the olives between two toothed discs – one stationary and one that rotates. The major advantage of these two modern systems is their speed and continuous operation, which translate into high output, compact size and low cost. However, the main drawback of hammer and disc crushers is the formation of emulsions, which impedes oil-water separation, and generates higher temperatures[116,117]. An important transformation in the phenolic profile occurs during the crushing step. In fact, oleuropein and ligstroside present in olive fruits are hydrolysed by the action of endogenous *β*-glucosidase leading to the formation of their aglycone forms[45]. In addition, phenolic release in olive oil was found to be higher when crushing is carried out using metallic crushers instead of a stone crusher[111].

The new technology in olive oil production is olive depitting. This ensures that the paste consists solely of the fleshy part of the olive (mesocarp), without the stone or pit (endocarp) that holds the seed. The destoner consists of a cylindrical perforated stationary grill and a rotary shaft. The olives are pushed by centrifugal force towards the perforated grill. Olive tissue crosses the grill whilst the kernel remains inside the cylinder. Using this method the grinding of pulp tissues is not drastic[114]. The effect of this operation on olive oil phenolic profile was also studied[118]. Indeed, a higher concentration of phenolic compounds, such as oleuropein aglycone and its decarboxymethylated form, was found in olive oils from destoned olives than in oils obtained from normal olives.

[115] Preziuso, S. M.; Di Serio, M. G.; Biasone, A.; Vito, R.; Mucciarella, M. R.; Di Giovacchino, L. *Eur. J. Lipid Sci. Technol.* **2010**, *112*, 1345–1355.
[116] Caponio, F.; Gomes, T.; Summo, C.; Pasqualone, A. *Eur. J. Lipid Sci. Technol.* **2003**, *105*, 201–206.
[117] Caponio, F.; Catalano, P. *Eur. Food Res. Technol.* **2001**, *213*, 219–224.
[118] Ranalli, A.; Marchegiani, D.; Pardi, D.; Contento, S.; Pardi, D.; Girardi, F.; Kotti, F. *Food Bioprocess Technol.* **2008**, *2*, 322–327.

(Stone mill) (Disc crusher) (Hammer crusher)

Figure 5. Crusher equipment.

4.2.4 Malaxation

During malaxation, olive paste is subjected to a slow continuous kneading, aimed at breaking off the emulsions formed during the crushing process and facilitating adequate coalescence[119]. For many years, malaxing machines were mainly characterized by a cradle shape and a non-hermetic closure consisting of a stainless steel grill. However, different limitations on the use of this equipment were found, mainly the lack of a hermitic seal, which produces a considerable loss of olive oil phenolic compounds[120]. Because of this, improvements in malaxer machine technology have included new models with inert gas processing (nitrogen or argon) and oxygen concentration control using a hermetic cover cap[119,121]. It was observed that reducing oxygen contact with olive paste during malaxation enables polyphenol oxidase activity to be slowed, resulting in an increase in olive oil phenolic content[122].

Recently, an innovative mixer for the malaxation process was also proposed. This new machine has been developed to improve the process of kneading and heating the olive paste, thereby increasing the heat transfer surface in order to reduce the mixing time. To achieve this goal, the innovative mixer has

[119] Leone, A.; Romaniello, R.; Zagaria, R.; Tamborrino, A. *Biosyst. Eng.* **2014**, *118*, 95–104.
[120] Clodoveo, M. L. *Trends Food Sci. Technol.* **2012**, *25*, 13–23.
[121] Masella, P.; Parenti, A.; Spugnoli, P.; Calamai, L. *J. Am. Oil Chem. Soc.* **2010**, *88*, 871–875.
[122] Migliorini, M.; Mugelli, M.; Cherubini, C.; Viti, P.; Zanoni, B. *J. Sci. Food Agric.* **2006**, *86*, 2140–2146.

been designed with a circular and spiral-shaped interspace that covers the whole internal longitudinal surface of the tank. This means that paste can be conveyed and maintained at the desired temperature more quickly and effectively. A new set of blades provides a bidirectional thrust of the paste, which causes its rotation, and continuously brings new sections of paste into contact with the heating walls[120,123] **(Figure 6)**.

Many studies were carried out in order to establish the relationship between the temperature and time of malaxation and olive oil phenolic composition. When studying the effect of malaxation temperature, ranging from 20 to 35 ºC, on olive oil phenolic compounds, a positive relationship was found between the temperature and the concentration of secoiridoids such as decarboxymethyl oleuropein and ligstroside aglycones[124]. In another study in which the partition of phenolic compounds during malaxation was investigated at different times (t= 0, t= 15, t= 45 min)[125], it was observed that malaxation time had an important effect on phenolic alcohols and secoiridoids. Indeed, hydroxytyrosol and tyrosol decreased in oil phase as the malaxation time increased and their hydrophilic character was proved through their presence in the wet pomace and wastewater. In addition, transformations in secoiridoids under enzymatic actions were observed. The new innovations introduced in the malaxation equipment allowed better control of parameters such as temperature, time and the atmosphere in contact with the paste during malaxation[126].

[123] Tamborrino, A.; Clodoveo, M. L.; Leone, A.; Amirante, P.; Paice, A. G. *The Malaxation Process: Influence on Olive Oil Quality and the Effect of the Control of Oxygen Concentration in Virgin Olive Oil*; Elsevier, 2010; pp. 77–83.
[124] Taticchi, A.; Esposto, S.; Veneziani, G.; Urbani, S.; Selvaggini, R.; Servili, M. *Food Chem.* **2013**, *136*, 975–983.
[125] Artajo, L.-S.; Romero, M.-P.; Suárez, M.; Motilva, M.-J. *Eur. Food Res. Technol.* **2006**, *225*, 617–625.
[126] Selvaggini, R.; Esposto, S.; Taticchi, A.; Urbani, S.; Veneziani, G.; Maio, I. Di; Sordini, B.; Servili, M.; Agrarie, S.; Ambientali, A.; Costanzo, V. S. *J. Agric. Food Chem.* **2014**, *62*, 3813–3822.

Figure 6. Olive paste malaxer.

4.2.5 Liquid and solid-phase separation

Another step that affects the final phenolic content of olive oil is liquid and solid-phase separation. The oil can be extracted from olive paste by pressing, and centrifugal decanters[111,127,128]. Pressing is one of the oldest methods of oil extraction. The olive paste of 2-3 cm thickness obtained from malaxation is placed uniformly in synthetic fibre draining diaphragms that operate as filters. The diaphragms are placed in moving units (trolleys) with a central shaft. A metal tray and a diaphragm without paste are placed after every three to four diaphragms to obtain a uniform application and a more stable load. The moving unit along with its load is placed under a hydraulic pressure unit (**Figure 7**). When applying the pressure, the liquid phases (oil and water) run through the olive cake with the help of the drainage effect of the mats and stone fragments. A pressure system does not require the addition of water to olive paste, and it is considered as a phenolic saving system. However, if ripe olives are processed in such a system, washing the tower with water after squeezing may be required[128,129.] The addition of water was reported to negatively affect the *o*-diphenol and total phenol content in olive oils obtained using the pressure system[128]. In addition, when a pressure

[127] Caponio, F.; Summo, C.; Paradiso, V. M.; Pasqualone, A. *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 1626–1633.
[128] Di Giovacchino, L.; Solinas, M.; Miccoli, M. *J. Am. Oil Chem. Soc.* **1994**, *71*, 1189–1194.
[129]Amirante, P.; Clodoveo, M. L.; Leone, A.; Tamborrino, A.; Patel, V. B. *Influence of Different Centrifugal Extraction Systems on Antioxidant Content and Stability of Virgin Olive Oil*; Elsevier, 2010; pp. 85–93.

system is used, the paste is exposed for a long time to air, thereby increasing the degree of oxidation, which affects the olive oil phenolic profile[130].

Figure 7. Traditional olive press with fibre discs.

In the following years, the researches aimed to replace the pressing strength with a centrifugal one[131]. This objective was attained in 1965 after a period of 60 years of research, when the first horizontal centrifugal extractor was manufactured. This technology is based on the difference in density of the olive paste constituents (olive oil, water and insoluble solids). In a three-phase system, the separation occurs after adding water to the received paste. Centrifugal force moves the heavier solid materials to the outside; a lighter water layer is formed in the middle, with the lightest oil layer on the inside. This system is called a three-phase system because the centrifugal decanter allows for the separation of three flows of matter: the olive oil, pomace (solid remains of olive) and wastewater (**Figure 8**). However, the addition of water to the olive pastes modifies the distribution of hydrophilic phenols between oil and water, improving their release in the water phase, and affecting the final olive oil phenolic content[132,133]. In addition, a considerable volume of wastewater is produced, which causes serious environmental problems[129]. For

[130] Ranalli, A.; Martinelli, N. *Grasas y Aceites.* **1995**, *46*, 255–263.
[131] Altieri, G.; Di Renzo, G. C.; Genovese, F. *J. Food Eng.* **2013**, *119*, 561–572.
[132] Ammar, S.; Zribi, A.; Mansour, A. Ben; Ayadi, M.; Abdelhedi, R.; Bouaziz, M. *J. Oleo Sci.* **2014**, *63*, 311–323.
[133] Ranalli, A.; Gomes, T.; Delcuratolo, D.; Contento, S.; Lucera, L. *J. Agric. Food Chem.* **2003**, *51*, 2597–2602.

this reason, research continued in order to resolve the problems in question. In 1992, several olive oil plant manufacturers introduced a new model called a two-phase decanter, which enables the oil phase to be separated from the malaxed olive paste without the addition of warm water. This decanter has two exits producing oil and wet pomace only, and because of this it is called a "two-phase decanter"[129].

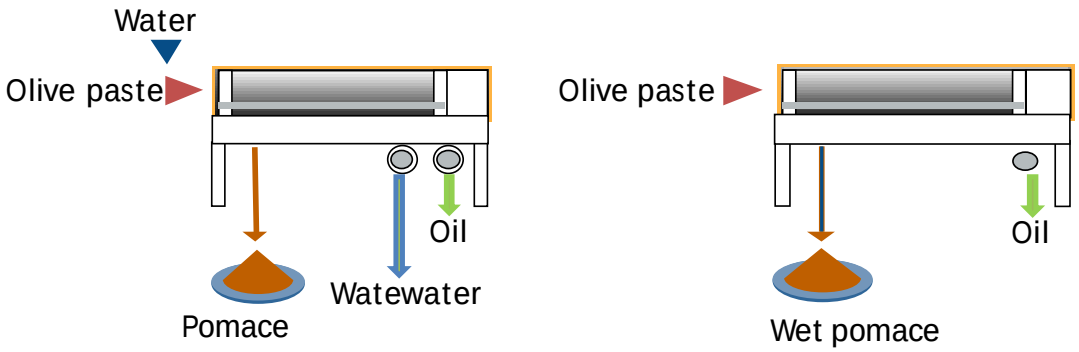

Figure 8. Three- and two-phase decanters.

In an effort to evaluate the efficiency of a two-phase decanter in saving olive oil phenolic compounds, a comparative study between olive oils obtained from three- and two-phase decanters in terms of phenolic composition was reported[134]. In fact, it was found that the oils extracted using the two-phase decanter showed higher concentrations in all the quantified phenolic compounds than the oils obtained from the three-phase decanter. In particular, the highest differences were observed for oleuropein aglycone and its decarboxymethylated form. Recently, different generations of decanters have also been designed and set up[127].

4.2.6 Separation of liquid phases

This further cleaning step is generally possible by natural decantation or by centrifugation (**Figure 9**)[135]. The old natural decantation method is a slow process in which the oil is in contact with vegetable water for a long time with the consequent risk of contamination, and phenolic oxidation in the

[134] De Stefano, G.; Piacquadio, P.; Servili, M.; Di Giovacchino, L.; Sciancalepore, V. *Lipid – Fett.* **1999**, *101*, 328–332.
[135] Altieri, G.; Di Renzo, G. C.; Genovese, F.; Tauriello, A.; D'Auria, M.; Racioppi, R.; Viggiani, L. *Biosyst. Eng.* **2014**, *122*, 99–114.

presence of light and oxygen. This method was formerly used in mills equipped with traditional olive presses. However, centrifugation using vertical centrifuge, with a rotary speed of 6500-7000 rpm, is a very quick operation that requires little labour and separates oil effectively from impurities. Different vertical centrifuges were manufactured in order to improve the efficacy of this separation step, especially the automatic cleaning of the cones, which become clogged with the residual solids. During the centrifugation, fresh warm water is usually added to clean the oil, creating a greater separation of the phases[111,136,137]. The effect of vertical centrifugation on olive oil phenolic content was found to be related mainly to the warm water added to achieve the separation of the oily must components. The water produces a loss in olive oil phenolic compounds. In fact, compounds belonging to phenolic alcohols and secoiridoids were reported to be identified in wash water used in the vertical centrifuge[138].

Recently, given that there is a great interest from a commercial point of view, and on the basis of consumer requests, in minimally processed olive oil a modern decantation chain was developed using vertical columns and volumetric pumps to achieve a good cleaning of olive oil. The composition of olive oil obtained using modern decantation was compared with that obtained using vertical centrifuge[135]. The results showed higher total phenolic content in olive oils separated by the modern decantation system.

[136] Parenti, A.; Spugnoli, P.; Masella, P.; Calamai, L. *Eur. J. Lipid Sci. Technol.* **2007**, *109*, 1180–1185.
[137] Masella, P.; Parenti, A.; Spugnoli, P.; Calamai, L. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 1094–1096.
[138] García, A.; Brenes, M.; Martínez, F.; Alba, J.; García, P.; Garrido, A. *J. Am. Oil Chem. Soc.* **2001**, *78*, 625–629.

Figure 9. Different liquid-phase separation systems.

4.2.7 Storage

Like many products, olive oil is produced in a limited period of time, but it is consumed throughout the year, it must be stored, and the storage conditions determine its commercial life. Among the tanks used for the storage of olive oil, we can find in the market metallic drums lined with epoxy resins, iron tanks, polyester-glass fibre tanks and stainless steel tanks. It is believed that the ideal tank capacity is 50,000 kg, because this facilitates quality control and traceability. The bottom of the tanks must be conical or sloped (5%) to facilitate the accumulation and removal of solids and water[49,139,140]. Monitoring the phenolic compound status of olive oil over the storage is mandatory to establish the main parameters that affect their concentration. In fact, parameters such as temperature, oxygen, light and time were reported to have an effect on the phenolic composition of olive oil during storage. Higher storage temperatures were found to produce a decrease in secoiridoids, and increase hydroxytyrosol content[141]. In addition, it was observed that decarboxymethyl ligstroside aglycone concentration decreased

[139] Leone, A.; Romaniello, R.; Tamborrino, A. *Trans. ASABE.* **2013**, *56*, 1017–1024.
[140] Lozano-Sánchez, J.; Giambanelli, E.; Quirantes-Pin, R.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fern, A. *J. Agric. Food Chem.* **2011**, *59*, 11491–11500.
[141] Lerma-García, M. J.; Simó-Alfonso, E. F.; Chiavaro, E.; Bendini, A.; Lercker, G.; Cerretani, L. *J. Agric. Food Chem.* **2009**, *57*, 7834–7840.

from 90 mg/kg to 56 mg/kg (37%) after 10 months' exposure to both light and oxygen. Nevertheless, its content only decreased by 15% (90 mg/kg to 76 mg/kg) after the same storage time without exposure to light and oxygen[142].

The control of temperature was achieved in olive mills by conditioning the storage environment. The effect of light was easily avoided by using an adequate storage material or choosing a dark place in the factory to store olive oil. However, the oxygen contact with olive oil was very difficult to control. In fact, its presence in the headspace of storage tanks was problematic and unavoidable[143]. Recently, researchers have become interested in developing new storage systems in order to overcome this problem[139]. Therefore, the tanks were connected to a source of nitrogen N_2 with the objective of adjusting the atmosphere of their headspace. Interesting results were obtained using this system: it was possible to maintain the oxygen percentage only between 0.7 % and 0.9 %. The total phenolic content was higher in olive oil stored under this atmospheric composition than olive oil from the control tank that was not connected to the N_2 generator. Nitrogen gas is most usually added through a valve lower in the tank and allowed to bubble through olive oil to the top of the tank where it displaces the air that is forced out through a relief valve.

The **Figure 10** shows stainless steel tanks used for the storage of olive oil.

[142] Cicerale, S.; Conlan, X. a.; Barnett, N. W.; Keast, R. S. J. *Food Res. Int.* **2013**, *50*, 597–602.
[143] Ricca, M.; Foderà, V.; Vetri, V.; Buscarino, G.; Montalbano, M.; Leone, M. *J. Food Sci.* **2012**, *77*, 1084–1089.

Figure 10. Olive oil storage on an industrial scale**.**

4.2.8 Filtration

The final steps of the elaboration process also affect olive oil composition, and consequently its phenolic content. In spite of the previous separation techniques applied to olive oil (vertical centrifugation or decantation), the final product is still turbid and opalescent and contains impurities such as water in emulsion, pieces of fruit or stone and mucilage. Consequently, filtration as a final step before bottling was included in the olive oil process in many factories. The objective was to remove the suspended solids and moisture and make the olive oil more brilliant for consumer acceptance[144]. In this regard, olive oil companies have different traditional filtration systems that are currently being improved in some cases and replaced by new systems in others. As this step has been studied in this thesis, a detailed description of it will be given in the following sections. Thus, olive oil filtration can be divided into conventional filtration systems and new ones[145].

[144] Bendini, A.; Valli, E.; Rocculi, P.; Romani, S.; Cerretani, L.; Gallina Toschi, T. *Curr. Nutr. Food Sci.* **2013**, *9*, 43–51.
[145] Lozano-Sánchez, J.; Cerretani, L.; Bendini, A.; Gallina-Toschi, T.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *J. Agric. Food Chem.* **2012**, *60*, 3754–3762.

4.2.8.1 Conventional filtration systems

These processes can be carried out with various materials in combination with filtration hardware to improve the filtration performance. These materials, known as filter aids, can be produced from a wide variety of raw materials and their use depends on the final purpose[146]. Principally, the conventional filtration system includes two steps: first, the removal of suspended solids, and second, the elimination of moisture.

- **Filtration process to remove suspended solids**

This is indicated for olive oils with a high content of solid particles. There are different types of equipment for this operation, mostly horizontal and vertical filters. However, currently the vertical filter seems most popular, especially the one with candles, as it has various advantages, such as its easy cleaning and the possibility of completely exhausting filter aids before disposal, losing a minimal amount of oil; and finally there is no need to disassemble it in order to remove the filter aids.

This step requires a preliminary phase during which the surface of the filtration equipment is covered with a filter aid[147]. Through this procedure the support filters are protected, allowing the full removal of residual solids present in olive oil and ensuring an almost instantaneous olive oil clarification at the steady state. For the precoat deposition, a required amount of filter aid is mixed with olive oil in a slurry tank. The slurry is circulated through the filter and back to the slurry tank. The filter aid is retained on the filtration equipment and circulation is continued until the precoat is formed and the effluent runs clear[148]. After achieving the formation of the precoat, olive oil filtration is carried out under a constant flow and increasing differential pressure. Once the filter cake has been completely saturated by suspended solids and moisture, the system (filter tank) reaches a maximum pressure

[146] Lozano-Sánchez, J.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Food Chem.* **2011**, *124*, 1146–1150.
[147] Lozano-Sánchez, J.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Trends Food Sci. Technol.* **2010**, *21*, 201–211.
[148] Masella, P.; Parenti, A.; Spugnoli, P.; Baldi, F.; Mattei, A. *Sep. Sci. Technol.* **2011**, *46*, 1709–1715.

(4 x10^5 Pa) and the filtration cycle is completed. Normally, 50,000 L of cloudy olive oil can be filtered with 100 kg of diatomaceous earth, but the final amount of olive oil to be filtered depends on its composition (solids and moisture)[147]. In some cases, a periodic addition of filter aids with higher porosity than those used previously as the precoat is recommended, with the objective of prolonging the filtration cycle. **Figure 11** shows the filtration equipment and its different parts.

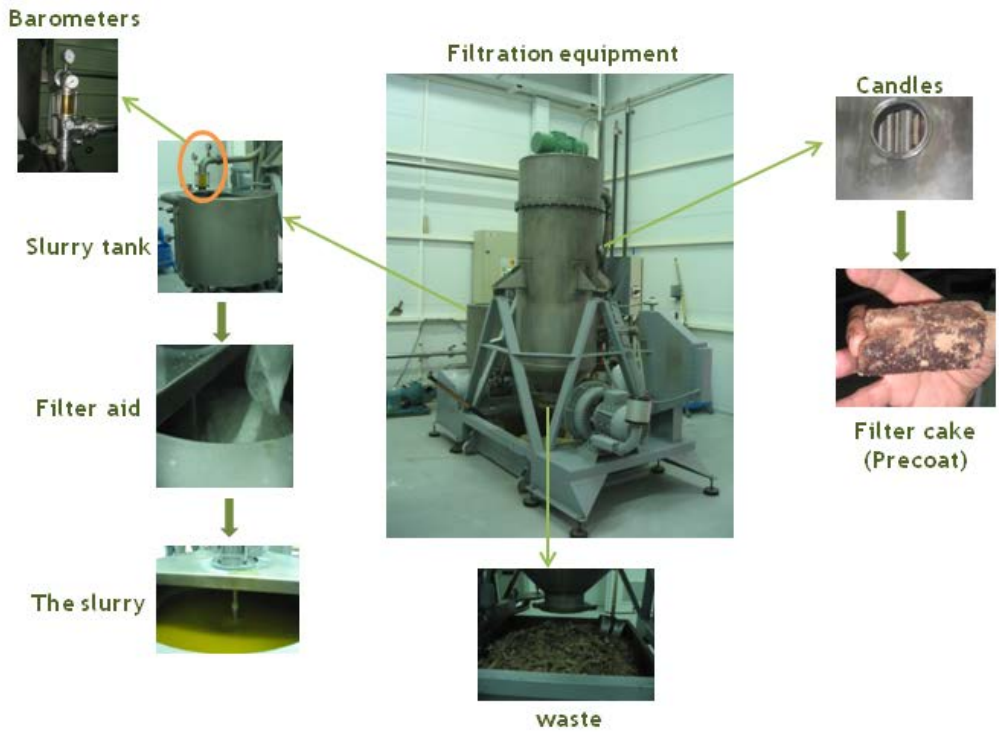

Figure 11. Industrial filtration process to remove suspended solids from olive oil.

Filter aids for precoat filtration can be produced from a wide variety of raw materials. The filter aids utilized commonly include diatomite with different particle sizes, and consequently different permeabilities.

Diatomite, also known as diatomaceous earth, is a silica mineral that is composed of the fossilized skeletal remains of microscopic single-celled aquatic plants called diatoms. Over 10,000 species of these microscopic algae have been recognized, each with its own distinct shape, ranging in size from below 5 microns to over 100 microns. Normally, the diatomite deposits consist of diatom shells only, but actually these deposits contain other sediments like

clay, inorganic carbonates, iron oxides and fine sand. High-grade diatomaceous earth normally contains a minimum of about 95% diatomite, a clay content of not more than 3–4 % and other impurities not exceeding 1–2 %[149,150].

The diatomaceous earth can be classified depending on its elaboration process, which is divided into the following steps: drying, crushing, screening, sorting and calcinations. The specific procedure depends on the raw material and the final product desired. In this way, two products can be obtained: natural diatomaceous earth and a calcined one[151]. The natural diatomaceous earth is obtained only by mechanical means, and it is rarely used as a filter aid due to its low filtration efficiency. However, the calcined one is obtained by the calcination of the natural diatomaceous earth in a rotary kiln at 870–1100 °C (depending on the properties of the raw material and the method of production)[152,153]. The advantages of calcinations are: removing the impurities, and increasing the particle size, which means an increase in flow rate during filtration[149]. The **Figure 12** shows two kinds of commercial diatomaceous earth used in olive oil filtration.

Figure 12. Commercial diatomaceous earth (1, Ciarcel; 2, Celite).

In recent years, filter aids based on organic fibrous materials have become increasingly popular. Depending on their production process, they can be

[149] Goren, R.; Baykara, T.; Marsoglu, M. *Scand. J. Metall.* **2002**, *31*, 115–119.
[150] Ibrahim, S. S.; Selim, A. Q. *J. ORE Dress. 12*, 24–32.
[151] Martinovic, S.; Vlahovic, M.; Boljanac, T.; Pavlovic, L. *Int. J. Miner. Process.* **2006**, *80*, 255–260.
[152] Chaisena, A.; Rangsriwatananon, K. *J. Sci. Technol.* **2004**, *11*, 289–299.
[153] Ediz, N.; Bentli, İ.; Tatar, İ. *Int. J. Miner. Process.* **2010**, *94*, 129–134.

classified into two subclasses: natural wood or plant fibres, and fibres from highly pure cellulose. From a chemical point of view, filter aids from natural wood or plant fibres consist principally of a mixture of cellulose, hemicelluloses and lignin. The pre-shredded raw material is ground to the desired fineness through a completely mechanical process without the addition of chemicals or bleaching. Hereby, the technological processing determines the filter aid's specific filtration properties. The second subclass (fibres from highly pure cellulose) are obtained through chemical methods, in which decorticated and pre-shredded woodchips are treated under high pressure (5 atm) and high temperature (140-180 ºC) for a long period of time with $Ca(HSO_3)_2/H_2SO_3$ or with a mixture of $NaOH/Na_2S/Na_2CO_3/Na_2SO_4$, before bleaching with oxidizing chemicals. Through this process, lignin, hemicelluloses and other possible impurities present in the raw material are dissolved. Therefore, the finished product (cellulose) is distinguished by its high purity[154,155].

The new tendency to use organic filter aids instead of diatomite in olive mills to carry out olive oil filtration is attributed to a series of advantages that organic aids offer to the user:

1- As a result of a lower filter cake density, the specific consumption can be up to 70% less than with mineral filter aids.

2- Due to their structure, rough surface and large porosity, higher flow rates and longer cycle times can often be obtained.

3- Since the plant and cellulose fibres are soft and not abrasive, materials, pumps, pipes and the conveyor are well protected.

4- Due to their structure, they act elastically against pressure drops.

5- The lack of harmful crystalline components also insures against health hazards under normal usage conditions.

[154] Bolio-López, G. I.; Cadenas-Madrigal, G.; Veleva, L.; Falconi, R.; De la Cruz-Burelo, P.; Hernández-Villegas, M. M.; Pelayo-Muñoz, L. *Int. J. Innov. Sci. Eng. Technol.* **2015**, *2*, 977–981.
[155] Gerdes, E. *Filtr. Sep.* **1997**, *34*, 1040–1043.

6- Their disposal is environmentally friendly.

The **Figure 13** shows two commercial filter aids obtained from organic raw material.

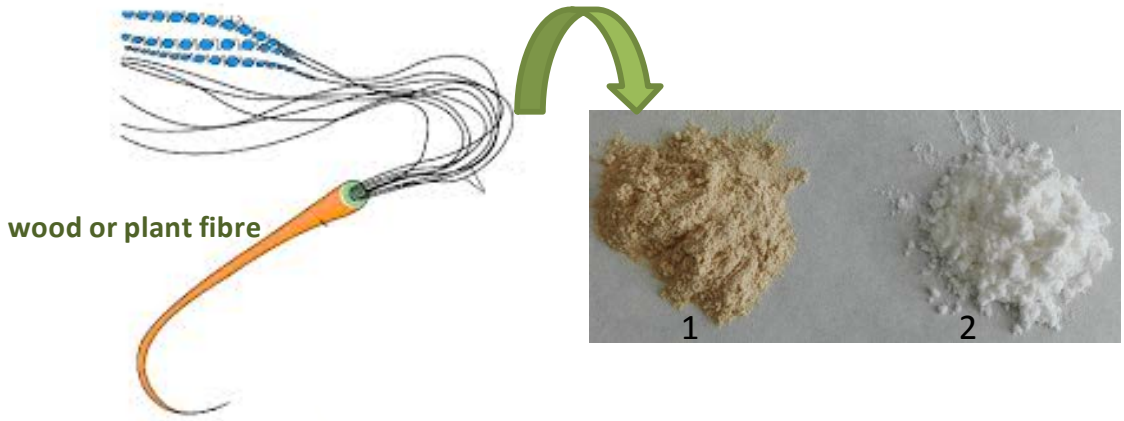

Figure 13. Commercial organic filter aids (1, Filtracel; 2, Arbocel).

❖ **Filtration to eliminate moisture**

This step is carried out to make the oil brilliant and it offers an impeccable commercial presentation. For this purpose, traditional filter presses are used, and the filter aid in this case is not a powder but consists of compact cotton or cellulose paper sheets. Normally, this process is carried out after the filtration step to remove suspended solids. The filter press consists of filter plates used to constitute different chambers in which the filter sheets are introduced (**Figure 14**). The number of filter plates determines the filtration capacity of the equipment[156]. Sometimes this kind of filter can have an additional slurry tank in which powdered filter aids of different natures can be mixed in order to increase the efficiency of the process.

[156] Guerrini, L.; Masella, P.; Migliorini, M.; Cherubini, C.; Parenti, A. *J. Food Eng.* **2015**, *157*, 84–87.

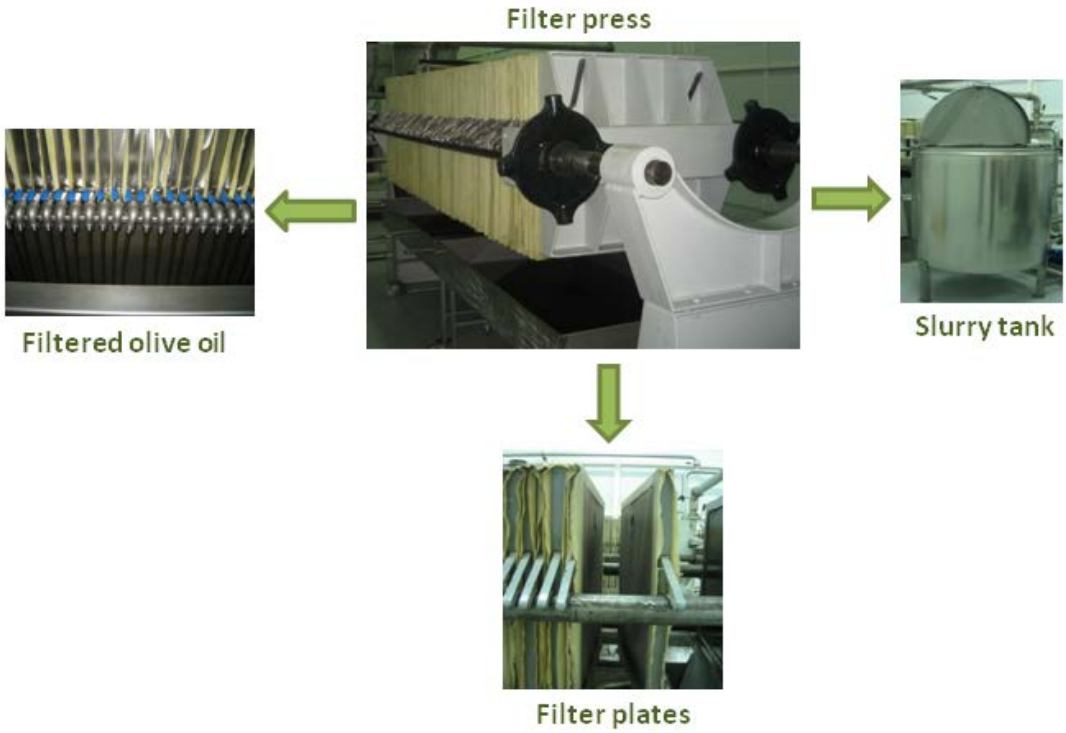

Figure 14. Filter press and its different parts.

The effect of the conventional filtration systems, especially the ones using filter aids, on the composition of olive oil phenolic compounds has been evaluated on a laboratory scale[146]. In fact, olive oil was filtered using different filter aids (organic and inorganic). After that, the total phenol content was determined using a spectrophotometric method in unfiltered and filtered olive oil, and individual characterization of phenolic compounds in filter aids was carried out by high-performance liquid chromatography coupled to mass spectrometry. It was observed that filtration produced a decrease in the total phenol content in all filtered olive oils, and a large number of phenolic compounds belonging to phenolic alcohols, secoiridoids, flavones, lignans and phenolic acids were identified in the filter aids after filtration. The retentive power of filter aids to phenolic compounds was confirmed at industrial scale filtration system[157].

[157] Lozano-Sánchez, J.; Castro-Puyana, M.; Mendiola, J. A.; Segura-Carretero, A.; Cifuentes, A.; Ibáñez, E. *Int. J. Mol. Sci.* **2014**, *15*, 16270–16283.

4.2.8.2 New filtration systems

❖ **Cross-flow filtration system**

This has been proposed as an interesting and alternative method that involves olive oil flow parallel to the membrane instead of the perpendicular flow in conventional filtration methods. In particular, there are important applications in the microfiltration and ultrafiltration processes, using commercial membranes of different types, with tubular mono- or multi-channel configuration[158]. The separation characteristics of the membranes are dependent not only on their physicochemical properties, but also on the feed characteristics and operating variables such as velocity, temperature and pressure. The first applications of membranes in the filtration of olive oil showed that this system allows impurities to be removed without involving olive oil loss and possible olive oil contamination by filter aids as happens in the conventional systems[159]. Unfortunately, there are no data on using this system in olive oil factories; all the reported filtration experiments were carried out in a laboratory and on pilot scale.

❖ **Filter bag system**

This consists of two compartments: a cylindrical tube and a filter bag. The filter bag is made up of a unique bag (usually of polypropylene) and its support **(Figure 15)**. The filter bag is introduced into the cylindrical tube and the system is pressurized by a hydraulic closure. Olive oil is directly conducted from storage tanks to filtration equipment. It passes across the filter bag and suspended solids are removed. Occasionally, similarly to the other filtration systems described, different materials selected by the manufacturer may be used as filter aids in order to improve the filtration process. The main advantages of this system are its wide versatility and easy maintenance, which permits an optimal level of oil limpidity. However, the problem common to all filter bags is that their fairly lightweight construction

[158] Bottino, A.; Capannelli, G.; Mattei, A.; Rovellini, P.; Zunin, P. *Eur. J. Lipid Sci. Technol.* **2008**, *110*, 1109–1115.
[159] Bottino, A.; Capannelli, G.; Comite, A.; Ferrari, F.; Marotta, F.; Mattei, A.; Turchini, A. *J. Food Eng.* **2004**, *65*, 303–309

and high flexibility often make them difficult to insert into the retaining cage or basket without wrinkling and collapsing[145,147].

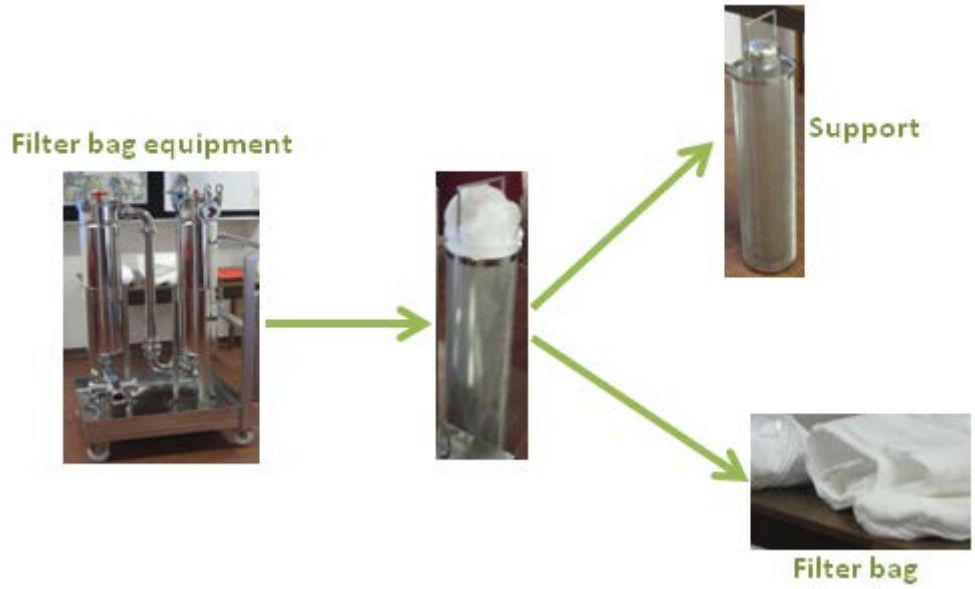

Figure 15. Filter bag equipment and its different parts.

- **Filtration using inert gas**

In this case, the filtration equipment comprises two different modules. The first module consists of a typical tank, where olive oil is introduced and the filtration process is carried out. The second module is the storage tank of inert gas utilized as a filter aid (nitrogen or argon). This module has an insertion device connected to the bottom of the filter tank that introduces a constant inert gas flow directly into the centre of the olive oil mass. The gas insertion generates a circular movement of the olive oil, resulting easily in the precipitation of the suspended solids. It is important to underline that clarification by an inert gas flow avoids the use of organic materials coming into contact with the olive oil**.** Commercial equipment is available with capacities ranging from 50 to 300 litres/h[144,145,147].

The effect of the new filtration systems on olive oil phenolic content has also been studied. On a laboratory scale, it was reported that the application of cross-flow microfiltration and ultrafiltration with different commercial membranes to olive oil decreased strongly the total phenolic compounds and

secoiridoids in filtered olive oil, in comparison to the unfiltered one[158]. The highest decrease was observed using a Carbosep M1 membrane. Furthermore, an interesting study was carried out on an industrial scale, in which olive oil was filtered by filter bag and inert gas filtration systems[145]. Phenolic compounds were characterized in filtered and unfiltered olive oils, and the results showed an apparent increase of secoiridoid content in filtered olive oils obtained using both filtration systems. However, the rest of the phenolic families behaved differently depending on the filter aids used. While phenolic alcohols, lignans and flavones decreased after filtration using a polypropylene filter bag, some of these compounds did not decrease when using the inert gas flow filtration system.

4.2.9 Bottling

Bottling is the last step before olive oil commercialization. Generally, this process is carried out in a complete line in which filler equipment can be put in line with a capper and labeller. The automated filling lines have a variety of options but one of the most useful is filling by weight or exact volume. They are usually designed to customer specifications. Some filler equipment can accommodate very small bottles and odd shapes. Others blow air into the bottles first to clean them. Automated lines can also offer the option of injecting nitrogen into the bottle during the filling so that the olive oil will be protected from deterioration. Once the bottles are filled, they pass on to capping and labelling processes conducted in the same line. The **Figure 16** shows the complete line of olive oil bottling.

Figure 16. Olive oil bottling process.

The container chosen for olive oil packaging has to be able to protect it from different factors that can produce olive oil deterioration during storage and distribution, such as oxygen, light, high temperatures and trace metals. The materials most commonly used for olive oil packaging are: glass, plastic, tinplate and Tetra Brik[160]-[162]. The variation in phenolic content of olive oil stored in different containers was a concern of many searches reported in the literature. In fact, in an effort to evaluate the stability of olive oil during storage, different containers such as clear and dark glass, polyethylene (PE) and tinplate bottles were used[161]. The olive oil samples were stored under light at room temperature. After 75 days of storage, it was observed that total phenolic content decreased significantly from 363 to 285 mg/kg in olive oils stored in PE, and from 363 to 305 mg/kg in olive oils stored in clear glass bottles. However, in the other materials, it decreased slightly from 363 to 342 mg/kg in the tinplate container and from 363 to 337 mg/kg in the dark glass bottle during the same period of storage. The differences observed among the materials used were attributed by the authors to the joint action of light and the permeability of the PE container to the oxygen that catalyses the oxidation reaction. Nevertheless, the phenomenon was less pronounced in the oil stored in the tinplate container and the dark glass bottle. In another study, Tetra Brik was found to be a good material for preserving olive oil phenolic compounds from the action of oxygen and light[163].

5. Extraction and analytical techniques for olive oil phenolic determination

5.1 Extraction of olive oil phenolic compounds

Extraction is a necessary step prior to the analysis, in which isolation of a specific fraction or target compound is carried out using different procedures. It is aimed at the preparation of a sample extract uniformly enriched in all compounds of interest and free from interfering matrix components. The selection of the appropriate extraction procedure depends on the nature of

[160] Samaniego-Sánchez, C.; Oliveras-López, M. J.; Quesada-Granados, J. J.; Villalón-Mir, M.; Serrana, H. L.-G. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 194–204.
[161] Gargouri, B.; Zribi, A.; Bouaziz, M. *J. Food Sci. Technol.* **2014**, *52*, 1948–1959.
[162] Pristouri, G.; Badeka, A.; Kontominas, M. G. *Food Control.* **2010**, *21*, 412–418.
[163] Méndez, A. I.; Falqué, E. *Food Control.* **2007**, *18*, 521–529.

the matrix, the chemical structure of the compounds, interactions between both matrix and target compounds, and the concentration level at which the analysis needs to be carried out. Among the extraction methods used for the extraction of phenolic compounds from olive oil, liquid-liquid extraction (LLE) and solid-phase extraction (SPE) systems are the most reported ones in the literature.

5.1.1 Liquid-liquid extraction

LLE is based on transference of the phenolic fraction from olive oil to a more hydrophilic phase such as methanol or methanol–water mixtures (MeOH:H_2O) with different percentages of water ranging from 0 to 40 %[164]. The use of organic extraction solvents other than methanol was reported and these included ethanol, tetrahydrofuran and N,N-dimethylformamide[165]. They were used as pure solvents or mixed with water. In an effort to improve the recovery efficiency, sometimes different tensioactives such as Tween20 have been used to make the release of phenolic compounds easier[166]. After extraction, clean-up and/or pre-concentration steps can be implemented to achieve the appropriate selectivity and sensitivity, respectively. One alternative to remove potential interferences is to store extracts overnight at a subambient temperature followed by filtration or centrifugation[167]. The clean-up step can also be carried out with solvents such as hexane, petroleum ether and chloroform[168]. With regard to the concentration of the extract, it is usually performed by evaporation of the extractant under vacuum or a nitrogen stream at ambient or moderate temperature to avoid degradation.

[164] Gómez-Caravaca, A. M.; Carrasco Pancorbo, A.; Cañabate Díaz, B.; Segura Carretero, A.; Fernández Gutiérrez, A. *Electrophoresis.* **2005**, *26*, 3538–3551.
[165] Pizarro, M. L.; Becerra, M.; Sayago, A.; Beltrán, M.; Beltrán, R. *Food Anal. Methods.* **2013**, *6*, 123–132.
[166] Carrasco-Pancorbo, A.; Cerretani, L.; Bendini, A.; Segura-Carretero, A.; Gallina-Toschi, T.; Fernández-Gutiérrez, A. *J. Sep. Sci.* **2005**, *28*, 837–858.
[167] Angerosa, F.; D'Alessandro, N.; Konstantinou, P.; Di Giacinto, L. *J. Agric. Food Chem.* **1995**, *43*, 1802–1807.
[168] Montedoro, G.; Servili, M.; Baldioli, M.; Miniati, E. *J. Agric. Food Chem.* **1992**, *40*, 1571–1576.

Depending on the volume of the sample and solvent employed, LLE can be divided into large and small scale. In fact, the former was generally carried out with 60 g oil diluted with 60 mL hexane and extraction was performed with 4 x 20 mL portions of MeOH:H_2O 60:40 (v/v). The latter was performed with 2 g oil diluted with 1 mL of hexane and the extraction step, using 2 mL of MeOH:H_2O 60:40 (v/v), was replicated three times[169]. Other miniaturized LLE techniques using smaller solvent volumes have even been recently proposed for olive oil sample preparation[170]. In sipte of the controversial data reported in the literature about the best extraction conditions[164,171], generally LLE achieves a high recovery of phenolic compounds from olive oil.

5.1.2 Solid-phase extraction

The main advantage of SPE, as compared with LLE, is its dual action associated with isolation of the target compounds: clean-up and preconcentration. The proved versatility of this technique is based on the range of sorbents with different characteristics that can be used as well as eluents. The first applications of SPE for isolation of phenolic compounds from olive oil were based on the use of C18 and C8 as sorbents. However, incomplete extraction of the phenolic fraction has been reported[166,172]. In spite of the common assumption that C18 phase is less suitable for the isolation of polar components from a non-polar matrix, C18 cartridges have often been tested for isolation of polyphenols from olive oil. The application of normal-phase SPE instead of reversed-phase SPE was proposed for olive oil phenolic extraction. In fact, the sorbent in this case consisted of silica modified with diol groups. Using this new sorbent, a high recovery (>90%) of all major olive phenolic compounds was achieved[173]. Furthermore, the comparative studies carried out among the different sorbents previously

[169] Laura Capriotti, A.; Cavaliere, C.; Crescenzi, C.; Foglia, P.; Nescatelli, R.; Samperi, R.; Laganà, A. *Food Chem.* **2014**, *158*, 392–400.
[170] Godoy-Caballero, M. D. P.; Acedo-Valenzuela, M. I.; Galeano-Díaz, T. *J. Chromatogr. A* **2013**, *1313*, 291–301.
[171] Hrncirik, K.; Fritsche, S. *Eur. J. Lipid Sci. Technol.* **2004**, *106*, 540–549.
[172] Servili, M.; Baldioli, M.; Selvaggini, R.; Miniati, E.; Macchioni, A.; Montedoro, G. *J. Am. Oil Chem. Soc.* **1999**, *76*, 873–882.
[173] Mateos, R.; Espartero, J. L.; Trujillo, M.; Ríos, J. J.; León-Camacho, M.; Alcudia, F.; Cert, A. *J. Agric. Food Chem.* **2001**, *49*, 2185–2192.

mentioned showed that diol-phase cartridges are the best in terms of recovery efficiency[164,174]. Methanol or methanol-water mixtures and hexane were the main solvents used through SPE steps[173]. SPE includes conditioning, sample addition, washing and elution. **Figure 17** shows SPE step by step.

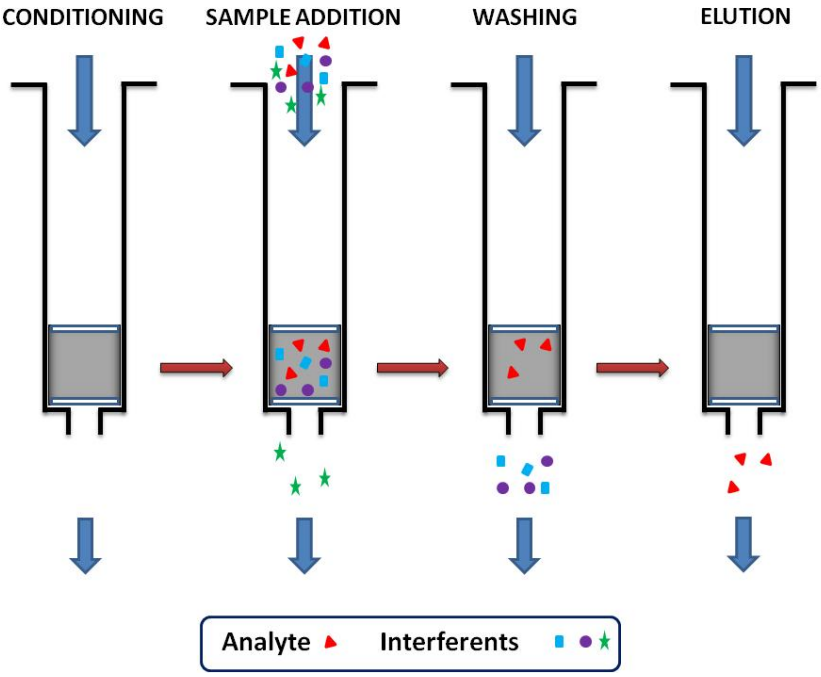

Figure 17. Solid-phase extraction steps.

5.2 Analytical techniques used for separating phenolic compounds

In order to separate the components of complex mixtures such as olive oil phenolic extracts, analytical chemistry has many techniques based on differences in the physicochemical properties of the various components of the extract. Continuous techniques are generally used, in which analytes are detected continuously (online) after separation. Among these techniques we can distinguish two large groups: chromatographic, such as gas (GC) and liquid (LC) chromatography, and non-chromatographic, such as capillary electrophoresis (CE). GC is less common because a derivatization step is necessary[167]. The results obtained by CE are very attractive, with short analysis times and high peak separation efficiency, but the downside of this

[174] Bendini, A.; Bonoli, M.; Cerretani, L.; Biguzzi, B.; Lercker, G.; Toschi, T. G. *J. Chromatogr. A.* **2003**, *985*, 425–433.

technique is the low concentration sensitivity[174]. The usual technique for analysing the olive oil phenolic fraction is LC due to a series of advantages such as good resolution, short time analysis, high sensitivity and good performance. Because liquid chromatography was the separation technique used in this thesis, its characteristics will be developed in more detail in the next section.

5.2.1 Liquid chromatography

Different studies aimed at the characterization of olive oil phenolic compounds reported the different steps required to follow in the optimization of the chromatographic method and the best conditions for mobile phase composition, column particle size, flow rate and temperature to achieve a balance among high resolution, peak capacity and short analysis time[37,175].

Among the different types of LC, partition chromatography is the one used for the analysis of olive oil phenolic compounds. Within this category, two modes can be defined depending on the relative polarity of two phases (stationary and mobile phases): normal- and reversed-phase LC. In the past, phenolic analysis was carried out by normal-phase LC[176]. However, in recent years, reversed-phase LC has been the preferred technique for olive oil phenolic separation using, as stationary phase, mainly a non-polar octadecylsilane (C18) bonded phase. It was reported to offer the best results in terms of the reproducibility of retention time and separation[166].

Most applications use gradient elution[177], but isocratic chromatographic methods were also developed for the separation of phenolic compounds belonging to phenolic acid and phenolic alcohol families[178]. The elution is generally performed with a mobile phase of high polarity such as aqueous solutions used in a binary system with polar organic solvents such as

[175] García-Villalba, R.; Carrasco-Pancorbo, A.; Oliveras-Ferraros, C.; Vázquez-Martín, A.; Menéndez, J. a; Segura-Carretero, A.; Fernández-Gutiérrez, A. *J. Pharm. Biomed. Anal.* **2010**, *51*, 416–429.
[176] Cert, A.; Moreda, W.; Pérez-Camino, M. C. *J. Chromatogr. A.* **2000**, *881*, 131–148.
[177] Pirisi, F. M.; Cabras, P.; Cao, C. F.; Migliorini, M.; Muggelli, M. *J. Agric. Food Chem.* **2000**, *48*, 1191–1196.
[178] Akasbi, M.; Shoeman, D. M.; Saari Csallany, A. *J. Am. Oil Chem. Soc.* **1993**, *70*, 367–370.

acetonitrile or methanol[92,179]. The separation of the more polar phenolic compounds such as phenolic acids depends strongly on the pH of the mobile phase. The presence of weak acid in the mobile phase prevents these analytes from ionizing, thereby improving their separation using reversed-phase chromatography. Other less polar compounds like flavonoids have a lower tendency to ionize, and therefore they can be separated with neutral mobile phases, without the addition of acid. Among the acids used in the mobile phase for the separation of olive oil phenolic compounds, acetic acid and formic acid were the most reported ones[180,181].

The columns employed in reversed-phase chromatography for the separation of olive oil phenolic compounds differ mainly in their dimensions, i.e. length and diameter, and the particle size of the stationary phase. Traditionally, the analysis of phenolic compounds was carried out using columns with a particle size of between 3 and 5 microns[177,182.] However, good separation of the peaks was always accompanied by a long analysis time. Recently, the use of columns packed with very small particles (≤2 µm) and the application of high flow rates have allowed short analysis times and better resolution between peaks to be achieved[183]. However, working with such columns and high flows, it was necessary to develop new equipment that supports high pressures. Among them, rapid-resolution LC (RRLC) and ultra-performance LC (UPLC) were the most frequently reported equipment. While the former supports maximum pressures of 600 bars, the latter is able to achieve pressures of 1200 bars. The only improvement introduced in these two forms of equipment to achieve such pressures was the pumping system. The rest of the compartments are similar to those of conventional LC. The **Figure 18** presents a basic schema of commercial LC equipment, showing its commercial appearance.

[179] Baccouri, O.; Guerfel, M.; Baccouri, B.; Cerretani, L.; Bendini, A.; Lercker, G.; Zarrouk, M.; Daoud Ben Miled, D. *Food Chem.* **2008**, *109*, 743–754.
[180] Oliveras-López, M. J.; Innocenti, M.; Giaccherini, C.; Ieri, F.; Romani, A.; Mulinacci, N. *Talanta.* **2007**, *73*, 726–732.
[181] Carrasco-Pancorbo, A.; Neusüss, C.; Pelzing, M.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *Electrophoresis.* **2007**, *28*, 806–821.
[182] Selvaggini, R.; Servili, M.; Urbani, S.; Esposto, S.; Taticchi, A.; Monterdero, G. *J. Agric. Food Chem.* **2006**, *54*, 2832–2838.
[183] Fu, S.; Segura-Carretero, A.; Arráez-Román, D.; Menéndez, J. A.; De La Torre, A.; Fernández-Gutiérrez, A. *J. Agric. Food Chem.* **2009**, *57*, 11140–11147.

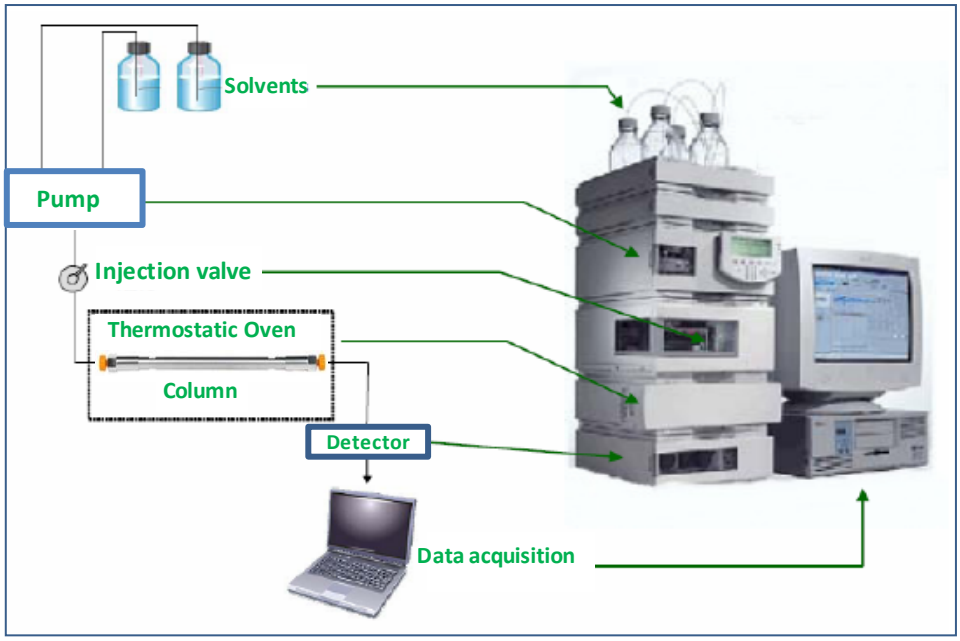

Figure 18. Simplified outline of a commercial LC.

The temperature of the analysis was also found to be of great importance in order to achieve a good and reproducible separation of olive oil phenolic compounds. Therefore, in order to maintain the temperature of the analysis, the LC is equipped with a thermostatic oven compartment.

5.3 Detectors

Different types of detectors can be coupled to LC. The choice of the ideal detector for each application is based on the nature and properties of the analytes to be determined and the sensitivity required and what information is sought (structural, quantitative, etc). Among the detectors used for the characterization of olive oil phenolic compounds, UV-visible, fluorescence, ion trap mass spectrometry (IT-MS), time-of-flight mass spectrometry (TOF-MS), quadrupole time-of-flight mass spectrometry (Q-TOF-MS) and hybrid quadrupole-orbitrap mass spectrometery (Q-orbitrap-MS) were the most frequently reported ones[50,169,173,183,184]. However, in the following sections we focus only on UV-visible and TOF-MS, as they were the two detectors used in this thesis.

[184] El Riachy, M.; Priego-Capote, F.; Rallo, L.; Luque-de Castro, M. D.; León, L. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 542–551.

5.3.1 UV-visible absorbance

The detection system based on the absorbance of the UV-visible radiation is the most commonly used in commercial LC equipment, due to its ability to determine a large number of compounds and its ease of use, although its sensitivity is much lower than that of other detection systems. Its simplicity and relatively low cost are its main advantages. The detection of analytes is based on the interaction between the UV-visible and the material that gives rise to absorption of certain wavelengths of radiation from such compounds. Three types of absorption detectors are available: fixed wavelength, variable wavelength and photo diode array. The former uses a light source that emits maximum light intensity at one or more discrete wavelengths (e.g. 254, 280 and 365 for a mercury lamp) that are isolated by appropriate filters. The second type of detector is variable wavelength, which works in the range of the UV-visible spectrum (190-650). This type of detector has been equipped with two lamps, one of deuterium for measurements at 190-360 nm and the other of tungsten for measurements in the visible spectrum from 360 to 650 nm, with a monochromator (a moveable grating controlled by a stepper motor to select the wavelength through an exit slit). Nowadays, with only a deuterium lamp the entire UV-visible spectrum is covered. Finally, a diode array detector is considered the most versatile. It allows simultaneous detection of a range of wavelengths in seconds in order to obtain complete UV-vis absorption spectra[55,185].

A diode array detector was reported to be used for the characterization of olive oil phenolic compounds in order to establish a comparative study between liquid-liquid and solid-phase extraction in terms of phenolic recovery effeciency[173]. Indeed, in the reported study, compounds belonging to phenolic alcohols, phenolic acids, secoiridoids, lignans and flavones were identified, using the spectral information, commercial standards and other phenolic compounds previously isolated from olive oil phenolic extract. In another study, 47 Turkish commercial olive oils were characterized using high-

[185] El Riachy, M.; Priego-Capote, F.; Rallo, L.; Luque-de Castro, M. D.; León, L. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 800–810.

performance liquid chromatography with a diode array detector[97]. Again, the use of commercial standards and spectral information was able to identify and quantify 17 phenolic compounds, and the obtained results were used for the classification of olive oil samples according to their geographical origin. The identification of olive oil phenolic compounds is generally carried out at 240 and 280 nm wavelengths. The **Table 7** includes the maximum absorption of some phenolic compounds and their derivatives present in olive oil.

Table 7. Maximum absorbance of the main olive oil phenolic compounds

Phenolic compounds	Wavelength (nm)
Vanillic acid	228/265/295
p-coumaric acid	230/310
Ferulic acid	240/295/325
Hydroxytyrosol	232/280
Hydroxytyrosol acetate	232/285
Tyrosol	232/276
Tyrosol acetate	232/285
Elenolic acid	240
Oleuropein aglycone	236/282
Decarboxymethyl oleuropein aglycone	235/280
Ligstroside aglycone	235/285
Decarboxymethyl ligstroside aglycone	235/285
Pinoresinol	232/280
Acetoxypinoresinol	232/280
Luteolin	255/350
Apigenin	230/270/340

The detection of UV-visible is very robust, making it one of the best ways to perform quantifications as was reported in the two studies mentioned above. However, it has the disadvantage of not providing structural information and

therefore it does not allow the identification of phenolic compounds if the commercial standards are not available. Consequently, in order to achieve a detailed characterization of olive oil phenolic compounds, detectors other than UV-visible are often necessary. Among them, mass spectrometry is the most frequently reported.

5.3.2 Mass spectrometry

Mass spectrometry is a powerful analytical technique that measures the molecular masses of individual compounds and atoms precisely by converting them into charged ions. Quite often, the structure of a molecule can also be deduced. Mass spectrometry is also uniquely qualified to provide quantitative information about an analyte at extraordinary levels of structure specificity and sensitivity. Thus, mass spectrometry is probably the most versatile and comprehensive analytical technique currently available to chemists and biochemists[175,186-188]. There are many types of mass spectrometry; however, all of them include the following elements: an ion source, a mass analyser and a detector. Recently the applications of mass spectrometry as a detection system coupled to various separation techniques, particularly LC for the characterization of olive oil phenolic compounds, have grown exponentially[17,181]. The **Figure 19** shows a combination of high-performance liquid chromatography with mass spectrometry.

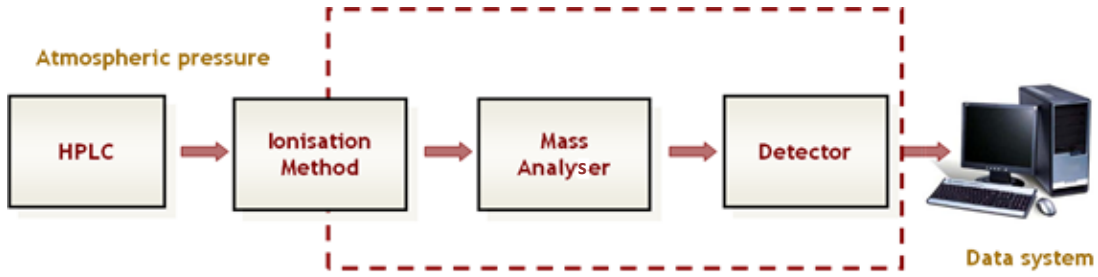

Figure 19. Combination of a separation technique (HPLC) with mass spectrometry.

[186] Finehout, E. J.; Lee, K. H. *Biochem. Mol. Biol. Educ.* **2004**, *32*, 93–100
[187] Ibáñez, M.; Sancho, J. V.; Bijlsma, L.; van Nuijs, A. L. N.; Covaci, A.; Hernández, F. *TrAC Trends Anal. Chem.* **2014**, *57*, 107–117.
[188] Abu-Reidah, I. M.; Contreras, M. M.; Arráez-Román, D.; Segura-Carretero, A.; Fernández-Gutiérrez, A. *J. Chromatogr. A.* **2013**, *1313*, 212–227.

The first step in mass spectrometry measurement is the conversion of neutral molecules to charged species (i.e. ions), which are then separated according to their mass-to-charge (*m*/*z*) ratio in a mass analyser. It is a fundamental requirement of mass spectrometry that the ions be in the gas phase before they can be separated according to their individual *m*/*z* values and detected. Many ionization systems have been used in olive oil phenolic studies (electrospray ionization (ESI), atmospheric pressure ionization (API), atmospheric pressure chemical ionization (APCI) and matrix-assisted laser desorption ionization (MALDI))[169,189,190.] However, more details will be given about ESI as the one coupled to TOF-MS in the experimental part of the thesis.

Electrospray ionization (ESI)

ESI is one of the most versatile ionization sources. In fact, it provides a simple, real-time means of analysing a wide range of polar molecules (100-200,000 dalton range), and it is a soft ionization method. ESI has become the method of choice for coupling LC with mass spectrometry for olive oil phenolic characterization[54,191].

The sample comes from the separation technique dissolved in a polar, volatile solvent and introduced through the nebulizer assembly into the spray chamber, where it is subjected to the ESI process by means of an electrical field between the inner chamber wall and the spray shield, with the aid of a nebulizer gas (N_2). Heated drying gas (N_2) delivered from the desolvation assembly, flowing in the opposite direction to the stream of droplets, enters the spray chamber, and is used to aid volatilization, and to carry away any uncharged material. Indeed, as the solvent evaporates, the droplet shrinks until it reaches the point where the surface tension can no longer sustain the charge (the Rayleigh limit), at which point a "Coulomb explosion" occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the

[189] Lerma-García, M. J.; Herrero-Martínez, J. M.; Simó-Alfonso, E. F.; Lercker, G.; Cerretani, L. *Anal. Bioanal. Chem.* **2009**, *395*, 1543–1550.
[190] Calvano, C. D.; Aresta, A.; Zambonin, C. G. *J. Mass Spectrom.* **2010**, *45*, 981–988.
[191] Gambacorta, G.; Faccia, M.; Previtali, M. a; Pati, S.; La Notte, E.; Baiano, A. *J. Food Sci.* **2010**, *75*, C229–35.

process until the final desolvation generates sample ions[192] **(Figure 20)**. During the ionization process, mono- or multicharged ions can be formed, which allows the detection of compounds with very high molecular weights using mass analysers working with a limited range of m/z[186]. In this step, the entrance of flows between 0.2 and 0.5 ml/min into the ESI unit is recommended in order to produce stable electrosprays. Consequently, the use of a splitter to reduce the flows coming from LC was found in many cases to be necessary.

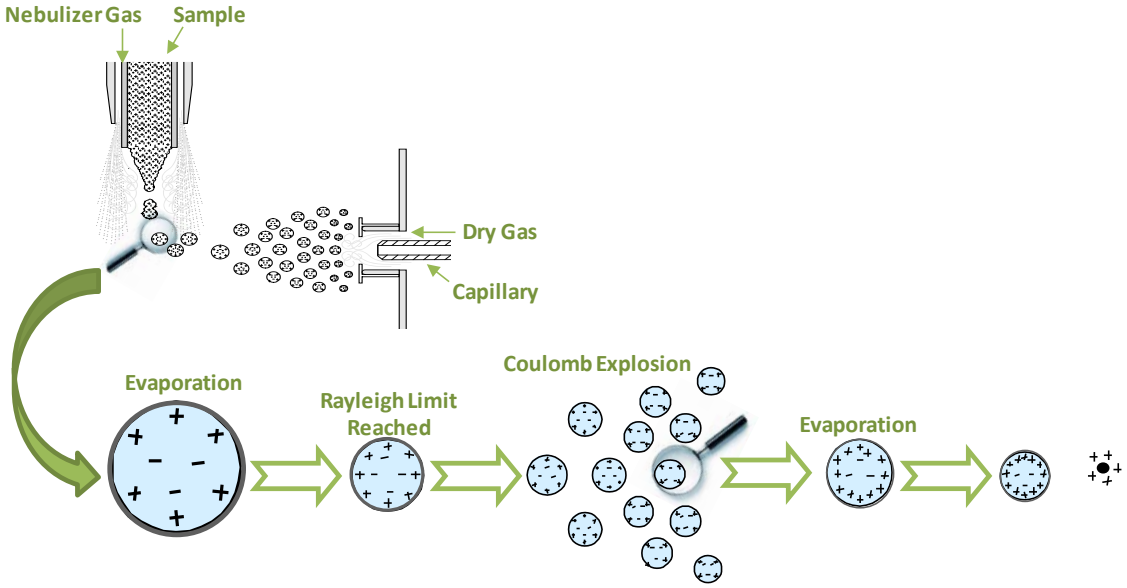

Figure 20. A schematic of the ion formation mechanism.

Time-of-flight mass analyser

This is a device that can separate and sort ions coming from the ion source according to their *m/z* values. A time-of-flight mass analyser (TOF-MS) uses electrical or magnetic fields, or a combination of the two, to move the ions from the region where they are produced to a detector, where they produce a signal, which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, the mass-to-charge ratio, and not only the mass,

[192] Forcisi, S.; Moritz, F.; Kanawati, B.; Tziotis, D.; Lehmann, R.; Schmitt-Kopplin, P. *J. Chromatogr. A.* **2013**, *1292*, 51–65.

is important[186]. The analyser is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield.

A TOF-MS uses the differences in transit time through a flight/drift zone to separate ions of different masses. The principle is that smaller ions, being lighter, will reach the detector faster than heavier ions. The resolution between the different *m/z* is better when the tube length is higher (there would be a major separation of ions in time).

The process of ion separation until their detection passes through different stages (**Figure 21**). First, the formed ions are attracted by the electrical field strength between the spray chamber and the negatively biased metal-coated glass capillary. The potential difference between the spray shield and the tip of the glass capillary acts as a further ion pull into the **ion transmission module**. The later consists of three units of high vacuum separated from each other by two skimmers. The hexapoles make the transfer of the ions to the zone of high vacuum, while the lenses determine the direction of those ions. After that, the ions reach the orthogonal acceleration stage, which deflects and transfers incoming ions to the **reflector** using an intermittent electric field. In fact, after leaving the orthogonal acceleration stage the ions pass through a flight tube in which they are separated as a result of their m/z ratio. Due to the different velocities and positions of the ions prior to orthogonal acceleration, slight differences in the final kinetic energy are observed. However, the reflector normalizes these energy differences, improving, as a consequence, the resolution. Ions of the same mass but of unequal kinetic energies will penetrate the reflector field to different depths, which compensates for their varying starting energies, before being reflected to the **detector.** The detector is of electronic impact which converts an ion impact into an electrical signal[186,192,193].

[193] Liu, Z.-Y. *J. Mass Spectrom.* **2012**, *47*, 1627–1642.

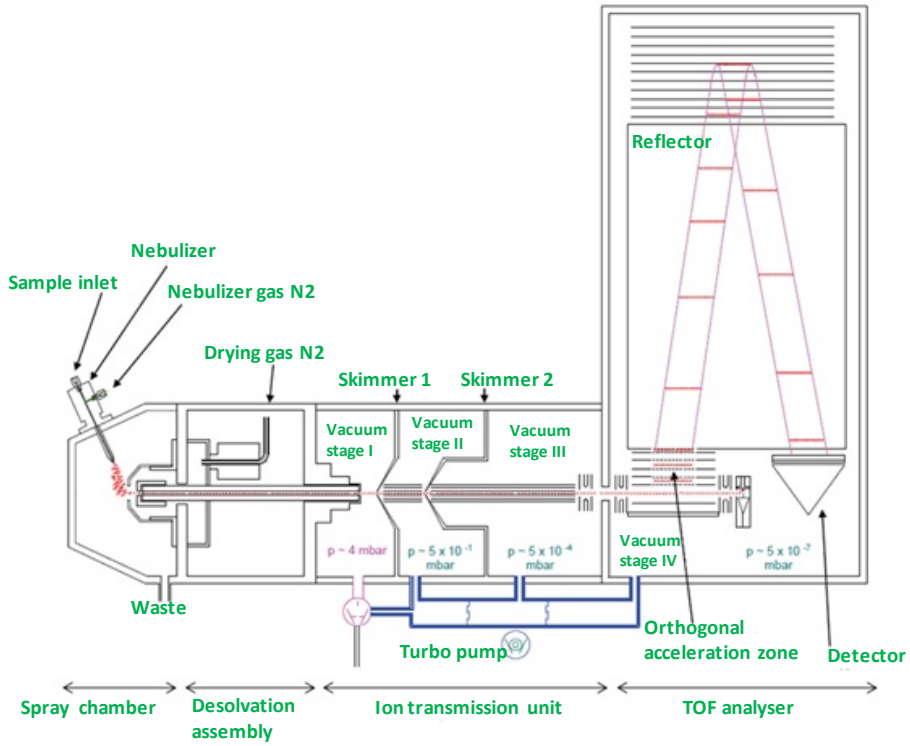

Figure 21. Schematic diagram of time-of-flight mass spectrometry.

Technical developments in coupling LC with MS, and in particular the introduction of the ESI technique, facilitated the separation, identification and structural determination of phenolic compounds in olive oil. ESI-TOF-MS was reported to be used for the characterization of olive oil phenolic compounds from six Tunisian olive varieties[89]. The information provided by mass spectrometry of accuracy mass and isotopic distribution made possible the determination of the molecular formula of the compounds. Using these data the authors identified 23 phenolic compounds belonging to different families. In addition, their individual quantification was possible using external calibration curves, which gave more information about the phenolic content of Tunisian olive varieties. ESI-TOF-MS was also found useful in another study, in which commercial olive oil of Arbequina, Koroneiki, Arbosana, Grappolo, Manzanilla, Coratina, Frantoio and MGS Mariense varieties from three different Brazilian states and two crops were analysed[54]. In this study, the authors were able to identify and quantify 19 phenolic compounds. In conclusion, it was reported that commercial Brazilian olive oils are rich in phenolic compounds, comparing the obtained values with those

previously reported in the literature for olive oils from other countries. The study of the variation in individual phenolic compounds during olive ripening and its repercussion in the produced olive oil was possible using ESI-TOF-MS[194]. Different behaviours among the characterized compounds were observed, and the obtained results were helpful in establishing the optimum harvest time for Chétoui olives in order to obtain olive oil with high-quality characteristics. Interesting data were also reported about the changes that occur in olive oil phenolic compounds during storage using ESI-TOF-MS as a detector[49]. First, a detailed characterization of individual phenolic compounds in olive oil samples was carried out mainly using the information provided by the detector. After that, the quantification of the identified phenolic compounds showed that secoiridoids were the main group responsible for the changes in the olive oil phenolic profile during storage. In fact, these compounds underwent alterations due mainly to hydrolysis, oxidation and an increase of decarboxymethylated derivatives. The application of ESI-TOF-MS as a detector coupled to LC was also reported in studies aimed at olive oil phenolic bioactivity and insights into olive oil secoiridoids, which are considered to be the most complex phenolic group[50,183,195].

Negative ionization mode was the one applied in the mentioned applications. Moreover, the source and transfer parameters were established for a good sensitivity and reasonable resolution of the mass range for the compounds of interest (50-1000 m/z), taking into consideration the flows entering into MS from LC[37].

[194] Ben Youssef, N.; Zarrouk, W.; Carrasco-Pancorbo, A.; Ouni, Y.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Daoud, D.; Zarrouk, M. *J. Sci. Food Agric.* **2010**, *90*, 199–204.

[195] Fernández-Arroyo, S.; Gómez-Martínez, A.; Rocamora-Reverte, L.; Quirantes-Piné, R.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Ferragut, J. A. *J. Pharm. Biomed. Anal.* **2012**, *63*, 128–134.

EXPERIMENTAL PART

SECTION I

Changes in virgin olive oil phenolic profile according to geographical area of olive cultivation and agronomic practices

Chapter 1

Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia

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Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia

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Abstract

The aim of this work was to characterize Arbequina extra-virgin olive oils (EVOOs) from different locations in southern Catalonia (Spain) in terms of their phenolic profile, to show the classification of oil samples with respect to geographical area. The phenolic compounds present in 32 olive-oil samples were analyzed by a rapid and effective HPLC–ESI-TOF/MS method, and 18 phenolic compounds belonging to different phenolic types were identified. The results showed no qualitative differences in the phenolic fractions among EVOO from different geographical region. However, quantitative differences were observed in a wide number of phenolic compounds. In all olive-oil samples studied, secoiridoids were the most abundant, followed by lignans, phenolic alcohols, and flavonoids, respectively. Multivariate data were analysed by canonical discriminant analyses. Seventeen variables were used without a variable reduction step. Phenolic content of extra-virgin olive oils was found to depend highly on geographical area.

Keywords: HPLC-ESI-TOF/MS, Olive oil, Phenolic compounds, Geographical area, Discriminant Analysis.

1. Introduction

The olive tree (*Olea europaea* L.) is cultivated mostly in the Mediterranean region (Spain, Italy, Greece, Tunisia, Turkey, Morocco and Algeria) for climatic reasons. Traditionally, olive oil has been a basic element of the Mediterranean diet, and in recent years increasing worldwide popularity has resulted from its health benefits, such as its high content in mono-unsaturated fatty acids and its minor components (Ocakoglu, Tokatli, Ozen, & Korel, 2009; Tura et al., 2007). Spain, which has made great efforts in the last decades to improve olive-oil quality, is the leading olive-oil-producing country in the world (Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012).

One of the main Spanish olive varieties, which is well known in the international oil market for its excellent taste and flavour, is Arbequina (Nieves, Ramón, José, & Paz, 2004; Ramón, Paz, & Motilva, 2006). This cultivar owes its name to the municipal district of Arbeca (Lleida, Catalonia, Spain), where it was grown for the first time. It is characterized by frost resistance, low vigour, small-sized fruit, and high productivity. The oval-shaped olive has a low flesh-to-stone ratio. Because of its small size, an average of 1.9 g, it is difficult to harvest mechanically, but it is very highly regarded because the trees produce a large amount of fruit with a relatively high oil yield of 20.5%. The resulting oil is dense and fluid, tasting of orchard fruit (Nieves et al., 2004).

Extra-virgin olive-oil (EVOO) composition determines its intrinsic quality and could be influenced by several factors, agronomical and technological factors, such as olive cultivar (Tura et al., 2007), the climate, degree of maturation (Cerretani et al., 2006; Lazzez et al., 2008), crop season (Rodney, Ayton, & Graham, 2010) and the production process (Lozano-Sánchez, Cerretani, Bendini, Segura-Carretero, & Fernández-Gutiérrez, 2010; Servili et al., 2007). However, geographical area is greatly responsible for the specific characteristics of olive oil (Petrakis, Agiomyrgianaki, Christophoridou, Spyros, & Dais, 2008). The relationship between geographical area and quality indices as well as chemical composition of other EVOO varieties has also been studied

by several authors. In this way, many studies have classified olive oils according to geographical origin by means of statistical analysis applied to fatty acids and sterol compositions (Longobardi et al., 2012) volatile compositions (Kotti, Cerretani, Gargouri, Chiavaro, & Bendini, 2009; Ouni et al., 2011) and minor components, especially phenolic compounds. Several significant differences between the total phenolic content calculated by spectrophotometric method based on Folin-Ciocalteau reactive have been reported in the literature (Salvador, Aranda, Gomez-Alonso, & Fregapane, 2003). However, these differences were not observed among all samples under studies which were from different geographical area. Consequently, it was not possible to classify the olive oils according to the geographical origin. The individual quantitation of the phenolic compounds using chromatographic determination in combination with several quality index, like oxidative stability has made it capable of classifying the EVOO samples according to their respective production area (Alkan, Tokatli, & Ozen, 2012; Taamalli, Arraez Roman, Zarrouk, Segura-Carretero, & Fernandez-Gutierrez, 2011; Taamalli, Gómez-Caravaca, Zarrouk, Segura-Carretero, & FernándezGutiérrez, 2010). It should be taken into account that in most cases the EVOOs from different geographical area used in these studies were not from the same olive fruit varieties. The combination of the individual quantitation of the phenolic profile by high performance liquid chromatography coupled to time-of-flight mass spectrometry (HPLC-EST-TOF) with statistical treatment like discriminant analysis could be a powerful tool to classify monovarietal Arbequina EVOOs from different regions of southern Catalonia.

Polyphenols are an important group of natural compounds, which are produced in the secondary metabolism of many plants in nature. The main classes of phenolic compounds described in Arbequina EVOO are phenolic acids, phenolic alcohols, secoiridoids, lignans, and flavonoids. These compounds affect the sensory and health properties of EVOO (Bendini et al., 2007; Christophoridou & Dais, 2009; Fu et al., 2009; Servili et al., 2009). Phenolic acids, phenolic alcohols that include the (3,4-dihydroxyphenyl)ethanol (3,4-DHPEA or hydroxytyrosol) and (*p*-

hydroxyphenyl)ethanol (*p*-HPEA or tyrosol), and flavonoids such as luteolin and apigenin are present in small amounts, while secoiridoids and lignans are the most concentrated phenolic compounds (García, Brenes, García, Romero, & Garrido, 2003; Gómez-Rico, Fregapane, & Salvador, 2008; Oliveras-López et al., 2007). The most abundant secoiridoides of Arbequina EVOO are the dialdehydic form of decarboxymethyl elenolic acid linked to (3, 4 dihydroxyphenyl) ethanol or (*p*-hydroxyphenyl) ethanol. Oleuropein and ligstroside aglycones were also detected but were found in low concentration (Lozano-Sánchez, Segura-Carretero et al., 2010). Concerning lignans, their concentrations in Arbequina olive oil are relatively high, especially acetoxypinoresinol (García-González, Tena, & Aparicio, 2010).

The marketing of EVOO is increasingly directed towards the differentiation and characterization of products from different geographical areas. The aim of the present work is to analyse the qualitative and quantitative differences of phenolic compounds in commercial virgin olive oils from Arbequina variety cultivated in different olive-growing regions in southern Catalonia using HPLC-ESI-TOF/MS. The findings of this study may provide ways for classifying Arbequina olive oils according to their phenolic profiles as the geographical indicators. To our knowledge, this is the first time that the monovarietal Arbequina EVOOs have been classified according to their geographical origin.

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Methanol and *n*-hexane, reagents used for extracting the phenolic compounds from the olive-oil samples, and sodium hydroxide used to prepare the calibration solution, were purchased from Panreac (Barcelona, Spain). Acetic acid from Fluka and Sigma-Aldrich (Steinheim, Germany), and methanol were used for preparing the mobile phase. Water was deionized using a Milli-Q-system (Millipore, Bedford, MA, USA). Standard compounds such as hydroxytyrosol, tyrosol, luteolin, apigenin, and quinic acid were purchased by Sigma-Aldrich

(St. Louis, MO, USA), and pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein was purchased 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 °C.

2.2. Samples

The EVOOs used in this study were of the Arbequina variety from different producing regions in southern Catalonia. Samples were classified in three groups (group 1, group 2, and group 3) according to their geographical origin, which were demarcated taking into account the edaphological characteristics and orography (**Fig. 1**). A total of 32 olive-oil samples were obtained from different mills: eleven EVOOs produced in the first region (group 1), fourteen in the second region (group 2) and seven in the third one (group 3) (September 2011). The oils were extracted from monovarietal Arbequina olive fruit by continuous industrial plants equipped with a hammer crusher, a horizontal malaxator, and a two-phase decanter. Polar phenols, were isolated from the EVOOs using solid-phase extraction (SPE) with Diol-cartridges (bed weight 1000 mg, 6 mL of tube size), following the method described by Lozano-Sánchez, Segura-Carretero, et al. (2010). EVOO (60 g) was dissolved and loaded onto the column. The cartridge was washed with 15 mL of *n*-hexane, which was then discarded in order to remove the nonpolar fraction of the oil. Finally, the sample was recovered by passing through 40 mL of methanol and the solvent was evaporated under vacuum at 35 °C. The residue was dissolved with 2 mL of methanol and filtered through 0.25 *μ*m filter before the HPLC analysis. The olive-oil extracts were diluted (1:10, v: v) with methanol.

2.3. Chromatographic separation

HPLC analyses were made with an Agilent 1200 series rapid resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). Separation was carried out with a 150 mm× 4.6 mm, 1.8 μm, Zorbax Eclipse Plus RP-C18 analytical column (Agilent Technologies, Palo Alto, CA, USA). Gradient elution

was conducted using water with 0.25% acetic acid as eluent A and methanol as eluent B at a constant flow rate of 0.80 mL/min using the following gradient: 0 min, 5% B; 7 min, 35% B; 12 min, 45% B; 17 min, 50% B; 22 min, 60% B; 25 min, 95% B, 27 min, 5% B, and finally a conditioning cycle of 5 min with the same conditions for the next analysis. The injection volume in the HPLC system was 10 µL and the analysis was made at room temperature (Lozano-Sánchez, Segura-Carretero et al., 2010).

2.4. ESI-TOF/MS conditions

TOF-MS was conducted using a microTOF™ (Bruker Daltonics, Bremen, Germany) orthogonal-accelerated TOF mass spectrometer equipped with electrospray ionization (ESI) interface. The parameters for analysis were set using negative-ion mode with spectra acquired over a mass range of 50–1000 m/z. The optimum values of source parameters were: capillary voltage of +4 kV; drying gas temperature, 190 °C; drying gas flow, 9 L/min; nebulizing gas pressure, 2 bar, and end plate offset, −0.5 kV. The values of transfer parameters were: capillary exit, −120 V; skimmer 1, −40 V; hexapole 1, −23 V; RF hexapole, 50 Vpp, and skimmer 2, −22.5 V (Lozano-Sánchez, Segura-Carretero et al., 2010).

The flow delivered into the MS detector from HPLC was split using a flow splitter (1:3) to achieve stable electrospray ionization and reproducible results. External mass-spectrometer calibration was performed using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA) containing sodium acetate clusters solution (5 mM sodium hydroxide and water:2-propanol 1:1 (v/v) with 0.2% of acetic acid). The calibration solution was injected at the beginning of the run, and all the spectra were calibrated prior to polyphenol identification. The accurate mass data for the molecular ions were processed using the software Data Analysis 4.0 (Bruker Daltonik), which provided with a list of possible elemental formulas by using the Generate Molecular Formula Editor. The latter uses a CHNO algorithm providing standard functionalities such as minimum/maximum elemental

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

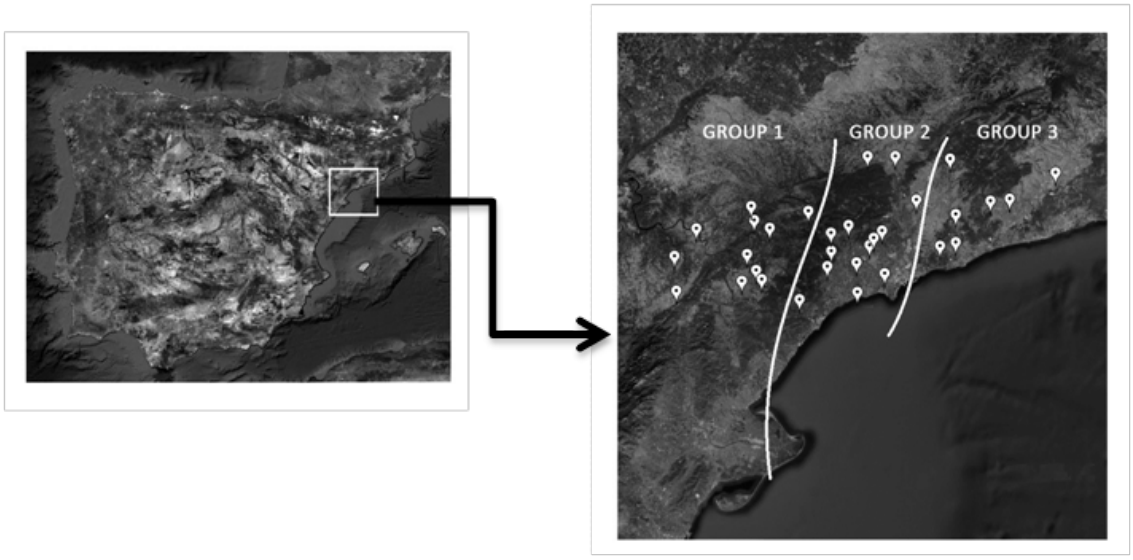

Fig. 1. Map showing the distribution of oil samples from the south of Catalonia

2.5. Statistical analysis

The statistical procedure was based on discriminant analysis (DA). This statistical technique is based on the extraction of discriminant functions of the independent variable by means of qualitative dependent variables and the quantitative independent variables. The procedure automatically chooses a first function that will separate the groups as much as possible. It then chooses a second function that is both uncorrelated with the first function, and provides as much further separation as possible. The procedure continues adding functions in this way until reaching the maximum number of functions as determined by the number of predictors and categories in the dependent variable. We used as statistical software SPSS 13.0 for windows (SPSS Inc., Chicago, IL, USA).

3. Results and discussions

3.1. Qualitative characterisation of olive-oil phenolic compounds

UV chromatograms obtained at 280 and 240 nm and base-peak chromatogram (BPC) of a representative Arbequina EVOO sample are presented in **Fig. 2**. The tentatively identified phenolic compounds are summarized in **Table 1** including retention times, experimental and calculated m/z, molecular formula, error, and msigma values.

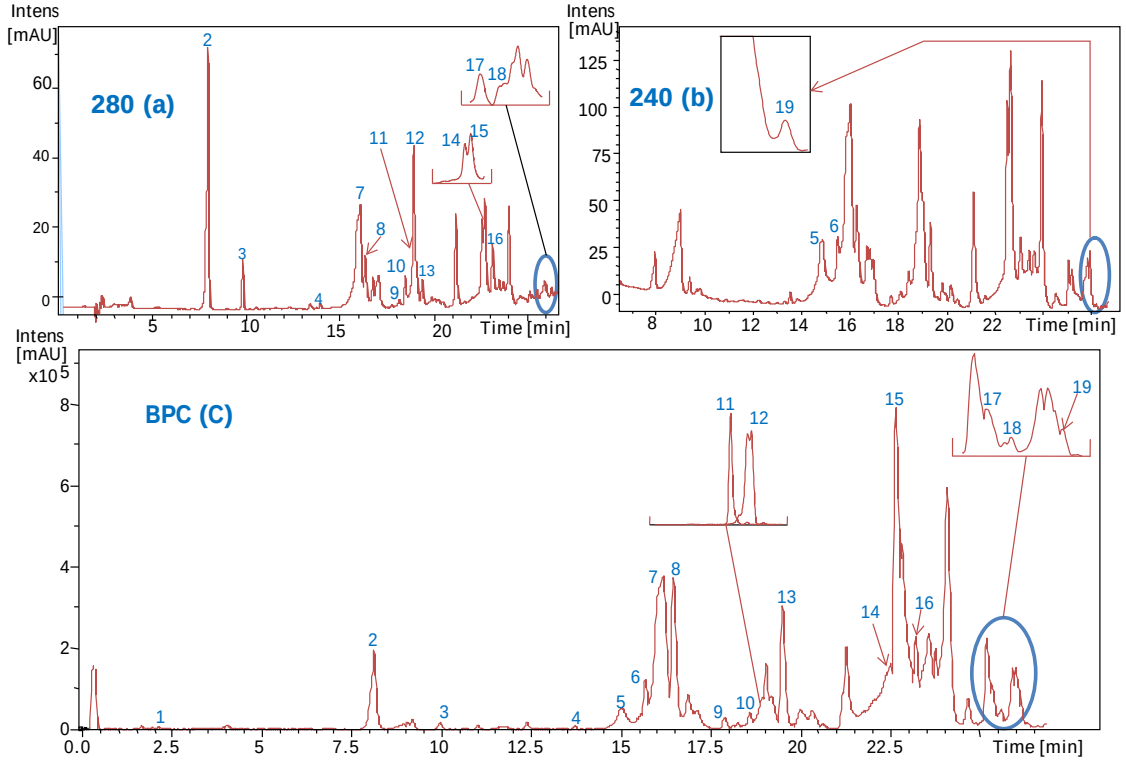

Fig. 2. HPLC-UV chromatograms detected at 280 (a) and 240 (b) nm of a representative Arbequina EVOO phenolic extract. Base-peak chromatogram (BPC) of the same EVOO phenolicextract (c) obtained by HPLC-ESI-TOF.

The phenolic compounds were identified by comparing both the retention times and the MS spectral data from olive-oil samples and standards detailed in the Materials and Methods section. The remaining compounds, for which no commercial standards were available, were identified by the interpretation of their mass spectral results provided by the TOF-MS and information previously reported (Lozano-Sánchez, Segura-Carretero et al., 2010). The analysis of

phenolic extracts enabled the identification of 18 phenolic compounds. The results showed no qualitative differences in the phenolic profile among the olive oils from different geographical regions.

Table 1. Main phenolic compounds identified in a representative extract of Arbequina EVOO variety by HPLC-ESI-TOF/MS.

Peaks	Compounds[a]	RT (min)[b]	Molecular Formula	m/z calcd [c]	m/z Exptl [d]	Error (ppm)	Msigma
1	Quinic acid	2.4	$C_7H_{11}O_6$	191.056	191.057	-1.0	8.6
2	HYTY	8.2	$C_8H_{10}O_3$	153.056	153.055	3.2	5.6
3	TY	10.0	$C_8H_{10}O_2$	137.060	137.062	-6.3	49.6
4	HYTY-Ac	14.1	$C_{10}H_{12}O_4$	195.066	195.067	-4.1	31.1
5	EA	15.0	$C_{11}H_{14}O_6$	241.072	241.072	1.2	9.2
6	H-EA	15.5	$C_{11}H_{14}O_7$	257.067	257.066	2.0	1.9
7	DOA	16.1	$C_{17}H_{20}O_6$	319.119	319.118	-2.4	1.60
8	H-DOA	16.4	$C_{17}H_{20}O_7$	335.114	335.114	-1.3	5.1
9	Syringaresinol	17.9	$C_{22}H_{26}O_8$	417.156	417.155	0.7	6.0
10	Pin	18.5	$C_{20}H_{22}O_6$	357.134	357.133	3.0	6.0
11	AcPin	19.0	$C_{22}H_{24}O_8$	415.140	415.139	2.0	8.8
12	D-Lig Agl	19.1	$C_{17}H_{20}O_5$	303.123	303.122	6.7	8.5
13	H-D-Lig Agl	19.4	$C_{17}H_{20}O_6$	319.119	319.118	3.2	4.3
14	10-H-Ol Agl	22.5	$C_{19}H_{22}O_9$	393.119	393.120	-1.0	1.5
15	Ol Agl	22.7	$C_{19}H_{22}O_8$	377.124	377.124	0.8	2.8
16	lut	23.1	$C_{15}H_{10}O_6$	285.041	285.040	2.2	1.9
17	Lig Agl	25.2	$C_{19}H_{22}O_7$	361.129	361.128	3.4	8.4
18	Apig	25.5	$C_{15}H_{10}O_5$	269.045	269.044	5.7	5.1
19	Methyl Ol Agl	26.0	$C_{20}H_{24}O_8$	391.140	391.139	1.8	3.8

[a] HYTY, hydroxytyrosol; TY, tyrosol; HYTY-Ac, hydroxytyrosol acetate; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-DOA, hydroxy decarboxymethyl oleuropein aglycone; Pin, pinoresinol; AcPin, acetoxypinoresinol; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; 10-H-Ol Agl, hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lut, luteolin; Lig Agl, ligstroside aglycone; Apig, apigenin; Methyl Ol Agl, Methyl oleuropein aglycone.

[b] RT, retention time

[c] m/z Calcd: Calculated mass; [d] m/z Exptl: Experimental mass

The main phenolic alcohols found in the Arbequina EVOO were hydroxytyrosol (HYTY) and tyrosol (TY). Moreover, in all olive-oil samples, hydroxytyrosol derivative previously described in the olive oil as hydroxytyrosol acetate was identified (HYTY-Ac) (Ouni et al., 2011).

In the secoiridoid group, both oleuropein aglycone (Ol Agl) and ligstroside aglycone (Lig Agl) were identified in the samples. Decarboxymethylated and hydroxylated derivatives were also detected: decarboxymethyl oleuropein aglycone (DOA), decarboxymethyl ligstroside aglycone (D-Lig Agl), hydroxy oleuropein aglycone (10-OH-Ol Agl), hydroxy decarboxymethyl ligstroside aglycone (H-DLig Agl) and hydroxy decarboxymethyl oleuropein aglycone (H-D-OL Agl). Elenolic acid (EA) and its derivative; hydroxylated form (H-EA) which may not be considered as polyphenols, but as secoiridoids derivatives were also identified.

With regard to lignans, pinoresinol (Pin), its derivative acetoxipenoresinol (AcPin), and another compound described in olive oil in recent years as syringaresinol also belonging to this group, were identified. Finally, in the flavonoid group, the flavones identified were apigenin (Apig) and luteolin (Lut). Among the other polar compounds, quinic acid was also identified in the EVOO extracts. **Fig. 3** shows extracted ion chromatogram (EIC) for the representative phenolic compounds identified in each family and its chemical structure.

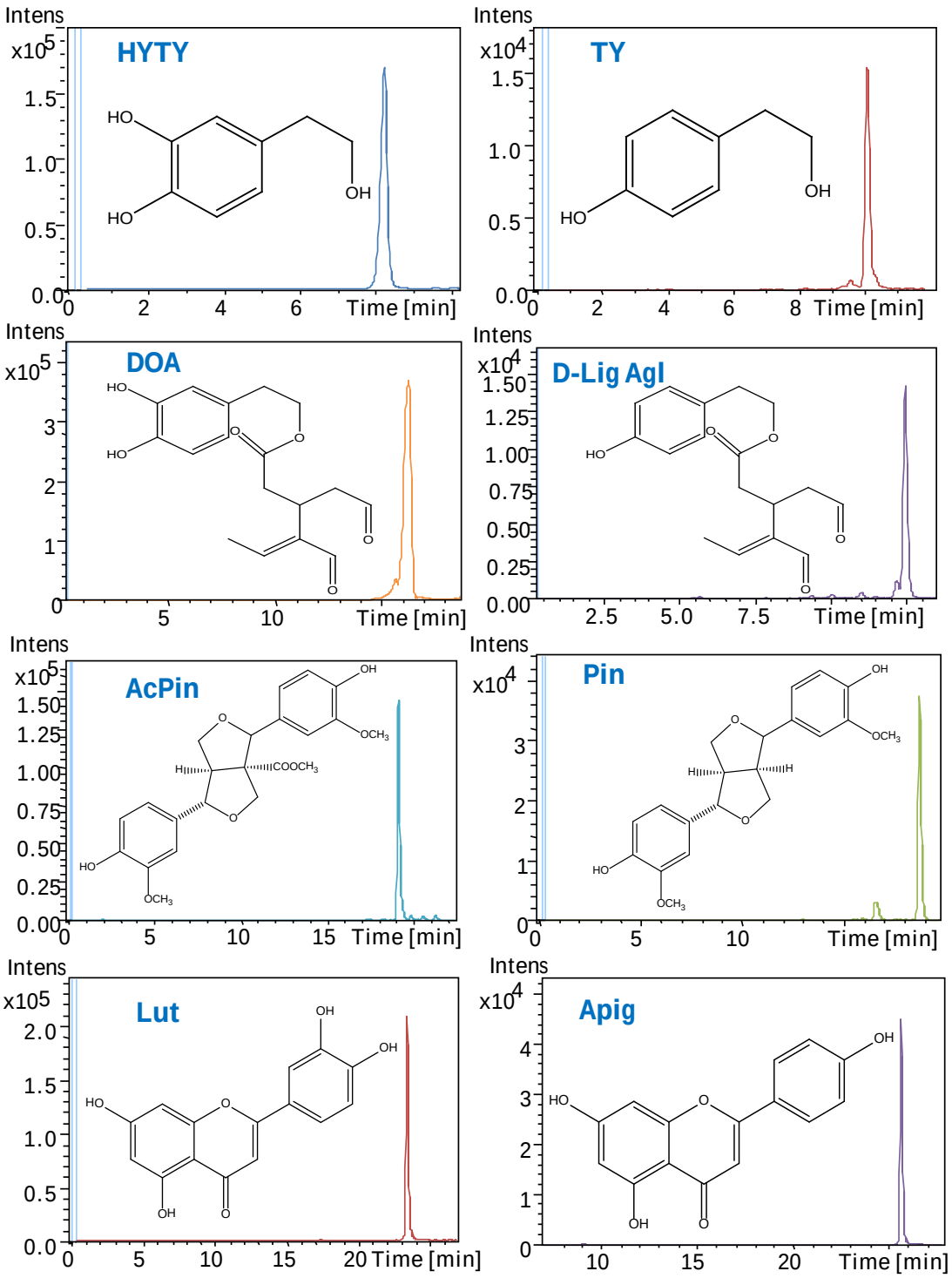

Fig. 3. Extracted ion chromatogram (EIC) of main phenolic compounds identified in Arbequina EVOO: (HYTY) hydroxytyrosol; (TY) tyrosol; (DOA) decarboxymethyl oleuropein aglycone; (D-Lig Agl) decarboxymethyl ligstroside aglycone; (AcPin) acetoxypinoresinol; (Pin) pinoresinol; (Lut) luteolin; (Apig) apigenin.

3.2. Quantitative characterisation of olive-oil phenolic compounds

The phenolic compounds were quantitated by HPLC-ESI-TOF/MS. Seven standard calibration curves of the main compounds found in the samples were prepared using seven commercial standards. All calibration curves showed good linearity over the study range (r^2> 0.991). The individual concentrations were determined using the area of each individual compound and by interpolation of the corresponding calibration curve shown in **Table 2**. Phenolic compounds hydroxytyrosol, tyrosol, luteolin, apigenin, and pinoresinol such as quinic acid (another polar compound) were quantitated by the calibration curves drawn from their respective commercial standards. The other phenolic compounds, which had no commercial standards, were tentatively quantitated on the basis of other compounds with similar structures. Hydroxytyrosol acetate was quantitated using a hydroxytyrosol calibration curve, acetoxypinoresinol, and syringaresinol using a pinoresinol calibration curve. Regarding secoiridoid group, all these compounds were quantitated with oleuropein standard. It should be taken into account that the response of the standards could differ from that of the analytes in the oil samples, and consequently the quantitation of these compounds is only an estimation of their actual concentrations.

Table 2. Standards curves

Commercial standards	Calibration curves	R^2
Quinic acid	y = 11056701.368x + 8205.227	0.996
Hydroxytyrosol	y = 6418598.422x + 12740.112	0.996
Tyrosol	y = 1576003.317x + 7054.169	0.995
Pinoresinol	y = 25 299 560.324x – 55.453	0.993
Oleuropein	y = 33528830.117x - 36149.051	0.995
Luteolin	y = 54196530.683x + 200221.620	0.994
Apigenin	y = 45565507.044x + 227298.586	0.992

The concentrations of phenolic compounds of olive-oil samples from different geographical area are given in **Table 3**. The analysis of the profiles of the different oils varied in the quantitative distribution of some phenolic

compounds detected. As a means of explaining those differences, the phenolic fraction was divided into four main groups (phenol alcohols, secoiridoid derivatives, lignan, and flavonoids).

In all olive-oil samples studied, secoiridoids were the most abundant, followed by lignans, phenolic alcohols and flavonoids, respectively. The percentage of each family in the total phenolic fraction is given in **Fig. 4**. The major secoiridoid compounds and their derivatives quantitated were decarboxymethylated form of oleuropein aglycone (DOA) and elenolic acid (EA). The former varied from 78.4 mg/kg (group 3) to 116 mg/kg (group 1) and the latter from 9.3 mg/kg (group 2) to 16.6 mg/kg (group 1). Oleuropein aglycone and other ligtroside aglycone secoiridoids identified were found in low concentrations. These compounds were also found in low concentrations in Arbequina olive oil in previous works (Lozano-Sánchez, Segura-Carretero et al., 2010). The highest concentration of the first component (Ol agl) was recorded in group 1 with (5.8 mg/kg) whereas the lowest was detected in group 2 (3.1 mg/kg). For the second component (Lig agl), EVOO from group 3 showed the lowest value (0.37 mg/kg) and the highest value was registered in group 2 (1.8 mg/kg). Hydroxylation product of dialdehydic form of decarboxymethyl oleuropein aglycone (H-DOA) and, decarboxylated form of ligtroside aglycone (D-Lig Agl) were found in relatively higher concentrations in the olive oils of group 1. Hydroxy elenolic acid and methyl oleuropein aglycone (H-EA, Methyl Ol Agl) were not quantitated in olive oils from group 2 and group 3 because their concentrations were between their detection and quantitation limits detailed previously (Lozano-Sánchez, Segura-Carretero et al., 2010).

Concerning the amounts of phenyl alcohols, HYTY and TY were the most abundant compounds quantified of this family. Their concentration ranged from 2.6 mg/kg (group 2) to 4.1 mg/kg (group 1) and from 1.1 mg/kg (group 2) to 1.57 mg/kg (group 1), respectively. HYTY-Ac, a derivative compound of HYTY, was also quantitated.

Lignans were also found in considerable amount, particularly acetoxypinoresinol (Ac Pin) and pinoresinol (Pin) which reached 22 mg/kg and 4.1 mg/kg, respectively, in olive oil from group 2. Meanwhile, syringaresinol was detected in low concentrations, not exceeding 0.41 mg/kg.

Table 3. Phenolics content in Arbequina EVOOs according to geographical region (mg/kg).

Compounds [a]	Group1	Group2	Group3
HYTY	4.1 ± 0.4	2.6 ± 0.2	2.9 ± 0.3
TY	1.57 ± 0.09	1.1 ± 0.1	1.46 ± 0.01
HYTY-Ac	0.71 ± 0.06	1.4 ± 0.1	1.5 ± 0.2
H-EA	1.1 ± 0.1	NQ [b]	NQ
D-Lig Agl	4.6 ± 0.1	3.3 ± 0.3	3.8 ± 0.1
DOA	116 ± 6	88 ± 6	78.4 ± 4.5
H-D-Lig Agl	2.6 ± 0.2	1.3 ± 0.1	1.61 ± 0.07
EA	16.6 ± 0.7	9.3 ± 0.7	11.9 ± 1.1
H-DOA	7.2 ± 0.3	5.1 ± 0.5	5.5 ± 0.5
Lig Agl	0.39 ± 0.03	1.8 ± 0.1	0.37 ± 0.03
Ol Agl	5.8 ± 0.3	3.1 ± 0.1	3.63 ± 0.01
Methyl Ol Agl	0.5 ± 0.2	NQ	NQ
Pin	2.94 ± 0.09	4.1 ± 0.4	3.9 ± 0.3
AcPin	18 ± 1	22 ± 2	19.6 ± 0.8
Syringaresinol	0.32 ± 0.03	0.41 ± 0.01	0.38 ± 0.02
Apig	0.79 ± 0.02	0.75 ± 0.04	0.82 ± 0.02
Lut	2.23 ± 0.02	2.3 ± 0.1	1.9 ± 0.2
Quinic acid	0.51 ± 0.03	0.76 ± 0.08	0.57 ± 0.05

[a] HYTY, hydroxytyrosol; TY, tyrosol; HYTY-Ac, hydroxytyrosol acetate; H-EA, hydroxy elenolic acid; D-Lig Agl, decarboxymethyl ligstroside aglycone; DOA, decarboxymethyl oleuropein aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; EA, elenolic acid; H-DOA, hydroxy decarboxymethyl oleuropein aglycone; Lig Agl, ligstroside aglycone; Ol Agl, oleuropein aglycone; Pin, pinoresinol; AcPin, acetoxypinoresinol; Lut, luteolin; Apig, apigenin.

[b] NQ: Not Quantitated

Regarding flavonoids such as luteolin and apigenin were quantitated in all the olive oils studied. The concentration of luteolin was higher than that of apigenin. The major concentrations of the former and latter were detected in

olive oil from groups 2 and 3, respectively. These results confirm this chemical class as a minor constituent of the polyphenol fraction, as previously described for other EVOO (García-Villalba et al., 2010). However, the behaviour of flavonoids differed from that of other families. This family showed a relative stability in concentration among geographical areas studied.

The EVOOs obtained from the three demarcated region shown differences related to the amounts of secoiridoids, phenolics alcohols, lignans and flavones. Taking into account that all EVOOs under study were obtained from Arbequina olive fruit variety and the same elaboration process, the differences could be attributed to the climate, edaphological characteristics and orography between the regions studied. The effects of these variables on the phenolic composition have also been mentioned by other authors (Alkan et al., 2012; Ocakoglu et al., 2009; Tura et al., 2007). Future researches are warranted to evaluate their effects on the phenolic composition of Arbequina EVOO variety.

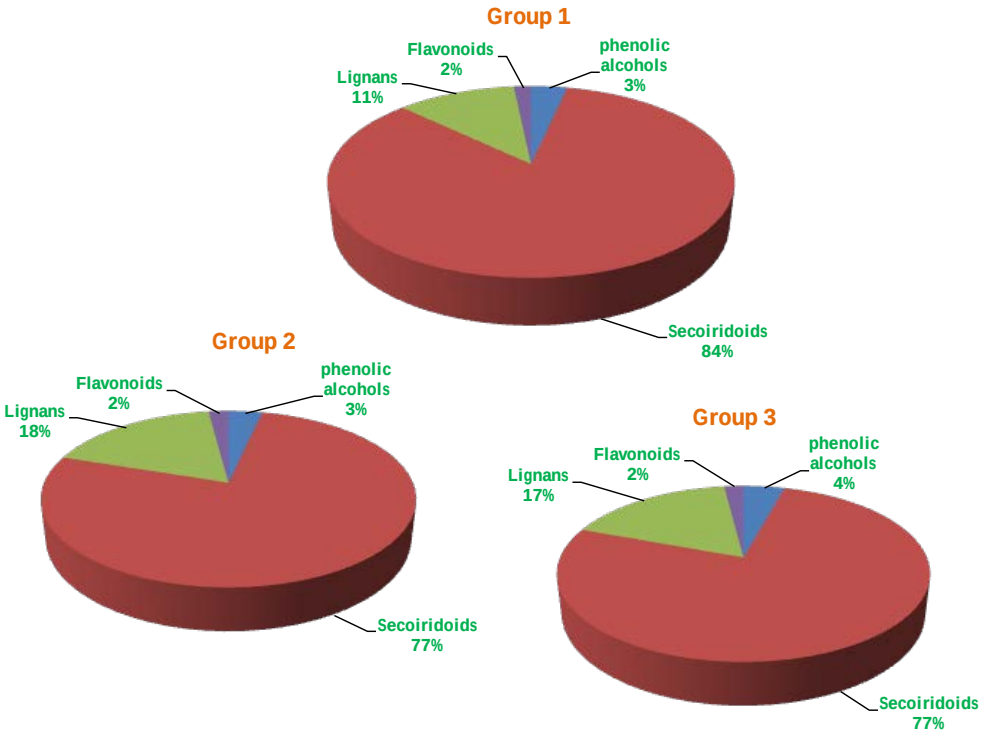

Fig. 4. Types of phenolic compounds in Arbequina EVOO according to geographical region (%).

3.3. Chemometrics

3.3.1. Discriminant analysis

To evaluate the possibility of differentiating Arbequina EVOOs according to their geographical area taking into account the phenolic fraction, we applied a discriminant analysis (DA) for the results of the HPLC–ESI-TOF-MS analyses of the quantitated phenolic profile. The amount of all the phenolic compounds was considered to identify the two main functions. The leave-out cross-validation method was used to test the ability for prediction classification. Classification parameters such as number of eigenvalue, canonical correlation, Wilk's lambda and Chi-square coefficients were also shown in **Table 4**. The first two discriminant functions are shown to account 74.3 and 25.7% of the total discriminating power (eigenvalue). The first was associated mainly with pinoresinol, elenolic acid, decarboxymethyl ligstroside aglycone, hydroxy decarboxymethyl ligstroside aglycone, syringaresinol, acetoxypenoresinol, ligstroside aglycone, hydroxylelenolic acid, hydroxy decarboxymethyl oleuropein aglycone, oleuropein aglycone, methyl oleuropein aglycone, hydroxytyrosol, and tyrosol. The second was associated primarily with hydroxytyrosol-acetate, luteolin, decarboxymethyl oleuropein aglycone, and apigenin. The canonical correlation measures the association between the discriminant scores and the groups; a high value (near 1) shows that the discriminant functions were correct. In **Table 4**, the canonical correlation showed 0.944 and 0.859 for the first and the second discriminant function, respectively. Wilk's Lamda indicates the proportion of the total variance in the discriminant scores not explained by differences among groups. A low lambda value (near 0) indicates that the group's mean discriminant scores differ. The sig ($p < 0.001$) is for the Chi-square test, which indicates a highly significant difference between the group's centroids. The predicted results provided a percentage of predicted membership values according to the geographical area of 100%, signifying that all the objects were correctly classified. When the cross-validation procedure was applied, the percentage (71.9%) was calculated.

Table 4. Classification parameters and results.

Eigenvalues					
Function	Eigenvalue	% of variance	% Cumulative	Canonical correlation	
1	8.174[a]	74.3	74.3	0.944	
2	2.823[a]	25.7	100	0.859	
[a] First 2 canonical discriminant functions were used in the analysis					
Wilks' Lamda					
Test of function (s)		Wilks' Lamda	Chi-square	df	Sig
1 through 2		0.029	74.706	34	0
2		0.262	28.163	16	0.03

Classification results[b,d]						
		Geographical area (samples groups)	Predicted group membership			Total
			1	2	3	
Original	Count	1	11	0	0	11
		2	0	14	0	14
		3	0	0	7	7
	%	1	100	0	0	100
		2	0	100	0	100
		3	0	0	100	100
Cross -Validated[c]	Count	1	7	2	2	11
		2	1	12	1	14
		3	2	1	4	7
	%	1	63.6	18.2	18.2	100
		2	7.1	85.7	7.1	100
		3	28.6	14.3	57.1	100

[b] 100% of original grouped cases correctly classified.

[c] Cross validation is done only for those cases in analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.

[d] 71.9% of cross-validated grouped cases correctly classified.

Fig. 5 showed a projection of the score corresponding to olive-oil samples in the two-dimensional space defined by the first and second discriminant functions. A good resolution among all the samples according to the geographical origin was achieved (group 1, group 2, group 3). The first function distinguishes between samples of group 1 and group 3, on one hand, the variables of which have positive coefficients, and those of group 2, on the other hand. Meanwhile, the latter separates samples of groups 1 and 2 with respect to those of group 3. According to the results found, we conclude that a good differentiation among observations was achieved with DA using all the variables under study and without selection of the most discriminant variables.

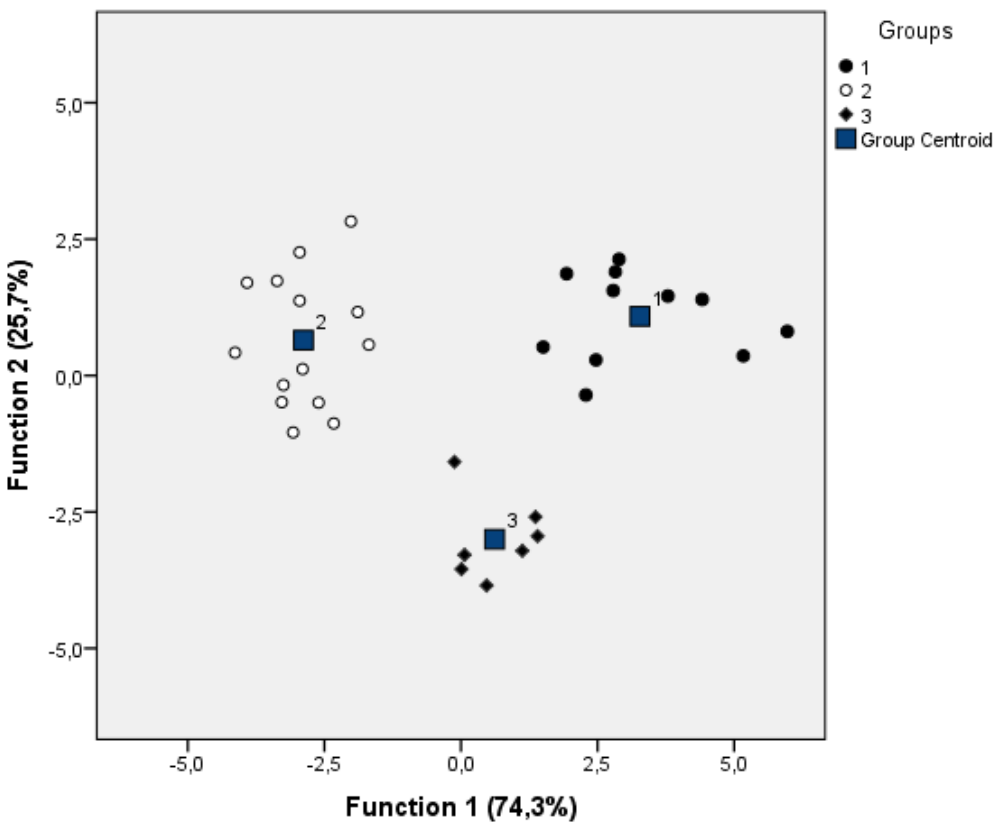

Fig. 5. Score plot on the plane of the two discriminant functions obtained to predict the geographical origin of EVOOs.

4. Conclusions

In this study, phenolic compounds of Arbequina EVOO from different regions of southern Catalonia were characterized by HPLC-ESI-TOF/MS. All the olive oils analysed showed very low concentrations of flavonoids and higher concentrations of secoidridoid compounds and derivatives. DA differentiated the olive oils according to geographical area, taking into consideration all the phenolic compounds quantitated without a variable reduction step. All these compounds could be used as a fingerprint to characterize and differentiate these olive oils based on geographical origin.

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Chapter 2

Changes in Chemlal olive oil chemical characteristics with regard to irrigation regime and harvest time

Changes in Chemlal olive oil chemical characteristics with regard to irrigation regime and harvest time

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Abstract

This study investigated the changes in Chemlal olive oil composition obtained under two different irrigation treatments on three harvest dates, corresponding to three ripeness indices. First, the olive grove was divided into two parts: one was under rain fed conditions (non-irrigated) and the other was irrigated with 100% of the crop evapotranspiration (ETc). Olive oil samples were obtained from irrigated and non-irrigated olives on three harvest dates for posterior analysis. According to the results, in all olive oils under study, the quality parameters were found to be within the limit established for extra virgin olive oil. Oil yield was positively affected by irrigation, whereas fatty acid composition was only slightly varied without any nutritional relevance. No significant differences were observed in chlorophyll content. Nevertheless, carotenoids were significantly higher in the olive oil from non-irrigated trees. In order to obtain detailed information about the phenolic behaviour, the characterisation of individual phenolic compounds was carried out using high performance liquid chromatography coupled with electrospray time-of-flight mass spectrometry (HPLC-ESI-TOF/MS). The range

of the total phenolic content was not highly different among olive oils from the two irrigation treatments. However, there was a clear tendency, in the most phenolic families, to increase the concentration as the irrigation was applied to the olive trees. Within each treatment, a significant effect of olive ripening was obtained in the analysed parameters, and the time course during maturation process, mainly of phenolic compounds, was found different between the two irrigation treatments. From the obtained results under the experimental conditions of this study, the optimum harvest period for the Chemlal variety was dependent on the agronomic conditions, being earlier when the trees were under irrigation.

Keywords: Chemlal olive oil composition, non-irrigated, irrigated, ripening, phenolic compounds, HPLC-ESI-TOF/MS.

1. Introduction

The olive (*Olea europea* L.) is native to the coastal areas of the eastern Mediterranean basin. These include southeast Europe, western Asia and North Africa. Historically, olives were produced under dry land conditions, with widely spaced trees, to take full advantage of the water stored in the soil during winter rains for the next spring and summer's growth (Issaoui et al., 2013). It is considered one of the best-adapted species to the semiarid environment. The drought resistance of olive trees is attributed to the well developed root system (which become wider if the soil is dry), and the particular leaf function (Dabbou et al., 2011b). However, under these conditions, there is usually a decrease in photosynthesis that limits its growth and yield. Recently, due to the well-known health properties of olive oil, its demand in the international market increased, encouraging growth in the amount of land devoted to olive production. As a result, growers are showing increased interest in improving the productivity of their orchards. Irrigation is a vital factor in improving both production and productivity (Caruso et al., 2014; Naor et al., 2012).

In this sense, many irrigation strategies have been proposed. The first was a full irrigation system, in which the amount of water supplemented to the olive tree is equal to the amount that is estimated to be lost via crop evapotranspiration (ETc). Second, deficit irrigation strategies were proposed, in which the amount of water supplemented to the olive tree is less than that estimated as lost by ETc (Dabbou et al., 2010). The applicability of each strategy depends on local circumstances and the need to limit the amount of water used. The reported studies have shown differences in yield and chemical composition of olive oil from irrigated and rain fed olive trees. However, the studies differ in the volume of water applied to trees, as well as the olive varieties used in each study (Dabbou et al., 2010; Dag et al., 2008; Stefanoudaki et al., 2009). As it is well known, there is a strong varietal differences in the response of olive trees to water status in soil (Stefanoudaki et al., 2009).

In Algeria, olive oil production is relatively low in comparison to the rest of the Mediterranean countries. This was due to the fact that 90% of Algerian orchards are traditional, located in the mountain areas and marginal lands, characterised by tree aging and shortage of different agronomic practices such as pruning, phytosanitary treatments, and irrigation. Irrigation is only practiced in 10% of Algerian orchards. Besides low production, the produced olive oil is considered as unsuitable for human consumption, limiting its market share (Hadjou et al., 2013). In an effort to overcome this difficult situation, intervention of the Algerian government was necessary. Different programmes were proposed aimed at increasing productivity and improving the Algerian olive oil quality.

Within these programmes, many experts were asked to find a solution for the olive oil sector, studying the chemical composition of Algerian olive oil and the possible factors that could affect its production and quality. In a previous study, we published data in which the best period of harvesting was established for the Chemlal variety (Bengana et al., 2013). However, the trees were grown under irrigation conditions, which make them non-representative, taking into account that 90% of Algerian orchards are grown under rain fed conditions. Therefore, the objective of this work was to study the changes in Chemlal olive oil's chemical composition from non-irrigated and irrigated trees obtained on three harvest dates, in order to obtain the maximum information about the time course of its component under both water status conditions. As it is the first time that a comparative study has been carried out, full irrigation with 100% of ETc was chosen as the irrigation programme.

2. Materials and methods

2.1. Samples

The experiment took place during the 2013–2014 crop season using Chemlal olive cultivar grown in the Akbou region, situated in the northeastern part of Algeria (latitude 36° 27' north and longitude 4° 33' east). The climate of the area is Mediterranean, with an approximate annual average rainfall of 569.7

mm, concentrated mainly from autumn to spring. The warmer months are July/August and the coldest are January/February. **Fig. 1** shows the monthly means of temperature and precipitation for 2013.

The orchard was planted in 2003, and the trees were 12 years old when the experiment was carried out. The surface of the orchard was 1 hectare, with spacing among the trees of 6 x 6 m. In half of the orchard, the trees received a water irrigation amount that was equivalent to 100% of crop evapotranspiration, using a flood irrigation technique once a week, from June to September. The standard FAO formula for crop evapotranspiration (ETc = ETo x Kc x Kr) was used to calculate irrigation level, where ETo is the Penman-Monteith reference evapotranspiration over grass, Kc is the single crop coefficient, and Kr is the coefficient of reduction associated with percentage crop cover (Dabbou et al., 2011a). The other half of the orchard was not irrigated in order to carry out the comparative study.

The olives were harvested on three dates (D1, 24 October 2013; D2, 17 November 2013; D3, 13 December 2013). For each harvest, we randomly chose nine irrigated trees and nine non-irrigated ones. Approximately 1500g of olives were harvested manually from each tree. After that, we gathered the harvest of each set of three trees to form a sample of 4500 kg. Therefore, for each harvest date, we obtained three samples from irrigated trees and three samples from non-irrigated ones. A total of 18 samples were collected on the harvest dates under study. In addition, the olive ripeness index (RI) was determined according to the method previously reported in the literature (Morelló et al., 2004). This method was based on the assessment of the colour of the olive skin and pulp. RI values ranged from 0 (very green skin 100%) to 7 (100% purple flesh and black skin).

Immediately after each harvest, olive oil samples were obtained at the laboratory scale using the Abencor system (S.I.O.L. 20240 GHISONACCIA, France) equipped with a hammer crusher, malaxer, and centrifuge. Prior to the crushing step, the olives were manually sorted and cleaned, removing damaged fruits, leaves, and other debris. The clean and healthy olives were

crushed and were slowly mixed for 30 min at 25°C. Then, the resulting paste was subjected to centrifugal separation for 3 min at 3000 rpm. The liquid phase (oil and waste) was allowed to decant naturally into the specimens. The top oil layer was removed, stored in glass bottles at room temperature, and kept away from light until its analysis.

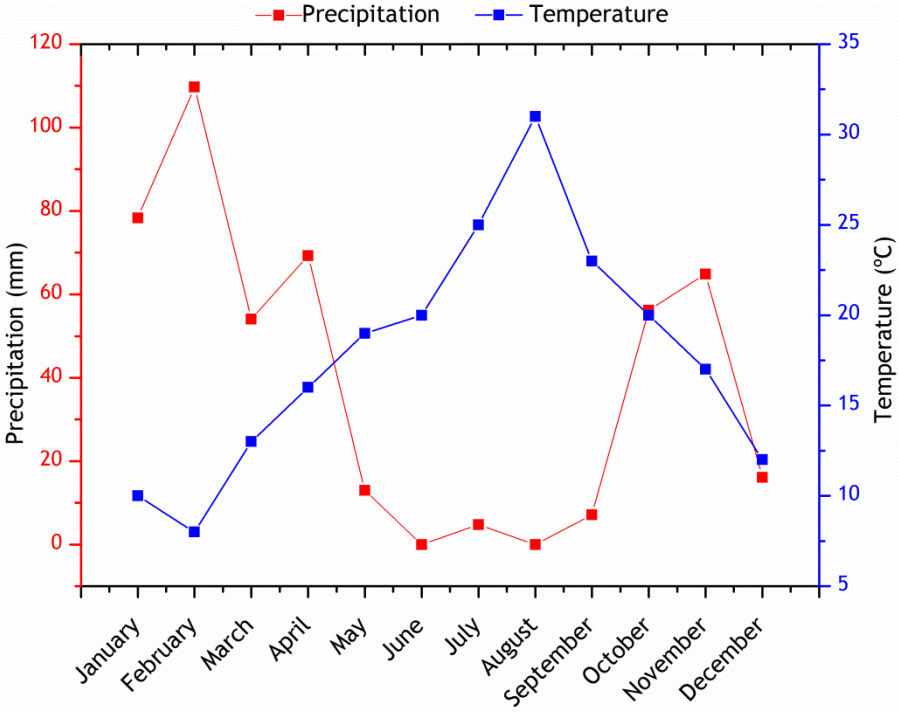

Fig. 1. Monthly means of temperature and precipitation during 2013**.**

2.2. Chemicals and apparatus

All chemicals were of analytical reagent grade. Cyclohexan, potassium hydroxide, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium hydroxide, isopropanol, *n*-hexane, and methanol were purchased from Merck (Darmstadt, Germany). Double deionised water with conductivity less than 18.2 MΩ.cm was obtained via a Milli-Q system (Millipore, Bedford, MA, USA). Standards of hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric acid, and vanillic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein and dihydrocaffeic acid were purchased from Extrasynthese (Lyon, France).

2.3. Oil yield

The olive oil content was determined according to the analytical method described in European Union Commission Regulation 2568/91, applied to olive pomace and the following amendments (EUC, 1991). The olives were dried at 80°C, and were then ground in a mortar. Afterwards, 10 g of the ground material was used for oil extraction in a Soxhlet apparatus for 8 h using n-hexane at 80°C. At the end of the extraction, a rotary evaporator separated the solvent.

2. 4. Moisture content

The moisture content of olive fruits was determined using an oven drying method at 105 ± 1 °C until a constant weight was achieved. The moisture content of the fruit was calculated as a percentage of loss of the fruit weight (Stefanoudaki et al., 2009).

2.5. Analytical determination of the quality parameters

Free fatty acids (FFA) (%), peroxide value (PV), and spectrometric UV (K_{232} and K_{270} nm) were determined according to the analytical methods described in European Union Commission Regulations 2568/91 and the following amendments (EUC, 1991). FFA was given as a percentage of oleic acid and PV expressed in milliequivalents of active oxygen per kg of oil (meq O2/kg). Spectrophotometric determinations were made using a UV mini-1800 instrument (Shimadzu Co., Kyoto, Japan). The K_{232} and K_{270} extinction coefficients were calculated from absorption at 232 and 270 nm, respectively.

2.6. Fatty acid composition

The fatty acid (FA) composition was determined according to the method described previously (Bengana et al., 2013). Prior to the chromatographic analysis, the fatty acids were converted to methyl esters using the following method: 0.2g of olive oil was added to 3 ml of *n*-hexane and 0.4 ml of methanolic potassium hydroxide 2N, followed by stirring. A gas chromatograph (GC) Chrompack CP 9002 (Les Ulis, France), equipped with a split/splitless

injector and flame-ionisation detector (FID), was used for this determination. The analytes were separated on a DB23 (50% cyanopropyl) capillary column (30m x 0.32 mm i.d., 0.25 µm film thickness; Agilent Technologies, Palo Alto, CA, USA) with nitrogen as the carrier gas (Linear velocity, 0.5 cm/min; split ratio of 1:30, v/v). The injection volume into a split GC port was 0.8µl. The temperature of the column was held constant throughout the entire separation at 200°C. The FID and the injector temperatures were 280, and 250°C, respectively.

2.7. Chlorophyll and carotenoids

Pigment amounts were calculated using the specific extinction values, via the method reported previously (Minguez-Mosquera et al., 1991), dissolving 7.5 g of olive oil in 25 ml of cyclohexane. The extinction coefficients applied were E0 = 613 for pheophytin 'a' and E0 = 2000 for lutein. Thus, pigment contents were calculated as follows:

[chlorophyll] (mg/kg) = (A_{670} x10^6)/ (613 x 100 x d)

[carotenoid] (mg/kg) = (A_{470} x10^6)/ (2000 x 100 x d)

where A is the absorbance and d is the spectrophotometer cell thickness (1 cm).

2.8. Phenolic compounds

Phenolic compounds were isolated from Chemlal olive oils using a liquid–liquid extraction system, following the method reported previously (Bakhouche et al., 2015). Olive oil, with 50 µL of internal standard solution (25 mg L^{-1} dihydrocaffeic acid in methanol) added, was dissolved in *n*-hexane (2.5 g in 5 mL). Afterwards, 5 mL of methanol/water (60/40, v/v) was added, and the mixture was vortexed and then centrifuged at 3500 rpm for 10 min. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure at 35 °C. The residue was dissolved in 0.25 mL of methanol/water (50/50 v/v) and finally, was filtered through a 0.2 µm filter before the HPLC analysis.

The analysis to characterise the phenolic profile of Chemlal phenolic extracts was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump, and a diode array detector (DAD). The chromatographic separation of these compounds was performed on a 150 mm × 4.6 mm i.d., 1.8 µm, Zorbax Eclipse Plus RP-C18 column (Agilent Technologies, Palo Alto, CA, USA). The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The total run time was 27 min, using a previously reported multistep linear gradient (Lozano-Sánchez et al., 2010). The flow rate was 0.80 mL min^{-1} and, consequently, the use of a splitter was required for the coupling with the MS detector, as the flow that arrived at the TOF detector had to be 0.2 mL min^{-1} to ensure reproducible results and stable spray. HPLC was coupled to a time-of-flight mass spectrometer detector micrOTOF (Bruker Daltonik, Bremen, Germany), which was equipped with a model G1607A ESI interface (Agilent Technologies), operating in negative ion mode.

External mass-spectrometer calibration was performed using sodium acetate clusters (5 mM sodium hydroxide in water/isopropanol 1/1 (v/v), with 0.2% of acetic acid) in quadratic + high-precision calibration (HPC) regression mode. The optimum values of the source and transfer parameters were established according to the method published previously (Lozano-Sánchez et al., 2010). The widely accepted accuracy threshold for confirmation of elemental compositions was set at 10 ppm for most of the compounds. The phenolic compounds were identified by comparing both retention times and MS data from samples and standards. The remaining compounds for which no commercial standards were available were identified by the interpretation of the information generated by the DAD, TOF analyser, and the information reported in the literature (Bengana et al., 2013). Quantification was made by HPLC-ESI-TOF/MS. Eight standard calibration curves of the main compounds found in the samples were prepared using eight commercial standards. Stock solutions, at a concentration of 1000 mg L^{-1} for each phenolic compound, were first prepared by dissolving the appropriate amount of the compound in

methanol. Afterwards, the stock solutions were serially diluted to working concentrations. All calibration curves showed good linearity over the study range (r^2 = 0.994). The individual concentrations were determined using the area of each individual compound (three replicates) and by interpolation of the corresponding calibration curve. Results were given in mg of analyte per kg of olive oil.

2.9. Statistical analysis

The data were analysed using Origin (version Origin Pro 8 SR0, Northampton, MA, USA) to perform a one-way-analysis of variance (ANOVA) at a 95% confidence level $p \leq 0.05$. This was done to identify significant differences among all parameters analysed in Chemlal olive oil from irrigated and non-irrigated trees obtained on three harvest dates.

3. Results and discussion

3.1. Ripening index; oil and water content

As shown in **Fig. 2**, the ripeness index seems to be affected by irrigation treatment. Indeed, for the same harvest date, significant differences were observed, showing the highest values in fruits from non-irrigated trees. As previously reported in the literature, irrigation delays the maturation process of olive fruits (Motilva et al., 2000).Within the same irrigation treatment, the ripeness index was also affected by the harvest time. In fact, an increase in its values was observed from the first harvest date to the third one. This tendency was observed in olives from non-irrigated and irrigated trees. The obtained results are in agreement with those found in olive oil from other varieties (Yorulmaz et al., 2013).

Oil yield (expressed as % dry weight) was significantly higher in fruit from irrigated trees, which was observed in all harvest dates under study **(Fig. 2).** In fact, differences of 24%, 20%, and 22% between irrigated and non-irrigated olives for the first (D1), second (D2) and third (D3) harvest dates were observed, respectively. The lower oil content in fruits from non-irrigated

trees in comparison to the irrigated ones can be explained by hydric stress of trees during the dry period (summer), which affected the accumulation of oil in the fruit. As observed in **Fig. 1**, high temperatures and low precipitation were registered in July and August. The high oil yield produced from irrigated trees was also obtained in other studies (Ramos and Santos, 2010; Stefanoudaki et al., 2009). Within the same irrigation treatment (non-irrigated or irrigated), an increase was found in oil yield, showing the lowest values at D1. After D2, the oil yield showed minimal increase and even remained constant between D2 and D3. The observed differences were not significant among the harvest dates within both irrigation treatments. These results confirm those previously published for other varieties (Bakhouche et al., 2015; Morelló et al., 2004).

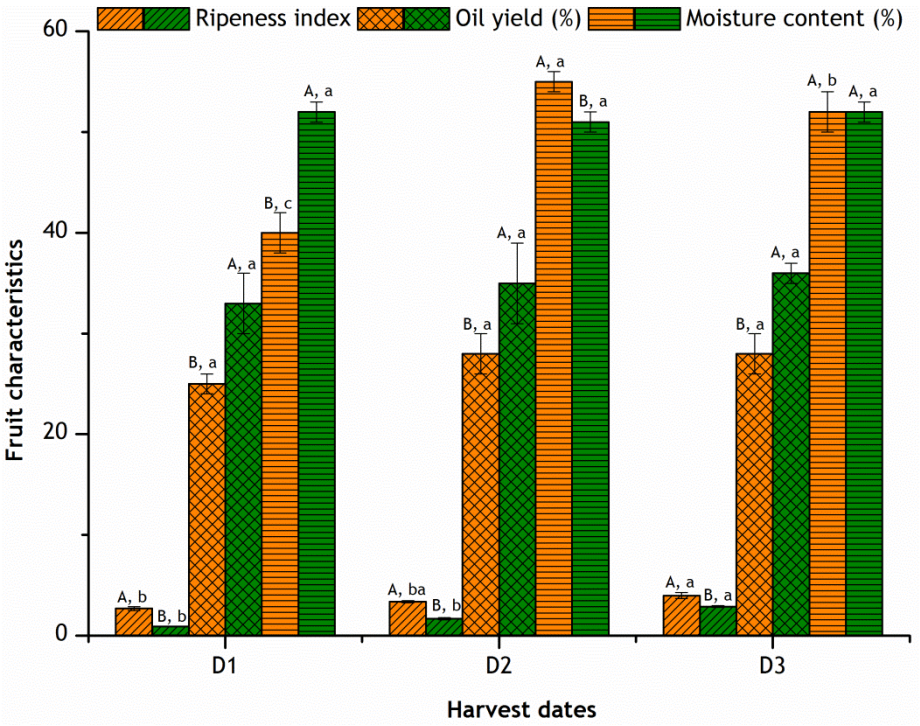

Fig. 2. Olive fruit characteristics obtained from non-irrigated and irrigated trees on three harvest dates: Orange colour, non-irrigated; green colour, irrigated; D1, D2, D3, harvest dates; different letters within histogram (A–C) indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to irrigation treatment in each sampling; different letters within the histogram (a–c) indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to ripeness index for each treatment.

Concerning water content, at D1, fruits from irrigated trees showed higher water content than those obtained from the non-irrigated ones; even a significant difference was observed (23%) **(Fig. 2)**. This result was expected because of the hydric stress suffered by the non-irrigated trees (Dabbou et al., 2011b; Motilva et al., 2000). However, at D2, a strong increase was observed in the water content of fruits from non-irrigated trees, while only a very slight change occurred in the fruits from irrigated ones. This can be explained by the precipitation received in September and October, which hydrated the fruit from non-irrigated trees **(Fig. 1)** (Fernandes-Silva et al., 2010; Stefanoudaki et al., 2009). In fact, the non-irrigated trees more efficiently used the water received from precipitation than did the irrigated ones. After 26 days, the efficiency of water absorption from the soil decreased, which explains the results obtained at D3, where water content was the same in fruits from non-irrigated and irrigated trees. Within the same irrigation treatment, no significant differences were found among the harvest dates studied in fruits from irrigated trees. Taking into consideration that the ripeness index changes with the change of harvest date, the water content in the fruit also seems to not be affected by the ripening stage. However, significant differences were observed in water content among D1, D2 and D3 in fruits from non-irrigated trees. This result supports the explanation given above, relating the changes in water content in fruits from non-irrigated trees to the efficiency of water use received from precipitation.

3.2. Quality parameters

Overall olive oil quality characteristics are shown in **Table 1**. The values obtained for free fatty acids (FFA), the peroxide index (PI), K_{232} and K_{270} were well within the limits of extra virgin olive oil established by EC regulations in all samples studied. Actually, FFA ranged from 0.31 to 0.33% and PI values ranged from 3.3 to 5.5 meq O_2 kg^{-1}. These two parameters were not affected by the application of water to trees during the dry period, as the values obtained were not significantly different among the olive oil samples from irrigated and non-irrigated trees. However, K_{232} and K_{270}, with values ranging from 1.8 to 2.3 and 0.12 to 0.17, respectively, showed, in some cases,

significant differences between olive oils from non-irrigated and irrigated trees.

If we analyse the time course of quality parameters within each irrigation treatment, we can observe that, while FFA and PI values tend to increase from D1 to D3, K_{232} and K_{270} showed an opposite trend in olive oils from non-irrigated trees. However, the differences were not significant among the harvest dates under study (Rotondi et al., 2004). The same behaviour was observed in olive oils from irrigated trees, with the exception of K_{270}, which showed a significant decrease at more advanced harvest dates. The changes found in olive oil quality parameters within each treatment can be attributed to the maturity process of the fruits (Bakhouche et al., 2015; Ben Youssef et al., 2010).

3.3. Chlorophyll and carotenoids

Pigments are responsible for olive oil colour, which is one of the factors that influence selection by consumers. Also, they are important because of their antioxidant properties (Gandul-Rojas and Minguez-Mosquera, 1996; Tura et al., 2007). Chlorophyll values varied from 1.1 to 5.06 mg/kg in all olive oil samples under study (**Table 1**). The irrigation seems to have an effect on chlorophyll content, as the higher values were obtained in olive oils from irrigated trees for the same harvest dates. These results can be explained by the low ripeness index obtained in fruits from irrigated olive trees. However, the differences between olive oils from non-irrigated and irrigated trees were not significant. Carotenoid values ranged from 0.70 to 2.9 mg/kg in the olive oil samples. In fact, significant differences were found between the irrigation treatments, being higher in olive oils from non-irrigated trees. It was reported that the synthesis of carotenoids is stimulated by water stress, which may explain the results found in this study (Beltrán et al., 2005).

Table 1. Analytical characteristics of Chemlal olive oils obtained under two irrigation treatments at three harvest dates.

[e]Parameters	[f]NIR			[g]IR			legal limits
	[h]D1	D2	D3	D1	D2	D3	
Free fatty acids (%)	$0.31\pm0.03^{A,a}$	$0.32\pm0.01^{A,a}$	$0.33\pm0.03^{A,a}$	$0.31\pm0.02^{A,a}$	$0.33\pm0.03^{A,a}$	$0.33\pm0.03^{A,a}$	≤ 0.8
Peroxid value (meq O2/kg)	$5.2\pm0.3^{A,a}$	$5.5\pm0.2^{A,a}$	$5.5\pm0.5^{A,a}$	$3.3\pm0.2^{A,a}$	$3.8\pm0.3^{A,a}$	$5.5\pm0.4^{A,a}$	≤ 20
K_{232}	$2.3\pm0.2^{A,a}$	$2.3\pm0.1^{A,a}$	$2.0\pm0.1^{A,a}$	$2.1\pm0.1^{A,a}$	$1.91\pm0.02^{B,a}$	$1.8\pm0.1^{A,a}$	≤ 2.5
K_{270}	$0.18\pm0.01^{A,a}$	$0.17\pm0.01^{A,a}$	$0.17\pm0.01^{A,a}$	$0.20\pm0.01^{A,a}$	$0.14\pm0.01^{B,b}$	$0.12\pm0.01^{B,c}$	≤ 0.22
Chlorophyll (mg/kg)	$4.3\pm0.3^{A,a}$	$2.3\pm0.2^{A,b}$	$1.1\pm0.1^{A,c}$	$5.06\pm0.01^{A,a}$	$2.9\pm0.2^{A,b}$	$1.3\pm0.1^{A,c}$	
Carotenoids (mg/kg)	$2.9\pm0.2^{A,a}$	$2.1\pm0.2^{A,b}$	$0.92\pm0.05^{A,c}$	$2.5\pm0.2^{B,a}$	$1.6\pm0.1^{B,b}$	$0.70\pm0.04^{A,c}$	
Palmitic acid (%)	$18\pm1^{A,a}$	$18\pm1^{A,a}$	$18\pm1^{A,a}$	$18\pm1^{A,a}$	$19.3\pm0.5^{A,a}$	$18.5\pm0.2^{A,a}$	7.5-20
Palmitoleic acid (%)	$2.41\pm0.03^{A,c}$	$3.4\pm0.2^{A,a}$	$2.82\pm0.03^{A,b}$	$2.2\pm0.1^{A,bc}$	$2.6\pm0.1^{B,ac}$	$2.5\pm0.2^{A,c}$	0.3-3.5
Stearic acid (%)	$2.3\pm0.2^{A,a}$	$2.8\pm0.1^{A,b}$	$2.80\pm0.01^{A,b}$	$1.9\pm0.1^{A,a}$	$2.0\pm0.2^{B,a}$	$1.7\pm0.1^{B,a}$	0.5-5
Oleic acid (%)	$66\pm1^{A,a}$	$64\pm2^{A,a}$	$63\pm2^{A,a}$	$66\pm1^{A,a}$	$65\pm1^{A,a}$	$64\pm5^{A,a}$	55-83
Linoleic acid (%)	$11\pm1^{A,a}$	$12\pm1^{A,a}$	$11.1\pm0.3^{A,a}$	$10\pm1^{A,ab}$	$11.4\pm0.2^{A,a}$	$9.3\pm0.4^{B,b}$	3.5-21
Linolenic acid (%)	$0.54\pm0.03^{A,a}$	$0.53\pm0.04^{A,a}$	$0.65\pm0.02^{A,a}$	$0.48\pm0.04^{A,a}$	$0.73\pm0.03^{A,a}$	$0.57\pm0.01^{A,a}$	≤1
MUFA/PUFA	$7.3\pm0.5^{A,a}$	$5.2\pm0.2^{A,c}$	$5.9\pm0.1^{B,b}$	$6.5\pm0.3^{B,a}$	$5.6\pm0.1^{A,b}$	$6.8\pm0.2^{A,a}$	

[e]MUFA/PUFA, monounsaturated fatty acids/polyunsaturated fatty acids

[f]NIR, non-irrigated; [g]IR, irrigated; [h]D1, D2, D3, harvest dates

Different letters (A–C) within the same line indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to irrigation treatment in each sampling

Different letters (a-c) within the same line indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to ripeness index for each treatment

The time course of the two pigments within the same irrigation treatment showed a significant decrease in their values in olive oils from non-irrigated trees. Indeed, the highest value was obtained at D1 and the lowest one at the more advanced harvest date (D3), corresponding to a higher ripeness index. The chlorophyll content was higher than that of the carotenoids, and its decrease was more marked for all harvest dates. The same tendency was observed in olive oils from irrigated trees among the studied harvest dates. It was documented in other studies that pigments, such as chlorophyll and carotenoids, tend to decrease as fruit maturity increases, which support the results obtained in our study within each irrigation treatment (Baccouri et al., 2008; Motilva et al., 2000). During the ripening process, photosynthetic activity decreases and the concentrations of chloroplast pigments, chlorophylls and carotenoids, also decrease progressively.

3.4. Fatty acid composition

The fatty acid composition of the Chemlal olive oils is reported in **Table 1.** The variability of the fatty acid composition of the samples covered the normal range expected for extra virgin olive oils. Overall, the fatty acid composition was only slightly affected by the application of water to trees during the dry period, and no consistent pattern of change was observed in response to tree water status. In fact, the two main olive oil fatty acids, oleic and palmitic fatty acids, showed no significant differences among olive oils from irrigated and non-irrigated trees for the same harvest dates. The rest of fatty acids are present in olive oil at a lower concentration. While palmitoleic, stearic, and linoleic acids seemed to be higher in olive oil from non-irrigated trees, showing significant differences in some analysed samples, the variation in linolenic acid content was not significant. Comparing the MUFA/PUFA ratio obtained in olive oils from irrigated and non-irrigated trees, we can observe changes in its values. However, these changes are slight and do not possess any nutritional relevance. Discrepancies among the data published about the effect of water application to trees on olive oil fatty acid composition were observed in the previously published studies for other varieties (Caruso et al., 2014; Stefanoudaki et al., 2009). This was explained

by the strong varietal differences in the response of olive trees to water stress.

Analysing the time course of fatty acid composition within each irrigation treatment, variations were found among the three harvest dates. In olive oils from irrigated trees, these variations were only significant for palmitoleic and linoleic acid. In fact, their highest values were found at D2. The evolution of fatty acid composition in olive oil from non-irrigated trees among the harvest dates was very similar to olive oil from irrigated trees. However, in olive oils from non-irrigated trees, the observed significant differences were in palmitoleic and stearic acids contents. With regard to MUFA/PUFA ratio, there was a decrease at earlier harvest dates (D1, D2) in olive oils from irrigated trees. Nevertheless, its value increased at D3. The same tendency was found in olive oil from non-irrigated trees. The obtained results for fatty acid composition within each treatment (irrigated or non-irrigated) among the harvest dates may be due to the enzymatic actions that are part of the anabolic and catabolic reactions of fatty acid syntheses during olive ripening (Benito et al., 2013; García et al., 2013; Gómez-Rico et al., 2007).

3.5. Phenolic characterisation in olive oil samples

3.5.1. Qualitative characterisation

No qualitative differences were obtained in the phenolic profile of all the olive oil samples under study. **Table 2** lists the compounds identified in the representative Chemlal phenolic extract, including the information provided by the mass spectrometer: retention time, molecular formula, experimental and calculated mass, error, and msigma. A total of 23 phenolic compounds, belonging to phenolic alcohols, phenolic acids, secoiridoids, lignans, and flavones, were tentatively identified in the extract.

Table 2. Main phenolic compounds identified in a representative Chemlal olive oil phenolic extract obtained by HPLC-ESI-TOF/MS.

Compounds[a]	Retention Time(min)	Molecular formula	m/z calcd[b]	m/z Exptl[c]	Error (ppm)	msigma
H- HYTY	3.92	$C_8H_8O_3$	151.0401	151.0398	1.9	2.0
HYTY	8.00	$C_8H_{10}O_3$	153.0557	153.0562	-3.4	3.8
Secoiridoid derivative	8.11	$C_{17}H_{28}O_{11}$	407.1559	407.1569	-2.4	3.0
TY	9.83	$C_8H_{10}O_2$	137.0608	137.0600	5.9	16.6
Vanillic acid	10.86	$C_8H_8O_4$	167.0350	167.0351	-0.7	6.2
DEA	10.92	$C_9H_{12}O_4$	183.0663	183.0658	2.5	4.8
H-D-Ol Agl or isomer	11.42	$C_{17}H_{20}O_7$	335.1136	335.1138	-0.4	4.1
p-coumaric acid	13.16	$C_9H_8O_3$	163.0401	163.0398	1.4	19.0
EA	14.72	$C_{11}H_{14}O_6$	241.0718	241.0717	0.2	2.5
H-EA	15.54	$C_{11}H_{14}O_7$	257.0667	257.0669	-0.7	1.6
DOA	15.91	$C_{17}H_{20}O_6$	319.1187	319.1196	-2.7	2.0
H-D-Ol Agl or isomer	16.29	$C_{17}H_{20}O_7$	335.1136	335.1132	1.4	3.7
Syringaresinol	17.83	$C_{22}H_{26}O_8$	417.1555	417.1538	4.0	22.4
Pin	18.50	$C_{20}H_{22}O_6$	357.1344	357.1352	-2.4	3.1
D-Lig Agl	18.88	$C_{17}H_{20}O_5$	303.1238	303.1245	-2.2	4.5
AcPin	19.02	$C_{22}H_{24}O_8$	415.1398	415.1416	-4.3	12.8
H-D-Lig Agl	19.47	$C_{17}H_{20}O_6$	319.1187	319.1189	-0.6	11.2
H-Ol Agl	22.85	$C_{19}H_{22}O_9$	393.1191	393.1179	3.2	1.9
Ol Agl	22.90	$C_{19}H_{22}O_8$	377.1242	377.1256	-3.6	4.0
Lut	23.26	$C_{15}H_{10}O_6$	285.0405	285.0409	-1.5	0.1
Lig Agl	25.44	$C_{19}H_{22}O_7$	361.1293	361.1311	5.1	6.7
Apig	25.64	$C_{15}H_{10}O_5$	269.0455	269.0454	0.7	8.5
Methyl Ol Agl	25.70	$C_{20}H_{24}O_8$	391.1398	391.1401	-0.6	26.2

[a] H-HYTY, oxidized hydroxytyrosol; HYTY, hydroxytyrosol; TY, tyrosol; DEA, decarboxymethylated form of elenolic acid; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; Pin, pinoresinol; D-Lig Agl, decarboxymethyl ligstroside aglycone; AcPin, acetoxypinoresinol; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; H-Ol Agl, hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lut, luteolin; Lig Agl, ligstroside aglycone; Apig, apigenin; Methyl Ol Agl, methyl oleuropein aglycone.

[b] m/z calcd: calculated mass. [c] m/z exptl: experimental mass.

3.5.2. Quantitative characterisation

The use of HPLC-ESI-TOF/MS allowed for initial evaluation of the changes that occur in individual phenolic compounds of Chemlal olive oils obtained under two irrigation conditions.

Table 3 includes all of the quantitative results of phenolic compounds in the olive oil samples under study. The total phenol content and the contribution of each phenolic compound's family were tentatively calculated by adding together the individual phenolic compound concentrations. **Fig. 3** shows the time course of the total phenolic content, as well as the trend of each phenolic family in olive oils from non-irrigated and irrigated trees obtained at three harvest times D1, D2, and D3.

Total phenol content ranged from 156 to 274 mg/kg and 185 to 267 mg/kg for olive oils from non-irrigated and irrigated trees, respectively. Taking into account this range of concentration, there were no high differences in olive oil total phenolic content between the two irrigation treatments. However, if we analyse the results obtained for each phenolic family, the obtained range of concentration for phenolic alcohols, phenolic acids, flavones, and lignans was higher in olive oils from irrigated trees (**Table 3**). The concentration range of secoiridoids can be considered as not being very different between olive oils from the two irrigation treatments. The higher concentration observed in phenolic families of olive oils from irrigated trees can be explained partly by the low ripeness index of olives from where they were obtained. However, if we observe the results obtained in olive oil from non-irrigated and irrigated trees at D1 and D3 (with similar ripeness index: 2.7 and 2.9), respectively, we can observe that all phenolic families showed higher values in olive oils from irrigated trees. The obtained data are not in agreement with the majority of the previously published studies, which showed that reducing water availability induces a higher accumulation of total phenols in olives and the resulting oils (Dag et al., 2008; Servili et al., 2007). The reported discrepancies can be explained by the different environmental conditions of cultivation, and the differences in the response

of each cultivar to water stress. It should be taken into consideration that increasing phenolic content in olive oils from irrigated olive grove was also found by some authors (Dabbou et al., 2010; Dag et al., 2015).

Analysing the changes in olive oil phenolic content within each irrigation treatment, we can clearly observe that the total phenol content was significantly different among the harvest dates under study (**Fig. 3**). In olive oils obtained from the non-irrigated olive grove, the total phenolic content increased at earlier harvest dates, from 156 mg/kg to 274 mg/kg at D1 and D2, respectively. After that, a decrease in its value was observed at D3, showing a value of 200 mg/kg. However, the total phenolic content in olive oils from irrigated trees showed its highest value at D1 (267 mg/kg), and decreased progressively to 190 and 185 mg/kg at later harvest dates, D2 and D3, respectively. These results confirm the effect of olive ripening on olive oil phenolic content previously reported in other studies (Morelló et al., 2004). The difference obtained of total phenolic compound over time between olive oils from the two irrigation treatments during ripening may partly explain the discrepancies reported in the literature regarding the effect of olive maturation on olive phenolic content. In fact, some authors found the same trend as the one observed in our study for olive oils from non-irrigated trees (Ben Youssef et al., 2010), while others reported a linear decrease in phenolic content as olive maturation progresses, the same trend observed in our results for olive oils from irrigated trees (Bakhouche et al., 2015; Bengana et al., 2013). Therefore, under which irrigation treatment the olives and corresponding olive oils were obtained is a key important factor to consider when making comparisons between reported data from studies related to olive ripening and phenolic content.

Table 3. Concentration of phenolic compounds in Chemlal olive oils obtained from non-irrigated and irrigated trees at three harvest dates.

Parameters[e]	[f]NIR			[g]IR		
	[h]D1	D2	D3	D1	D2	D3
Ripeness index	2.7±0.2^{A,b}	3.39±0.1^{A,ba}	3.99±0.3^{A,a}	0.92±0.05^{B,b}	1.7±0.1^{B,b}	2.9±0.1^{B,a}
H- HYTY	0.71±0.03^{B,a}	2.12±0.01^{A,bc}	2.3±0.2^{A,c}	1.4±0.1^{A,ac}	1.0±0.1^{B,bc}	1.31±0.02^{B,c}
HYTY	2.759±0.001^{B,c}	4.89±0.04^{A,b}	6.4±0.5^{A,a}	6.3±0.1^{A,a}	3.6±0.2^{B,b}	3.0±0.3^{B,b}
TY	2.40±0.04^{B,c}	5.8±0.2^{B,b}	8.8±0.4^{A,a}	16.2±0.4^{A,a}	9.3±0.5^{A,b}	7.3±0.5^{A,b}
Phenolic alcohols	**5.876±0.002^{B,c}**	**12.8±0.2^{A,b}**	**17±1^{A,a}**	**24.0±0.5^{A,a}**	**14±1^{A,b}**	**11.6±0.2^{B,c}**
Vanillic acid	0.71±0.06^{B,ba}	0.38±0.02^{B,b}	0.90±0.08^{B,a}	3.8±0.3^{A,a}	2.1±0.1^{A,b}	2.2±0.1^{A,b}
p-coumaric acid	0.398±0.002^{B,ba}	0.308±0.003^{B,b}	0.48±0.02^{A,a}	0.82±0.03^{A,a}	0.45±0.04^{A,c}	0.54±0.02^{A,b}
Phenolic acids	**1.1±0.1^{B,ba}**	**0.69±0.02^{B,b}**	**1.4±0.1^{B,a}**	**4.6±0.3^{A,a}**	**2.6±0.1^{A,b}**	**2.7±0.1^{A,b}**
Secoiridoid derivative	0.73±0.05^{A,b}	1.137±0.002^{B,a}	2.2±0.2^{A,a}	1.4±0.1^{A,ba}	2.5±0.1^{A,a}	1.3±0.1^{B,b}
DEA	NQ	0.857±0.004^{A,a}	0.561±0.005^{A,b}	NQ	0.61±0.04^{B,a}	0.12±0.01^{B,b}
H-D-OI Agl or isomer	0.17±0.01^{B,c}	1.51±0.04^{A,a}	0.891±0.004^{b}	0.321±0.002^{A,a}	0.071±0.004^{B,b}	NQ
EA	8.6±0.7^{B,a}	8.2±0.1^{B,a}	12.2±0.3^{B,a}	36±2^{A,a}	38±3^{A,a}	32±1^{A,a}
H-EA	0.12±0.01^{B,a}	0.177±0.004^{A,a}	0.26±0.02^{A,a}	0.52±0.05^{A,ac}	0.24±0.01^{A,bc}	0.34±0.01^{A,c}
DOA	6.6±0.4^{B,c}	29±1^{A,a}	19±1^{A,b}	26±1^{A,a}	4.8±0.4^{B,b}	7.3±0.1^{B,b}
H-D-OI Agl or isomer	0.46±0.03^{B,b}	0.60±0.01^{A,a}	0.31±0.01^{B,c}	0.67±0.03^{A,a}	0.34±0.03^{B,b}	0.54±0.02^{A,c}
D-Lig Agl	1.3±0.1^{A,b}	2.23±0.02^{A,a}	1.3±0.1^{A,b}	1.2±0.1^{A,a}	0.94±0.02^{B,a}	1.2±0.1^{A,a}
H-D-Lig Agl	NQ	0.183±0.001^{A}	NQ	0.16±0.01^{a}	0.10±0.01^{A,a}	0.13±0.01^{a}
H-OI Agl	0.65±0.01^{B,a}	1.37±0.02^{A,a}	1.32±0.02^{A,a}	2.2±0.2^{A,a}	1.5±0.1^{A,a}	1.9±0.1^{A,a}
OI Agl	118±4^{A,b}	165.7±0.2^{A,a}	124±2^{A,b}	133±1^{A,a}	104±2^{B,b}	105±10^{B,b}
Lig Agl	8.3±0.5^{B,b}	46±2^{A,a}	11.3±0.3^{A,b}	18±1^{A,a}	9.3±0.5^{B,b}	11±1^{A,b}

Parameters[e]	[f]NIR			[g]IR		
	[h]D1	D2	D3	D1	D2	D3
Methyl Ol Agl	0.13±0.01^{B,a}	0.141±0.002^{a}	0.32±0.03^{A,b}	0.21±0.02^{A,a}	NQ	0.11±0.01^{B,b}
Secoiridoids	**145±3^{B,c}**	**257±1^{A,a}**	**173±6^{A,b}**	**221±7^{A,a}**	**162±9^{B,b}**	**161±11^{A,b}**
Syringaresinol	0.50±0.05^{B,a}	0.271±0.002^{B,b}	0.44±0.04^{B,a}	0.90±0.05^{A,ac}	0.62±0.01^{A,bc}	0.77±0.04^{A,c}
Pin	1.5±0.1^{B,b}	1.499±0.002^{B,b}	3.8±0.1^{A,a}	6.3±0.4^{A,a}	5.3±0.3^{A,a}	3.1±0.2^{A,b}
AcPin	0.70±0.01^{B,b}	0.370±0.001^{B,b}	1.38±0.03^{A,a}	3.5±0.2^{A,a}	3.6±0.1^{A,a}	3.5±0.3^{A,a}
Lignans	**2.7±0.2^{B,b}**	**2.140±0.001^{B,b}**	**5.7±0.1^{A,a}**	**11±1^{A,a}**	**9.5±0.2^{A,ab}**	**7.4±0.2^{A,b}**
Lut	1.1±0.1^{B,b}	1.12±0.02^{B,b}	2.3±0.2^{A,a}	5.8±0.5^{A,a}	2.29±0.03^{A,b}	2.6±0.1^{A,b}
Apig	0.34±0.02^{B,a}	NQ	0.16±0.01^{A,a}	1.2±0.1^{A,a}	0.31±0.03^{b}	0.38±0.03^{A,b}
Flavones	**1.4±0.1^{B,ba}**	**1.12±0.02^{B,b}**	**2.5±0.2^{A,a}**	**7.0±0.7^{A,a}**	**2.6±0.1^{A,b}**	**3.0±0.3^{A,b}**
Total phenols	**156±3^{B,c}**	**274±1^{A,a}**	**200±7^{A,b}**	**267±7^{A,a}**	**190±10^{B,b}**	**185±12^{A,b}**

[e]H-HYTY, oxidized hydroxytyrosol; HYTY, hydroxytyrosol; TY, tyrosol; DEA, decarboxymethylated form of elenolic acid; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; H-Ol Agl, hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lig Agl, ligstroside aglycone; Methyl Ol Agl, methyl oleuropein aglycone; Pin, pinoresinol; AcPin, acetoxypinoresinol; ; Lut, luteolin; Apig, apigenin.

[f]NIR, non-irrigated; [g]IR, irrigated; [h]D1, D2, D3, harvest dates

Different letters (A–C) within the same line indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to irrigation treatment in each sampling

Different letters (a-c) within the same line indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to ripeness index for each treatment

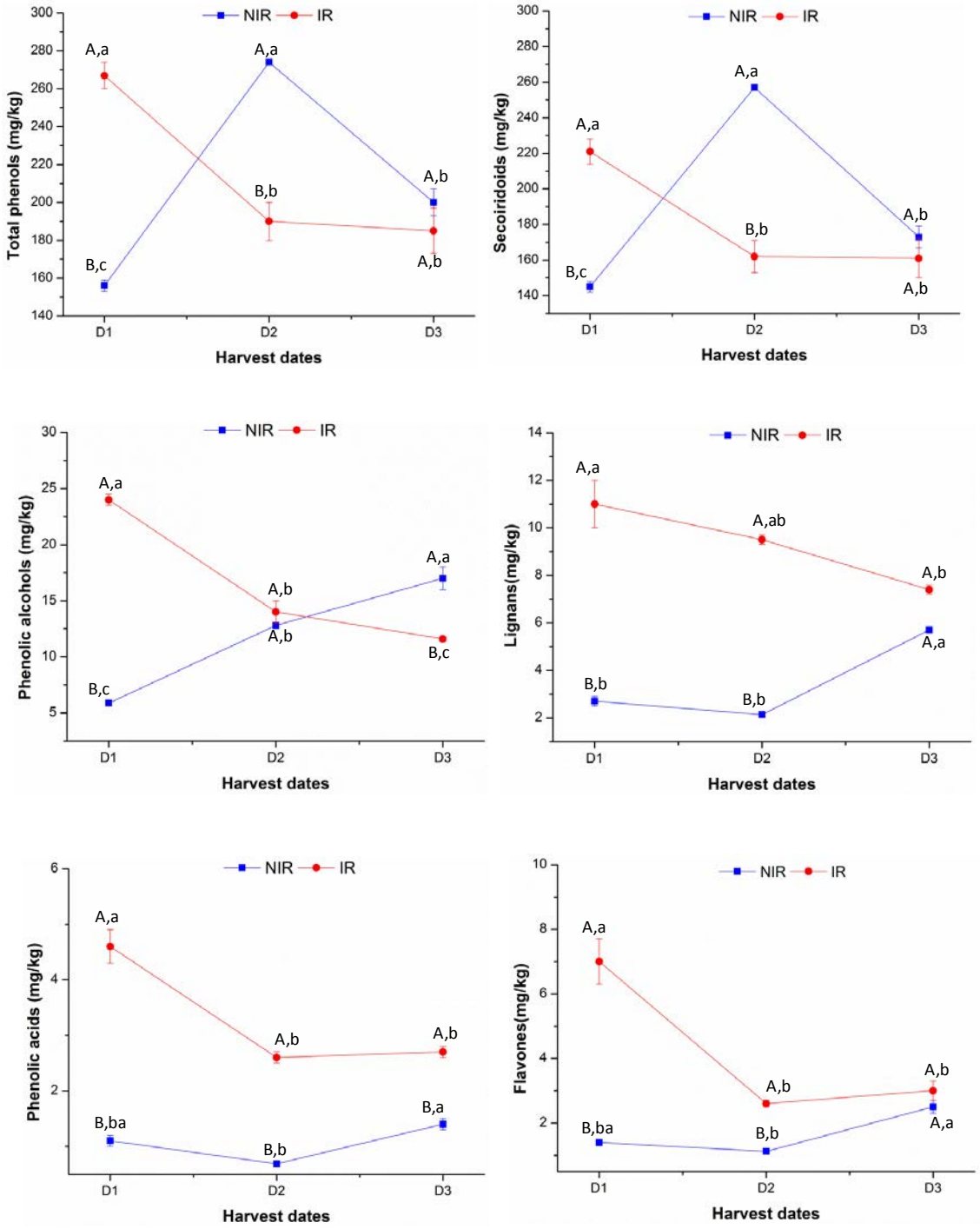

Fig. 3. Time course of total phenols and phenolic families in olive oils obtained under two irrigation treatments on three harvest dates: NIR, non-irrigated; IR, irrigated; different letters (A–C) indicate significant differences at a 95% confidence level ($p \leq 0.05$), with respect to irrigation treatment in each sampling; different letters (a–c) indicate significant differences at a 95% confidence level ($p \leq 0.05$) with respect to ripeness index for each treatment.

The time course relative to the total phenolic content on the three harvest dates within each irrigation treatment was the result of the behaviour of the different phenolic families, mainly, secoiridoids. As shown in **Fig. 3**, the trend of secoiridoids was similar to that of total phenols among the harvest dates under study. Indeed, their highest concentration was obtained at D2 (257 mg/kg) and D1 (221 mg/kg) in olive oils from non-irrigated and irrigated trees, respectively. Oleuropein algycone, decarboymethyl oleuropein aglycone, and ligstroside aglycone were the major secoiridoids quantified in olive oil from non-irrigated trees. A strong increase was observed in their content at early harvest dates (D1 and D2). After that, their content decreased significantly at D3, which explains the behaviour of the secoirdoid content during ripening in olive oils from non-irrigated trees. These compounds were also found at a high concentration in olive oils from irrigated trees. However, their evolution among the harvest dates was characterised by a significant decrease as maturation progressed. Elenolic acid, which is not considered a secoiridoid, but rather, a derivative, was found at concentrations that were relatively high in the olive oil samples under study. Nevertheless, its evolution within each treatment at different harvest dates was not significant. The rest of the secoiridoids and derivatives were found at low concentrations in the olive oil samples, and their content within each irrigation treatment was affected by olive ripening. Their time course during ripening was different between olive oils from non-irrigated and irrigated trees.

The changes observed in secoiridoids and derivatives during ripening within each treatment can be explained by the action of the different enzymes, such as *β*-glucosidase, esterase and others involved in the anabolic and catabolic pathways of phenolic compound biosynthesis during ripening (El Riachy et al., 2011; Gutierrez-Rosales et al., 2010). The way that these reactions take place seems to be affected by the water status of the tree, as the time course of secoiridoids during ripening was different in olive oils from non-irrigated and irrigated trees.

With regard to phenolic alcohols, a significant increase was obtained as the fruit ripening progressed in olive oils from non-irrigated trees, showing the lowest value at D1 (5.876 mg/kg) and the highest one at D3 (17 mg/kg). Hydroxytyrosol and tyrosol were the major compounds quantified in this family, and they were responsible for its behaviour during ripening. The increase observed in the content of these compounds from the more advanced harvest time can be explained by the catalytic action of enzymes, such as esterase, exerted on major secoiridoids during olive ripening. Nevertheless, at earlier harvest dates (D1 and D2), the content of the precursors of hydroxytyrosol and tyrosol (oleuropein and ligstroside aglycones) increased. Consequently, the obtained results for phenolic alcohol can be explained by the fact that the anabolic reactions of oleuropein and ligstroside aglycones were higher compared to the catabolic ones during olive ripening or by other biosynthetic intermediates that were not considered in this study (ligstroside and oleuropein) (Gutierrez-Rosales et al., 2010). The time course of phenolic alcohols in olive oils from irrigated trees during ripening was opposite to that observed in olive oils from non-irrigated trees. In fact, a significant linear decrease in their content was obtained, showing the highest value at D1 (24 mg/kg) and the lowest one at D3 (11.6 mg/kg). Again, hydroxytyrosol and tyrosol were the major phenolic compounds and they were responsible for this behaviour.

Concerning lignans, their content in olive oil from non-irrigated trees showed a slight decrease in value on the early harvest dates, however, this was not significant (D1, 2.7 mg/kg; D2, 2.14 mg/kg). After this, a strong increase was observed at D3 (5.7 mg/kg**)**. Nevertheless, in olive oils from irrigated trees, lignans showed a linear decrease in their content, being not significant between D2 (9.5 mg/ kg) and D3 (7.4 mg/kg). In all samples studied, pinoresinol was the compound responsible for the observed behaviour of this family during ripening.

Regarding phenolic acids and flavones, their content was significantly affected by olive ripening within each irrigation treatment (non-irrigated or irrigated). However, their tendency during olive ripening was similar between

olive oils from non-irrigated and irrigated trees (**Fig. 3**). Vanillic acid and luteolin were the major compounds quantified in these two families.

4. Conclusions

Taking into account the obtained results in this research, irrigation produced a slight variation in olive oil composition in comparison to the one obtained under rain fed conditions. The only clear difference was observed in oil yield, being higher in olive oil from irrigated trees. Although the concentration range of the total phenolic content was not highly different between the two treatments, the most phenolic families showed a higher content in olive oils from irrigated trees. The more important achievement of this study was the differences observed in the time course of the olive oil component, mainly phenolic compounds, within each treatment (non-irrigated or irrigated) among the harvest dates. Therefore, in order to establish the optimum harvest period for the Chemlal variety, it is of great importance to consider agronomic practices, such as irrigation, that can affect the olive ripening process, and consequently the olive oil optimal composition. From the results obtained under the experimental condition of this study, the optimum harvest period for non-irrigated Chemlal trees seems to be D2, and the optimal harvest time for the same variety under irrigation conditions seems to be D1. These data can be considered helpful to the olive oil sector. Finally, future comparative studies with water application that is less than 100% of ETc would be interesting for a better water management.

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SECTION II

Virgin olive oil elaboration process: Effect on phenolic composition

Chapter 3

Literature review on production process to obtain extra virgin olive oil enriched in bioactive compounds. Potential use of byproducts as alternative sources of polyphenols

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Review

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Literature Review on Production Process To Obtain Extra Virgin Olive Oil Enriched in Bioactive Compounds. Potential Use of Byproducts as Alternative Sources of Polyphenols

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Abstract

This review describes the olive oil production process to obtain extra virgin olive oil (EVOO) enriched in polyphenol and byproducts generated as sources of antioxidants. EVOO is obtained exclusively by mechanical and physical processes including collecting, washing, and crushing of olives, malaxation of olive paste, centrifugation, storage, and filtration. The effect of each step is discussed to minimize losses of polyphenols from large quantities of wastes. Phenolic compounds including phenolic acids, alcohols, secoiridoids, lignans, and flavonoids are characterized in olive oil mill wastewater, olive pomace, storage byproducts, and filter cake. Different industrial pilot plant processes are developed to recover phenolic compounds from olive oil byproducts with antioxidant and bioactive properties. The technological information compiled in this review will help olive oil producers to improve EVOO quality and establish new processes to obtain valuable extracts enriched in polyphenols from byproducts with food ingredient applications.

Keywords: EVOO, byproducts, polyphenol, production process, antioxidants, bioactive

1. Introduction

The traditional Mediterranean diet is characterized by preferential consumption of vegetables, legumes, fruit, nuts, and cereals, as well as olive oil being the main fat in the diet[1–3]. Extra virgin olive oil (EVOO), extracted from fresh and healthy olive fruits (*Olea europaea* L.), properly processed and stored at low temperatures, is characterized by increased oxidative stability and unique aroma highly appreciated by consumers[4–7].

EVOO is a natural juice obtained exclusively by mechanical and physical processes, in contrast to other edible oils, namely, sunflower and soybean oils, which must be refined before consumption, thus changing their original composition during this process[8]. The consumption of EVOO is associated with a low incidence of cardiovascular diseases, neurological disorders, and breast cancer[9–11]. Recently, several minor components have been related to the olive oil healthy properties, mainly polyphenols. These compounds are also associated with the oxidative stability and flavor characteristics of virgin olive oil[12–14]. However, the phenolic composition of EVOO is influenced by complex multivariate interactions from genotype, agronomical, environmental, and technological factors[15]. The qualitative and quantitative phenolic composition of EVOO is widely affected by many variables related to production processes, from the ripening stage of olive fruits to storage conditions[16]. The steps of the production process include collecting, washing, and crushing of olives, malaxation of olive paste, centrifugation, storage, and filtration. Most quality attributes of EVOO are determined by the chemical composition and biochemical status of the olive fruit[17]. Milling and malaxation are considered as the most critical steps during olive processing and oil extraction as the most important changes in EVOO phenolic composition[15]. However, qualitative and quantitative changes take place in olive oil polyphenols during storage and filtration[18–20]. Consequently, rigorous controls of all olive oil processes are recommended to produce olive oil of high phenolic quality[21]. Unfortunately, the production of EVOO is associated with the generation of large quantities of wastes,[22,23] and it is associated with the loss of olive polyphenols in olive oil byproducts. The well-known bioactivity of olive polyphenols[24] has stimulated

qualitative and quantitative characterization of the phenolic profile in these wastes.

The aim of this review is focused on (a) the effect of different production processing steps taking into account the latest technological innovations to establish the best flow diagram for EVOO enriched in polyphenols, (b) phenolic composition and its bioactivity of some byproducts generated during the EVOO production process, and (c) development of pilot plant and industrial processes to recover polyphenols from olive oil byproducts.

2. Extra-virgin olive oil production process: Technological alternative to obtain olive oil enriched in phenolic compounds

2.1. Harvest period

The olive ripening stages that include harvest time and maturity index are the most important factors associated with the quality evaluation of olive oil. Indeed, during the ripening, several metabolic processes take place in olives followed by variations in the phenolic composition due to different biosyntheses and biotransformation pathways of phenolic compounds. Different anabolic and catabolic pathways in olive fruits were established from Arbequina and Hojiblanca cultivars,[17] related to the oleuropein and its derivatives and to the activity of *β*-glucosidase during the growth and ripening of olive fruits. The main phenolic compounds and derivatives, including hydroxytyrosol, ligstroside aglycon, oleuropein aglycon, acetoxy-pinoresinol, and elenolic acid, showed an increase in EVOO at the early stages of olive harvest, followed by a reduction of their concentrations at more advanced stages of maturity[25]. Consequently, early harvested fruit produces olive oil with high polyphenol content and high oxidative stability. It is well-known and widely accepted that both phenolic amount and oxidative stability are linked to the antioxidant capacity of EVOO polyphenols. However, these compounds have also been associated with the flavor characteristics, and harvesting too early produces olive oils that have occasionally unacceptable sensory quality due to excessive polyphenol concentrations. It was suggested that the majority of olive oil produced does not have the best commercial quality

because the fruit has not been picked at the optimal harvest time[26]. There is therefore a need to determine the most appropriate maturation stage of each olive cultivar before processing the olive fruit. According to literature reports, it is possible to establish that the best harvest time is carried out early, when the fruit reaches optimum ripeness, when the "envero" or the change in color of olives starts taking place. From the point of view of phenolic composition, oxidative stability, and organoleptic properties, the best olive oil was shown to be obtained with maturity index values between 2.5 and 3.5 in Nostrana di Brisighella cultivar cultivated in Jaen, Spain[27].

2.3. Crushing

Crushing of olives is a physical process used to break the fruits' tissues and release the oil drops contained in the vegetable cell vacuoles. The olive paste preparation is currently performed in industrial oil mills either with the traditional discontinuous stone-mill or with the continuous hammer crusher. The latter is mainly used in the olive oil industry where the oil extraction is usually performed by centrifugation[28].

Olive crushing is one of the most important steps that affect the phenolic profile of EVOO produced[29]. Indeed, after olive crushing, several enzymes that can be activated are involved in the generation and transformation of phenolic compounds. Secoiridoid aglycons such as the elenolic acid linked to hydroxytyrosol and the decarboxymethylated form of elenolic acid linked to hydroxytyrosol and to tyrosol are produced during crushing, by hydrolysis and loss of carboxymethyl groups of oleuropein, dimethyl-oleuropein, and ligstroside. These changes take place when the reaction is catalyzed by the endogenous *β*-glucosidases followed by other chemical reactions[30].

The systems used and crushing conditions have an influence on these reactions and the partitioning behavior of polyphenols. A comparative study carried out between both systems described above, traditional discontinuous stone-mill and continuous hammer crusher, showed that phenolic compounds were better preserved in olive oil obtained with the continuous system[31]. These results were associated with the homogeneous and smaller sizes of the

solid fragments obtained by the continuous hammer crusher, favoring the substance exchange process between the oily phase and the aqueous phase of the olive paste. The crushing conditions, which have been evaluated by several authors, include the use of a hammer crusher, the grid hole size, and rotation speed. Stronger conditions using smaller grid holes and higher rotation speed increase the final phenolic content of EVOO, this effect being higher for the hydroxytyrosol than for tyrosol[29].

2.4. Malaxation

Malaxation of the olive paste is carried out with a stainless steel device made of a semicylindrical vat with a horizontal shaft, rotating arms, and blades of different shapes and sizes. This vat is equipped with a heating jacket, circulating hot water to warm the olive paste[32]. The efficiency of malaxation depends on the rheological characteristics of the olive paste and the technological parameters of the operation, such as temperature and time[33].

Regarding the phenolic composition, temperature, time, and the activity of several enzymes are involved in the evolution of these compounds during the malaxation step. On one hand, it has been described that increasing malaxation temperature from 15 to 30 to 37 to 42 °C and times from 20 to 45 to 60 min improved the phenol contents and oxidative stability of EVOO. On the other hand, a longer malaxation time more than 60 min apparently affected the phenol contents negatively[34]. However, in another study the secoiridoid group showed a quasi-linear increment of concentrations with increasing temperature up to 30 °C, followed by a corresponding marked decrease with the highest malaxation temperatures (33 and 36 °C)[35]. Furthermore, increasing the temperature during the olive paste malaxation process increases the activity of oxidoreductase enzymes such as polyphenol oxidase present in olive fruit which is rather high at 35 °C. The lipoxygenases that catalyze the formation of hydroperoxides could also be responsible for an indirect oxidation of secoiridoids. Another active enzyme, *β*-glucosidase, could play a role in the production of phenol aglycons (secoiridoids) by hydrolysis of the oleuropein and dimethyloleuropein[32]. These enzymatic

activities explain also the lineal increase of hydroytyrosol and tyrosol obtained by degradation of complex phenolic compounds during malaxation[36].

The most important losses of different phenolic groups present in olive paste occur in the solid phase (wet pomace) and aqueous phase by the low lipophilic behavior of the phenolic structures that led to a low concentration in EVOO[37]. However, in some EVOOs, a low phenolic concentration may improve their sensory quality. Cornicabra EVOO obtained under malaxation conditions of temperature below 28 °C and a time longer than 60 min improved its bitterness by reducing phenolic content, and the expected decrease in oxidative stability would not affect its shelf life[38].

2.5. Centrifugation

Centrifugation is usually applied for a primary separation of the olive oil fraction from the vegetable solid material and vegetation water. This step may be carried out using the combination of two different systems: horizontal centrifugation (three- and two-phase decanter) and vertical centrifugation. Horizontal centrifugation using three-phase decanter requires the addition of warm water to dilute the olive paste to facilitate the separation described above[39] while the two-phase decanter consists of "no-water" centrifugation plants for separating the oily phase from malaxed pastes without requiring adding warm water. It should be considered that the two-phase decanter requires a minimal moisture value on the olive paste (50%) to facilitate the separation process. When this value is not reached, a low amount of water is loaded into the decanter[40,41]. This decanter has the advantage of recovering more complex hydrophilic phenolic compounds and preserving them more efficiently in EVOO than by the threephase method[34,39,42]. Concerning vertical centrifugation, this system is used to separate the oily must obtained from horizontal centrifugation. Phenolic composition of the wash water added during this step has also been characterized. Hydroxytyrosol, tyrosol, and the dialdehydic form of elenolic acid linked to hydroxytyrosol were the most representative phenolic compounds identified[43]. However, another study showed a slight variation in the concentrations of phenolic compounds when

the comparison between olive oil composition before and after vertical centrifugation process has been carried out. It could be attributed to the small water amounts added in the experimental conditions developed by the research[44]. Therefore, the best way to reduce the loss of phenolic compounds during horizontal centrifugation (two and three phases) and vertical centrifugation is established by the equilibrium between the volume of water added and by a good separation of phases.

2.6. Storage

In the Mediterranean area, olive oil is generally produced from September to February and stored in the mill until filtration and commercialization. Several studies have focused on the possible hydrolytic and oxidative degradation of phenolic compounds present in EVOO over the shelf life in commercial containers. As expected, after storage for 9 months the peroxide values increased and the total phenol content and oxidative stability of olive oil decreased[45]. With the aim of understanding chemical changes produced in EVOO polyphenols, different stress conditions have been applied. In this way, EVOO samples were kept in the dark at 60 °C for up to 7 weeks and removed every week from the oven to carry out the analysis. The results showed that secoiridoids were apparently oxidized[46]. In a more recent study, EVOO samples were stored in different commercial containers (glass, polyethylene terephthalate, and Tetra-Brik1) at room temperature (20 °C) and refrigerated temperature (4 °C). After 9 months of storage, the smallest decrease in phenolic content was in EVOOs stored in Tetra-Brik, due to a minor degradation process by preventing the passage of light and oxygen[47].

The oxidation and storage conditions applied in these studies do not exactly reflect the real storage conditions of EVOO in the mill companies until the oil is sold. The phenolic patterns discerned could depend on storage conditions including time, temperature, oxygen availability, and industrial or commercial containers[48]. The formation of oxidized and hydrolyzed products and changes in the phenolic patterns of EVOO after storage for 10 months in industrial tanks without headspace at room temperature in the dark have also been

evaluated. Degradation pathways were proposed based on the half-life, elimination, and appearance rate of the complex phenolic compounds, and their oxidized and hydrolyzed products[49]. In fact, when the correlation for the pair oleuropein aglycon or decarboxymethyl oleuropein aglycon and their hydrolyzed derivatives was evaluated, the determination coefficients of the mathematical function proved higher than 0.950.

From all studies carried out, the higher phenolic contents and oxidative stability were obtained when the EVOO was stored for shorter times under the best conditions of temperature, light, and oxygen.

2.7. Filtration

Filtration is a special important final step to remove suspended solids and moisture to produce a brilliant olive oil for consumer acceptance. Different filtration systems have been applied in the pilot plant and olive oil industry, including conventional filtration systems (filter tanks and filter presses), cross-flow filtration (tangential flow filtration), inert gas flow filtration systems, and filter bags. Controversial results were published by different authors on how filtration affects the phenolic composition of EVOO. A laboratory scale study[50] showed that similar amounts of phenolic compounds were found with almost all cultivars in unfiltered as in filtered EVOO. Another laboratory scale study[18] showed that the hydroxytyrosol concentration decreased after filtration with cotton compared to the unfiltered olive oils. However, an apparent increase in hydroxytyrosol was produced in EVOOs after filtration with paper plus anhydrous sodium sulfate. A pilot plant scale study[20] using inert gas flow filtration systems and filter bags showed that the concentration of the most phenolic compounds seemed to increase after filtration. Among these, mainly secoiridoids were responsible for the apparent increase in the total phenolic content. Regarding the oxidative stability, it was reduced after filtration by the effects of water content on the polyphenolic antioxidant capacity. However, filtration of olive oil in the presence of inert gases did not decrease the main positive sensory attributes.

Filtration is therefore recommended because moisture reduction improves the quality of EVOO. Furthermore, the higher polar phase content in unfiltered olive oils may augment the degradation process and reduce the shelf life. From the phenolic composition point of view, the effects of this step are controversial. Indeed, in polar matrix such as unfiltered EVOO, the affinity of phenols to solvent extraction is lower than in a filtered EVOO, and the majority of phenolic compounds located around water droplets remain in unfiltered olive oil. Future investigations are warranted to develop a new analytical methodology taking into account the different water content in unfiltered and filtered EVOO and its effect on the extraction process used to qualitative and quantitative characterization of these compounds.**Table 1** shows the best process conditions to produce EVOO with high phenolic content.

Table 1. Best process conditions to produce EVOO with high phenolic content.

Steps	Control parameters	Conditions	Systems	Refs
Ripening	Maturity index or harvesting time	Early harvest time with low value of maturity index	-	17, 25
Crushing	Techniques, grid hole diameter, and rotation speed	Small grid holes and high rotation speed	Hammer crusher	29, 31
Malaxation	Temperature and time of malaxation	Temperature lower than 30 ºC and time shorter than 60 min	-	34-36
Horizontal Centrifugation	Two and three phase-decanter	Without addition of warm water	Two phase-decanter	40,42
Vertical Centrifugation	Water	Small water amounts added	-	44
Storage	Time, temperature, oxygene, and light	Short time, room temperature, darkness and absence of oxygen	-	49
Filtration	Filter aids	Nitrogen gas flow	Filtration using inert gas	20

3. Olive oil byproducts

A huge quantity of olive byproducts produced from olive processing of different kinds have been described in the literature according to the

extraction, filtration, and storage systems. Traditionally, large volumes of water are used in the three-phase mill to aid the separation of olive oil and generate two byproducts. The first byproduct of liquid waste is known as olive mill wastewater, vegetation water, or alpechin. The second byproduct is a solid waste called pomace or orujo. The use of a modern two-phase processing technique, in which no water is added, generates a new byproduct called alperujo or pomace and includes a combination of liquid and solid waste[51]. Other olive oil byproducts generated by storage and filtration of EVOO are composed of solid and liquid storage wastes and cakes used for EVOO filtration[52,53]. **Fig. 1** shows the many processing steps of olive oil producing food and waste byproducts, including crushing, malaxations, two centrifugations, destoning, storage, filtration, and bottling.

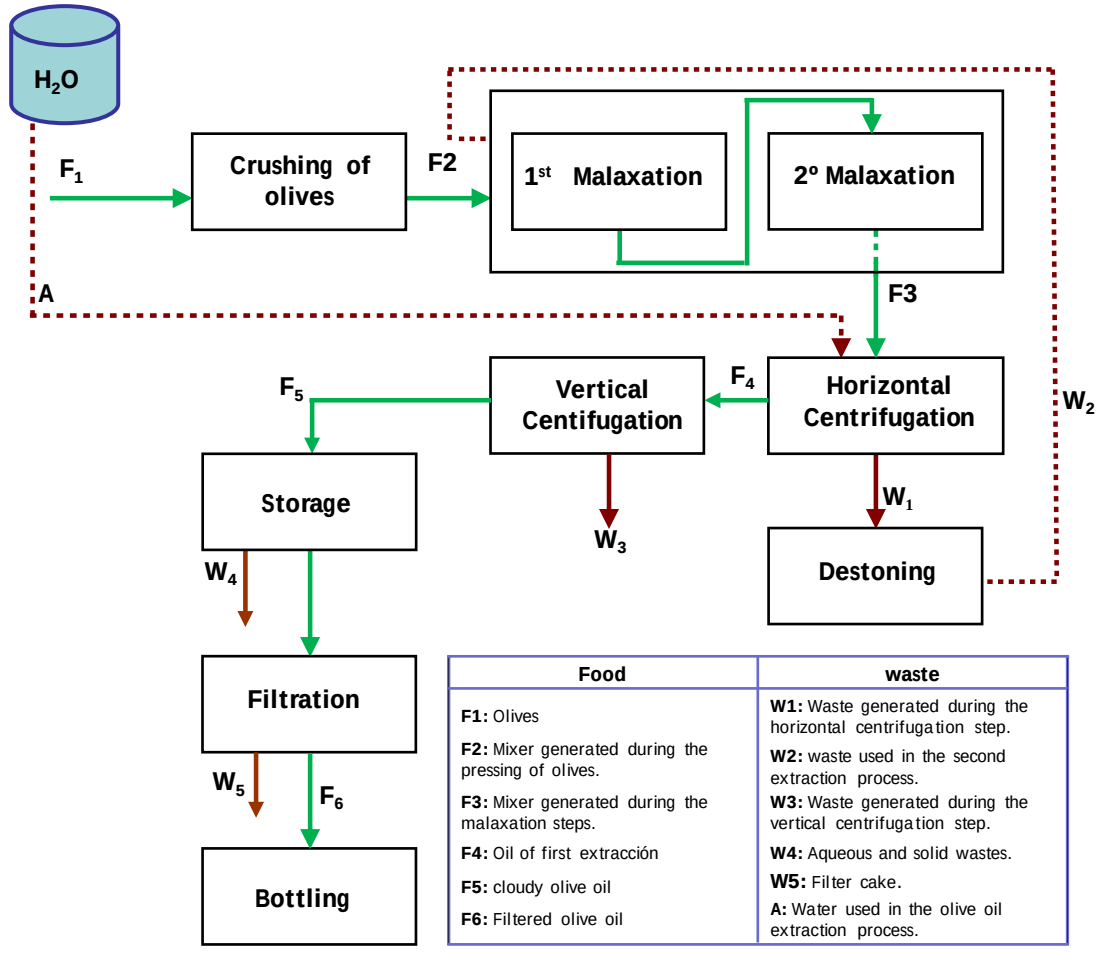

Fig. 1. Flow diagram of EVOO production industrial process.

3.1. Olive mill wastewater

The olive mill wastewater (OMWW) is a liquid of violet to dark brown color with a strong smell of olive oil[54]. This byproduct is composed of vegetable water from the fruit and the water used in different stages of oil extraction that contain olive pulp, mucilage, pectin, oil, and other suspended components in a relatively stable emulsion[55]. The chemical composition of OMWW is variable depending on olive cultivars, growing techniques, harvesting period, and especially the technology used for oil extraction[56,57]. Olive wastewater is characterized by diverse specific components and a high degree of organic pollution [chemical oxygen demand (COD) and biological oxygen demand (BOD)], acidic pH, high electrical conductivity, and phenolic content. Many published physical–chemical characteristics of OMWW show wide variations in pH, electrical conductivity, chemical and biological oxygen demand, and total phenolic compounds[58–63]. The composition and amounts of the OMWW are serious environmental problems in the Mediterranean areas, and the discharge of large quantities of these pollutants in the sewage system is not possible without any treatment. Different biological and chemical/physical methods have been proposed to reduce the organic matter, polyphenols, and tannins present in OMWW to detoxify their effects on the environment[64–66].

On the other hand, phenolic extracts from OMWW can be used as natural alternatives to commercial synthetic antioxidants with applications in food as well as the development of nutraceutical and medical products[67–69]. According to the extraction process, the partitioning behavior of polyphenols and their distribution between the oil and waste fractions are affected by the processing temperature and the quantity of water used for extraction. Although the partitioning of polyphenols into the oil is increased at higher temperatures, more amphiphilic polyphenols are lost in the wastewater if more water is added[70].

Different analytical extraction methods have been used to recover the phenolic components of OMWW which include oleuropein aglycon derivatives,

elenolic acid, luteolin 7-glucoside, quercetin, and phenolic alcohols[71]. Moreover, 20 phenolic compounds have been identified and 16 were quantified in the olive wastewater of Canino olives using high performance liquid chromatography (HPLC) coupled to mass spectrometry (MS)[72]. Phenolic compounds were also recovered by liquid–liquid extraction from centrifuged OMWW and characterized by gas chromatography coupled to MS[73]. The phenolic compounds identified in samples include hydroxytyrosol as the major component (66.5%), together with tyrosol, cafeic acid, *p*-coumaric acid, homovanillic acid, protocatechuic acid, 3,4-dihydroxymandelic acid, vanillic acid, and ferulic acid. Furthermore, the phenolic compounds were identified and quantitated in two different OMWW samples; hydroxytyrosol was the most abundant compound and represented about 70% and 55% of the total phenolic concentration of both OMWW extracts[74] Other phenolic compounds which have also been characterized in OMWW include verbascoside, isoverbascoside, *β*-hydroxyverbascoside, and various oxidized phenolic compounds[75,76].

3.2. Olive pomace

Olive pomace consists of olive pulp, skin, stone, and water. Different terms may be given depending on factors such as composition and oil content (crude or extracted olive pomace), stones, or moisture (fresh or dry olive pomace)[77]. The different olive oil extraction procedures and resulting byproducts have recently been documented[51]. The olive pomace obtained from the two-phase extraction procedure may be differentiated by the higher moisture and the lower oil content than from the three-phase centrifugation procedure, resulting in a more efficient and environmentally friendly centrifugation process, compared to the traditional three-phase system. Thus, olive pomace is an inexpensive biomass that is generated in large quantities in Mediterranean countries that also represents serious environmental problems[79]. Many studies have been aimed at reducing the environmental impact of olive pomace and/or harnessing its potential economic value. Olive pomace has been used as fuel, fertilizer, or animal feed[80,81]. Nevertheless, the profitability of olive pomace treatment plants is still in doubt because these activities only represent a very small percentage of the olive pomace

produced. **Table 2** shows the composition of olive pomace produced by three- and two-phase decanter[7].

Table 2. Quantity and characteristics of olive pomace obtained with different extraction systems for olive oil.

Measurements	3-phase decanter	2-phase decanter
Quantity (kg/t olives)	450–550	800–850
Moisture (%)	45–55	65–75
Oil (% on fresh pomace)	3.5–4.5	3–4
Pulp (%)	15–25	10–15
Stones (%)	20–28	12–18
Ash (%)	2–4	3–4
Nitrogen (mg/100 g)	200–300	250–350
Phosphorous (mg/100 g)	30–40	40–50
Potassium (mg/100 g)	100–150	150–250
Total phenolic Compounds (mg/100 g)	200–300	400–600

Evidently, there is a demand for alternative benefits from olive pomace, which is characterized by high contents of polyphenols[78]. The potential antioxidative activity of polyphenols in olive pomace would provide a cheap source of natural antioxidants in concentrations up to 100 times higher than in EVOO[82]. In fact, many scientific studies have been published on the phenolic characterization of olive pomace[83–86]. Methanolic extracts of olive pomace (two-phase extraction) were analyzed by HPLC–MS. Phenolic compounds identified included phenolic alcohols, flavonoids, and secoiridoids, including 10-hydroxyoleuropein, identified for the first time. In the same study, the comparison between olive pulp and olive pomace showed a change in phenolic structure. Because some phenolic compounds were not degraded during olive oil extraction, the olive pomace from the two-phase system may be considered as a good source of these compounds as olive pulp[87].

Different studies were carried out to use phenolic extracts from olive pomace to develop potential applications as food antioxidants. Edible oils and other foods were enriched with polyphenols extracted from olive pomace, and many phenolic compounds have been identified[88,89]. Studies of the optimization,

characterization, and quantification of phenolic compounds in olive pomace showed that the highest yield of total phenolic compounds was achieved by extraction with methanol at 70 °C for 12 h[85]. The major bound phenolic compounds in full-fat olive pomace included syringic acid (22%), protocatechuic acid (21%), caffeic acid (14%), sinapic acid (13%), and rutin (12%). In defatted olive pomace the relative concentrations were 23%, 14%, 11%, 17%, and 8% respectively.

3.3. Olive oil byproducts generated by storage and filtration of EVOO

The potential use of waste generated during the storage of EVOO as a natural source of phenolic antioxidant compounds has been evaluated[52] by solid–liquid and liquid–liquid extraction processes followed by rapid resolution liquid chromatography (RRLC) coupled to electrospray time-of-flight and ion trap mass spectrometry (TOF/IT-MS). Several degradation pathways of phenolic compounds were proposed based on hydrolysis, oxidation, hydration, and loss of the carboxylic group. These reactions occur during storage time, and the byproducts generated may be considered an important natural source of secoiridoid derivatives and flavones, mainly hydroxytyrosol, tyrosol, decarboxymethyl oleuropein aglycon, and luteolin. In solid waste, the dialdehyde form of decarboxymethyl-elenolic acid was the most abundant derivative, followed by hydroxytyrosol, luteolin, vanillin, and decarboxymethyl-oleuropein aglycon. Although the aqueous waste contained a small amount of phenolic compounds, it contained the highest amounts of phenolic alcohols.

Filtration may be carried out with various materials or filter aids in combination with filtration hardware to improve performance. Filter cake used during filtration could be used as a source of bioactive compounds. The hydrophilic phenolic compounds retained in different organic and inorganic filter aids included phenolic acids and alcohols, secoiridoids, lignans and flavones, vanillin, vanillic, ferulic, and *p*-coumaric acids, tyrosol, and hydroxytyrosol[53]. Although the healthy properties of the polyphenols have been identified in the wastes and in the byproducts and filter cake,[90–93]

additional investigations are needed to evaluate their applications as food antioxidant and nutraceutical products.

Fig. 2 shows the structure of the main phenolic compounds from each family identified in olive oil byproducts.

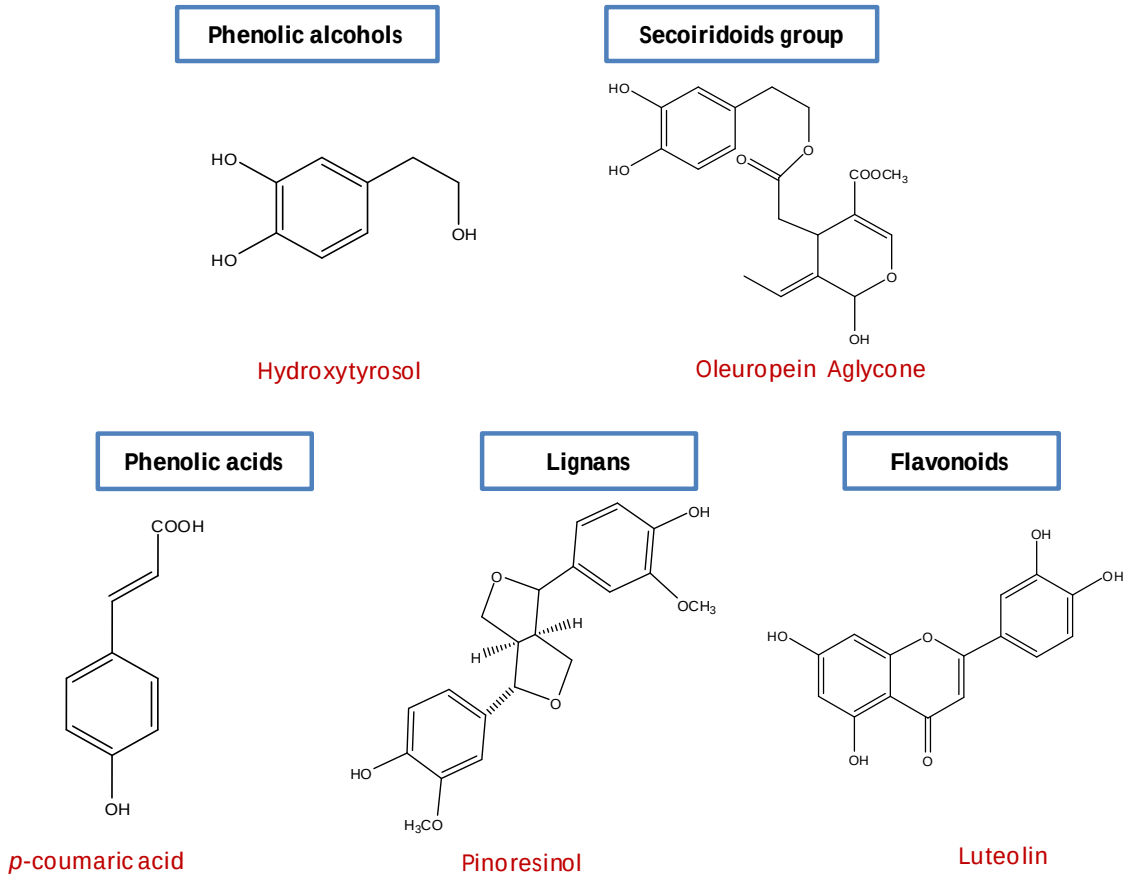

Fig. 2. Structures of the main phenolic compounds from each family identified in olive oil byproducts.

3.4. Biological activities and potential antioxidants of olive mill wastewater polyphenols

Several in vitro and in vivo studies showed that OMWW phenolic compounds exert potent biological activities including, but not limited to, antioxidant and free radical scavenging properties[24,90,94]. Hydroxytyrosol from OMWW is one of the most promising compounds as a potent inhibitor of copper and peroxyl radical-induced oxidation of low-density lipoprotein (LDL), representing one of the initial steps in the onset of atherosclerosis. Hydroxytyrosol may also

scavenge free radicals and modulate several enzymatic activities linked to cardiovascular diseases. The superoxide anion scavenging activity of four OMWW phenolic extracts in cultured human promonocyte cells (THP-1) has also been described,[95] being attributed to hydroxytyrosol as the most active component responsible. Moreover, the administration of OMWW extract fractions and purified hydroxytyrosol to diabetic rats caused a decrease in the glucose level in plasma[96–98].

On the other hand, the efficacy of a hydroxytyrosol-rich OMWW extract to attenuate Fe^{2+} and nitric oxide (NO) induced cytotoxicity in murine-dissociated brain cells was supported by ex vivo data providing the first evidence of neuroprotective effects of oral hydroxytyrosol intake[99]. Besides, to better understand the absorption potential for verbascoside and its derivatives recovered from OMWW, both in vitro digestion and Caco-2 human intestinal cell absorption studies were carried out to establish digestive stability and recovery (bioaccessibility) and efficiency of intestinal uptake/accumulation. During the experiment carried out, verbascoside was found to be moderately stable to in vitro digestive conditions with recovery of 53%, and its uptake by highly differentiated Caco-2 monolayers was rapid with peak accumulation occurring after 30 min. The total accumulation efficiency was 0.1% of the original amount of verbascosides present in a partially purified phenolic fraction of OMWW[75]. The verbascoside derivatives present in OMWW were also shown to provide a rationale in subsequent bioavailability and bioactivity studies. In anotherstudy, individual verbascoside from OMWW was active as a scavenger of reactive oxygen species and as a chemopreventive agent protecting LDL from oxidative damage[76].

3.5.Biological activities and potential antioxidant of olive pomace

The antioxidant activity of olive pomace due to its phenolic content has been evaluated and demonstrated by several authors. A positive correlation was reported between olive pomace total phenolic content and the antioxidant activity; these results suggest that the phenolic compounds in olive pomace could be used at different concentrations as antioxidant foods over the shelf

life[85]. It has recently been reported that the oxidative stability of EVOO and other edible oils was improved by using phenolic compounds extracted from olive pomace[88,89]. Finally, the analysis of rat tissues obtained after administration of a phenolic extract from olive pomace[83] showed a wide distribution of phenolic compounds and their metabolites, with a main detoxification route through the kidneys. The free forms of some phenolic compounds, such as oleuropein derivative, were quantitated in plasma and brain, luteolin in kidney, testicle, and heart, and hydroxytyrosol in plasma, kidney, and testicle.

3.6. Pilot plant and industrial processes to recover phenolic compounds from olive oil byproducts

Taking into account the phenolic composition of olive oil byproducts and their biological activity, these wastes may be used as valuable sources of components for nutraceuticals, food, and pharmaceutical preparations or in the cosmetics industry[100]. Although diverse synthetic procedures have been developed for the production of hydroxytyrosol and other phenolic compounds, the technological processes proposed so far are expensive and/or produce low yields[101]. Consequently, other types of natural compounds that could be used as antioxidants are urgently needed. Several extraction and purification technologies have been reported to obtain polyphenol enriched extracts, mainly in hydroxytyrosol, from olive oil byproducts. The main systems proposed to recover the phenolic compounds from olive waste include resin chromatography, microfiltration, ultrafiltration, nanofiltration reverse osmosis, and solid–liquid or liquid–liquid solvent extractions[102].

A patented system proposed to purify hydroxytyrosol from OMWW[103] includes passing the liquid source of hydroxytyrosol through an ion-exchange resin to trap the antioxidant and eluting with water, followed by adsorption through an XADtype nonionic resin. This matrix is washed with mixtures of methanol or ethanol and water (30–33%), to produce a solution containing at least 75% of hydroxytyrosol, followed by removal of the polar organic solvent to produce a solid containing 95% by weight of hydroxytyrosol, plus significant

fractions reaching up to 99% of purity. Another patented process is claimed104 for totally recovering the polyphenolic compounds in OMWW to reuse the concentrate residues in the production of fertilizers, biogas, and highly purified aqueous products that may also be used as a basic component of beverages. The process includes adjusting the pH of the freshly produced wastewater to within an acidic range, and an enzymatic hydrolysis followed by separation of the permeate streams obtained, by means of centrifugation and subsequent treatments with membrane technologies, consisting of microfiltration, ultrafiltration, nanofiltration, and reverse osmosis.

Another pilot scale system for the treatment of OMWW was developed for the recovery of valuable polyphenols and reduction of environmental problems[105]. The treatment consists of four steps: (a) successive filtration stages to gradually reduce and decolorize water suspended solids, (b) passage of the filtered wastewater through adsorbent resins to deodorize and decolorize the wastewater and recover the polyphenol and lactone components, (c) thermal evaporation and recovery of the organic solvent mixtures used to regenerate the resin, and (d) separation of the polyphenols and other organic substances by fast centrifuge partition chromatography. This procedure is claimed to reduce 99.99% of the polyphenols and 98% of chemical oxygen demand (COD) and to produce an extract rich in polyphenols and lactones of high antioxidant activity and added value, and an extract containing the coloring substances of olive fruit, and pure hydroxytyrosol. The extracts and pure compounds obtained are claimed to be useful not only for the pharmaceutical and cosmetic industries but also to produce wastewater free of polyphenols.

In another study, the application of a novel process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final liquid phase that contained a high concentration of simple phenolic compounds[106]. During thermal treatment, either 10 or 20 kg of alperujo was loaded into a 100 L reactor and for different heating times (15–90 min) was evaluated at 160 °C. The wet material was then centrifuged at 4700g to separate solids and liquids. After centrifugation, 10 L of the liquid phase from each treatment was concentrated to 1 L by rotary vacuum evaporation at 30 °C. A maximum

concentration of phenolic extract (11 g/kg) was finally obtained after 75 min of thermal treatment.

A new filtration process of EVOO to produce a filter cake enriched in polyphenols, which may be used as ingredient in functional foods and nutraceuticals, has been recently developed[107]. The filtration systems consist of using native starch as filter aid with filter tanks and filter presses. The composition of the final byproduct is based on native starch enriched in polyphenols, mainly hydroxytyrosol yields about 30 times higher than that from EVOO.

The interests in the healthy benefits of EVOO polyphenols have been increased due to recent different studies supporting their biological properties in reducing oxidative stress, especially when they are derived from more concentrated olive oil sources than in EVOO. Therefore, researching for the best ways of concentrating phenolic compounds in EVOO and recovering them from its byproducts could be very promising. To achieve this goal, many studies about the effects of olive oil process steps on final olive oil phenolic composition have been summarized to establish the best conditions to obtain EVOO with higher phenolic content. Taking into account the literature used in this review, harvesting too early, crushing olives using hammer crusher equipped with small grid holes and high rotation speed, malaxation of paste at temperature lower than 30 °C and time shorter than 60 min, centrifugation of paste using two-phase decanter followed by vertical centrifugation with a minimum water added, storage of EVOO at short time and low temperature, and filtration using inert gases, contributed to obtain EVOO enriched in phenolic compounds and to conserve its positive sensory attributes.

The next aim of this review was to summarize the qualitative and quantitative characterization of the phenolic compounds in olive oil byproducts. Phenolic alcohols, consisting of hydroxytyrosol, phenolic acids, secoiridoids, lignans, and flavonoids, were found as the main phenolic families. Furthermore, the antioxidant and biological activity of phenolic extracts from OMWW and olive pomace showed interesting results in all works carried out and summarized in

the review. However, phenolic extracts from waste generated during storage of olive oil and filter cake have not been evaluated, and future investigations are needed to evaluate their applications in food antioxidant and nutraceutical products. Finally, different pilot plant and industrial processes employed to recover phenolic compounds from olive oil byproducts have been widely reviewed.

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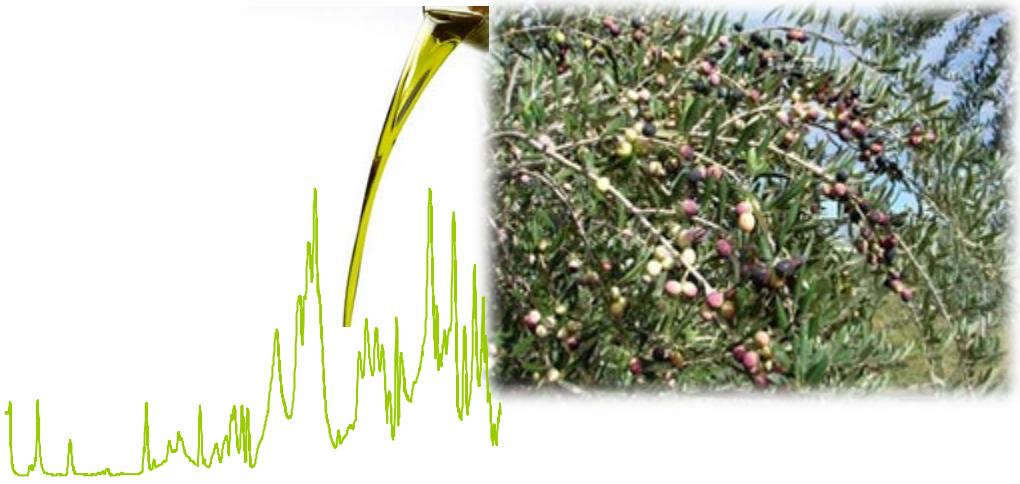

Chapter 4

Time course of Algerian Azeradj extra-virgin olive oil quality during olive ripening

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Research Article

Time course of Algerian Azeradj extra-virgin olive oil quality during olive ripening

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Abstract

The aim of this work was the chemical characterization of Algerian Azeradj extra-virgin olive oil (EVOO) at different harvest dates in terms of oil yield, quality indices, fatty acids, pigments, polyphenols, and oxidative stability, in order to establish the best harvesting period for this variety. For this purpose, Azeradj EVOO samples were taken at three harvest dates corresponding to three ripening stages. Except for K_{270}, the analysis of EVOOs showed a significant increase in the values of all quality parameters as olive ripening progresses (free fatty acids, peroxide index, and K_{232}), while no significant variation was found for oil yield.Moreover, a significant decrease was observed for the monounsaturated fatty acid: polyunsaturated fatty acid (MUFA: PUFA) ratio, chlorophyll, carotenoids, total phenols, and oxidative stability in EVOOs produced at the advanced ripening stage. For better monitoring of the behavior of the phenolic fraction in EVOOs during ripening, for the first time in this work, individual phenolic compounds of Azeradj EVOOs was characterized using HPLC-ESI-TOF/MS. A total of 21 phenolic compounds belonging to different families were identified in Azeradj EVOOs. The highest concentrations of secoiridoids and phenolic alcohols were found at the beginning of November (D1); however, lignans and flavones concentrations were higher at the end of November (D2). EVOO obtained at the last harvest date (D3) showed the lowest content on all phenolic families.

Finally, according to the results of the analyses performed in this work, early harvesting provides Azeradj EVOO with excellent chemical characteristics.

Practical applications: The Algerian olive oil sector is one of the least competitive in the Mediterranean region even if the Algerian olive oil displays some potential assets. Therefore, the chemical characterization of olive oil from one of the main varieties (Azeradj) cultivated in Algeria is of a great importance. The results will be available to all Algerian olive oil producers, and should be helpful in choosing the optimal harvest period to obtain Azeradj EVOO of high chemical quality.

*Keywords***:** Azeradj EVOO, Chemical characterization, HPLC-ESI-TOF/MS, Phenolic compounds, ripening

1. Introduction

Extra-virgin olive oil (EVOO) is the main source of fat in the Mediterranean region. The healthy proprieties of this oil were attributed firstly to its high content on monounsaturated fatty acids (MUFA), presented by oleic acid as the most abundant one [1]. However, recent studies demonstrated that the minor fraction as polyphenols also make a major contribution to healthy EVOO properties [2, 3]. Due to these characteristics, EVOO consumption, as a healthy food, is increasing considerably throughout the world. Nevertheless, an increasing demand for EVOO cannot be ascribed exclusively to its beneficial effects on health, but also to its unique aroma and taste that distinguish it from other edible vegetable oils [4]. In addition to its health and organoleptic properties, the oxidative stability of EVOO is high, due to its high ratio of monounsaturated: polyunsaturated (MUFA: PUFA) fatty acids, and the presence of polyphenols, chlorophyll, and carotenoids [5, 6].

Algeria is a Mediterranean country where olive-oil production began under the Imperial Roman dominance. Olive orchards are mostly traditional (90%), located in the mountain areas and marginal lands, characterized by a rainfall average of 400 to 900 mm/year. The orchards are dominated by three main varieties: Chemlal, Bouchouk, and Azeradj. Although Algeria ranks seventh worldwide in olive-oil production, most of olive oil produced in the country is still considered unsuitable for human consumption, limiting its market share. Due to this low competitiveness of Algerian olive oils, the enhancement of its quality in order to guarantee its nutritional and organoleptic proprieties has become a priority of the country. For this, the study of the factors having a direct impact on olive-oil quality is fundamental [7, 8].

Olive-oil composition is well known to be affected by several factors related to its agronomic conditions of cultivation and its extraction process [9, 10]. Among the agronomic factors, the degree of olive ripening is a key to take into consideration before the harvesting. During ripening, chemical changes occur inside the drupe in relation to the synthesis of triglycerides and other enzymatic activities. These changes are reflected in olive oil quality [11, 12].

Previous studies have suggested that the majority of olive oil produced does not belong to the best commercial quality as the fruit is not picked at the optimal harvest time. This illustrates the need to determine an appropriate maturation stage of each olive variety before processing [13]. Consequently, the objective of this work was the chemical characterization of Algerian Azeradj EVOO obtained at different harvest dates, in order to determine its optimal harvest period. Notably, a detailed characterization of individual phenolic compounds in EVOOs from this variety, using HPLC-ESI-TOF/MS, was carried out for the first time in this work.

2. Materials and methods

2.1. Chemicals and apparatus

All chemicals were of analytical reagent grade. Cyclohexan, potassium hydroxide, and acetic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium hydroxide, isopropanol, *n*-hexane, and methanol were purchased from Merck (Darmstadt, Germany). Double-deionized water with conductivity less than 18 MΩ.cm was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Standards of hydroxytyrosol, tyrosol, luteolin, apigenin, *p*-coumaric acid, and quinic acid were purchased by Sigma–Aldrich (St. Louis, MO, USA), and pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein and dihydrocaffeic acid were purchased from Extrasynthese (Lyon, France).

2.2. Samples

Olive fruits from the Azeradj variety were manually collected on different dates (D1, 03 November 2013; D2, 27 November 2013; and D3, 21 December 2013) from trees cultivated in the same area (Haizar), in north-central Algeria. The trees were under the same agricultural practices and climatic conditions (temperature and precipitation). For the sampling, nine trees were randomly assigned to different groups. From each group, 1200 g of olives was collected for each harvest date, making three homogeneous samples per harvest, which were used for olive-oil extraction. In addition, the

olive-maturity index (MI) was determined according to the method previously reported in the literature [14]. This method was based on the assessment of the color of the olive skin and pulp. MI values ranged from 0 (very green skin 100%) to 7 (100% purple flesh and black skin).

Olive-oil samples were made at the laboratory scale using the Abencor system (S.I.O.L. 20240 GHISONACCIA, France) equipped with a hammer crusher, malaxer, and centrifuge. Prior to the crushing step, the olives were manually sorted and cleaned, removing damaged fruit, leaves, and other debris. The clean and healthy olives were crushed and were slowly mixed for 30 min at 25°C. Then, the resulting paste was subjected to centrifugal separation for 3 min at 3000 rpm. The liquid phase (oil and waste) was allowed to decant naturally into specimens. The top oil layer was removed, stored in glass bottles at room temperature, and kept away from light until its analysis.

2.3. Oil yield

The olive-oil content was determined according to the analytical method described in EEC Regulation 2568/91 applied to olive pomace and the following amendments [15]. Olives dried at 80°C, were ground in a mortar. Afterwards, 10 g of the ground material was used for oil extraction in a Soxhlet apparatus for 8 h using n-hexane at 80°C. At the end of the extraction the solvent was separated by a rotary evaporator**.**

2.4. Analytical determination of the quality parameters

Free fatty acids (FFA) (%), peroxide value (PV), and spectrometric UV (K_{232} and K_{270} nm) were determined according to analytical methods described in EEC Regulations 2568/91 and the following amendments [15]. All parameters were determined in triplicate for each sample. FFA was given as a percentage of oleic acid and PV expressed in milliequivalents of active oxygen per kilogram of oil (meq O2/kg). Spectrophotometric determinations were made using an UV mini-1800 instrument (Shimadzu Co., Kyoto, Japan). The K_{232} and K_{270} extinction coefficients were calculated from absorption at 232 and 270 nm, respectively.

2.5. Fatty acid composition

The fatty acid (FA) composition was determined according to the method described previously [8]. Prior to chromatographic analysis, the fatty acids were converted to methyl esters using the following method: 0.2 g of olive oil was added to 3 mL of *n*-hexane and 0.4 mL of methanolic potassium hydroxide 2N, followed by stirring. A gas chromatograph (GC) Chrompack CP 9002 (Les Ulis, France) equipped with split/splitless injector, and flame-ionization detector (FID) was used for this determination. The analytes were separated on DB23 (50% cyanopropyl) capillary column (30 m 0.32 mm i.d., 0.25 mm film thickness; Agilent Technologies, Palo Alto, CA, USA) and nitrogen as the carrier gas (Linear velocity, 0.5 cm/min; split ratio of 1:30, v/v). The injection volume into a split GC port was 0.8 ml. The temperature of the column was held constant throughout the entire separation at 200°C. The FID and the injector temperatures were 280, and 250°C, respectively. Three replicates were prepared and analyzed per sample.

2.6. Chlorophyll and carotenoids

Pigment amounts were calculated using the specific extinction values, by the method reported previously [16], dissolving 7.5 g of olive oil in 25 mL of cyclohexane. The extinction coefficients applied were E_0 = 613 for pheophytin 'a' and E_0 = 2000 for lutein. Thus, pigment contents were calculated as follows:

[chlorophyll] (mg/kg) = (A_{670} x10^6)/ (613 x 100 x d)

[carotenoid] (mg/kg) = (A_{470} x10^6)/ (2000 x 100 x d)

where A is the absorbance and *d* is the spectrophotometer cell thickness (1 cm).

2.7. Phenolic compounds

Phenolic compounds were isolated from Azeradj EVOOs using a liquid-liquid extraction system following the method reported previously [9], with some modifications. EVOO, with 50 mL of internal standard solution (25 mg L^{-1} dihydrocaffeic acid in methanol) added, was dissolved in *n*-hexane (2.5 g in 5 mL). Afterwards, 5 mL of methanol/water (60/40, v/v) was added, and the mixture was vortexed and then centrifuged at 3500 rpm for 10 min. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure at 35°C. The residue was dissolved in 0.25 mL of methanol/water (50/50 v/v) and finally filtered through a 0.2 µm filter before the HPLC analysis.

The analysis to characterize the phenolic profile of Azeradj EVOOs extracts was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump, and a diode array detector (DAD). The chromatographic separation of these compounds was performed on a 150 mm x 4.6 mm i.d., 1.8 mm, Zorbax Eclipse Plus RP-C18 column (Agilent Technologies, Palo Alto, CA, USA). The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The total run time was 27 min using a previously reported multistep linear gradient [17]. The flow rate was 0.80 mL min^{-1} and, consequently, the use of a splitter was required for the coupling with the MS detector, as the flow which arrived to the TOF detector had to be 0.2 mL min^{-1} to ensure reproducible results and stable spray. HPLC was coupled to a time-of-flight mass spectrometer detector micrOTOF (Bruker Daltonik, Bremen, Germany), which was equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative ion mode.

External mass-spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/isopropanol 1/1 (v/v), with 0.2% of acetic acid) in quadratic + high-precision calibration (HPC) regression mode. The optimum values of the source and transfer parameters were established according to the method published previously [17]. The widely accepted

accuracy threshold for confirmation of elemental compositions was set at 10 ppm for most of the compounds. The phenolic compounds were identified by comparing both retention times and MS data from samples and standards. The remaining compounds for which no commercial standards were available were identified by the interpretation of the information generated by the TOF analyzer, and the information reported in the literature [9, 17, 18]. Quantification was made by HPLC-ESI-TOF/MS. Eight standard calibration curves of the main compounds found in the samples were prepared using eight commercial standards. Stock solutions at a concentration of 1000 mg L^{-1} for each phenolic compound were first prepared by dissolving the appropriate amount of the compound in methanol. Afterwards, the stock solutions were serially diluted to working concentrations. All calibration curves showed good linearity over the study range (r^2 = 0.996). The individual concentrations were determined using the area of each individual compound (three replicates) and by interpolation of the corresponding calibration curve. Results were given in milligram of analyte per kilogram of EVOO.

2.8. Oxidative stability

The olive-oil oxidative stability was measured using rancimat (Metrohm Applications) following the method proposed previously [19]. The oil sample was subjected to an air stream at 10 L/h and temperature of 102°C ± 1.6°C. The results were expressed as oxidative induction time (hours). The oxidative stability was tested three times per sample.

2.9. Statistical analysis

The data were analyzed using Origin (version Origin Pro 8 SR0, Northampton, MA, USA) to perform a one-way analysis of variance (ANOVA) with $p \leq 0.05$ to identify significant differences among all parameters analyzed in Azeradj EVOOs from different harvest dates.

3. Results and discussion

3.1. Oil content

Fruit color serves as a common marker for maturation level, expressed as maturity index (MI). As shown in **Table 1**, the temporal progression of the harvest season was accompanied by a significant increase in MI. Indeed, the lowest value (2.5) was found in olives harvested early corresponding to the harvest date D1. However, the highest value (5.0) corresponded to olives harvested later (D3). As previously reported, the increase in the maturity index changed the olive-oil content expressed as a percentage of dry matter [20]. Analyzing the results in this study, we found that olive-oil yield values firstly tended to increase from 37% to 40% for harvest dates D1 and D2, respectively. Later, olive-oil yield tended to decrease to 37% at the last harvest date (D3). Nevertheless, no significant variation in olive-oil yield was found during the maturity process. These results corroborate those of previous studies [8, 21].

3.2. Quality parameters

According to the results obtained for FFA, PV, and specific extinctions, K_{232} and K_{270}, all the samples analyzed can be categorized as extra-virgin olive oil. FFA designates the degree of hydrolytic deterioration, reflecting the health status of the olive fruit. Its values varied slightly in EVOO for harvest dates (D1) and (D2). However, a significant increase was found in EVOO belonging to the last harvest date (D3). This tendency in FFA during ripening could be explained by the enzymatic activity, especially lipolytic ones [22]. PV is the most method commonly used to measure the total hydroperoxides as the primary oxidation products. Although the values for these parameters found in this work were much lower than the limit fixed for the EVOO category, PV tended to increase during ripening. Nevertheless, this increase proved significant only in EVOO produced from olives harvested in December (D3). The increase of PV during ripening was found in previous studies, and was attributed to the high lipoxygenase activity [4, 23]. K_{232} also considered an indicator of olive-oil primary oxidation. The same tendency of K_{232} as PV was

found during ripening. Indeed, its highest value was found for the last harvest date (D3). Finally, the analysis of K_{270} gives information on secondary oxidation of olive oil. The results for this parameter did not significantly differ among harvest dates. With respect to the results for PV, K_{232}, and K_{270}, the EVOOs made at the advanced maturity stage corresponding to the harvest date D3 were slightly oxidized. However, secondary oxidation did not occur.

Table 1. Analytical characteristics of Azeradj extra-virgin olives oils obtained at different olives harvest dates.

Parameters[d]	D1[k]	D2	D3	legal limits
Maturity index	2.5^{c}±0.2	3.3^{b}±0.3	5.0^{a}±0.1	
Oil yield (% dry matter)	37^{a}±3	40^{a}±2	37^{a}±3	
Free fatty acids (% oleic acid)	0.32^{b}±0.01	0.34^{b}±0.02	0.44^{a}±0.01	≤ 0.8
Peroxid value (meq O_2/kg)	4.5^{b}±0.3	5.3^{b}±0.3	6.5^{a}±0.2	≤ 20
K_{232}	2.17^{b}±0.02	2.2^{b}±0.2	2.5^{a}±0.21	≤ 2.5
K_{270}	0.15^{a}±0.01	0.11^{a}±0.01	0.11^{a}±0.01	≤ 0.22
Chlorophyll (mg/kg)	3.3^{a}±0.1	0.79^{b}±0.04	0.62^{b}±0.04	
Carotenoids (mg/kg)	1.8^{a}±0.1	0.74^{b}±0.02	0.71^{b}±0.03	
Fatty acids Composition	**D1**	**D2**	**D3**	
Palmitic acid	13^{a} ±1	$12.3^{a,b}$±0.3	11.5^{b}±0.2	7.5-20
Palmitoleic acid	0.96^{a}±0.02	0.76^{b}±0.01	$0.92^{a,b}$±0.04	0.3-3.5
Stearic acid	4.0^{a}±0.4	$3.50^{a,b}$±0.03	3.44^{b}±0.03	0.5-5
Oleic acid	75^{a}±1	75^{a}±1	72^{a}±3	55-83
Linoleic acid	8.1^{b}±0.1	9.1^{b}±0.3	12^{a}±1	3.5-21
Linolenic acid	NQ	0.61^{a}±0.04	0.66^{a}±0.01	≤1
MUFA/PUFA	9.3^{a}±0.3	7.8^{b}±0.2	6^{c}±1	

[d] MUFA/PUFA: monounsaturated fatty acids/polyunsaturated fatty acids.

[k] D1, D2, D3: harvest dates.

Values with the same letter in a line are not significantly different at a 95% confidence level ($p \leq 0.05$).

3.3. Fatty acids

The change in fatty acid composition of Azeradj EVOO during ripening is given in **Table 1**. As the olive ripening progressed, palmitic acid, the main saturated fatty acid in EVOO, significantly declined in content at the last harvest date (D3) together with stearic acid. A slight decrease in oleic acid content was

observed in EVOO produced from olives harvested later (D3). However, it did not significantly vary among the harvest dates under study. Linoleic acid content tended to increase during ripening, and its increase was significant only in EVOO belonging to the last harvest date. The behavior of linoleic acid during ripening has been attributed in previous studies to the activity of the enzyme oleate desaturase, which transforms oleic acid into linoleic acid during triacylglycerol biosynthesis [24, 25]. However, analyzing our results, we found that between the first and second harvest dates linoleic acid content tended to increase while oleic acid content remained constant. These results could be explained by the equilibrium between oleic acid synthesis and its transformation to linoleic acid. Concerning linolenic acid, it was identified in Azeradj EVOO obtained only on the second (D2) and third (D3) harvest dates, its content did not vary significantly between the two dates. Due to the great importance of fatty acid composition on EVOO oxidative stability [6], the ratio of MUFA: PUFA was calculated for different harvest dates (Table 1). This ratio decreased significantly during olive ripening. Indeed, the highest value (9.3) was found in EVOO belonging to the first harvest date (D1). Nevertheless, its lowest value (6) resulted in EVOO from olives harvested later (D3). This tendency for fatty acid composition during ripening could involve a rise in EVOO oxidative susceptibility at more advanced ripening stages, corresponding to the last harvest date.

3.4. Pigments (chlorophylls and carotenoids)

Chlorophyll and carotenoid play an important role in olive-oil sensory properties, by determining its color, which is one of the factors that influence consumer selection. These pigments were considered responsible mainly for the variation on olive-oil color from yellowish green to greenish gold. Furthermore, pigments are also involved in olive-oil oxidative stability [5,16]. The degree of olives ripening is crucial for pigment (chlorophyll and carotenoid) concentrations in olive oil. The highest concentration value of chlorophyll in EVOOs under study was found in the early olive-harvesting period (D1, 3.3 mg kg^{-1}). After 24 days, the chlorophyll content significantly declined (D2, 0.79 mg kg^{-1}), resulting in a reduction of 78% from the initial

content recorded on the first harvest date. Finally, the decrease in this content was lower, and no significant variation was found between EVOO from D2 and D3, respectively. A similar trend was found for the carotenoid content during the ripening process **(Table 1)**. These results agree with those reported previously in the literature for other cultivars [8, 13, 22].

3.5. Characterization of phenolic and other polar compounds in Azeradj EVOOs

3.5.1. Qualitative characterization

The base-peak chromatogram (BPC) of Azeradj EVOO phenolic extract, determined using HPLC-ESI-TOF/MS in negative ionization mode, is shown in **Fig. 1**. The tentatively identified compounds are summarized in **Table 2**, including retention times, calculated and experimental mass m/z, molecular formula, error, and msigma values. A total of 21 phenolic compounds and another polar compound (quinic acid) were characterized in the samples. The phenolic compounds identified are classified into five groups: phenolic alcohols, phenolic acids, secoiridoids, lignans, and flavones. The results provided by HPLC-ESI-TOF/MS showed no qualitative differences in phenolic profile among Azeradj EVOOs from different harvest dates under study.

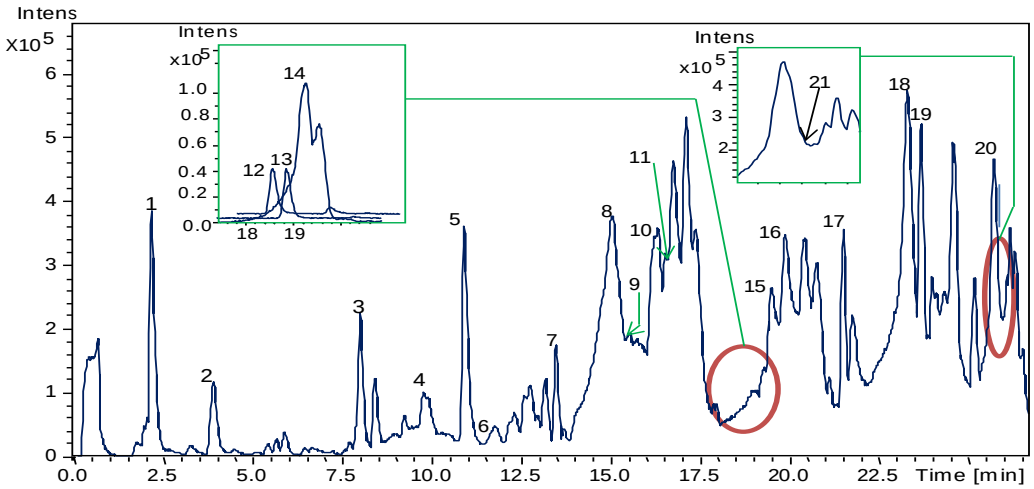

Fig. 1. Base-peak chromatogram (BPC) of Azeradj EVOO phenolic extract, using HPLC-ESI-TOF/MS. Proposed phenolic compounds have been numbered by elution order and the peak number has been included in Table 2.

Table 2. Main phenolic and other polar compounds identified in a representative Azeradj extra-virgin olive oil extract obtained by HPLC-ESI-TOF/MS.

Peak number	Compounds[a]	RT(min)[b]	Molecular formula	m/z calcd[c]	m/z Exptl[d]	Error (ppm)	msigma
1	Quinic acid	2.19	$C_7H_{12}O_6$	191.0561	191.0563	-0.9	2.7
2	H- HYTY	3.92	$C_8H_8O_3$	151.0401	151.0395	3.5	1.8
3	HYTY	8.00	$C_8H_{10}O_3$	153.0557	153.0554	2.2	3.9
4	TY	9.83	$C_8H_{10}O_2$	137.0608	137.0596	8.5	4.7
5	DEA	10.90	$C_9H_{12}O_4$	183.0663	183.0655	4.3	3.1
6	H-D-Ol Agl or isomer	11.74	$C_{17}H_{20}O_7$	335.1136	335.1116	5.9	14.0
7	*p*-coumaric acid	13.45	$C_9H_8O_3$	163.0401	163.0395	3.7	12.1
8	EA	15.00	$C_{11}H_{14}O_6$	241.0718	241.0713	1.8	4.6
9	H-EA	15.62	$C_{11}H_{14}O_7$	257.0667	257.0656	4.3	1.5
10	DOA	16.29	$C_{17}H_{20}O_6$	319.1187	319.1182	1.6	3.5
11	H-D-Ol Agl or isomer	16.62	$C_{17}H_{20}O_7$	335.1136	335.1122	4.1	17.0
12	Syringaresinol	18.21	$C_{22}H_{26}O_8$	417.1555	417.1525	7.2	9.4
13	Pin	18.92	$C_{20}H_{22}O_6$	357.1344	357.1322	6.0	4.1
14	D-Lig Agl	19.27	$C_{17}H_{20}O_5$	303.1238	303.1235	0.80	4.2
15	AcPin	19.42	$C_{22}H_{24}O_8$	415.1398	415.1380	4.4	3.2
16	H-D-Lig Agl	19.84	$C_{17}H_{20}O_6$	319.1187	319.1177	3.1	18.8
17	Dehydro Ol Agl	21.61	$C_{19}H_{20}O_8$	375.1085	375.1057	2.8	14.3
18	Ol Agl	23.26	$C_{19}H_{22}O_8$	377.1242	377.1208	8.9	7.4
19	Lut	23.65	$C_{15}H_{10}O_6$	285.0405	285.0382	8.0	2.5
20	Lig Agl	25.65	$C_{19}H_{22}O_7$	361.1293	361.1272	5.9	2.5
21	Apig	25.90	$C_{15}H_{10}O_5$	269.0455	269.0445	3.8	4.6

[a]H-HYTY, Oxidized hydroxytyrosol; HYTY, hydroxytyrosol; TY, tyrosol; DEA, decarboxymethylated form of elenolic acid; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; Pin, pinoresinol; D-Lig Agl, decarboxymethyl ligstroside aglycone; AcPin, acetoxypinoresinol; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone; Ol Agl, oleuropein aglycone; Lut, luteolin; Lig Agl, ligstroside aglycone; Apig, apigenin.

[b]RT: retention time

[c]m/z calcd: calculated mass.

[d]m/z exptl: experimental mass.

3.5.2. Quantitative characterization

Differences in phenolic content were detected among Azeradj EVOOs from different harvest dates. As shown in **Table 3**, phenolic alcohols decreased in EVOO as ripening progressed. Indeed, the highest concentration in EVOO corresponded to the first harvest date (29 mg kg^{-1}, D1). A sharp decrease (71%) was registered on the phenolic alcohols content between EVOOs from the first (D1) and second (D2) harvest dates, respectively. The lowest content on this group of compounds was found in EVOOs from the last harvest date (D3), corresponding to the most advanced maturity index. Secoiridoids was the most abundant phenolic group quantified in the samples under study, a similar trend as phenolic alcohols was observed in its concentration during olive ripening. Although their decline was not high among the harvest dates studied, secoiridoids content varied between 186 mg kg^{-1} and 168 mg kg^{-1}.

As opposed to phenolic alcohols and secoiridoids, lignans increased significantly in concentration, and its highest value was found in EVOO from the second harvest date (4.7 mg kg^{-1}, D2). Afterwards, lignans concentration declined significantly to reach 3.3 mg kg^{-1} at the last harvest date (D3). The same trend as lignans was detected in the flavones content during ripening.

For a clearer understanding of the trends of phenolic compounds during ripening and for a fuller analysis of the results, the monitoring of individual concentrations provided by HPLC-ESI-TOF/MS is of great importance. **Table 3** lists the phenolic compounds quantified and the individual concentration of each one at different harvest dates. Tyrosol seemed to be the most abundant phenolic alcohol in Azeradj EVOO, followed by hydroxytyrosol and its oxidized form. The concentration of these three compounds was higher in early harvested samples (D1). However, the hydroxytyrosol concentration declined by 87% from the first harvest date (D1) to the last one (D3). This data is important due to the reported role of hydroxytyrosol as antioxidant [26]. The loss on its content during ripening could affect the oxidative stability of olive oil. A sharp reduction on tyrosol content was also found (91%) between D1 and D3 harvest dates. The trend of the content of these compounds during

ripening has been reported in olive oils from other olive cultivars [8, 27, 28]. The behavior of phenolic alcohols content during ripening could be attributed to the decrease on their synthesis. Previous study [11] showed that the active phenol synthesis takes place at the first ripening stages in fruit, and once the massive phase of polyphenol synthesis is complete, the biosynthetic capacity is reduced and there is a sudden decrease on polyphenols levels.

Table 3. Phenolic and other polar compounds content and oxidative stability of Azeradj extra-virgin olive oil at different harvest dates.

Parameters [d]	D1 [k]	D2	D3
H-HYTY	2.06[a]±0.01	0.70[b]±0.01	0.48[c]±0.01
HYTY	5.9[a]±0.1	2.5[b]±0.2	0.76[c]±0.05
TY	21[a]±1	5.2[b]±0.1	1.8[c]±0.1
Phenolic alcohols	**29[a]±2**	**8.5[b]±0.3**	**3.0[c]±0.2**
P-coumaric acid	1.9[a]±0.1	1.3[b]±0.1	0.21[c]±0.01
DEA	0.60[a]±0.01	0.70[a]±0.01	0.20[b]±0.02
EA	53[a]±1	30[b]±2	25[c]±1
DOA	52[a]±5	46[b]±3	37.1[c]±0.3
H-D-Ol Agl or Isomer	8.51[b]±0.02	21[a]±1	27[a]±2
D-Lig Agl	18.0[a]±0.1	7.0[b]±0.2	7.2[b]±0.3
H-D-Lig Agl	20[b]±2	25[a]±2	23[a]±1
Dehydro-Ol Agl	10.4[b]±0.4	24[a]±1	25[a]±1
Ol Agl	15.6[a]±0.3	17[a]±1	17.58[a]±0.02
Lig Agl	8.0[a] ±0.4	7.8[a]±0.4	7.3[a]±0.1
Secoiridoids	**186[a]±1**	**178[b]±14**	**168[c]±9**
Syringaresinol	0.46[b]±0.03	0.49[a]±0.04	**NQ**
Pin	3.7[b]±0.1	4.2[a]±0.1	3.3[b]±0.3
Lignans	**4.15[b]±0.04**	**4.7[a]±0.1**	**3.3[c]±0.3**
Lut	1.5[b]±0.1	2.7[a]±0.2	1.04[b]±0.01
Apig	0.16[b]±0.01	0.33[a]±0.01	0.25[a,b]±0.02
Flavones	**1.7[b]±0.1**	**3.0[a]±0.3**	**1.3[b]±0.1**
Quinic acid	2.11[a]±0.03	0.66[b]±0.01	0.97[b]±0.04
Total phenols	**222[a]±1**	**196[b]±6**	**176[c]±9**
Oxidative stability	32[a]±3	31[a]±3	15.26[b]±0.03

[d]H-HYTY, oxidized hydroxytyrosol; HYTY, hydroxytyrosol; TY, tyrosol; DEA, decarboxymethylated form of elenolic acid; EA, elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone;

Ol Agl, oleuropein aglycone; Lig Agl, ligstroside aglycone; Pin, pinoresinol; Lut, luteolin; Apig, apigenin; [K]D1, D2, and D3: harvest dates; values with the same letter in a line are not significantly different at a 95% confidence level ($p \leq 0.05$).

Concerning phenolic acids, the only compound which was identified and quantified in this group was p-coumaric acid. Its concentration tended to decrease during ripening, resulting on a reduction of 89% between the first (D1) and the last (D3) harvest dates.

Regarding secoiridoids, oleuropein aglycone, ligstroside aglycone, and their derivatives were the main secoiridoids quantified in Azeradj EVOOs. The trend of these compounds and their derivatives is linked to their precursors (phenolic alcohols and elenolic acid derivatives) as well as the activity of some enzymes over the ripening and extraction step in the olive oil production process. Analyzing the results for EVOOs at different harvest dates, we found that the decrease in decarboxymethyl oleuropein aglycone and decarboxymethyl ligstroside aglycone content during ripening was accompanied by an increase on their hydroxylated forms. These results could be explained by the rise in the activity of polyphenol oxidase at more advanced ripening stages, previously reported in the literature [29]. It should be taken into account that these compounds could also be produced during the olive-oil extraction process (crushing and malaxation steps), which may change their concentration in the final product (olive oil) [30]. Oleuropein aglycone and ligstroside aglycone are the results of oleuropein and ligstroside hydrolysis produced by the enzyme b-glucosidase in the olive fruits during ripening. No significant variation in their content was found among the harvest dates under study, results that could be explained by the equilibrium between the anabolic and catabolic pathways of oleuropein and ligstroside synthesis according to the cycle proposed in a previous study [11]. Elenolic acid and its decarboxymethylated form also declined during ripening. Indeed, their lowest concentration was found in EVOOs belonging to the last harvest date (D3). The decline in elenolic acid content could be attributed to the depressed hydrolytic reaction of oleoside-11-methyl ester catalyzed by the enzyme *β*-glucosidase [11]. Finally, the trend observed in

decarboxymethylated form of elenolic acid in EVOO from the last harvest date (D3) may be explained by the decrease in its precursors such as decarboxymethyl oleuropein aglycone and elenolic acid during ripening.

In terms of lignan concentrations, pinoresinol and syringaresinol increased significantly during the early harvest dates. Indeed, the highest concentration of both compounds was recorded in EVOOs from the second harvest date (D2). Nevertheless, the concentration decreased significantly at more advanced ripening stages. It should be taken into consideration that syringaresinol was not quantified in EVOOs from the last harvest date (D3), because its concentration was between the detection and quantification limits [17]. Finally, flavones presented by luteolin and apigenin showed the same trend as pinoresinol and syringaresinol during ripening. These results are consistent with the changes found previously in lignans and flavones during ripening [8].

3.6. Total phenols

In this study, the total phenols shown in **Table 3** were determined by adding together the individual concentrations of each phenolic compound from different phenolic families in Azeradj EVOOs samples identified using HPLC-ESI-TOF/MS. During ripening, the total phenol content decreased from 222 mg kg^{-1} in EVOO from the first harvest date (D1), to reach 196 mg kg^{-1} and 176 mg kg^{-1} in EVOOs from the second (D2) and the last (D3) harvest dates, respectively. This trend in total phenol content during ripening agrees with previously reported data [8]. As a result of the decreasing on total phenols in EVOO from olives harvested later (D2 and D3), nutritional and sensorial quality of the oil could be affected.

3.7. Oxidative stability

Although it is not considered a standard parameter of quality, oxidative stability provides useful information on olive-oil shelf life. It reveals the beginning of the oxidation process characterized by free-radical reactions. Several authors have reported a clear correlation among oxidative stability and fatty acid composition, phenolic compounds, and pigments [24, 31].

Analyzing the results for this parameter, we found that oxidative stability reached its highest value in EVOOs belonging to the first harvest date (32, D1). Despite the significant decrease on MUFA: PUFA ratio, total phenols and pigment content found between the first and the second harvest dates (D1 and D2), the oxidative stability value did not significantly change and even remained constant **(Table 3)**. This trend in oxidative stability could be explained by the behavior of some individual phenolic compounds during ripening. As shown in Table 3, the decrease in hydroxytyrosol in EVOO corresponding to the second harvest date was accompanied by a greater lignan and flavone content such as syringaresinol, pinoresinol, apeginin, and luteolin. This later showed antioxidant activity similar to that of hydoroxytyrosol previously reported in the literature [32], which may compensate for the loss in hydroxytyrosol. In addition, oleuropein aglycone is known to be a strong antioxidant in olive oil [33], and its content did not vary significantly between EVOOs from the first and second harvest dates. Finally, a sharp decrease (51%) in oxidative stability was found between the second and the last harvest dates (D2 and D3). This behavior could be explained by a general decline in all phenolic groups together with pigments, and MUFA:PUFA ratio, responsible for maintaining of olive- oil oxidative stability.

4. Conclusions

Changes in the chemical composition of Azeradj EVOOs were found during olive ripening. The increase in the maturity index was accompanied by an increase in some quality parameters such as free fatty acids, peroxide values, and K_{232}. However, these values were well within the legal limits for the EVOO category. In addition, the decrease on the MUFA: PUFA ratio, pigments and polyphenols, diminished the oxidative stability of Azeradj EVOOs at advanced ripening stages corresponding to the last harvest date (D3). The current data can be considered useful for determining the ideal harvest period for olives used to produce oil. From the analytical results of this work, early harvesting produces Azeradj EVOO with excellent chemical characteristics. Moreover, HPLC-ESI-TOF/MS was successfully employed for the first time in the

characterization of Azeradj phenolic profile, which will be available to all those researchers involved in EVOO chemical characterization.

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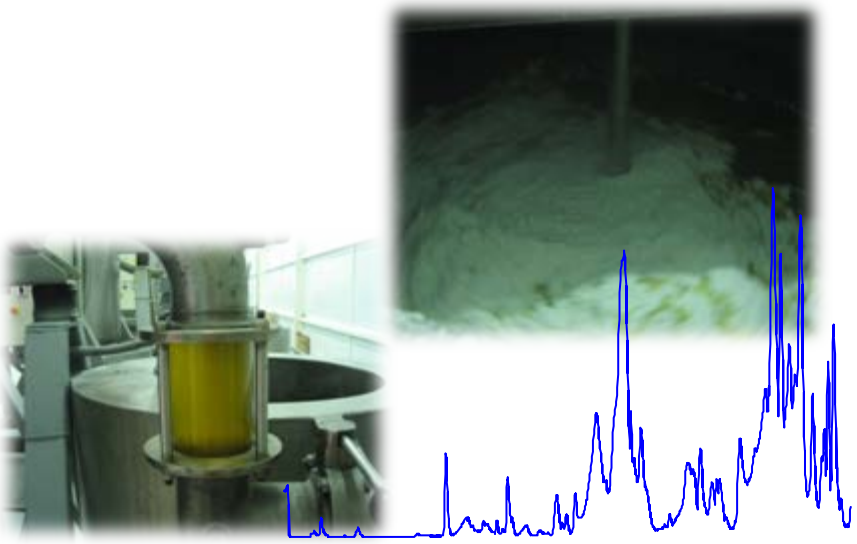

Chapter 5

Monitoring the moisture reduction and status of bioactive compounds in extra-virgin olive oil over the industrial filtration process

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Monitoring the moisture reduction and status of bioactive compounds in extra-virgin olive oil over the industrial filtration process

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Abstract

The aim of this study was to evaluate extra-virgin olive oil (EVOO) moisture and phenolic compounds content during industrial filtration, which is widely applied in the most olive-oil industries of the main producing countries of the Mediterranean as a final step prior to selling the oil. For this purpose, conventional filtration process was performed in duplicate using two lots (lot 1 and lot 2), for a total amount of 45,000 kg of EVOO each. The EVOOs were from the main Spanish olive varieties (Hojiblanca, Manzanilla, Picual, and Arbequina). Cloudy EVOOs were filtered using Vitacel® L-90 and Filtracel® EFC-950 as filter aids together with filtration tank. The moisture content was determined in unfiltered and filtered EVOOs. In addition, the individual phenolic compounds were qualitatively and quantitatively characterized by HPLC-ESI-TOF/MS. The results clearly showed that filtration sharply decreased moisture. Nevertheless, the time course of phenolic compounds during filtration differed for each family. Whereas phenolic alcohols and flavones decreased during filtration, secoiridoids tended to increase, while lignans were the least affected group. Although filtration can make EVOO brilliant and can increase its shelf life by reducing its moisture content, filtration sacrifices certain phenolic compounds which could affect EVOO oxidative

stability and its nutritional quality. Consequently, to maintain olive-oil quality, producers need to take into account both moisture loss as well as the antioxidant content during EVOO filtration.

Keywords: Extra-virgin olive oil, Filtration, Moisture, Phenolic compounds Quality

1. Introduction

Extra-virgin olive oil (EVOO) is a natural product obtained from pressing olive fruit (*Olea europaea*) (Gordillo, Ciaccheri, Mignani, Gonzalez-Miret, & Heredia, 2011). The characteristic aroma, taste, and color of this oil distinguish it from other edible vegetable oils. The excellent organoleptic and nutritional properties of EVOO, together with the current tendency of consumers to select minimally processed foods, have prompted a re-assessment of its consumption in the daily diet (Fregapane, Lavelli, León, Kapuralin, & Desamparados Salvador, 2006). Furthermore, the increase of EVOO intake has also been related to the healthier properties of some minor constituents, such as phenolic compounds (Tripoli et al., 2005).

EVOO quality has been related to its composition, and the moisture content is considered one of the main parameters to evaluate this quality (Gordillo et al., 2011; Hatzakis & Dais, 2008; Ruiz-Domínguez, Raigón, & Prohens, 2013). Freshly produced EVOO is naturally turbid, containing micro-droplets of vegetation water and solid particles from olive fruits (Brkić Bubola, Koprivnjak, & Sladonja, 2012). Although, it could be considered by some consumers less processed, the higher water content in water-in-oil emulsion maintains the stability of suspended solids for several weeks or even months until complete deposition (Gordillo et al., 2011). Indeed, the high polar phase content (water) may augment the alteration of EVOO during storage in milling companies and throughout the market period, by increasing the hydrolytic rate of the triacylglycerols. This process increases free acidity, exposing EVOO to oxidation in the presence of oxygen, light or high temperature (Yun & Surh, 2012). It is well known that oxidation leads to the formation of volatile products, which not only change the initial flavor of EVOO but also decrease the nutritional quality and may even lead to the formation of toxic products (Bendini et al., 2013; Stefanoudaki, Williams, & Harwood, 2010). Additionally, the acidity of cloudy EVOO affects the time course of phenolic compounds over storage by increasing the degradation of the secoiridoid group (Brenes, García, García, & Garrido, 2001). On the other hand, the cloudy aspect makes

sales difficult in some new markets, where consumers tend to prefer brilliant EVOO.

In recent years, most companies prior to bottling and sales apply a filtration step to remove suspended solids and reduce EVOO moisture content. The objective is to maintain EVOO quality and increase its shelf life before consumption. Besides protecting EVOO from chemical degradation by reducing its water content, the filtration step makes it more brilliant for consumer acceptance. In this sense, different filtration systems have been applied in the olive-oil industry: conventional filtration systems (filter tanks and filter presses), cross-flow filtration (tangential-flow filtration), inert-gas-flow filtration systems, and filter bags (Frankel, Bakhouche, Lozano-Sánchez, Segura-Carretero, & Fernández-Gutiérrez, 2013; Lozano-Sánchez et al., 2012).

Nevertheless, reducing the moisture content could affect the polar fraction of EVOO responsible of its oxidative stability. Phenolic compounds are the main components of this fraction with a strong antioxidant effect (Alacón Flores, Romero-González, Garrido Frenich, & Martínez Vidal, 2012; Anastasopoulos et al., 2011; Žanetić et al., 2013). Therefore, the time course of these two parameters during filtration needs to be studied. The aim of this work was to monitor moisture and individual phenolic compounds during EVOO filtration step at industrial scale. This is the first study available in which the effect of the industrial filtration process on phenolic compounds has been evaluated step by step using a high-performance liquid chromatography (HPLC) coupled to electrospray time-of-flight mass spectrometry (TOF-MS). The industrial filtration system evaluated is widely applied in most EVOO industries of the main producing countries of the Mediterranean.

2. Materials and methods

2.1. Samples

The EVOOs used in this study were obtained in November 2012 from industrial mills equipped with a hammer crusher, a horizontal malaxator, and a two-phase decanter (Oleoestepa S.L., Seville, Spain). For this work, the industrial filtration was performed in duplicate using two lots (lot 1 and lot 2), with a total amount of 45,000 kg of EVOOs each. The first consisted of EVOOs from the olive varieties Hojiblanca (52%), and Manzanilla (48%) and the second one from Hojiblanca (40%), Picual (40%), and Arbequina (20%) olive varieties. Both mixtures of cloudy EVOOs were filtered at room temperature using the following organic filter aids: Vitacel® L-90 (30 kg, composed of 100% cellulose) and Filtracel® EFC-950 (60 kg, composed of 70% cellulose and 30% lignin). The cake layer was performed in conjunction with filter tank. For the filtration, each lot underwent a preliminary phase of filtering through specially prepared combinations of filter aids and EVOO. In this preliminary step, the filtration equipment was covered with organic filter aids and the cake layer was formed. Afterward, filtration was conducted under a constant flow and increasing differential pressure. A total of 48 filtered and unfiltered samples were collected from both lots 1 and 2 following the procedure depicted in **Fig. 1**. As a means of achieving representative results and eliminating confounding factors which could affect EVOO composition, the moisture content measured and the phenolic fraction was isolated from samples without storage.

2.2. Chemicals and reagents

All chemicals were of analytical reagent grade. Methanol, *n*-hexane, sodium hydroxide and isopropanol were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from Fluka, Sigma-Aldrich (Steinheim, Germany). Double-deionized water with conductivity less than 18.2 MΩ·cm was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Standards of hydroxytyrosol, tyrosol, luteolin, apigenin, and quinic acid were purchased by SigmaeAldrich (St. Louis, MO, USA), and pinoresinol was acquired from Arbo

Nova (Turku, Finland). Oleuropein was purchased from Extrasynthese (Lyon, France).

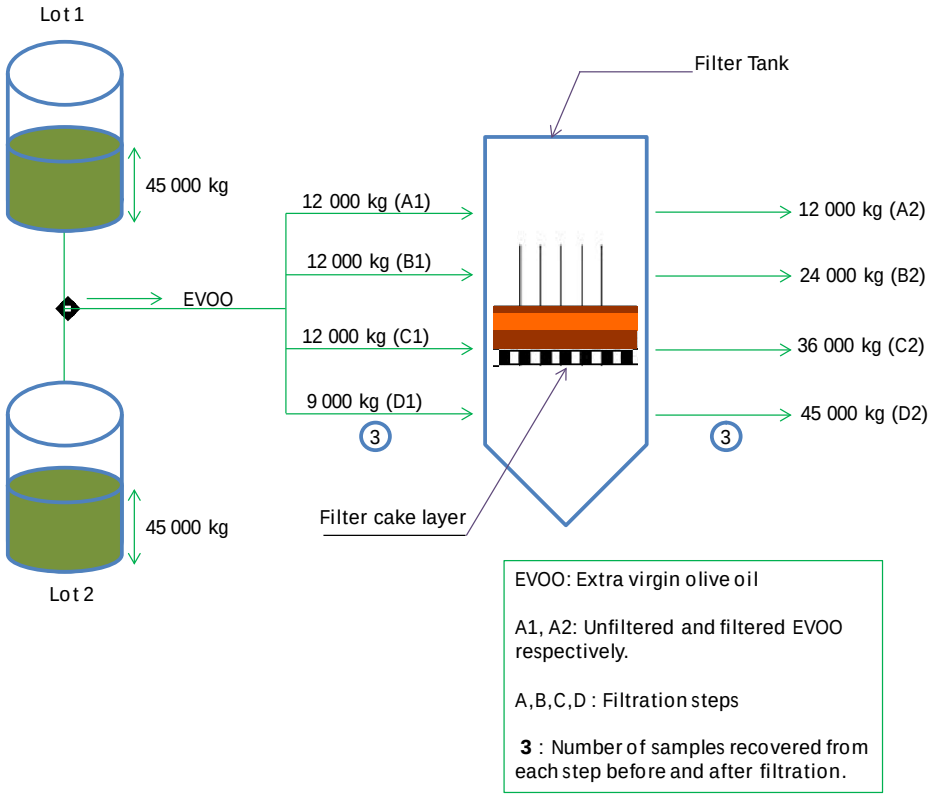

Fig. 1. Industrial filtration diagram

2.3. Moisture content

The moisture content was determined following the norms of the Spanish Association for Standardisation and Certification (AENOR). Briefly, in a capsule, previously dried at 105 ºC and cooled, 10 g of completely homogenized sample were weighed. The samples were placed in an oven (Memmert GmbH + Co. KG, Schwabach, Germany) at 105 ºC for 21 h, after which the samples were removed and weighed. Next, they were returned in the oven and the operation was repeated until the weight was constant. The moisture content was calculated as the difference in weights (AENOR, 1973).

2.4. Phenolic extraction

Phenolic compounds were isolated from the EVOO using liquid-liquid extraction. Briefly, 2.5 g of EVOO sample was weighed and dissolved in 5 mL

of *n*-hexane. After that, 5 mL of methanol:water (60/40, v/v) was added, the mixture was vortexed and then centrifuged at 3500 rpm for 10 min. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure and a temperature of 35 ºC. The residue was dissolved in 0.25 mL of methanol/water (50:50 v/v) and finally filtered through a 0.45 mm filter before the HPLC analysis (Taamalli, Arráez Román, Zarrouk, Segura-Carretero, & Fernández-Gutiérrez, 2012).

2.5. HPLC-ESI-TOF/MS phenolic compound analysis

The analysis to characterize the phenolic profile in filtered and unfiltered EVOOs was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump, and a diode array detector (DAD). The chromatographic separation of these compounds was performed on a 150 mm x 4.6 mm i.d., 1.8 mm, Zorbax Eclipse Plus RP-C18 column (Agilent Technologies, Palo Alto, CA, USA). The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The total run time was 27 min using a previously reported multistep linear gradient (Lozano-Sánchez et al., 2010). The flow rate was 0.80 mL/min and, consequently, the use of a splitter was required for the coupling with the MS detector, as the flow which arrived to the TOF detector had to be 0.2 mL/min to ensure reproducible results and stable spray. HPLC was coupled to a time-of-flight mass-spectrometer detector microTOF (Bruker Daltonik, Bremen, Germany), which was equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative ion mode.

External mass-spectrometer calibration was performed with sodium acetate clusters [5 mM sodium hydroxide in water/isopropanol 1/1 (v/ v), with 0.2% of acetic acid] in quadratic + high precision calibration (HPC) regression mode. The optimum values of the source and transfer parameters were established according to Lozano-Sánchez et al. (2010). The widely accepted accuracy threshold for confirmation of elemental compositions was set at 10 ppm for most of the compounds. The phenolic compounds were identified by

comparing both retention times and MS data from samples and standards. The remaining compounds for which no commercial standards were available were identified by the interpretation of the information generated by the TOF analyzer, and the information reported in the literature (most compounds have previously been described in EVOO). Quantification was made by HPLC-ESI-TOF-MS. Seven standard calibration curves of the main compounds found in the samples were prepared using seven commercial standards. Stock solutions at a concentration of 1000 mg/L for each phenolic compound were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations. All calibration curves showed good linearity over the study range (r^2= 0.996). The individual concentrations were determined using the area of each individual compound (three replicates) and by interpolation of the corresponding calibration curve. Results were given in mg of analyte per kg of EVOO.

2.6. Statistical analysis

The data were analyzed using Origin (version Origin Pro 8 SR0, Northampton, MA, USA) to perform a one-way-analysis of variance (ANOVA) at a 95% confidence level $p \leq 0.05$, in order to identify significant differences among the parameters analyzed in unfiltered and filtered EVOOs**.**

3. Results and discussion

3.1. Time course of moisture content during the filtration process

The time course of moisture content in unfiltered and filtered EVOOs is presented in **Fig. 2**. As expected, unfiltered EVOO had the highest moisture content during all filtration steps carried out (A, B, C, and D) for both lots 1 and 2. The results indicated that filtration using Vitacel® L-90 and Filtracel® EFC-950 as the filter cake in conjunction with the filter tank significantly reduces the moisture content, due to the high absorption and retentive power of water. As a difference between filtered and unfiltered EVOO, the reduction effect for all filtration steps ranged from 20 to 36% and from 20 to 29% in

EVOO for lot 1 and lot 2, respectively. Finally, the results establish the efficiency of organic material powder used to produce brilliant EVOO.

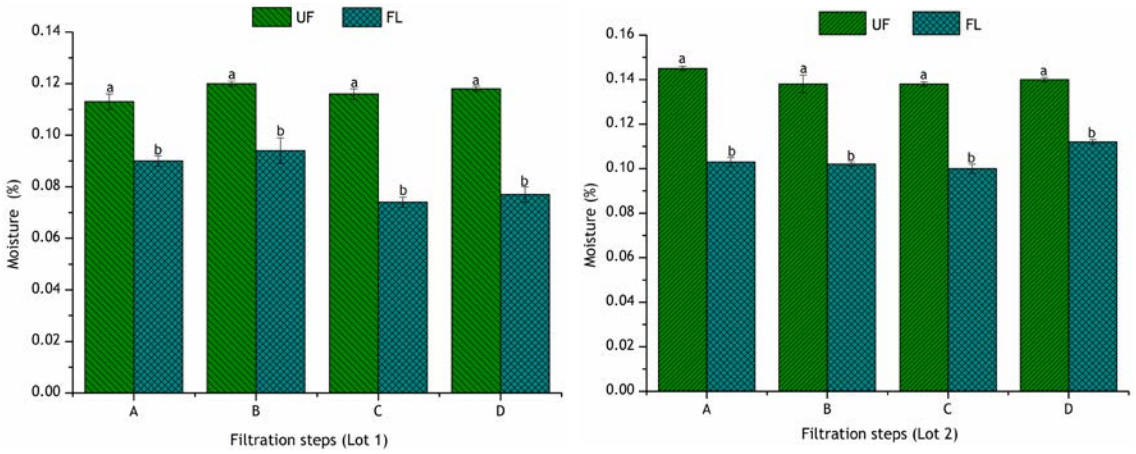

Fig. 2. Time course of EVOO moisture content during filtration: UF, unfiltered; FL, filtered; values with different letters are significantly different at a 95% confidence level ($p \leq 0.05$).

3.2. Qualitative characterization of phenolic and other polar compounds in EVOOs

No qualitative differences in the phenolic profile were detected, between EVOOs under study. **Fig. 3** shows the representative base peak chromatogram of the phenolic extract provided by HPLC-ESITOF/MS. The main phenolic compounds identified in samples and the information generated by the TOF analyzer are included in **Table 1**. A total of 22 compounds were characterized in EVOOs. Among these, 4 compounds belong to the phenolic alcohol group, 10 compounds were secoiridoids, 2 elenolic-acid derivatives, 3 lignans, and 2 flavones.

Peaks 3 and 4 were identified as phenolic alcohols hydroxytyrosol and tyrosol, respectively. Peak 2 was characterized as oxidation product of hydroxytyrosol. This oxidized form, being more polar than its nonoxidized derivative, elutes earlier. Peak 6 had a deprotonated molecule at m/z 195, corresponding to hydroxytyrosol derivative (hydroxytyrosol acetate).

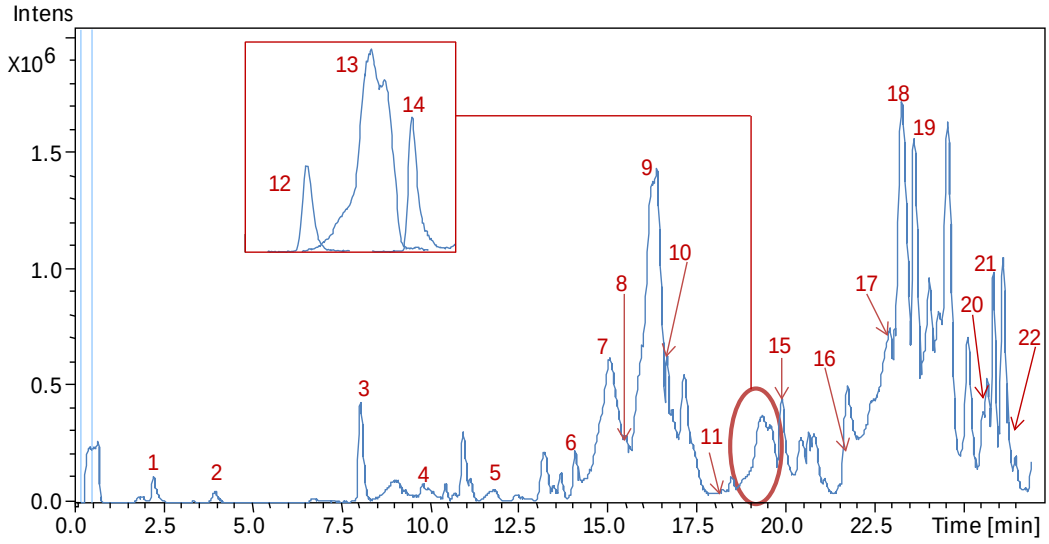

Fig. 3. Base-peak chromatogram (BPC) of representative unfiltered EVOO phenolic extract, by HPLC-ESI-TOF/MS. 1, Quinic acid; 2, oxidized hydroxytyrosol; 3, hydroxytyrosol; 4, tyrosol; 5, hydroxy decarboxymethyl oleuropein aglycone or isomer; 6, hydroxytyrosol acetate; 7, elenolic acid; 8, hydroxy elenolic acid; 9, decarboxymethyl oleuropein aglycone; 10, hydroxy decarboxymethyl oleuropein aglycone or isomer; 11, Syringaresinol; 12, pinoresinol; 13, decarboxymethyl ligstroside aglycone; 14, acetoxypinoresinol; 15, hydroxyl decarboxymethyl ligstroside aglycone; 16, dehydro-oleuropein aglycone; 17, 10-hydroxy oleuropein aglycone; 18, oleuropein aglycone; 19, luteolin; 20, ligstroside aglycone; 21, apigenin; 22, methyl oleuropein aglycone.

The main secoiridoids identified in samples were oleuropein aglycone (peak 18), ligstroside aglycone (peak 20), and their decarboxymethylated, hydroxylated, dehydrated, and methylated forms (peaks 5, 9, 10, 13, 15, 16, 17, and 22). Elenolic acid and its hydroxylated form (peaks 7 and 8), which may not be considered as phenolic compounds, but as secoiridoids derivatives, were also identified in the samples. Concerning lignans, the spectra generated for peaks 11, 12, and 14 yielded deprotonated molecules at m/z 417, 357, and 415, which were attributed to syringaresinol, pinoresinol, and acetoxypinoresinol, respectively.

Finally, flavones were also characterized in the EVOO samples analyzed. Peaks 19 and 21 had a deprotonated molecule at 285 and 269 m/z corresponding to luteolin and apigenin, respectively. Regarding the presence of other polar

compounds, quinic acid (peak 1) was detected in all the samples. The phenolic compounds identified in these samples have previously been reported in EVOOs from olive varieties under study (Bakhouche et al., 2013; Lozano-Sánchez et al., 2013).

Table 1. Main phenolic compounds identified in a representative unfiltered extra-virgin olive-oil phenolic extract obtained by HPLC-ESI-TOF/MS.

Compounds[a]	Retention Time(min)	Molecular formula	m/z calcd[b]	m/z Exptl[c]	Error (ppm)	msigma
Quinic acid	2.20	$C_7H_{12}O_6$	191.0561	191.0569	-4.3	6.3
H- HYTY	3.91	$C_8H_8O_3$	151.0403	151.0401	-1.5	12.4
HYTY	8.01	$C_8H_{10}O_3$	153.0557	153.0565	-5.0	2.4
TY	9.85	$C_8H_{10}O_2$	137.0608	137.0617	-6.2	11.1
H-D-Ol Agl or isomer	11.77	$C_{17}H_{20}O_7$	335.1136	335.1134	0.7	3.2
HYTY-Ac	14.03	$C_{10}H_{12}O_4$	195.0663	195.0670	-3.9	5.5
EA	15.02	$C_{11}H_{14}O_6$	241.0718	241.0731	-5.4	9.0
H-EA	15.59	$C_{11}H_{14}O_7$	257.0667	257.0669	-0.7	3.2
DOA	16.31	$C_{17}H_{20}O_6$	319.1187	319.1197	-3.0	3.1
H-D-Ol Agl or isomer	16.62	$C_{17}H_{20}O_7$	335.1136	335.1143	-2.0	2.9
Syringaresinol	18.18	$C_{22}H_{26}O_8$	417.1555	417.1543	2.9	17.4
Pin	18.90	$C_{20}H_{22}O_6$	357.1344	357.1343	0.1	18.5
D-Lig Agl	19.30	$C_{17}H_{20}O_5$	303.1238	303.1242	-1.4	2.5
AcPin	19.40	$C_{22}H_{24}O_8$	415.1398	415.1404	-1.4	3.4
H-D-Lig Agl	19.82	$C_{17}H_{20}O_6$	319.1187	319.1176	3.4	12.9
Dehydro Ol Agl	21.61	$C_{19}H_{20}O_8$	375.1085	375.1090	-1.2	39.3
10-H-Ol Agl	23.01	$C_{19}H_{22}O_9$	393.1191	393.1192	-0.1	3.1
Ol Agl	23.20	$C_{19}H_{22}O_8$	377.1242	377.1242	0.1	1.0
Lut	23.55	$C_{15}H_{10}O_6$	285.0405	285.0408	-1.3	5.7
Lig Agl	25.60	$C_{19}H_{22}O_7$	361.1293	361.1296	-0.1	2.2
Apig	25.79	$C_{15}H_{10}O_5$	269.0455	269.0462	-2.6	1.4
Methyl Ol Agl	26.24	$C_{20}H_{24}O_8$	391.1398	391.1409	-2.7	13.8

[a] H-HYTY, Oxidized hydroxytyrosol; HYTY, hydroxytyrosol; TY, tyrosol; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; HYTY-Ac, hydroxytyrosol acetate; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; Pin, pinoresinol; D-Lig Agl, decarboxymethyl ligstroside aglycone; AcPin, acetoxypinoresinol; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone;

Dehydro Ol Agl, dehydro-oleuropein aglycone; 10-H-Ol Agl, 10-hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lut, luteolin; Lig Agl, ligstroside aglycone; Apig, apigenin; Methyl Ol Agl, methyl oleuropein aglycone; [b] m/z calcd: calculated mass; [c] m/z exptl: experimental mass.

3.3. Behavior of phenolic compounds during filtration process

The total phenol content and the contribution of each family have tentatively been calculated by adding together the individual phenolic compound concentrations. **Fig. 4** shows the time course, step by step, of total phenolic content as well as the trend of each family. Concerning the total phenolic content, differences among unfiltered and filtered EVOOs were found for all filtration steps. However, these differences were statistically significant only in samples belonging to A, B, C, and D steps for lot 1 and the final step for lot 2. The trend in the total phenolic content was linked to the behavior of the different phenolic compound families detected in samples. As shown in **Fig. 4**, in terms of the filtration response, the different chemical families of phenolic compounds could be divided into three groups: phenolic alcohols and flavones; secoiridoids; and lignans. In both of the two industrial filtration processes used, phenolic alcohols tended to decrease in concentration during all the filtration steps (A, B, C, and D), the greatest loss of these compounds occurring at the first step. Indeed, their concentrations were reduced by 20% and 56% in filtered EVOOs for the lots 1 and 2, respectively. Flavones followed the same trend, with a reduction of the total amount of 28% and 52% in filtered EVOO for lots 1 and 2, respectively.

Secoiridoids showed the highest concentration in the EVOO samples under study. Their concentration increased during filtration, this increase being significant in all filtered EVOO for lot 1 and the two last filtration steps (C and D) for lot 2. This trend has been reported in previous studies at the laboratory scale using cotton as the filter medium (Gómez-Caravaca et al., 2007), and gas-flow filtration as filter aids at the pilot plant scale (Lozano-Sánchez et al., 2012). Lignans appeared to be the most stable phenolic family during filtration. Indeed, no significant differences were found between filtered and

unfiltered EVOO for either of the lots tested, with exception of some filtration steps, such as step B for lot 1 and step D for lot 2, in which cases the variation was very low. The lignan content in olive oil is affected mainly by agronomic conditions for cultivating trees while technological parameters of the extraction process have only a marginal impact on the olive-oil lignan concentration (Servili et al., 2009).

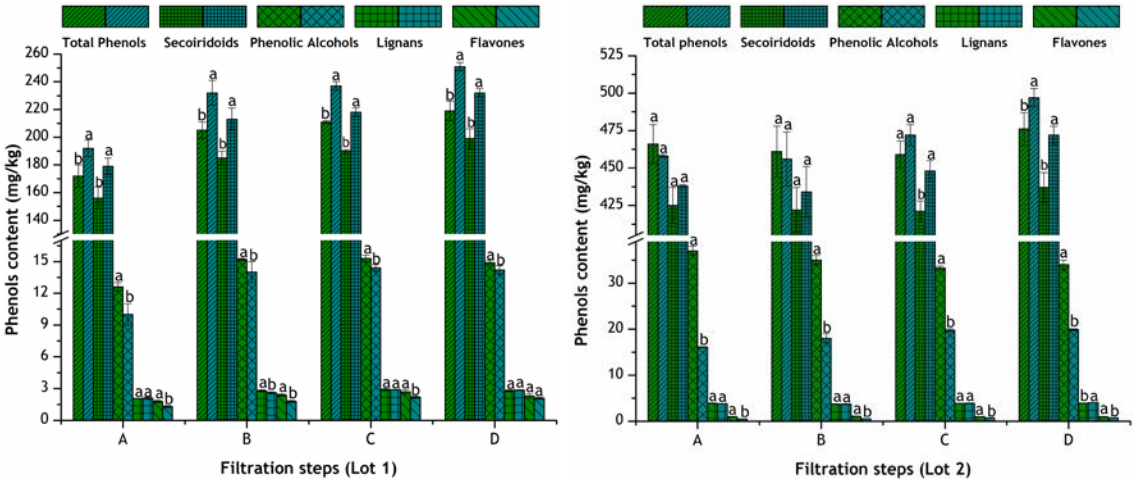

Fig. 4. Time course of the different phenolic families during EVOO filtration: green, unfiltered EVOO; dark cyan, filtered EVOO; values with the same letter are not significantly different at a 95% confidence level ($p \leq 0.05$).

The analysis of the individual concentrations indicated that within the same family, the filtration effect was different for each compound. This confirms the importance of studying the phenolic profile of EVOO using HPLC-ESI-TOF/MS, providing better understanding of the effect of the filtration process on this polar fraction. Hydroxytyrosol and tyrosol were the main phenolic alcohols affected during EVOO filtration. Their concentration proved lower in filtered EVOO from both lots 1 and 2, although hydroxytyrosol was more affected than tyrosol (**Fig. 5**). The loss of these two compounds was greater in filtered EVOO from lot 2 than filtered EVOO from lot 1. These differences may be attributed to the higher moisture content in unfiltered EVOO from lot 2, which resulted in a greater loss of their concentration in water after filtration. According to the literature, these compounds, and especially hydroxytyrosol, can be considered strong antioxidants (Bendini et al., 2007;

Servili et al., 2009). Therefore, losing them could affect nutritional proprieties as well as the oxidative stability of the oil. With regard to flavones, the main compounds quantified in EVOO samples were luteolin and apigenin. Their concentration tended to decrease during filtration. The heaviest loss in the luteolin content occurred during the first filtration step (A), where the reduction on filtered EVOO was 27% and 60% from lots 1 and 2, respectively. Apigenin showed the same trend during filtration but the effect of filtration on the concentration of this compound was weaker than in the case of luteolin (**Fig. 5**).

Analyzing the time course of the secoiridoid group (**Fig. 5**), we found that oleuropein aglycone, decarboxymethyl oleuropein aglycone, and decarboxymethyl ligstroside aglycone were the main compounds responsible for the apparent increase in secoiridoids and total phenolic content after filtration, as mentioned above. However, Lozano-Sánchez, Segura-Carretero, and Fernández-Gutiérrez (2011) confirmed that some of these phenolic compounds are retained in the same filter aids as used in this study, therefore the increase of those phenolic compounds observed after filtration could be explained by the extraction method used to recover these compounds. Indeed, extraction of phenolic compounds in samples with high water content does not allow for a complete recovery of these analytes. Given that these compounds are located at the water/oil interface, when the analytes are in a more polar matrix (unfiltered EVOO) their affinity to the extraction solvent is low. However, if the extraction with hydroalcoholic solution is done after the partial elimination of water (filtered EVOO), these compounds are more available to the extraction solvent, resulting in the apparent increase in their concentration in filtered EVOO (Gómez-Caravaca et al., 2007). Concerning lignans, only a slight variation was found in their concentration after filtration for both lots under study, being significant in samples belonging to lot2 (A, B and C filtration steps) for syringaresinol. However, pinoresinol showed a significant variation during filtration steps C and D for the same lot (**Fig. 5**).

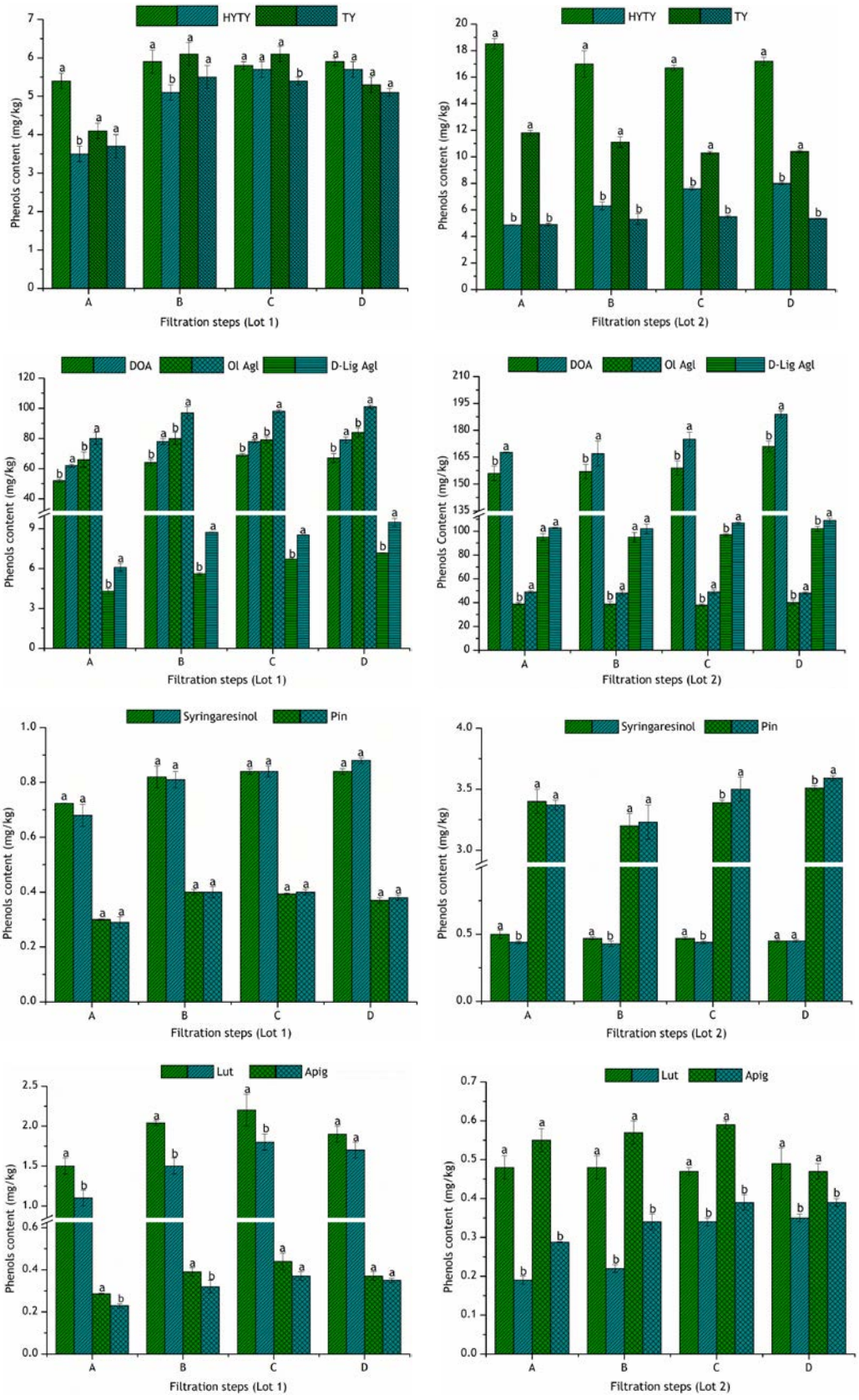

Fig. 5. Time course of the individual concentration of the main compounds from different phenolic families during EVOO filtration: HYTY, hydroxytyrosol;

TY, tyrosol; D-Lig Agl, decarboxymethyl ligstroside aglycone; DOA, decarboxymethyl oleuropein aglycone; Ol Agl, oleuropein aglycone; Pin, pinoresinol; Lut, luteolin; Apig, apigenin; green, unfiltered EVOO; dark cyan, filtered EVOO; values with the same letter are not significantly different at a 95% confidence level ($p \leq 0.05$).

The different behavior among phenolic compounds of each phenolic family during filtration could be explained by the difference in their chemical structure, which affects their partition between water fraction and oil, in some cases resulting in a loss of those polar phenols during the filtration process, associated with the reduction of water content. However, it should be taken into account that the interactions of phenolic compounds with filter aids could also affect the variations of these compounds in EVOO; the composition of filter aids used for EVOO filtration (cellulose and lignin) facilitates the hydrophobic interaction between aromatic rings of phenylpropyl alcohols as well as phenolic compounds (Lozano-Sánchez et al., 2011).

4. Conclusions

According to the results of this work, filtration could make EVOO more brilliant for marketing on one hand, and increase its shelf life on the other hand, by reducing moisture content. Nevertheless, filtration diminishes the EVOO antioxidant content, mainly phenolic alcohols. Consequently, during filtration, the equilibrium between losing moisture and antioxidant content is needed to achieve high-quality EVOO. The apparent increase of secoiridoids in filtered EVOO made it difficult to understand the real effect of filtration on this group of compounds. Consequently, future investigations are warranted to develop a new analytical methodology that takes into account the different water content in filtered and unfiltered EVOO and its effect on the extraction process used in the qualitative and quantitative characterization of these compounds.

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Supporting information

Table 1. Moisture values obtained in unfiltered and filtered extra-virgin olive oil

	A[e]		B		C		D	
	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
Moisture (%) Lot 1	0,113^{a}±0,003	0,090^{b}±0,002	0,120^{a}±0,001	0,094^{b}±0,003	0,116^{a}±0,002	0,074^{b}±0,002	0,118^{a}±0,001	0,077^{b}±0,003
Moisture (%) Lot 2	0,145^{a}±0,001	0,103^{b}±0,002	0,138^{a}±0,004	0,102^{b}±0,001	0,138^{a}±0,001	0,100^{b}±0,002	0,140^{a}±0,001	0,112^{b}±0,001

[e]Fitration steps (A, B, C, D).

Values with different letters in a line are not significantly different at a 95% confidence level ($p \leq 0.05$).

Table 2. Phenolic content (mg/kg) of extra-virgin olive oil from Lot1

Lot 1								
Compounds[c]	**A**[e]		**B**		**C**		**D**	
	UF[k]	**FL**[k]	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
HYTY	5.4[a]±0.2	3.5[b]±0.2	5.9[a]±0.3	5.1[b]±0.2	5.8[a]±0.1	5.7[a]±0.2	5.9[a]±0.1	5.7[a]±0.2
TY	4.1[a]±0.2	3.7[a]±0.3	6.1[a]±0.3	5.5[a]±0.3	6.1[a]±0.2	5.4[b]±0.1	5.3[a]±0.2	5.1[a]±0.1
HYTY-Ac	3.1[a]±0.1	3.1[a]±0.1	3.2[a]±0.2	3.5[a]±0.1	3.4[a]±0.1	3.36[a]±0.02	3.66[a]±0.04	3.4[b]±0.1
Phenolic alcohols	**12.6[a]±0.4**	**10[b]±1**	**15.2[a]±0.1**	**14[b]±1**	**15.3[a]±0.3**	**14.4[b]±0.3**	**14.9[a]±0.2**	**14.2[b]±0.4**
EA	23[a]±1	18[b]±1	23[a]±1	17[b]±1	23.6[a]±0.4	21.6[b]±0.3	29.9[a]±0.3	30[a]±1
H-EA	0.59[a]±0.03	0.55[a]±0.03	0.9[a]±0.01	0.43[b]±0.02	0.84[a]±0.02	0.39[b]±0.02	0.56[a]±0.02	0.45[b]±0.03
DOA	52[b]±1	62[a]±1	64[b]±2	78[a]±2	69[b]±1	78[a]±1	67[b]±3	79[a]±2
H-D-Ol Agl or Isomer	1.95[a]±0.04	1.6[b]±0.1	2.5[a]±0.1	1.8[b]±0.1	2.39[a]±0.01	1.79[b]±0.03	2.2[a]±0.2	2.07[a]±0.03
D-Lig Agl	4.3[b]±0.2	6.1[a]±0.3	5.6[b]±0.1	8.74[a]±0.02	6.72[b]±0.02	8.52[a]±0.02	7.17[b]±0.02	9.5[a]±0.3
H-D-Lig Agl	1.2[a]±0.1	1.2[a]±0.1	1.4[a]±0.1	1.4[a]±0.1	1.51[a]±0.04	1.3[b]±0.1	1.3[a]±0.1	1.4[a]±0.1
10-H-Ol Agl	2.6[a]±0.2	2.6[a]±0.1	2.9[a]±0.2	2.7[a]±0.2	2.8[a]±0.1	2.4[b]±0.1	2.6[a]±0.1	2.8[a]±0.1
Ol Agl	66[b]±5	80[a]±4	80[b]±4	97[a]±4	79[b]±2	98[a]±1	84[b]±3	101[a]±1
Lig Agl	3.2[b]±0.2	4.5[a]±0.2	3.1[b]±0.1	4.5[a]±0.1	3.1[b]±0.1	4.29[a]±0.03	3.5[b]±0.1	4.6[a]±0.1
Methyl Ol Agl	0.91[b]±0.03	1.5[a]±0.1	0.9[b]±0.01	1.7[a]±0.1	0.97[b]±0.04	1.5[a]±0.1	0.91[b]±0.04	1.5[a]±0.1
Secoiridoids	**156[b]±8**	**179[a]±6**	**185[b]±5**	**213[a]±8**	**190[b]±1**	**218[a]±3**	**199[b]±7**	**232[a]±3**
Syringaresinol	0.724[a]±0.001	0.68[a]±0.04	0.82[a]±0.04	0.81[a]±0.03	0.84[a]±0.01	0.84[a]±0.02	0.84[a]±0.01	0.88[a]±0.01
Pin	0.300[a]±0.002	0.29[a]±0.02	0.40[a]±0.01	0.40[a]±0.02	0.394[a]±0.003	0.40[a]±0.01	0.37[a]±0.01	0.38[a]±0.01
AcPin	1.06[a]±0.02	1.1[a]±0.1	1.6[a]±0.1	1.43[b]±0.02	1.61[a]±0.04	1.65[a]±0.02	1.6[a]±0.1	1.59[a]±0.03
Lignans	**2.08[a]±0.02**	**2.1[a]±0.1**	**2.8[a]±0.1**	**2.6[b]±0.1**	**2.9[a]±0.1**	**2.89[a]±0.03**	**2.8[a]±0.1**	**2.85[a]±0.04**

Lot 1								
Compounds[c]	**A**[e]		**B**		**C**		**D**	
	UF[k]	**FL**[k]	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
Lut	1.5[a]±0.1	1.1[b]±0.1	2.04[a]±0.03	1.5[b]±0.1	2.2[a]±0.2	1.8[b]±0.1	1.9[a]±0.1	1.7[a]±0.1
Apig	0.286[a]±0.003	0.23[b]±0.01	0.39[a]±0.02	0.32[b]±0.03	0.44[a]±0.04	0.37[a]±0.02	0.37[a]±0.02	0.35[a]±0.01
Flavonoids	**1.8[a]±0.1**	**1.3[b]±0.1**	**2.4[a]±0.1**	**1.8[b]±0.1**	**2.7[a]±0.2**	**2.2[b]±0.1**	**2.3[a]±0.2**	**2.1[a]±0.1**
Quinic Acid	0.20±0.01	NQ	0.4±0.02	NQ	0.48±0.04	NQ	0.71±0.02	NQ
Total phenols	**172[b]±8**	**192[a]±6**	**205[b]±6**	**232[a]±9**	**211[b]±1**	**237[a]±3**	**219[b]±7**	**251[a]±3**

[c] HYTY, hydroxytyrosol; TY, tyrosol; HYTY-Ac, hydroxytyrosol acetate; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or Isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; 10-H-Ol Agl, 10-hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lig Agl, ligstroside aglycone; Methyl Ol Agl, methyl oleuropein aglycone; Pin, pinoresinol; AcPin, acetoxypinoresinol; Lut, luteolin; Apig, apigenin.

[e] Filtration steps (A, B, C, D).

[k] UF, Unfiltered olive oil; FL, Filtered olive oil.

Values with the same letter in a line are not significantly different at a 95% confidence level ($p \leq 0.05$).

Table 3. Phenolic content (mg/kg) of extra-virgin olive oil from Lot 2.

Lot 2								
Compounds[c]	**A**[e]		**B**		**C**		**D**	
	UF[k]	**FL**[k]	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
HYTY	18.5[a]±0.4	4.87[b]±0.02	17[a]±1	6.3[b]±0.3	16.7[a]±0.2	7.6[b]±0.1	17.2[a]±0.3	8.0[b]±0.1
TY	11.8[a]±0.2	4.9[b]±0.1	11.1[a]±0.4	5.3[b]±0.4	10.3[a]±0.1	5.5[b]±0.1	10.4[a]±0.1	5.35[b]±0.02
HYTY-Ac	6.4[a]±0.1	6.4[a]±0.1	6.2[a]±0.2	6.5[a]±0.3	6.4[a]±0.1	6.7[a]±0.1	6.4[a]±0.1	6.6[a]±0.1
Phenolic alcohols	**37[a]±1**	**16.1[b]±0.1**	**35[a]±1**	**18[b]±1**	**33.3[a]±0.4**	**19.8[b]±0.3**	**34[a]±1**	**19.9[b]±0.2**
EA	89[a]±3	84[b]±1	86[a]±3	81[a]±2	81[a]±1	79[a]±1	83[a]±2	85[a]±2
DOA	156[b]±4	167.7[a]±0.2	157[b]±4	167[a]±7	159[b]±4	175[a]±4	171[b]±3	189[a]±2
H-D-OI AgI or Isomer	12[a]±1	9.40[b]±0.01	13[a]±1	9.1[b]±0.4	12.6[a]±0.2	9.8[b]±0.2	11.9[a]±0.2	9.8[b]±0.2
D-Lig AgI	95[a]±3	102.9[a]±0.4	95[a]±4	102[a]±4	97[b]±1	107[a]±1	102[b]±2	109[a]±2
Dehydro OI AgI	19[a]±1	13.6[b]±0.2	17[a]±1	13[b]±1	16.2[a]±0.3	13.3[b]±0.4	14.2[b]±0.4	15.2[a]±0.3
OI agli	39[b]±1	49[a]±1	39[b]±2	48[a]±2	38[b]±1	49[a]±2	40[b]±2	48[a]±1
Lig AgI	7.8[a]±0.4	6.9[a]±0.1	8.2[a]±0.2	7.5[a]±0.3	9.4[a]±0.2	10[a]±1	8.2[a]±0.1	10[a]±1
methyl OI AgI	7.3[a]±0.3	4.9[b]±0.2	6.9[a]±0.4	5.7[b]±0.2	7.0[a]±0.1	6.3[b]±0.1	6.7[a]±0.3	6.4[a]±0.1
Secoiridoids	**425[a]±12**	**438[a]±1**	**422[a]±15**	**434[a]±17**	**421[b]±7**	**448[a]±7**	**437[b]±10**	**472[a]±6**
syringaresinol	0.50[a]±0.03	0.44[b]±0.01	0.47[a]±0.01	0.43[b]±0.02	0.47[a]±0.01	0.44[b]±0.01	0.45[a]±0.01	0.45[a]±0.01
Pin	3.4[a] ± 0.1	3.37[a] ± 0.04	3.2[a]± 0.1	3.23[a] ± 0.14	3.39[b]±0.02	3.5[a]±0.1	3.51[b]±0.02	3.59[a]±0.02
Lignans	**3.9[a]±0.1**	**3.81[a]±0.04**	**3.7[a]±0.1**	**3.7[a]±0.1**	**3.87[a]±0.03**	**3.9[a]±0.1**	**3.96[b]±0.03**	**4.04[a]±0.02**

Lot 2								
Compounds[c]	**A**[e]		**B**		**C**		**D**	
	UF[k]	**FL**[k]	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
Lut	0.48[a]±0.03	0.19[b]±0.01	0.48[a]±0.03	0.22[b]±0.01	0.47[a]±0.01	0.34[b]±0.01	0.49[a]±0.04	0.35[b]±0.01
Apig	0.55[a]±0.03	0.288[b]±0.001	0.57[a]±0.03	0.34[b]±0.02	0.59[a]±0.01	0.39[b]±0.02	0.47[a]±0.02	0.39[b]±0.01
Flavonoids	**1.0[a]±0.1**	**0.48[b]±0.01**	**1.1[a]±0.1**	**0.55[b]±0.03**	**1.06[a]±0.01**	**0.73[b]±0.02**	**1.0[a]±0.1**	**0.74[b]±0.02**
Quinic acid	1.23±0.02	NQ	1.27±0.01	NQ	1.55±0.02	NQ	1.4±0.1	NQ
Total Phenols	**466[a]±13**	**458[a]±1**	**461[a]±17**	**456[a]±18**	**459[a]±9**	**472[a]±7**	**476[b]±11**	**497[a]±6**

[c] HYTY, hydroxytyrosol; TY, tyrosol; HYTY-Ac, hydroxytyrosol acetate; EA, elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or Isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone; Ol Agl, oleuropein aglycone; Lig Agl, ligstroside aglycone; Methyl Ol Agl, methyl oleuropein aglycone; Pin, pinoresinol; Lut, luteolin; Apig, apigenin.

[e] Filtration steps (A, B, C, D).

[k] UF, Unfiltered olive oil; FL, Filtered olive oil.

Values with the same letter in a line are not significantly different at a 95% confidence level ($p \leq 0.05$).

SECTION III

Analytical procedures for the determination of virgin olive oil phenolic composition

Chapter 6

Trends in chemical characterization of virgin olive oil phenolic profile: An overview and new challenges

Trends in chemical characterization of virgin olive oil phenolic profile: An overview and new challenges

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Abstract

Due to the important role played by phenolic compounds in the nutritional value of virgin olive oil (VOO), it is necessary to develop efficient and accurate analytical methods for their qualitative and quantitative analysis. This review presents an overview of different analytical approaches to the determination of phenolic compounds in VOO. In principle, the analytical procedure for the determination of individual phenolic compounds in VOO involves three basic steps: extraction from the oil sample, chromatographic separation, and characterization. The extraction systems in widest use are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Among the separation techniques reported, high performance liquid chromatography (HPLC) was the most widespread technique applied for the analysis of phenolic compounds. However, it was demonstrated that gas chromatography (GC) and capillary electrophoresis (CE) are able to achieve the same aims as HPLC while providing alternative methodologies for the characterization of phenolic compounds in VOO. The optimized parameters, advantages and

disadvantages of each technique are reported in this review. In addition, the different detectors coupled to the separation techniques are reviewed. Finally, the current analytical problems in the determination of phenolic compounds in VOO are also presented. In order to overcome these problems, researchers have to take into consideration the drawbacks of the previous methods. The future challenge will be to establish one single method for application to all VOO studies relating to phenolic compounds.

Keywords: VOO, phenolic compounds, extraction, separation techniques, analytical problems.

1. Introduction

Virgin olive oil (VOO) is increasingly popular worldwide, not only because of its unique sensory characteristics but also because of the beneficial health effects associated with its consumption, particularly as part of the Mediterranean diet. The health-promoting effects of olive oil have been attributed to its fatty acid profile, as well as to the presence of many bioactive components such as tocopherols, phospholipids and phenolic compounds. In fact, several biological functions and properties have been ascribed to phenolic compounds. In human studies, olive oil rich in phenolic compounds has been shown to improve antioxidant and anti-inflammatory effects and to reduce the proliferation of cell adhesion molecules compared with low-phenolic compound olive oils (Covas 2007; Fitó and de la Torre *et al*., 2007). In 2011, the European Food Safety Authority (EFSA) endorsed a claim regarding the effectiveness of olive oil phenolic compounds (5 mg/day) in protecting blood lipids from oxidative damage (Franco and Galeano-Díaz *et al*., 2014).

At least thirty-six structurally distinct phenolic compounds have been identified in VOO. Hydrophilic phenols, such as phenolic alcohols, phenolic acids, lignans, flavonoids and secoiridoids, are the most important class of natural antioxidants found in VOOs. Unfortunately, their concentration is not constant in VOO but varies depending on many factors including olive cultivar, fruit ripening stage, irrigation management, and pedoclimatic conditions in the growing area (Bajoub and Carrasco-Pancorbo *et al*., 2015; Bakhouche and Lozano-Sánchez *et al*., 2015; Dabbou and Chehab *et al*., 2010). Additionally, several studies have reported the effect of different stages of VOO processing, such as crushing, malaxation, centrifugation, storage and filtration, on VOO phenol composition (Bakhouche and Lozano-Sánchez, *et al*., 2014a; Frankel and Bakhouche *et al*., 2013).

Owing to the continuous variation in phenolic compounds in VOO under the effects of the factors mentioned above and to the need for correct discrimination of the richest VOOs from the poorest ones in terms of phenolic

compound content, several analytical methods have been proposed to determine phenolic compounds using different extraction, separation and qualitative and quantitative characterization techniques. Two main methods have been employed for phenolic recovery: liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Different solvent mixtures have been tested in the former method and different types of sorbent in the latter to maximize the recovery of phenolic compounds from VOO (Bendini and Bonoli *et al*., 2003). In the case of phenolic characterization, high performance liquid chromatography (HPLC) is the main technique used for the separation of phenolic compounds (Bayram and Esatbeyoglu *et al*., 2012). Other techniques such as gas chromatography (GC) and capillary electrophoresis (CE) have also been reported (Ballus and Meinhart *et al*., 2011; García-Villalba and Pacchiarotta *et al*., 2011). The separation techniques cited are coupled to different detectors. UV–visible diode array detection (DAD) is the standard method used for phenolic compounds and, together with mass spectrometry, is the predominant system nowadays (Bakhouche and Lozano-Sánchez *et al*., 2013). Nuclear magnetic resonance spectroscopy (NMR) has also been used as a detector in the characterization of phenolic compounds in VOO; however, due to its high cost, it is only available at a limited number of institutions (Etrakis and Giomyrgianaki *et al*., 2008).

Although many methods have been optimized to determine the concentration of phenolic compounds in VOO, direct comparison of the data available in the literature is still difficult because the reported concentrations often differ greatly. The explanations put forward, for instance that various agronomical and technological factors might affect VOO phenolic concentration, may account for these discrepancies, but only in part. However, some authors have suggested that the discrepancies observed in VOO concentrations might be caused by the analytical methods used (Bakhouche and Lozano-Sánchez *et al*., 2014b; Karkoula and Skantzari *et al*., 2012). Consequently, the objectives of this review were: first, to provide an overview of the main extraction and separation methods used in the analysis of phenolic compounds in VOO; second, to highlight the drawbacks of the most cited methods in order for

them to be taken into account in future studies of phenolic compounds in VOO.

2. Methods for the extraction of phenolic compounds from virgin olive oil

Isolation from the sample matrix is generally a prerequisite for any comprehensive analysis scheme. Its main objective is to prepare a sample extract that is uniformly enriched in all the compounds concerned and free from the interfering matrix component. LLE and SPE are the two systems reported the most for the extraction of phenolic compounds from VOO. These systems vary not only in the solvents and/or solid-phase sorbents used but also in the quantities of sample needed for analysis, and the volumes of the solvents.

2.1. Liquid-liquid extraction

The phenolic compounds in olive oil have mostly been isolated by extracting an oil solution with methanol or methanol/water mixtures. Before extraction, the liquid-liquid procedure involves a pre-step in which the VOO is dissolved with an apolar solvent in order to remove the lipid fraction and make it easier to extract the phenolic compounds with the polar solvent. Hexane, petroleum ether, and chloroform are used for this purpose; however, hexane is the most widely reported (Lerma-García and Lantano *et al*., 2009; Montedoro and Servili *et al*., 1992). In the case of extraction solvents, controversial data have been reported in the literature as regards the best solvent for the complete recovery of the phenolic compounds from VOO. Initially, extraction with methanol/water 80:20 seemed to give better results than absolute methanol or methanol/water 60:40 (Montedoro and Servili *et al*., 1992). However, five years later, absolute methanol was chosen to extract phenolic compounds from VOO instead of methanol/water owing to incomplete extraction of some phenolic compounds when the latter mixture was used as extraction solvent. This hypothesis could be attributed to the considerable formation of emulsion between the water and oil (Angerosa and D'Alessandro *et al*., 1995). Other studies demonstrated that reducing the percentage of methanol to 60% increased the recovery efficiency of phenolic compounds

(Ballus and Meinhart *et al*., 2014; Pirisi and Cabras *et al*., 2000). The use of organic solvents other than methanol in LLE has also been reported. Examples are ethanol, acetonitrile, and N, N dimethylformamide (DMF), the last of which seemed to show interesting results in terms of recovery efficiency (Brenes and Garcı *et al*., 2000).

The LLE system could be divided into different categories depending on the amount of sample and solvent used in the extraction of phenolic compounds from VOO. The conventional system was characterized by the use of large amounts of sample and organic solvents, which made it laborious, expensive and time-consuming (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). Due to these disadvantages, a new LLE system named liquid–liquid microextraction (LLME) was developed in place of the conventional one. It could be considered a miniature version of conventional LLE because it required a smaller amount of sample and generated a smaller volume of residues; it was also faster. The application of this method to extract phenolic compounds from VOO was reported in the literature. In a comparative study, LLE and LLME were evaluated in terms of repeatability, reproducibility, and phenolic compound recovery. The results showed that both methods had a good repeatability and reproducibility. However, LLE gave lower values than LLME for the total phenols extracted from VOO (Pizarro and Becerra *et al*., 2013). In a more recent study, the same LLME was improved by reducing the volume of extractant from 1 ml to 0.5 ml in order to characterize the phenolic compounds in VOO using ultra high performance liquid chromatography-triple-quadrupole mass spectrometry (Becerra-Herrera and Sánchez-Astudillo *et al*., 2014). Other liquid-liquid extraction techniques have also been developed and applied for the extraction of phenolic compounds from VOO, such as dispersive liquid–liquid microextraction (DLLME), and reversed phase dispersive liquid–liquid microextraction (RP-DLLME) (Godoy-Caballero and Acedo-Valenzuela *et al*., 2013).

2.2. Solid-phase extraction

The SPE technique has become more popular in the last decade as a step for the isolation of phenolic compounds from VOO. It has been applied using several types of sorbents. For instance, C8 cartridges (500 mg, 3.5 mL, Alltech) were used for the isolation of phenolic compounds from VOO and were found to be fast and simple (Pirisi and Cabras *et al*., 2000). A year later, diol-bonded phase cartridges and amino-phase cartridges were compared to determine which type was the best for the extraction of these analytes. The authors ruled out the amino-phase cartridge due to the appearance of some compounds in the extract as a result of an artifact originated during extraction through interactions between the solvent and the amino phase (Mateos and Espartero *et al*., 2001). Two other commercial cartridges, octadecyl C18 (2g, 6ml) and octadecyl C18 EC (end capped; 2g, 6 ml) have been applied for the extraction of phenolic compounds from VOO. A comparative study of both cartridges was carried out. The results reported unsatisfactory recoveries with C18 EC whereas the C18 cartridges resulted in practically full quantitative recovery of all the compounds examined. The authors attributed the differing behavior of the C18 and C18 EC cartridges to the interaction between the sorbent material and the analyte. The mechanism behind the eluent-induced release of the analyte probably depends on the interaction between the residual Si-OH groups located on the surface of the silica and the absorbed compounds. In the case of C18 EC, the residual polar groups are suppressed and this feature apparently worsens the analyte release mechanism (Liberatore and Procida *et al*., 2001). In another interesting study, all the cartridges cited above (C8, C18 and diol) were compared in terms of their recovery efficiency of phenolic compounds from VOO. The results showed that extraction using diol cartridges gave higher recoveries of total phenols, o-diphenols, tyrosol, hydroxytyrosol and secoiridoids than the other extraction procedures (Bendini and Bonoli *et al*., 2003). In a more recent study, diol-SPE was also chosen as the most efficient cartridge in comparison with C18-SPE and Sax-SPE (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). Another important aspect to take into

account during SPE extraction is the equilibrium between the amount of VOO and the volume of sorbent used. In this respect, initially 30 g of VOO were spiked with the analytes, and SPE was performed using different capacity (500 mg and 1 g) diol cartridges. The results obtained showed that when 500 mg cartridges were employed, extraction recovery values were low. However, they recorded a substantial increase with the 1 g cartridges. Subsequently, in the same study, a 1 g diol cartridge was used to extract phenolic compounds from 10 g, 20 g and 30 g of VOO, the objective being to detect the saturation point of the diol cartridge used. The results showed that most of the compounds recorded a linear increase in peak area as the amount of olive oil increased; however, as of 20 g of VOO, a curvature of the signal, probably related to cartridge saturation, was observed for tyrosol, vanillic acid and syringic acid (Godoy-Caballero and Acedo-Valenzuela *et al*., 2012). It was reported that 500 mg are generally employed for small scale SPE, and 1 g for large scale (Laura Capriotti and Cavaliere *et al*., 2014).

Comparisons of both extraction techniques, SPE and LLE, have also been carried out and have led to the publication of controversial data. While many comparative studies have shown that LLE achieves a higher recovery than SPE (Bendini and Bonoli *et al*., 2003; Hrncirik and Fritsche, 2004), other authors have considered SPE to be the reference method for the extraction of phenolic compounds from VOO instead of LLE because of its ease of use and short extraction time (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005; Pirisi and Cabras *et al*., 2000). However, other research has reported the SPE method to be problematic because of its selectivity towards the individual phenolics, particularly the aglycone ones (Hrncirik and Fritsche, 2004). Furthermore, when studying the retention effects of oxidized phenolic compounds during analytical extraction of the phenolic compounds in VOO, the authors came to the conclusion that SPE only seems to be effective for fresh VOO because in the case of oxidized VOO, the stationary phase of the SPE columns interacts with the oxidized phenols. This interaction would lead to further nonselective retention of non-oxidized phenolic compounds, thus

reducing the total recovery of these analytes (Armaforte and Mancebo-Campos *et al*., 2007).

3. Separation techniques for the analysis of phenolic compounds in VOO

3.1. Liquid Chromatography

Liquid chromatography (LC) is considered the most popular and reliable technique for the separation of phenolic compounds. Normal phase LC and reverse phase LC are reported in the literature. The first uses silica gel as the stationary phase in the column and a non-polar solvent as eluent while the second uses a non-polar octadecylsilane (C18) bonded stationary phase and a polar solvent as the mobile phase. Reverse phase LC was reported to be the technique in greatest use due to its better reproducibility and separation of polar compounds (Carrasco-Pancorbo and Cerretani *et al*., 2005; Laura Capriotti and Cavaliere *et al*., 2014). A wide variety of columns are employed. The preferred columns are 100 to 250 mm in length, with 2-4.6 mm inner diameter and 1.8-5 µm particle size (Selvaggini and Servili *et al*., 2006; Taamalli and Abaza *et al*., 2013).

An isocratic elution was developed for the separation of phenolic compounds and an adequate resolution was achieved using a suitable composition of mobile phase (Akasbi and Shoeman *et al*., 1993). However, recent studies exclusively use gradient elution mode. This fact confirms the complexity of the phenolic profile which cannot be properly separated in isocratic elution mode. Numerous mobile phases have been employed, but binary systems comprising water and a less polar organic solvent such as acetonitrile or methanol remain common (Bakhouche and Lozano-Sánchez *et al*., 2015; De la Torre-Carbot and Jauregui *et al*., 2005). Acids such as acetic, formic, and perchloric acid are usually added to water to maintain a constant acid concentration during gradient runs (Bayram and Esatbeyoglu *et al*., 2012; De la Torre-Carbot and Jauregui *et al*., 2005; Taamalli and Abaza *et al*., 2013). Decreasing the pH partly helped to improve the resolution. Nevertheless, the lack of separation between peaks and the long analysis time are still the main drawbacks of the LC technique. In fact, the separation of complex VOO

phenolic compounds has required longer run times using conventional HPLC methods. However, the development of columns with small particle sizes in the stationary phase has enhanced resolution and reduced analysis time. The high pressures produced by the use of small particle sizes made it necessary to develop new equipment such as rapid resolution liquid chromatography (RRLC) and ultra performance liquid chromatography (UPLC) to support the higher pressures. The application of the new column for the separation of VOO phenolic compounds, together with the optimization of gradient, temperature, and flow rate were reported in the literature. In a recent study, a new RRLC method has been optimized (Column Zorbax C18: 4.6 mm×150 mm and 1.8 µm particle size) on the basis of the chromatographic conditions of a previous high performance liquid chromatography (HPLC) method (Gemini C18 column: 3 mm×250 mm, 5 µm particle size). Firstly, a gradient elution was optimized using water + 0.5% of acetic acid as mobile phase A, and acetonitrile as mobile phase B. In the next step, flow rate and temperature values ranging from 0.5 to 2 ml/min and 25 ºC to 40 ºC, respectively, were tested. The results showed that increasing the flow and temperature shortened the run time without excessively compromising resolution, but temperatures above 40 ºC led to overlapping of some peaks and loss of compounds. Finally, an optimum flow of 1.5 mL/min and temperature of 30 ºC were chosen for the analysis. With the optimized method, the run time could be reduced from 60 min (HPLC) to 20 min (RRLC), and a good resolution was obtained by using a column with a small particle size (García-Villalba and Carrasco-Pancorbo *et al*., 2010). In an even more recent study, optimum chromatographic separation was obtained with the same equipment (RRLC) using water + 0.25% acetic acid as mobile phase A and methanol as mobile phase B. The testing temperature and the flow rate were 25 ºC and 0.8 ml/min, respectively. Applying all these conditions, the phenolic compounds were correctly separated in only 27 min (Lozano-Sánchez and Segura-Carretero *et al*., 2010).

3.2. Gas Chromatography

Since it was invented, and especially when the fused-silica capillary column was introduced in gas chromatography (GC), this technique has become one of the most effective techniques in analytical chemistry on account of the significant improvement in separation quality (Carrasco-Pancorbo and Nevedomskaya *et al*., 2009). GC has been used to perform qualitative and quantitative determinations of the phenolic compounds in VOO (García-Villalba and Pacchiarotta *et al*., 2011; Saitta and Curto *et al*., 2002). However, its use has been restricted by the limited volatility of many phenolic compounds. In order to overcome this problem, the analysis requires a pre-step named derivatization in which the phenolic compounds are converted into more volatile compounds using different reagents. There are many derivatization methods, although trimethylsilylation is the most reported one (Angerosa and D'Alessandro *et al*., 1996; Zafra-Gómez and Luzón-Toro *et al*., 2010).

In a recent study, the derivatization step was carefully optimized by comparing different reagents and testing their efficiency in both standard solutions and real samples. Firstly, the derivatization step was optimized in a mixture of tyrosol and hydoxytyrosol standard solution, using N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA), N,O-Bis(trimethylsilyl) trifluoroacetamide + trimethylchlorosilane (BSTFA+TMCS), N,O-Bis(trimethylsilyl)acetamide + trimethylchlorosilane (BSA+TMCS), and tert-Butyldimethylchlorosilane (TBDMSCl) as derivatization reagents. Pyridine and ACN were compared as reaction solvents. Despite the good results obtained using BSTFA and BSTFA+TMCS, the mixture of BSA+TMCS showed the best efficiency. However, BSTFA was chosen for the analysis of phenolic compounds in VOO samples to avoid injection of TMCS, which can reduce column life (Purcaro and Codony *et al*., 2014).

The literature reports many attempts to achieve good separation using GC in the analysis of phenolic compounds. The GC methods use different columns, oven temperature programs, injection temperatures, injection modes, and

injection volumes of the extract (Angerosa and D'Alessandro *et al*., 1996; García-Villalba and Pacchiarotta *et al*., 2011; Ríos and Gil *et al*., 2005). In almost all the methods reported, helium was employed as the carrier gas with a linear velocity. Besides separation quality, shorter or longer analysis times were achieved in the published methods depending on the analysis parameters mentioned above. However, the need to perform derivatization makes this technique more laborious. Furthermore, incomplete derivatization may be accompanied by the formation of several chemical species from the same compound, giving confusing results. Another drawback of this technique is the use of high temperatures, which can damage the analytes (Carrasco-Pancorbo and Cerretani *et al*., 2005).

3.3. Capillary Electrophoresis

In recent years, capillary electrophoresis (CE) has proved to be a fast technique combining short analysis times and high separation efficiency for the analysis of food components. It has been used in particular for the analysis of phenolic compounds in VOO (Bendini and Bonoli *et al*., 2003). Different electrophoretic methods have been optimized in order to find the best separation conditions to perform the analysis in the shortest time with sufficient resolution. Typical parameters for optimization were the buffer (type, concentration and pH), capillary temperature, voltage, effective capillary diameter, and type of sample injection. The first optimized methods were laborious because of the need for individual optimization of all the parameters cited above (Bonoli and Montanucci *et al*., 2003; Carrasco-Pancorbo and Gómez-Caravaca *et al*., 2006a; Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). However, recently the use of multi-criteria methods, which simultaneously take into account all the critical separations, makes the development of new electrophoretic methods easy and fast (Ballus and Meinhart *et al*., 2011; Ballus and Meinhart *et al*., 2014). Furthermore, in order to reduce the number of steps involved in the analysis of phenolic compounds using capillary electrophoresis, the use of a new technique named non-aqueous capillary electrophoresis (NACE) has been reported. Its main advantage in comparison with the conventional technique is its ability to work

with a large variety of organic solvents (methanol, ethanol, 1-propanol, 2-propanol, or acetonitrile, among others). As it is known, LLE or SPE is often needed to achieve pre-concentration with organic solvents before the analysis of complex matrices. After pre-concentration, the analytes concerned are commonly diluted in an organic solvent. In aqueous CE, the extract is usually evaporated and the analytes are dissolved in aqueous media. However, this step can be bypassed when NACE is used (Godoy-Caballero and Acedo-Valenzuela *et al*., 2012b). In spite of the efforts of several research groups to develop reliable CE methods, only a few papers have been published on the application of this technique in the field of Olea europaea, probably because of some disadvantages of CE such as poor reproducibility, low UV detection sensitivity, and difficulties with the MS coupling (Carrasco-Pancorbo and Gómez-Caravaca *et al*., 2006a; Godoy-Caballero and Acedo-Valenzuela *et al*., 2012b).

4. Detectors

Phenolic compounds are commonly determined using UV/VIS and DAD detectors, particularly coupled to LC and CE. In LC phenolic studies, 280 nm is useful for routine analysis since most VOO phenolics absorb at this wavelength, whereas 240 nm is used for some secoiridoids and their derivatives, 310-320 nm for hydroxycinnamic acids, and 350 nm for flavones (Bakhouche and Lozano-Sánchez *et al*., 2013; Garcia and Coelho *et al*., 2013; Godoy-Caballero and Acedo-Valenzuela *et al*., 2012a). In the official phenolic analysis method published by the International Olive Council (IOC), UV-Vis is used for detection. The method establishes the maximum absorbance values of 27 different phenolic compounds and gives a procedure for the quantification of these analytes on the basis of the data provided by the UV detector (IOC 2009). UV detectors have also been used to study the variation of phenolic content in VOO under the influence of different agronomical and technological factors (Gómez-Rico and Salvador *et al*., 2006; Parenti and Spugnoli *et al*., 2008). With regard to CE analysis, the literature also reports the performance of UV detection at 200, 240, 280 and 330 nm for the

characterization of different phenolic groups in VOO, although diode-array detection was used over the range of 190–600 nm to achieve spectral data (Carrasco-Pancorbo and Gómez-Caravaca *et al*., 2006a). In more recent studies, CE coupled to a UV detector was used to develop a new electrophoretic method for the separation of the phenolic compounds of VOO extracts, to compare different extraction systems in terms of phenolic recovery and to study the phenolic composition of VOO obtained from different olive varieties (Ballus and Meinhart *et al*., 2014; Godoy-Caballero and Galeano-Díaz *et al*., 2012c; Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005).

In GC, the flame ionization detector (FID) is the most common detector coupled to this separation technique. In fact, GC-FID has been used to analyze the phenolic profiles of different oils, including VOO. The method proposed for this purpose has made it possible to estimate the phenolic content of sunflower oil, colza oil, and VOO (Farajzadeh and Yadeghari *et al*., 2014). Other authors have used GC-FID to analyze the total hydroxytyrosol and tyrosol content of extra virgin olive oils, after hydrolysis of the linked forms (Purcaro and Codony *et al*., 2014).

Recently, mass spectrometry detectors (MS) have been coupled to different separation techniques for further characterization of the phenolic compounds in VOO. Time-of-flight (TOF-MS), quadrupole time-of-flight (QTOF-MS), and ion trap (IT-MS) MS detectors are the types reported most widely in the literature (Bakhouche and Lozano-Sánchez *et al*., 2014b; Fu and Segura-Carretero *et al*., 2009; Laura Capriotti and Cavaliere *et al*., 2014). Apart from fast data acquisition and a wide mass detection range, another important characteristic of the MS detector is its great accuracy in mass measurements. It provides high selectivity in the determination of phenolic compounds using the extracted ion chromatogram (EIC) mode when there are overlapping peaks, and it permits rapid and efficient confirmation of the elemental composition of ions when fragmentation is carried out. Coupling LC, GC, or CE to a mass spectrometry detector has permitted detailed characterization of

the phenolic fraction in different studies on VOO quality (Bengana and Bakhouche *et al*., 2013; Carrasco-Pancorbo and Arráez-román *et al*., 2006b; Saitta and Curto *et al*., 2002). Furthermore, the use of a mass spectrometry detector enables investigation of the relationship between the chemical nature or concentration of individual phenolic compounds in VOO extracts and their ability to reduce some chronic diseases (García-Villalba and Carrasco-Pancorbo *et al*., 2010). Other detectors such as fluorescence and NMR detectors are also reported (Etrakis and Giomyrgianaki *et al*., 2008; Tena and García-González *et al*., 2009); however, they are not as common as UV and MS detectors.

5. Analytical problems of phenolic compound characterization in VOO

In all the studies aimed at quantification of the phenolic compounds in VOO, the concern of researchers has been to achieve the total recovery of these analytes from the matrix. Initially, a mixture of commercial standards spiked in refined peanut oil was used to compare the efficiency recovery of various isolation techniques. The spiked oil was then subjected to different extraction systems (LLE and SPE). The resultant extracts were analyzed by HPLC, and the amount of each standard was compared with that of the standard mixture that had not undergone any extraction procedure (Bendini and Bonoli *et al*., 2003). However, when considering the results obtained, it should be borne in mind that most of the naturally occurring phenolic compounds in olive oil are not commercially available. Consequently, research continued on the basis of other compounds having similar structures. The response of the standards can be different from that of the analytes present in the oil samples; hence, the recovery results could only be estimates. To overcome this problem, other authors proposed an extraction method based on spiking refined sunflower oil (phenolic-free) with an exact dose of an LLE-prepared phenolic extract of VOO. The spiked oil was extracted using SPE, and its recovery efficiency was calculated (Gómez-Caravaca and Carrasco Pancorbo *et al*., 2005). This approach provided a partial solution for estimating the recovery of phenolic compounds from VOO. The previous attempts where different extraction systems were compared in terms of recovery efficiency did not consider the

interactions between both matrix and target compounds. In fact, the effect of VOO water content on phenolic extraction should be taken into account in both systems (LLE and SPE). Several studies have demonstrated the relationship between water content and phenolic compounds. It is common knowledge that olive oil contains a small quantity of water; for this reason it can be considered a water-in-oil-emulsion. Phenolic compounds are located in the water/oil interface (Ambrosone and Cinelli *et al*., 2006; Frankel and Huang *et al*., 1994).

The effect of water content on the extraction of phenolic compounds from VOO was observed for the first time on a laboratory scale by studying the effect of the filtration system on VOO phenolic content (Gómez-Caravaca and Cerretani *et al*., 2007). In this research, LLE was used to extract the phenolic compounds. The results obtained by the authors showed that compounds belonging to the secoiridoid group, such as ligstroside aglycone and oleuropein aglycone, increased significantly after reducing water content by filtration with cotton in comparison with unfiltered VOO. In effect, in a water-in-oil emulsion, phenolic compounds are stabilized around water droplets, and the affinity of the phenolic compounds for solvent extraction is low in a more polar matrix (olive oils with high water content), making their recovery more difficult. However, the partial elimination of water during the filtration process makes more phenolic compounds available for extraction with the apolar solvent mixture, which results in the apparent increase in their concentration in filtered VOO. Five years later, the effect of water content on phenolic compound extraction from VOO samples was confirmed using SPE (Lozano-Sánchez and Cerretani *et al*., 2012). Working on a pilot-plant scale using filter bags, the authors found that secoiridoids in filtered VOO were responsible for the apparent increase in the total phenolic content. Lastly, the apparent increase in different compounds from the secoiridoid group due to the variation in water content in VOO was confirmed in a more recent study (Bakhouche and Lozano-Sánchez *et al*., 2014a). The chemical structures of the main compounds whose recovery was affected by the variation in the water content of VOO are shown in **Fig. 1**.

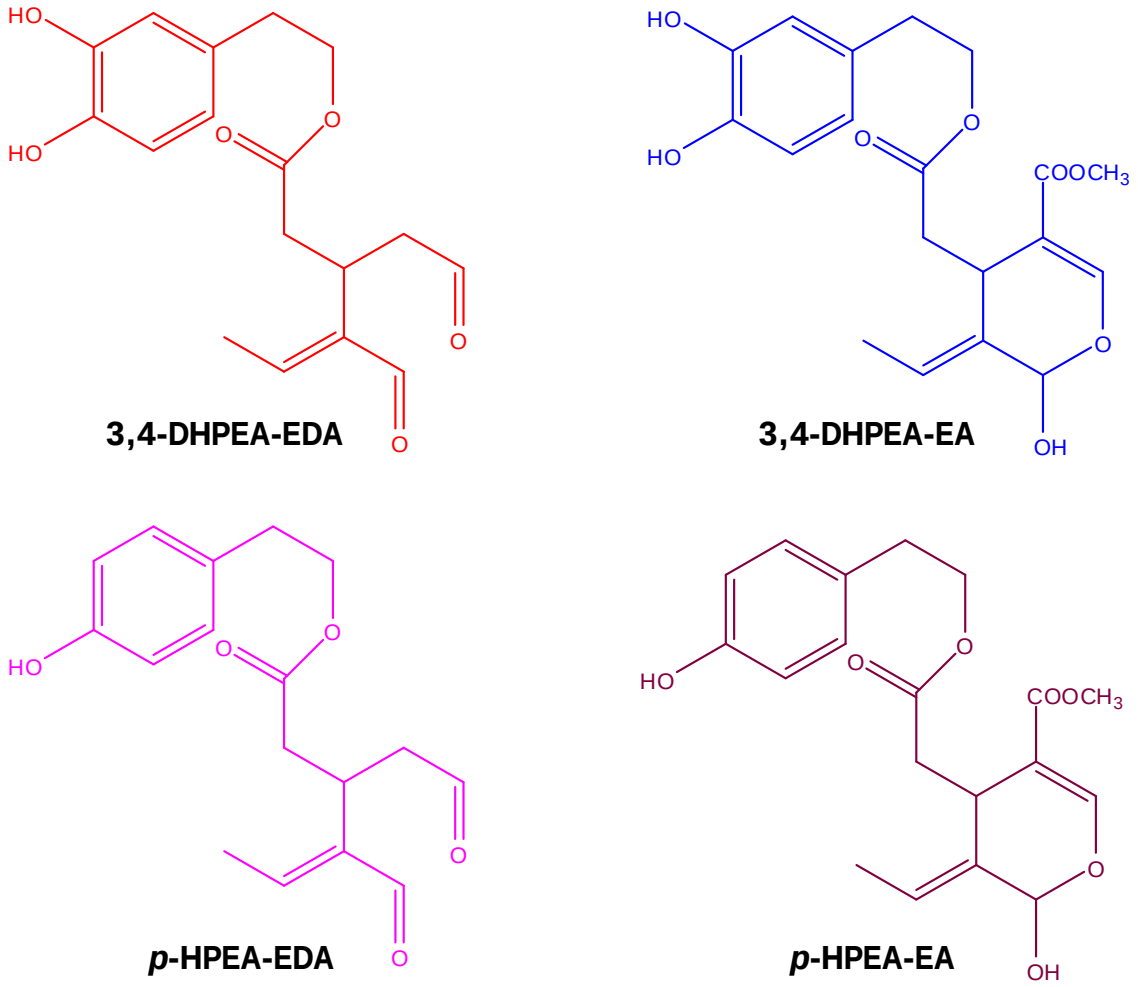

Fig. 1. Structure of some phenolic compounds affected by the variation in VOO water content during extraction: 3,4-DHPEA-EDA, decarboxymethyl oleuropein aglycone; 3,4-DHPEA-EA, oleuropein aglycone; *p*-HPEA-EDA, decarboxymethyl ligstroside aglycone; *p*-HPEA-EA, ligstroside aglycone.

These studies confirmed the effect of the filtration-induced variation in VOO water content on the extraction of phenolic compounds. However, it is common knowledge that VOO water content also varies according to fruit ripening stage, olive variety, and geographical area (Motilva and Tovar *et al*., 2000; Taamalli and Gómez-Caravaca *et al*., 2010). Many studies have used phenolic profiles as a fingerprint to distinguish between olive varieties as well as to classify VOOs according to their geographical origin (designation of origin) and to determine the best harvest period for obtaining high-phenolic VOO while other studies have been conducted on the bioactivity of VOO

phenolic compounds (García-Villalba and Carrasco-Pancorbo *et al*., 2010; Karkoula and Skantzari *et al*., 2012; Ouni and Taamalli *et al*., 2011; Rotondi and Bendini *et al*., 2004; Taamalli and Gómez-Caravaca *et al*., 2010). Unfortunately, in all of these studies, the variation in VOO water content during phenolic extraction was not considered. This could affect the accuracy of data reported in different publications for the concentration of these analytes in VOO. Recently, in an attempt to resolve this problem, a new approach was developed to correct the effect exerted by moisture reduction after VOO filtration on the recovery of phenolic compounds by using an internal standard during extraction. As a result, the apparent increase of secoiridoids was corrected and the phenolic compounds in filtered VOO were correctly quantified (Bakhouche and Lozano-Sánchez *et al*., 2014b). However, the optimization of the proposed method was based solely on the change in VOO moisture content after filtration. Consequently, future investigations are warranted to develop a new extraction method which can be applied to all kinds of VOO studies relating to the phenolic fraction and water content. Until then, the extraction of phenolic compounds from VOO will continue to be problematic.

As it is well known, after the isolation of the phenolic compounds from VOO, the next challenge is to draw up a reliable method for the analysis of the resultant extracts. Although powerful analytical equipment and methods have been developed, the total structural characterization of the phenolic fraction is still sometimes impossible because of the complexity of the wide group of secoiridoids. The main compounds identified in this group are oleuropein aglycone, ligstroside aglycone and their derivatives such as the hydroxylated, decarboxymethylated, dehydrated, and methylated forms. Today, the isomers of these compounds are the subject matter of scientific research. The first research characterized eleven isomers of oleuropein aglycone in Spanish VOO (Fu and Segura-Carretero *et al*., 2009) using rapid-resolution liquid chromatography coupled to electrospray time-of-flight and ion trap tandem mass spectrometry. In a more recent study, eighteen, seventeen and nine isomers were detected for oleuropein aglycone, ligstroside aglycone and

elenolic acid, respectively, using fused-core reverse phase chromatography coupled to high resolution mass spectrometry (HRMS) and high resolution tandem mass spectrometry (HRMS/MS), in positive and negative electrospray ionization (ESI) modes (Vichi and Cortés-Francisco *et al*., 2013). These isomers were considered to be the result of oleuropein and ligstroside isomerization after hydrolysis during olive ripening and olive oil processing. However, a study using HPLC-UV with reversed phase columns showed that isomers of decarboxymethyl ligstroside aglycone and decarboxymethyl oleuropein aglycone were formed by the reaction of these two compounds with water or methanol used as a mobile phase (Karkoula and Skantzari *et al*., 2012). In addition, in a more recent study, the artificial formation of oleuropein and ligstroside aglycone isomers was proved by the same authors (Karkoula and Skantzari *et al*., 2014). Therefore, isomers could also be formed during chromatographic analysis depending on the mobile phase used. This finding confirmed that classic chromatographic measurement of these compounds is problematic, especially in aqueous media, and that many of the previous measurements reported in the literature are more or less questionable. It should be taken into account that the validated and official methods proposed by several authors and international committees use water as the eluent for the mobile phase. This can affect the results owing to the interaction between water and some phenolic compounds, which casts doubts over the estimation of this fraction as shown for example in **Fig. 2** for the main isomers detected in VOO.

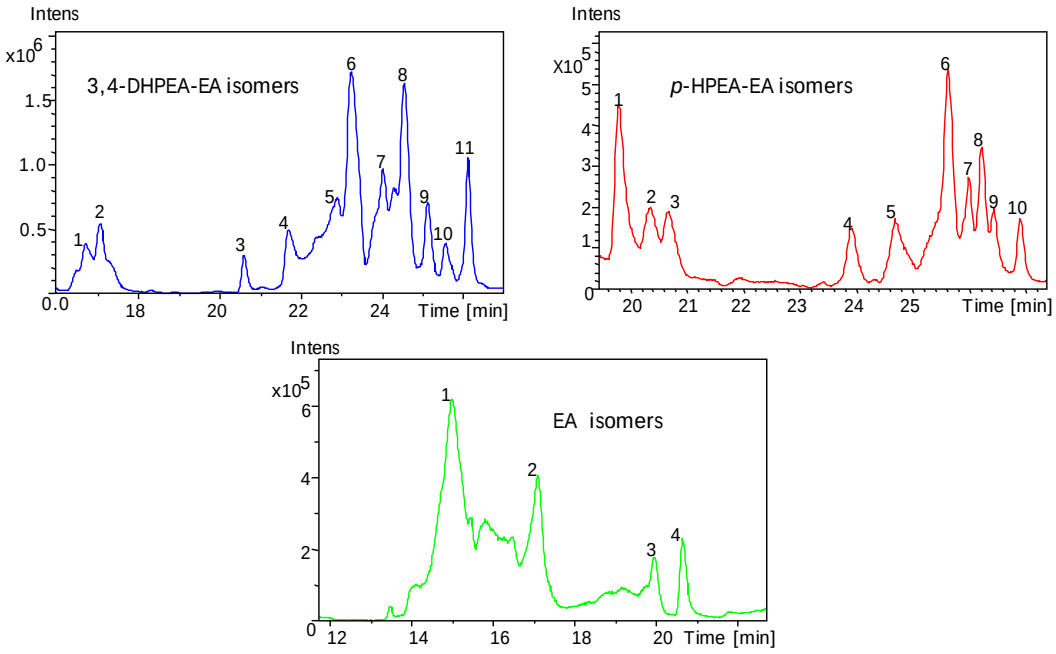

Fig. 2. Isomers of oleuropein aglycone (3,4-DHPEA-EA), ligstroside aglycone (*p*-HPEA-EA), and elenolic acid (EA) obtained using HPLC-ESI-TOF/MS.

6. Conclusions

The different methods for the isolation and separation of phenolic compounds resolved many problems related to the estimation of this fraction in VOO. Having done so, the nutritional value and healthy properties of VOO were easily proven. However, some aspects of the cited methods need to be improved. For instance, the controversial data reported in the literature make it difficult to compare VOOs produced in different parts of the world in terms of their phenolic content. New investigations are therefore warranted in order to devise accurate, harmonized methods aimed at avoiding confusion when different published data are compared. The improvements required need to take into account the current problems, especially the effect of VOO water content on the isolation of phenolic compounds, and the artificial formation of some isomers during chromatographic separation due to the mobile phase used.

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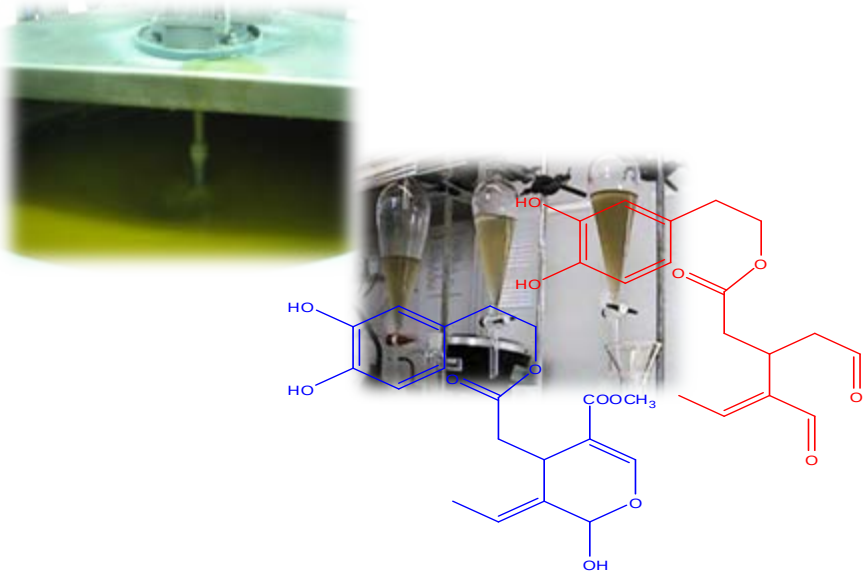

Chapter 7

A new extraction approach to correct the effect of apparent increase in the secoiridoid content after filtration of virgin olive oil

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A new extraction approach to correct the effect of apparent increase in the secoiridoid content after filtration of virgin olive oil

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Abstract

In the current study, a new approach has been developed for correcting the effect that moisture reduction after virgin olive oil (VOO) filtration exerts on the apparent increase of the secoiridoid content by using an internal standard during extraction. Firstly, two main Spanish varieties (Picual and Hojiblanca) were submitted to industrial filtration of VOOs. Afterwards, the moisture content was determined in unfiltered and filtered VOOs, and liquid–liquid extraction of phenolic compounds was performed using different internal standards. The resulting extracts were analyzed by HPLC-ESI-TOF/MS, in order to gain maximum information concerning the phenolic profiles of the samples under study. The reduction effect of filtration on the moisture content, phenolic alcohols, and flavones was confirmed at the industrial scale. Oleuropein was chosen as internal standard and, for the first time, the apparent increase of secoiridoids in filtered VOO was corrected, using a correction coefficient (Cc) calculated from the variation of internal standard area in filtered and unfiltered VOO during extraction. This approach gave the real concentration of secoiridoids in filtered VOO, and clarified the effect of the filtration step on the phenolic fraction. This finding is of great importance for future studies that seek to quantify phenolic compounds in VOOs.

Keywords: VOO; Filtration; Moisture; Phenolic compounds; Internal standard; HPLC-ESI-TOF/MS.

1. Introduction

Virgin olive oil (VOO) is a natural product obtained exclusively through mechanical and physical operations. The process begins by collecting and washing olives, followed by crushing them to tear the flesh cells and thus let the oil escape. The resulting olive paste has to be mixed; in this stage the droplets of oil merge into larger drops until they form a continuous liquid phase, and then the oil can be separated from the other phases by centrifugation [1-3] and [4]. Immediately after centrifugation, the VOO produced is turbid from suspended solid plant-tissue particles and vegetable water emulsified in the oil, which can deteriorate its quality by facilitating hydrolysis or oxidation of lipid matrix. Recently, filtration was included in VOO-production process as a final step before bottling in order to make VOO more brilliant and maintain its quality [1, 5] and [6].

Several filtration systems are used for VOO: conventional filtration systems, cross-flow filtration, and new patented approaches based on inert gas-flow filtration and filter bags [7] and [8]. At the industrial scale, the most widespread system is the conventional one, which employs filter aids in conjunction with filtration equipment (tanks or presses) to enhance or enable suspended solids and water–oil separation [5]. Filter aids for filter cake can be produced from a wide variety of raw materials. Traditionally, diatomite, known also as diatomaceous earth was used, the composition being largely silica (95–98%). Unfortunately, the sludge from this kind of filter cake represents a major source of pollution, and land disposal of this waste is forbidden. Consequently, in recent years, filter aids based on fibrous material are becoming more widely used. Normally, the fibrous products used to filter cloudy VOO are cellulose or mixtures of cellulose and lignin. Besides its ecological advantage, filtration by an organic filter aid is preferred due to its high performance in the filtration process [9-11].

The effect of this step on VOO composition, particularly on polar phenol fraction, has been studied by several authors, due to the importance of these peculiar compounds on VOO oxidative stability and organoleptic quality

[12-15]. Nevertheless, controversial results have been reported. On the one hand, no differences in the total polyphenol content have been found after VOO filtration, using gas-flow filtration as filter aids [6]. On the other, a laboratory study on the retentive power of inorganic and organic filter aids on phenolic compounds showed that a large number of polyphenols were retained in filter aids, lowering the total phenol content in filtered VOO [11]. The same trend for this fraction during filtration has been reported by other authors [16]. Furthermore, the effect of filtration on individual polyphenols in VOO has also been studied at laboratory scale [17]. The authors found that compounds belonging to the secoiridoid group, such as ligstroside aglycone and oleuropein aglycone, increased significantly after filtration with cotton in comparison to the unfiltered VOO. Five years afterwards, a pilot-plant-scale study using filter bags showed that secoiridoids in filtered VOO were responsible for the apparent increase in the total phenolic content [8]. Finally, the apparent increase in different compounds from the secoiridoid group after filtration was confirmed in a more recent study at the industrial scale [18]. The hypothesis proposed to explain secoiridoid behavior was that after filtration the reduction in VOO moisture content facilitated their extraction, triggering an apparent increase in the filtered VOO.

Thus, the objective of this work was to correct this apparent increase and then to evaluate what really happens to the phenolic compounds during VOO filtration. Taking into consideration the hypothesis proposed above, this work seeks to achieve the correction by using an internal standard in the extraction step, and then to quantify the real concentration of phenolic compounds in the filtered VOO. The analysis was made using HPLC-ESI-TOF/MS, which could provide information concerning the phenolic profile of the VOOs under study.

2. Materials and methods

2.1. Samples

The VOO samples used in this study were from Aceites Maeva Company (Aceites Maeva S.L., Granada, Spain). The extraction was made in November 2012 by a continuous industrial hammer crusher, a horizontal malaxator, and a two-phase decanter. For this work, 45,000 kg of VOO mixture was filtered using a conventional filtration process at the industrial scale. The mixture was from two of the main Spanish varieties, Picual 40% and Hojiblanca 60%. Cloudy VOOs were filtered at room temperature using the following organic filter aids: Vitacel® L-90 (30 kg, composed of 100 % cellulose) and Filtracel® EFC-950 (60 kg, composed of 70% cellulose and 30% lignin). The cake layer formed in conjunction with filter tank. For the filtration, a preliminary phase is required, during which a prepared combinations of filter aids and unfiltered VOO are mixed in a slurry tank. Afterwards, the slurry was circulated through filter tank and back to the slurry tank. The filter aids were kept in the filtration equipment and circulation continued until the cake layer formed and the effluent ran clear. Afterwards, filtration was conducted under a constant flow and increasing differential pressure in different steps designated as A, B, C, and D, with 12,000 kg each. During the last step (D) the filtrate was just 9000 kg, which depended on the availability of VOO in the company. A total of 24 unfiltered and filtered samples were collected for analysis (**Fig. 1**). To have representative results and eliminate confounding factors which could affect olive-oil composition, the moisture content and isolation of phenolic fraction from samples were determined without storage.

2.2. Chemicals and reagents

All chemicals were of analytical reagent grade. Methanol, *n*-hexane, sodium hydroxide and isopropanol were purchased from Merck (Darmstadt, Germany). Acetic acid was purchased from Fluka, Sigma-Aldrich (Steinheim, Germany). Double-deionized water with conductivity of less than 18.2 MΩ cm was obtained with a Milli-Q system (Millipore, Bedford, MA, USA). Standards of hydroxytyrosol, tyrosol, luteolin, apigenin, taxifolin and quinic acid were

purchased by Sigma-Aldrich St. Louis, MO, USA), and pinoresinol was acquired from Arbo Nova (Turku, Finland). Oleuropein, luteolin 7-glucoside and dihydrocaffeic acid were purchased from Extrasynthese (Lyon, France).

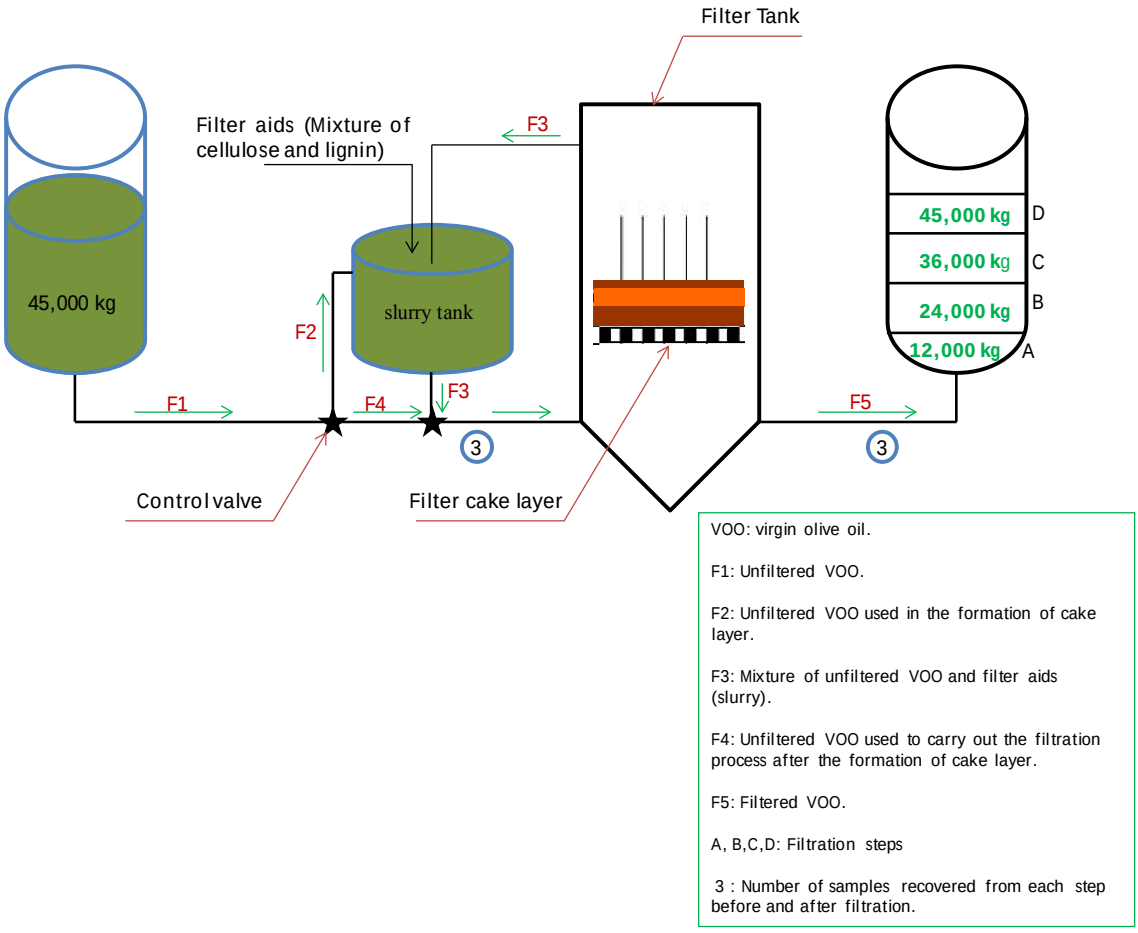

Fig. 1. Industrial filtration diagram

2.3. Moisture content

The moisture content was determined following the norms of the Spanish Association for Standardization and Certification (AENOR). Briefly, in a capsule, previously dried at 105 °C and cooled, 10 g of completely homogenized sample was weighed. The samples were placed in an oven (Memmert GmbH + CO.KG, Schwabach, Germany) at 105 °C for 21 h, after which the samples were removed and weighed. Next, they were returned in the oven and the operation was repeated until the weight was constant. The moisture content was calculated as the difference in weights [19].

2.4. Phenolic compound extraction

The phenolic compounds from the VOOs were extracted using a liquid–liquid extraction system following the method reported previously [18], with some modifications. As mentioned above, in an effort to correct the effect of moisture content on extraction of those analytes from the samples, different internal standards belonging to different phenolic families were tested: luteolin 7-glucoside, dihydrocaffeic acid, taxifolin, and oleuropein. Concentrations ranging from 5 mg L^{-1} to 15 mg L^{-1} of the internal standards were also tested. The extraction procedure was as described in the following protocol. VOO, with 50 µL of internal standard in methanol added, was dissolved in n-hexane (2.5 g in 5 mL). Afterwards, 5 mL of methanol/water (60/40, v/v) was added, and the mixture was vortexed and then centrifuged at 445.1 g during 10 min. The polar extract was evaporated to dryness in a rotary evaporator under reduced pressure at a temperature of 35 °C. The residue was dissolved in 0.25 mL of methanol/water (50/50 v/v) and finally filtered through a 0.2 µm filter before the HPLC analysis.

2.5. HPLC-ESI-TOF/MS phenolic analysis

The analysis to characterize the phenolic profile in filtered and unfiltered VOOs was performed in an Agilent 1200-HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, autosampler, a binary pump, and a diode array detector (DAD). The chromatographic separation of these compounds was performed on a 150 mm×4.6 mm i.d., 1.8 µm, Zorbax Eclipse Plus RP-C18 column (Agilent Technologies, Palo Alto, CA, USA). The mobile phases used were water with 0.25% acetic acid as eluent A and methanol as eluent B. The total run time was 27 min using a previously reported multistep linear gradient [20]. The flow rate was 0.80 mL min^{-1} and, consequently, the use of a splitter was required for the coupling with the MS detector, as the flow which arrived to the TOF detector had to be 0.2 mL min^{-1} to ensure reproducible results and stable spray. HPLC was coupled to a time-of-flight mass spectrometer detector micrOTOF (Bruker Daltonik,

Bremen, Germany), which was equipped with a model G1607A ESI interface (Agilent Technologies) operating in negative ion mode.

External mass-spectrometer calibration was performed with sodium acetate clusters (5 mM sodium hydroxide in water/isopropanol 1/1 (v/v), with 0.2% of acetic acid) in quadratic + high-precision calibration (HPC) regression mode. The optimum values of the source and transfer parameters were established according to the method published previously [20]. The widely accepted accuracy threshold for confirmation of elemental compositions was set at 10 ppm for most of the compounds. The phenolic compounds were identified by comparing both retention times and MS data from samples and standards. The remaining compounds for which no commercial standards were available were identified by the interpretation of the information generated by the TOF analyzer, and the information reported in the literature [21, 22] and [23]. Quantification was made by HPLC-ESI-TOF/MS. Seven standard calibration curves of the main compounds found in the samples were prepared using seven commercial standards. Stock solutions at a concentration of 1000 mg L^{-1} for each phenolic compound were first prepared by dissolving the appropriate amount of the compound in methanol and then serially diluted to working concentrations. All calibration curves showed good linearity over the study range (r^2= 0.993). The individual concentrations were determined using the area of each individual compound (three replicates) and by interpolation of the corresponding calibration curve. Regarding the secoiridoid group, their real concentration in filtered VOO was determined with a correction coefficient (Cc) calculated using the following equation:

$$Cc = \frac{A\,IS_{FL}}{A\,IS_{UF}}$$

where $A\,IS_{FL}$ is the area of the internal standard obtained in filtered VOO, and $A\,IS_{UF}$ is the area of the internal standard determined in the unfiltered VOO.

Afterwards, the area of all secoiridoids in filtered VOO was divided by the correction coefficient (Cc), and the quantification was performed as previously described using the new areas. Results were given in mg of analyte per kg of VOO.

2.6. Statistical analysis

The data were analyzed using Origin (version Origin Pro 8 SR0, Northampton, MA, USA) to perform a one-way-analysis of variance (ANOVA) at a 95% confidence level $p \leq 0.05$ to identify significant differences among the parameters analyzed in unfiltered and filtered VOOs.

3. Results and discussion

3.1. The time course of moisture content

As shown in **Table 1**, the moisture content was sharply reduced using organic filter aids. Meanwhile, the highest moisture values were registered in unfiltered VOO, which varied from 0.132 to 0.120%, the lowest ones being registered in filtered VOO, varying from 0.086 to 0.074%.

Table 1. Time course of virgin olive oil moisture content during filtration process.

Filtration Steps	Moisture (%)		Reduction percentage
	UF[c]	FL[e]	((UF-FL)/UF)x100
A	0.132[a]±0.001	0.074[b]±0.002	44 %
B	0.125[a]±0.002	0.077[b]±0.002	39 %
C	0.120[a]±0.003	0.083[b]±0.002	31 %
D	0.123[a]±0.004	0.086[b]±0.001	30 %

Values with different letters in a line are significantly different at a 95% confidence level ($p \leq 0.05$).

[a] Unfiltered.

[b] Filtered.

These results were analyzed further by calculating the difference on moisture content between unfiltered and filtered VOOs for each step. The results were expressed as a percentage reduction (**Table 1**). The initial trend for the difference in moisture content was a sharp decrease. The maximum values were found after filtering 12,000 kg (44%) corresponding to the first filtration step (A), to reach 31% of the VOO belonging to the filtration step (C). Next,

the moisture reduction showed minimal decreases and even remained constant in the last filtration step (D, 30%). The trend of this parameter during the filtration process could be explained by the saturation of filter cake. It should be taken into account that the cake layer used in this study was formed by 90 kg of organic filter aids; this amount did not change over the entire filtration cycle. Consequently, during filtration, the water absorption which raised the volume of filter-aid particles, and the trapped solid particles present in unfiltered VOO, lowered the volume and number of microscopic channels through which clarified oil could flow easily. This tendency during filtration affected the final moisture content in filtered VOO. Therefore, the monitoring of filter-cake saturation during filtration could be a key for determining the optimal time to add new filter aids in order to prolong the filtration cycle, increase the amount of VOO filtered per cycle, and maintain the moisture reduction stable.

3.2. Qualitative characterization of phenolic and other polar compounds in VOOs

Table 2 provides an overview of all the compounds characterized in a representative unfiltered VOO sample by HPLC-ESI-TOF/MS. These compounds are summarized together with their retention time, molecular formula, experimental and calculated mass (m/z), error, and msigma. **Fig. 2** shows the base peak chromatogram (BPC) of the VOO phenolic extract. In the present work, a total of 23 phenolic compounds and another polar one were characterized following the procedure reported above in Materials and methods.

Table 2. Main phenolic and other polar compounds identified in a representative extract of unfiltered virgin olive oil obtained by HPLC-ESI-TOF/MS.

Peak number	Compounds[a]	RT (min)[b]	Molecular formula	m/z calcd[c]	m/z Exptl[d]	Error (ppm)	msigma
1	Quinic acid	2.31	$C_7H_{12}O_6$	191.0561	191.0569	4.3	6.1
2	H- HYTY	3.94	$C_8H_8O_3$	151.0401	151.0398	1.6	7.2
3	HYTY	8.12	$C_8H_{10}O_3$	153.0557	153.0559	1.4	5.6
4	TY	9.90	$C_8H_{10}O_2$	137.0608	137.0605	2.2	9.4
5	H-D-Ol Agl or isomer	11.81	$C_{17}H_{20}O_7$	335.1136	335.1106	3.0	3.3
6	p-coumaric acid	13.51	$C_9H_8O_3$	163.0401	163.0384	1.7	6.7
7	HYTY-Ac	14.13	$C_{10}H_{12}O_4$	195.0663	195.0654	4.8	5.1
8	EA	15.14	$C_{11}H_{14}O_6$	241.0718	241.0709	3.7	2.4
9	H-EA	15.81	$C_{11}H_{14}O_7$	257.0667	257.0648	4.5	1.9
10	DOA	16.30	$C_{17}H_{20}O_6$	319.1187	319.1177	3.3	0.9
11	H-D-Ol Agl or isomer	16.64	$C_{17}H_{20}O_7$	335.1136	335.1114	2.3	2.4
12	Syringaresinol	18.22	$C_{22}H_{26}O_8$	417.1555	417.1533	2.2	4.4
13	Pin	18.91	$C_{20}H_{22}O_6$	357.1344	357.1349	1.6	9.2
14	D-Lig Agl	19.33	$C_{17}H_{20}O_5$	303.1238	303.1211	2.7	5.3
15	AcPin	19.41	$C_{22}H_{24}O_8$	415.1398	415.1373	2.5	4.3
16	H-D-Lig Agl	19.91	$C_{17}H_{20}O_6$	319.1187	319.1174	4.0	8.9
17	Dehydro Ol Agl	21.63	$C_{19}H_{20}O_8$	375.1085	375.1038	4.7	7.1
18	10-H-Ol Agl	23.02	$C_{19}H_{22}O_9$	393.1191	393.1170	2.1	2.4
19	Ol Agl	23.22	$C_{19}H_{22}O_8$	377.1242	377.1224	4.8	0.7
20	Lut	23.61	$C_{15}H_{10}O_6$	285.0405	285.0387	1.7	1.2
21	Lig Agl	25.60	$C_{19}H_{22}O_7$	361.1293	361.1259	9.3	7.0
22	Apig	25.91	$C_{15}H_{10}O_5$	269.0455	269.0435	2.0	1.7
23	Methyl Ol Agl	26.44	$C_{20}H_{24}O_8$	391.1398	391.1367	3.2	8.1

[a] H-HYTY, Oxidized hydroxytyrosol; HYTY, hydroxytyrosol; TY, tyrosol; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; HYTY-Ac, hydroxytyrosol acetate; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; Pin, pinoresinol; D-Lig Agl, decarboxymethyl ligstroside aglycone; AcPin, acetoxypinoresinol; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone; 10-H-Ol Agl, 10-hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lut, luteolin; Lig Agl, ligstroside aglycone; Apig, apigenin; Methyl Ol Agl, methyl oleuropein aglycone; [b] RT, retention time; [c] m/z calcd: calculated mass; [d] m/z exptl: experimental mass.

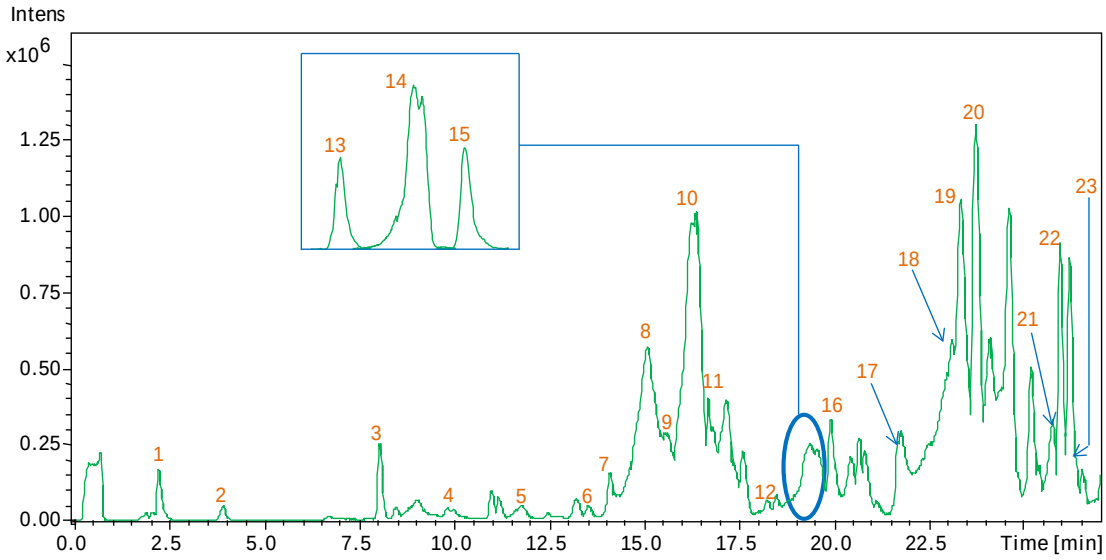

Fig. 2. Base-peak chromatogram (BPC) of representative unfiltered virgin olive oil phenolic extract obtained by HPLC-ESI-TOF/MS.

The phenolic compounds identified in the samples belong to different phenolic classes of phenolic alcohols, secoiridoids, lignans, flavones, and phenolic acids. The latter were represented only by *p*-coumaric acid eluted at a retention time of 13.51 min and yielded a deprotonated ion at m/z 163. Among the phenolic alcohols, oxidized form of hydroxytyrosol, hydroxytyrosol, and tyrosol were characterized, corresponding to the peaks (2), (3), and (4), respectively. The spectrum in the negative ionization mode also showed a deprotonated molecular ion at m/z 195 (peak 7), corresponding to hydroxytyrosol derivative (hydroxytyrosol acetate).

The most representative complex phenols identified in VOO were oleuropein aglycone, ligstroside aglycone, and their derivatives, which belong to secoiridoid group. Hydroxylated, decarboxymethylated, dehydrated, and methylated forms of oleuropein aglycone (peaks 5, 10, 11, 17, 18, 23) as well as a decarboxymethylated and hydroxylated forms of ligstroside aglycone (peaks 14 and 16) were found in VOO samples. Deprotonated molecular ions at *m/z* 241 and 257 were identified as elenolic acid and its hydroxylated form, respectively.

With regard to lignans, three compounds were detected in samples under study, namely syringaresinol (peak 12), pinoresinol (peak 13), and acetoxypinoresinol (peak 15), which yielded deprotonated molecules *m/z* 417, 357, and 415, respectively. The last phenolic group detected was composed of flavones. The most noteworthy compounds identified in this group were luteolin and apigenin, which had retention times of 23.61 and 25.91 min, respectively. With respect to the presence of other polar compounds, quinic acid (peak 1) was found in the VOO samples.

3.3. The time course of VOO phenolic content during filtration

It should be taken into account that the phenolic compounds in filtered and unfiltered VOOs were firstly quantified without taking into consideration the internal standard used during extraction.

Regarding the total phenol content, differences among unfiltered and filtered VOOs were found for all filtration steps. However, these differences were statistically significant only in samples belonging to filtration steps B and C. The trend in the total phenolic content was linked to the behavior of the different polyphenol families detected in samples. As shown in **Fig. 3**, phenolic alcohols significantly decreased their concentration in filtered VOO. Indeed, the highest loss in this family was found in VOO belong to the first filtration step (A, 19%). However, less decrease in its concentration was registered during the last filtration step (D, 10%). The concentration in flavones after filtration also diminished significantly during all filtration steps. Indeed, the greatest decrease was reached in filtered VOO from the second filtration step (B, 35%) and the least reduction during the third filtration step (C, 10%). These results confirm those of our previous study conducted under the same conditions [18]. While phenolic alcohols and flavones significantly decreased their concentration in filtered VOO, the variation in the secoiridoid content was not significant in any of the VOOs from filtration steps A, B, C, and D. Nevertheless, during the first filtration step (A), the secoiridoid content in filtered VOO tended to increase, but tended to decrease during the remaining steps. This trend could be explained by the balance between the

increase and the decrease of some compounds within the same family. Finally, no significant variations were found in lignan content after filtration in all the VOOs.

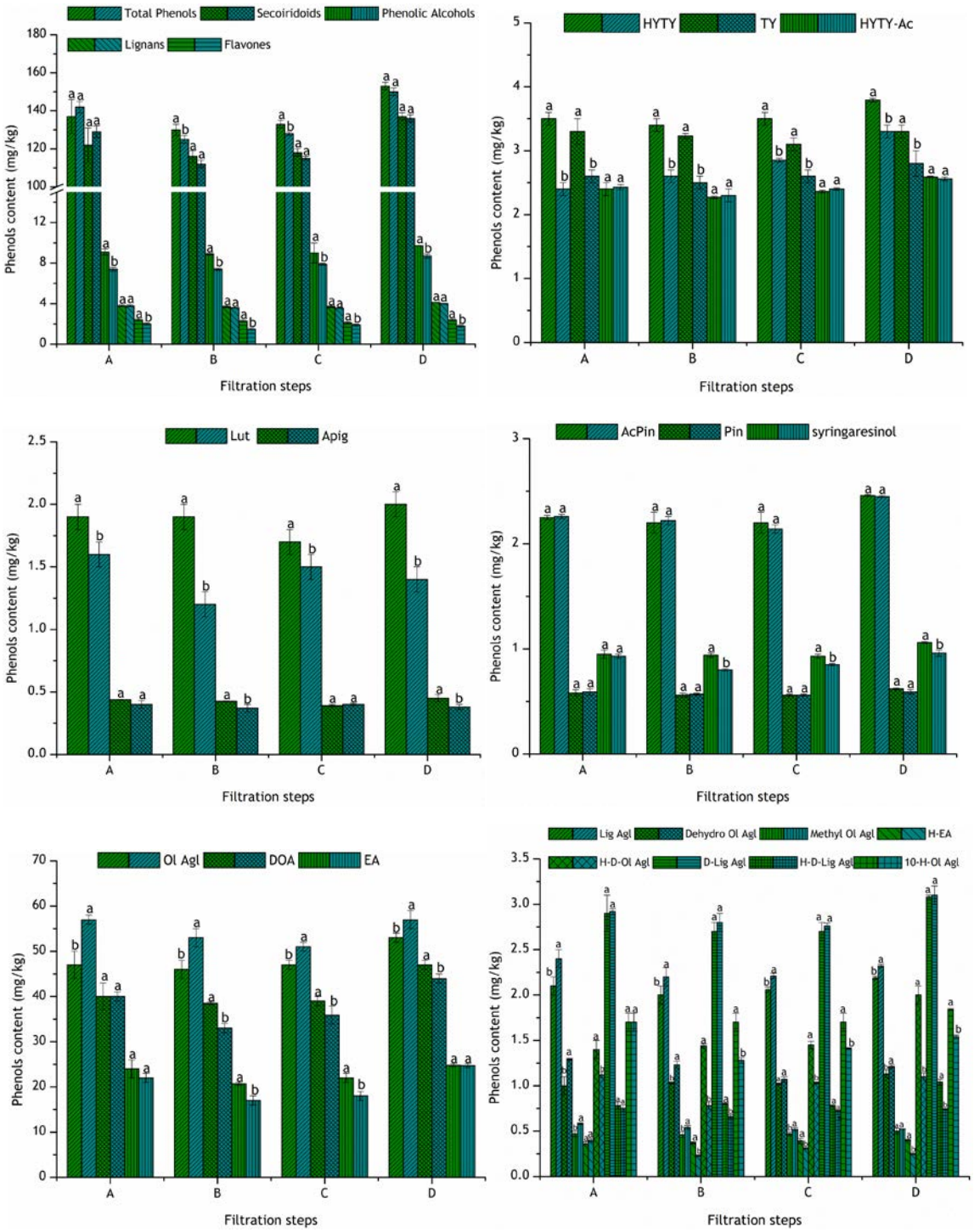

Fig. 3. Time course of virgin olive oil phenolic compounds during filtration process. HYTY, hydroxytyrosol; TY, tyrosol; HYTY-Ac, hydroxytyrosol acetate;

Lut, luteolin; Apig, apigenin; AcPin, acetoxypinoresinol; Pin, pinoresinol; Ol Agl, oleuropein aglycone; DOA, decarboxymethyl oleuropein aglycone; EA, elenolic acid; H-EA, hydroxy elenolic acid; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone; 10-H-Ol Agl, 10-hydroxy oleuropein aglycone; Lig Agl, ligstroside aglycone; Methyl Ol Agl, methyl oleuropein aglycone; green, unfiltered VOO; dark cyan, filtered VOO; values with the same letter are not significantly different at a 95% confidence level ($p \leq 0.05$).

The analysis of the individual concentrations indicated that hydroxytyrosol and tyrosol were the main phenolic compounds responsible for the decrease in the concentration of this group during filtration. The greatest loss was found in filtered VOOs belonging to filtration steps (A) and (B) for hydroxytyrosol and tyrosol, respectively. Hydroxytyrosol acetate, a derivative of hydoxytyrosol, showed no significant variation in its content during filtration. Concerning flavones, luteolin decreased significantly, and these results repeated in all the filtration steps under study. However, the variation on apigenin content was significant only during filtration steps (B) and (D). In addition, the reduction effect of filtration on luteolin content was higher than for apigenin. Acetoxypinoresinol and pinoresinol belonging to the lignan family showed no significant content variation in the filtered VOOs. However, syringaresinol decreased its concentration during filtration steps B, C, and D.

With regard to secoiridoids, 11 compounds were quantified in filtered and unfiltered VOOs. These compounds showed different trends during filtration. Dehydro-oleuropein aglycone, oleuropein aglycone, ligstroside aglycone, and methyl oleuropein aglycone, increased significantly in concentration after filtration in all filtration steps, with the exception of step C and D, where the variation was not significant for dehydro-oleuropein aglycone and methyl oleuropein aglycone, respectively. The apparent increase in these compounds found in a previous work [8] was attributed to the lack of extraction method used. In a water-in-oil emulsion, polyphenols are stabilized around water droplets, and the affinity of the phenolic compounds for solvent extraction is

lower than in a nonpolar matrix. However, the partial elimination of water during the filtration process permits a greater availability of polyphenols for extraction with a polar solvent mixture (methanol/water, 60/40) which results in the apparent increase in their concentration in filtered VOO. In the present experiment, the remaining compound belonging to this family as elenolic acid, decarboxymethyl oleuropein aglycone, and their hydroxylated forms, 10-hydroxy oleuropein aglycone and hydroxy decarboxymethyl ligstroside aglycone, maintained their concentration stable, with little variation in their content during some filtration steps. Nevertheless, their concentration decreased significantly during other filtration steps (**Fig. 3**). The results on the time course of this group of compounds during filtration proved unclear, due especially to the apparent increase in the concentration of some secoiridoids in the filtered VOO. Therefore, it is difficult to draw conclusions concerning the effect of this step on the VOO phenolic fraction, which is very important for VOO producers.

3.4. Correcting the effect of moisture reduction over the secoiridoids extraction from VOO

As a result of testing different internal standards in an effort to correct the effect of moisture content on extraction of phenolic compounds from the samples, luteolin 7-glucoside, taxifolin, and dihydrocaffeic acid were eliminated because their behavior was not similar to that of the compounds apparently increasing in filtered VOO, which made the correction impossible using these three standards. However, an apparent increase in oleuropein content was found in filtered VOO in comparison to the unfiltered one. Besides, the best results using oleuropein were found with a concentration of 10 mg L−1. In consideration of these results, oleuropein was chosen as internal standard for the correction of the secoiridoid concentration in filtered VOO. **Fig. 4** showed the time course of individual secoiridoid concentrations, the secoiridoid family, and total phenols during the industrial filtration process.

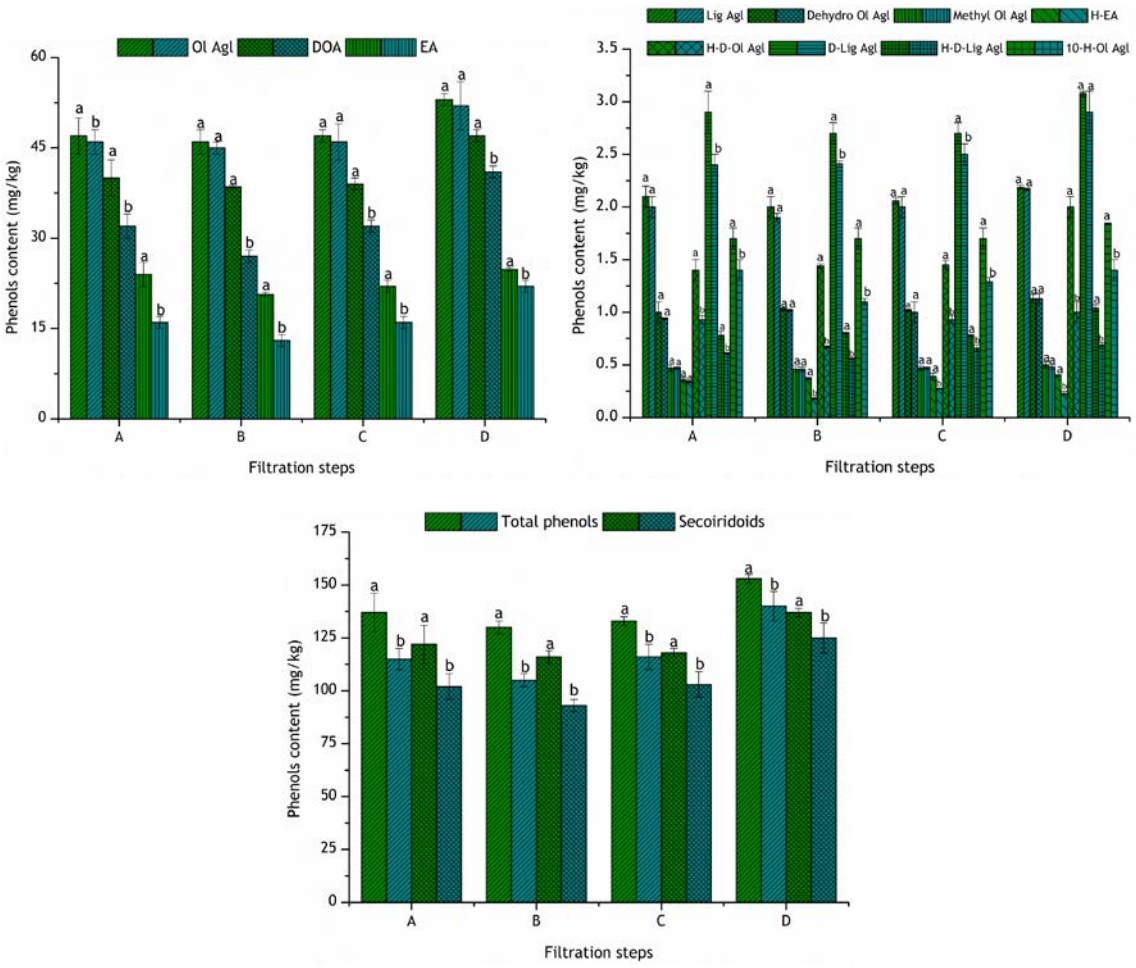

Fig. 4. Time course of secoiridoids and total phenols during filtration after correction using internal standard. Ol Agl, oleuropein aglycone; DOA, decarboxymethyl oleuropein aglycone; EA, elenolic acid; H-EA, hydroxy elenolic acid; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone; 10-H-Ol Agl, 10-hydroxy oleuropein aglycone; Lig Agl, ligstroside aglycone; Methyl Ol Agl, methyl oleuropein aglycone; green, unfiltered VOO; dark cyan, filtered VOO; values with the same letter are not significantly different at a 95% confidence level ($p \leq 0.05$).

The concentrations presented in this figure were those found after correction using oleuropein as internal standard during phenolic compound extraction. The use of the correction coefficient (Cc) allowed to observe that all the compounds belonging to this family tended to decrease after filtration. Indeed, oleuropein aglycone and ligstroside aglycone known as the main secoiridoids detected in VOO, showed no significant decrease on their content

in filtered VOO from filtration steps A, B, C, and D. The same results were found for methyl oleuropein aglycone and dehydro oleuropein aglycone. The effect of filtration was stronger on the rest of oleuropein and ligstroside aglycone derivatives, showing a significant decline in their content after filtration during all filtration steps carried out, with the exception to hydroxy elenolic acid and decarboxymethyl ligstroside aglycone, which showed no significant decrease in their content in filtered VOOs from the filtration steps A and D, respectively. As a sum of individual concentrations of the compounds belonging to this group, the secoiridoid family showed a significant decline after filtration. Indeed, the greatest decrease was found in VOO from filtration step (B, 20%), while the lowest decrease occurred during the last filtration step (D, 8%). Only by use of the correction coefficient (Cc) was it possible to discern the real behavior of the secoiridoid family during the VOO industrial filtration, which was masked until now by an apparent increase in those compounds that, in fact, was a result of an analytical artifact in the extraction step promoted by the moisture reduction in the filtered VOO.

Finally, in an effort to establish the effect of filtration on total phenols, the individual concentration of phenolic alcohols, lignans, flavones, and the corrected values of the secoiridoid concentration were summarized and presented as total phenols in **Fig. 4**. The results showed a significant decrease in total phenol content in filtered VOOs belonging to all filtration steps A, B, C, and D. These results confirm those found previously, using the same filter aids as used in this study but at a laboratory scale, where the retentive power of filter cake on some phenolic compounds caused a decrease in total phenols after filtration [11].

4. Conclusions

In this study, the effect of industrial filtration on the decrease in moisture content, phenolic alcohols, and flavones reported in previous works was confirmed at the industrial scale.

However, the most important achievement of this work is the proposal, for the first time, of a correction coefficient (Cc) that allowed the correction of the effect of moisture reduction on the apparent increase of secoiridoids such as dehydro-oleuropein aglycone, oleuropein aglycone, ligstroside aglycone, and methyl oleuropein aglycone in filtered VOO, using oleuropein as the internal standard during phenolic extraction. This is of great importance for future studies seeking to quantify the phenolic compounds in VOOs.

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Supporting information

Table 1. Time course of virgin olive oil phenolic compounds during filtration process.

	A[e]		B		C		D	
Compounds[c]	**UF[k]**	**FL[k]**	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
HYTY	$4^{a} \pm 1$	$2.4^{b}\pm0.1$	$3.4^{a}\pm0.1$	$2.6^{b}\pm0.1$	$3.5^{a}\pm0.1$	$2.85^{b}\pm0.03$	$3.79^{a}\pm0.03$	$3.3^{b}\pm0.1$
TY	$3.3^{a} \pm 0.2$	$2.6^{b}\pm0.1$	$3.23^{a}\pm0.04$	$2.5^{b}\pm0.1$	$3.1^{a}\pm0.1$	$2.6^{b}\pm0.1$	$3.3^{a}\pm0.1$	$2.8^{b}\pm0.2$
HYTY-Ac	$2.4^{a} \pm 0.1$	$2.43^{a}\pm0.04$	$2.27^{a}\pm0.02$	$2.3^{a}\pm0.1$	$2.36^{a}\pm0.02$	$2.40^{a}\pm0.02$	$2.59^{a}\pm0.01$	$2.56^{a}\pm0.03$
Phenolic Alcohols	**$9.1^{a} \pm 0.3$**	**$7.4^{b} \pm 0.2$**	**$8.9^{a}\pm0.1$**	**$7.4^{b}\pm0.1$**	**$9^{a}\pm1$**	**$7.9^{b}\pm0.1$**	**$9.72^{a}\pm0.02$**	**$8.7^{b}\pm0.2$**
EA	$24^{a}\pm2$	$22^{a}\pm1$	$20.7^{a}\pm0.3$	$17^{b}\pm1$	$22^{a}\pm1$	$18^{b}\pm1$	$24.8^{a}\pm0.3$	$24.7^{a}\pm0.4$
H-EA	$0.36^{a}\pm0.02$	$0.4^{a}\pm0.02$	$0.37^{a}\pm0.01$	$0.23^{b}\pm0.01$	$0.39^{a}\pm0.03$	$0.31^{b}\pm0.01$	$0.40^{a}\pm0.01$	$0.25^{b}\pm0.01$
DOA	$40^{a}\pm3$	$40^{a}\pm1$	$38.5^{a}\pm0.3$	$33^{b}\pm1$	$39^{a}\pm1$	$35.9^{b}\pm0.2$	$47^{a}\pm1$	$44^{b}\pm1$
H-D-OI AgI or Isomer	$1.4^{a}\pm0.1$	$1.12^{b}\pm0.03$	$1.44^{a}\pm0.02$	$0.78^{b}\pm0.04$	$1.45^{a}\pm0.04$	$1.03^{b}\pm0.01$	$2.0^{a}\pm0.1$	$1.10^{b}\pm0.04$
D-Lig AgI	$2.9^{a}\pm0.2$	$2.92^{a}\pm0.03$	$2.7^{a}\pm0.1$	$2.8^{a}\pm0.1$	$2.7^{a}\pm0.1$	$2.76^{a}\pm0.03$	$3.08^{a}\pm0.02$	$3.1^{a}\pm0.1$
H-D-Lig AgI	$0.78^{a}\pm0.04$	$0.75^{a} \pm0.04$	$0.80^{a}\pm0.01$	$0.66^{b}\pm0.02$	$0.78^{a}\pm0.01$	$0.72^{a}\pm0.01$	$1.04^{a}\pm0.03$	$0.74^{b}\pm0.01$
Dehydro OI AgI	$1.0^{b}\pm0.1$	$1.29^{a}\pm0.01$	$1.03^{b}\pm0.01$	$1.23^{a}\pm0.04$	$1.02^{a}\pm0.01$	$1.07^{a}\pm0.03$	$1.13^{b}\pm0.04$	$1.21^{a}\pm0.02$
10-H-OI AgI	$1.7^{a}\pm0.1$	$1.7^{a}\pm0.1$	$1.7^{a}\pm0.1$	$1.28^{b}\pm0.03$	$1.7^{a}\pm0.1$	$1.41^{b}\pm0.01$	$1.84^{a}\pm0.01$	$1.54^{b}\pm0.02$
OI AgI	$47^{b}\pm 3$	$57^{a}\pm1$	$46^{b}\pm2$	$53^{a}\pm2$	$47^{b}\pm1$	$51^{a}\pm1$	$53^{a}\pm1$	$57^{b}\pm2$
Lig AgI	$2.1^{b}\pm0.1$	$2.4^{a}\pm0.1$	$2^{b}\pm0.1$	$2.2^{a}\pm0.1$	$2.06^{b}\pm0.03$	$2.21^{a}\pm0.03$	$2.19^{b}\pm0.02$	$2.32^{a}\pm0.02$
Methyl OI AgI	$0.47^{b}\pm0.03$	$0.58^{a}\pm0.01$	$0.46^{b}\pm0.03$	$0.54^{a}\pm0.02$	$0.47^{b}\pm0.02$	$0.52^{a}\pm0.02$	$0.50^{a}\pm0.03$	$0.522^{a}\pm0.002$
Secoiridoids	**$122^{a}\pm9$**	**$129^{a}\pm3$**	**$116^{a}\pm3$**	**$112^{a}\pm2$**	**$118^{a}\pm2$**	**$115^{a}\pm1$**	**$137^{a}\pm2$**	**$136^{a}\pm2$**

	A[e]		B		C		D	
Compounds[c]	**UF[k]**	**FL[k]**	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
syringaresinol	0.95[a] ± 0.04	0.93[a]± 0.02	0.94[a]±0.02	0.80[b]±0.01	0.93[a]±0.02	0.85[b]±0.01	1.06[a]±0,01	0.96[b]±0.03
Pin	0.58[a]±0.03	0.59[a]±0.03	0.56[a]±0.02	0.57[a]±0.01	0.56[a]±0.01	0.56[a]±0.01	0.62[a]±0.01	0.59[a]±0.02
AcPin	2.25[a]±0.02	2.26[a]±0.02	2.2[a]±0.1	2.22[a]±0.04	2.2[a]±0.1	2.14[a]±0.04	2.46[a]±0.01	2.45[a]±0.01
Lignans	**3.8[a] ± 0.1**	**3.8[a]±0.1**	**3.7[a]±0.1**	**3.58[a]±0.04**	**3.7[a]±0.1**	**3.55[a]±0.03**	**4.138[a]±0.003**	**4.01[a]±0.03**
Lut	1.9[a]±0.1	1.6[b]±0.1	1.9[a]±0.1	1.2[b]±0.1	1.7[a]±0.1	1.5[b]±0.1	2.0[a]±0.1	1.4[b]±0.1
Apig	0.438[a]±0.003	0.40[a]±0.03	0.425[a]±0.003	0.37[b]±0.03	0.39[a]±0.01	0.40[a]±0.01	0.45[a]±0.03	0.38[b]±0.02
Flavones	**2.4[a] ± 0.1**	**2.0[b]±0.1**	**2.3[a]±0.1**	**1.5[b]±0.1**	**2.1[a]±0.1**	**1.9[b]±0.1**	**2.4[a]±0.1**	**1.8[b]±0.1**
Quinic Acid	0.78 ± 0.04	NQ	0.73±0.01	NQ	0.74±0.02	NQ	0.81±0.01	NQ
Total phenols	**137[a]±9**	**142[a]±3**	**130[a]±3**	**125[b]±2**	**133[a]±2**	**128[b]±1**	**153[a]±2**	**150[a]±2**

[c] HYTY, hydroxytyrosol; TY, tyrosol; HYTY-Ac, hydroxytyrosol acetate; EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-Ol Agl, hydroxy decarboxymethyl oleuropein aglycone or isomer; D-Lig Agl, decarboxymethyl ligstroside aglycone; H-D-Lig Agl, hydroxy decarboxymethyl ligstroside aglycone; Dehydro Ol Agl, dehydro-oleuropein aglycone; 10-H-Ol Agl, 10-hydroxy oleuropein aglycone; Ol Agl, oleuropein aglycone; Lig Agl, ligstroside aglycone; Methyl Ol Agl, methyl oleuropein aglycone; Pin, pinoresinol; AcPin, acetoxypinoresinol; Lut, luteolin; Apig, apigenin.

[e] Filtration steps (A, B, C, D).

[k] UF, Unfiltered; FL, Filtered.

Values with the same letter in a line are not significantly different at a 95 % confidence level ($p \leq 0.05$).

Table 2. Time course of secoiridoids and total phenols during filtration process after correction using internal standard.

	A[e]		B		C		D	
Compounds[c]	**UF [k]**	**FL[k]**	**UF**	**FL**	**UF**	**FL**	**UF**	**FL**
EA	24[a]±2	16[b]±1	20.7[a]±0.3	13[b]±1	22[a]±1	16[b]±1	24.8[a]±0.3	22[b]±1
H-EA	0.36[a]±0.02	0.34[a]±0.01	0.37[a]±0.01	0.18[b]±0.01	0.39[a]±0.03	0.274[b]±0.004	0.40[a]±0.01	0.23[b]±0.02
DOA	40[a]±3	32[b]±2	38.5[a]±0.3	27[b]±1	39[a]±1	32[b]±1	47[a]±1	41[b]±1
H-D-OI AgI or isomer	1.4[a]±0.1	0.93[b]±0.04	1.44[a]±0.02	0.67[b]±0.01	1.45[a]±0.04	0.93[b]±0.04	2.0[a]±0.1	1.0[b]±0.1
D-Lig AgI	2.9[a]±0.2	2.4[b]±0.1	2.7[a]±0.1	2.41[b]±0.03	2.7[a]±0.1	2.5[b]±0.1	3.08[a]±0.02	2.9[a]±0.2
H-D-Lig AgI	0.78[a]±0.04	0.61[b]±0.01	0.80[a]±0.01	0.56[b]±0.01	0.78[a]±0.01	0.66[b]±0.03	1.04[a]±0.03	0.69[b]±0.02
Dehydro OI AgI	1.0[a]±0.1	0.94[a]±0.01	1.03[a]±0.01	1.02[a]±0.01	1.02[a]±0.01	1.0[a]±0.1	1.13[a]±0.04	1.13[a]±0.05
10-H-OI AgI	1.7[a]±0.1	1.4[b]±0.1	1.7[a]±0.1	1.10[b]±0.03	1.7[a]±0.1	1.29[b]±0.04	1.84[a]±0.01	1.4[b]±0.1
OI AgI	47[a]± 3	46[b]±2	46[a]±2	45[a]±1	47[a]±1	46[a]±3	53[a]±1	52[a]±4
Lig AgI	2.1[a]±0.1	2.0[a]±0.1	2[a]±0.1	1.90[a]±0.04	2.06[a]±0.03	2.0[a]±0.1	2.19[a]±0.02	2.17[a]±0.01
Methyl OI AgI	0.47[a]±0.03	0.47[a]±0.01	0.46[a]±0.03	0.46[a]±0.02	0.47[a]±0.02	0.47[a]±0.01	0.50[a]±0.03	0.48[a]±0.02
Secoiridoids	**122[a]±9**	**102[b]±6**	**116[a]±3**	**93[b]±3**	**118[a]±2**	**103[b]±6**	**137[a]±2**	**125[b]±7**
Total phenols	**137[a]±9**	**115[b]±5**	**130[a]±3**	**105[b]±3**	**133[a]±2**	**116[b]±6**	**153[a]±2**	**140[b]±7**

[c] EA, elenolic acid; H-EA, hydroxy elenolic acid; DOA, decarboxymethyl oleuropein aglycone; H-D-OI AgI, hydroxy decarboxymethyl oleuropein aglycone or isomer; D-Lig AgI, decarboxymethyl ligstroside aglycone; H-D-Lig AgI, hydroxy decarboxymethyl ligstroside aglycone; Dehydro OI AgI, dehydro-oleuropein aglycone; 10-H-OI AgI, 10-hydroxy oleuropein aglycone; OI AgI, oleuropein aglycone; Lig AgI, ligstroside aglycone; Methyl OI AgI, methyl oleuropein aglycone; [e] Filtration steps (A, B, C, D); [k] UF, Unfiltered; FL, Filtered; Values with the same letter in a line are not significantly different at a 95 % confidence level ($p \leq 0.05$).

GENERAL CONCLUSIONS

SECTION I

1. In the first chapter the relationship between the geographical area of cultivation and virgin olive oil (VOO) phenolic composition was evaluated. In fact, the study of the phenolic profile of Arbequina olive oil using HPLC-ESI-TOF/MS showed no qualitative differences in the phenolic fractions among VOOs from different geographical areas of Southern Catalonia. However, quantitative differences were observed in a large number of phenolic compounds. In all Arbequina VOOs, secoiridoids showed the highest concentration followed by lignans, phenolic alcohols and flavones, respectively. The quantified phenolic compounds were able to classify Arbequina VOO samples according to their geographical origin through the discriminant analysis model.

2. This chapter includes the changes that occur in VOO composition under the effect of some agronomic practices. Irrigation was found to affect Chemlal oil yield and its chemical composition, mainly its phenolic content. In fact, although the range of concentration of total phenols was not very different between the two irrigation treatments, most phenolic families showed higher content in VOOs from irrigated trees. However, the most important achievement of this study was the differences observed in the time course of VOO components, mainly phenolic compounds, within each treatment (non-irrigated and irrigated) among the harvest dates. From the obtained results under the experimental conditions of the study, the optimum harvest period for the Chemlal variety differs depending on the water status of trees during the dry period.

SECTION II

3. In the third chapter, the scientific data previously reported in the literature were reviewed to establish the best conditions for obtaining VOO with the highest possible phenolic content. Taking into consideration these data, harvesting too early, crushing olives using a hammer crusher equipped with small grid holes and at high rotation speed, malaxation of paste at

temperatures lower than 30 °C and for times shorter than 60 min, centrifugation of paste using a two-phase decanter followed by vertical centrifugation with a minimum of water added, storage of VOO for short times and at low temperatures, and filtration using inert gases, all contributed to obtaining VOO enriched in phenolic compounds. In addition, qualitative and quantitative characterization studies of phenolic compounds in VOO by-products were summarized in the review. Phenolic alcohols, secoiridoids, lignans and flavonoids were the main characterized phenolic families. Interesting results were reported about the antioxidant and biological activity of phenolic extracts obtained from olive oil by-products. Finally, different pilot plant and industrial processes employed to recover phenolic compounds from olive oil by-products were widely reviewed.

4. This chapter includes an evaluation of the olive ripening effect on the final composition of VOO. To do this, the Algerian Azeradj variety was selected to perform the study. Indeed, an increase was observed in quality parameters as the maturity index increased. However, all the analysed samples were within the limits established for the extra-virgin olive oil (EVOO) category. In addition, the decrease in some components, mainly polyphenols, was accompanied by a decrease in the oxidative stability of Azeradj VOOs at advanced ripening stages corresponding to the last harvest date. The current data can be considered useful for determining the ideal harvest period for olives used to produce oil. Based on the analytical results of this work, early harvesting produces Azeradj VOO with excellent chemical characteristics.

5. In the fifth chapter, monitoring of the VOO moisture and phenolic content during the industrial filtration process was carried out. Filtration could make VOO more brilliant for marketing on the one hand, and increase its shelf life on the other, by reducing moisture content. Nevertheless, filtration produces a decrease in the VOO antioxidant content, such as phenolic alcohols and flavones, which can negatively affect its oxidative stability. Consequently, during filtration, an equilibrium between losing moisture and antioxidant content is needed to achieve high-quality VOO. In this study, the apparent

increase of secoiridoids in filtered VOO made it difficult to understand the real effect of filtration on this group of compounds.

SECTION III

6. This chapter includes a review of the different isolation, separation and detection methods used for the characterization of phenolic compounds. The proposed methods resolved many problems related to the estimation of this fraction in VOO. Having done so, the nutritional value and healthy properties of VOO were easily proven. However, some aspects of the cited methods need to be improved. For instance, the VOO water content was never taken into consideration during phenolic extraction, which can affect the recovery efficiency of these analytes. In addition, artificial formation of some isomers during chromatographic separation due to the mobile phase used, especially the aqueous one, made the previously reported measurement of phenolic compounds in VOO somewhat questionable. Therefore, the development of one single and accurate method for VOO phenolic analysis taking into consideration the current problems is necessary. In doing so, the confusion when different published data are compared will also be avoided.

7. In the last chapter, a new extraction approach was developed to establish the real effect of the filtration step on VOO phenolic composition. In fact, the decrease in phenolic alcohols and flavones after filtration was confirmed. However, the most important achievement was the proposal, for the first time, of a correction coefficient (Cc) using oleuropein as internal standard during phenolic extraction. This coefficient allowed the correction of the effect of moisture reduction on the apparent increase of secoiridoids such as dehydro-oleuropein aglycone, oleuropein aglycone, ligstroside aglycone and methyl oleuropein aglycone in filtered VOO. This approach made it easy to understand the correct behaviour of these analytes during VOO industrial filtration. In addition, the proposed extraction method is of great importance for future studies seeking to quantify the phenolic compounds in VOOs.

CONCLUSIONES GENERALES

SECCIÓN I

1. En el primer capítulo se estableció la relación existente entre el área geográfica de cultivo y la composición fenólica del aceite de oliva virgen (AOV). Los resultados derivados de este trabajo de investigación pusieron de manifiesto que no se detectaron diferencias cualitativas en la composición fenólica entre los AOVs de la variedad Arbequina procedentes de diferentes áreas geográficas del sur de Cataluña. Sin embargo, las muestras analizadas mostraron diferencias cuantitativas en un amplio número de compuestos. En efecto a pesar de que en todos los AOVs los seoiridoides fueron los polifenoles mayoritarios seguidos por los lignanos, alcoholes fenólicos y las flavonas, las diferencias observadas en los compuestos fenólicos individuales permitieron clasificar las muestras de AOV de acuerdo a su origen geográfico a través del uso de un modelo de análisis discriminante.

2. El segundo capítulo incluyó una evaluación de los cambios que se producen en la composición del AOV obtenidos de frutos sometidos a distinto estrés hídrico. Las diferentes condiciones de irrigación aplicadas a los cultivos afectaron al perfil fenólico del aceite de la variedad Chemlal. A pesar de que el rango de concentración de los fenoles totales no fue muy diferente entre los dos tratamientos de irrigación (secano y regadío), para la mayoría de las familias fenólicas identificadas en las muestras la concentración fue superior en los AOV obtenidos de frutos cuyos olivos fueron regados. Los resultados derivados de este estudio permitieron establecer el efecto simultáneo sobre la composición fenólica de este parámetro y el grado de maduración del fruto. Teniendo en cuenta los resultados obtenidos bajo las condiciones experimentales de este estudio, el periodo de recolección óptimo de la variedad Chemlal varía dependiendo de la disponibilidad del agua para los olivos.

SECCIÓN II

3. En el tercer capítulo, la revisión del estado del arte sobre el proceso de producción del aceite de oliva permitió establecer las mejores condiciones de producción para obtener AOV con alto contenido fenólico. Teniendo en cuenta estos datos, la recolección temprana, la molienda de las aceitunas usando molinos de martillo equipados con una criba que presentan orificios con un diámetro interno pequeño y trabajan a alta velocidad de rotación, el batido de la pasta a una temperatura inferior a 30º C y un tiempo inferior a 60 min, centrifugación de la pasta usando un decanter de dos fases seguido por una centrifugación vertical con una mínima adición de agua, almacenamiento del AOV durante periodos cortos de tiempo y a baja temperatura, y la filtración con gas inerte, contribuyen a la obtención AOV enriquecido en compuestos fenólicos. Además, y dada las pérdidas de polifenoles generadas durante el proceso de elaboración, en este trabajo se incluyó un estudio de la composición fenólica en los subproductos del AOV, sus propiedades antioxidantes así como tecnologías disponibles para recuperar estos compuestos para su uso potencial como compuestos bioactivos.

4. En el cuarto capítulo se determinó el efecto del grado de maduración de las aceitunas en la composición final de AOV. Los resultados derivados del análisis del aceite obtenido de la variedad Azeradj mostraron un incremento en los valores de los parámetros de calidad a medida que el grado de maduración del fruto incrementó quedando dentro de los límites establecidos para la categoría de aceite de oliva virgen extra (AOVE). De forma paralela, los aceites obtenidos con frutos con mayor grado de maduración mostraron una reducción en la estabilidad oxidativa derivada de la menor concentración en componentes con propiedades antioxidantes como los polifenoles. Estos datos pueden considerarse útiles para determinar el período óptimo de recolección para las aceitunas utilizadas en la producción de aceite. Teniendo en cuenta los resultados analíticos de este trabajo, la recolección temprana produce AOV de la variedad Azeradj con excelentes características químicas.

5. En el quinto capítulo se llevó a cabo la monitorización del contenido en humedad y polifenoles de AOV durante el proceso industrial de filtración. Los resultados derivados de este estudio verificaron que esta etapa del proceso reduce el contenido en agua dotando al aceite de un aspecto comercial más apto para los consumidores. Sin embargo, la monitorización de los polifenoles dio como resultado una reducción en el contenido de los antioxidantes de AOV, como los alcoholes fenólicos y las flavonas, que puede afectar negativamente a su estabilidad oxidativa. Además, el efecto de este proceso sobre el contenido en algunos compuestos fenólicos no pudo ser determinado con la metodología analítica propuesta hasta el momento.

SECCIÓN III

6. Este capítulo incluyó una revisión de los diferentes métodos de extracción, técnicas separativas y sistemas de detección empleados en la caracterización de los compuestos fenólicos. Las ventajas e inconvenientes que cada autor ha establecido para las distintas metodologías analíticas fueron resumidas para intentar establecer las mejores condiciones que permitan una caracterización de la fracción fenólica con diversas aplicaciones. Problemas analíticos que a día de hoy aún no se han solucionado fueron puestos de manifiesto, tal es el caso del efecto matriz, producido por el diferente contenido en agua del AOV Además teniendo en cuenta los análisis de esta fracción llevados a cabo por diferentes autores es importante resaltar la posible formación de compuestos durante la separación cromatográfica.

7. En el capítulo siete se desarrolló un nuevo procedimiento de extracción para intentar clarificar el efecto de la filtración sobre la composición fenólica del AOV. Esta nueva metodología consistió en utilizar un coeficiente de corrección (Cc) utilizando oleuropeína como patrón interno durante la extracción fenólica para eliminar el efecto del diferente contenido en humedad entre muestras sin filtrar y filtradas. Este coeficiente permitió la corrección del efecto de la reducción en humedad sobre el incremento aparente de los secoiridoides tales como dehidro-oleuropeína aglicona, oleuropeína aglicona, ligustrósido aglicona, metil oleuropeína aglicona.