PhD Thesis

COMPOSICIÓN FENÓLICA DEL ACEITE DE OLIVA: INFLUENCIA VARIETAL, EFECTOS DE FACTORES TECNOLÓGICOS Y ESTABILIDAD OXIDATIVA

OLIVE OIL PHENOLIC COMPOSITION: CULTIVAR VARIABILITY, EFFECTS OF TECHNOLOGICAL FACTORS AND OXIDATIVE STABILITY

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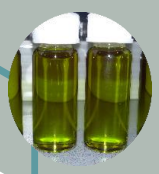
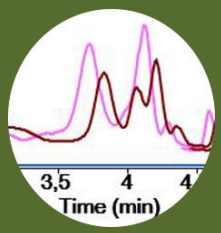
Cordoba, Spain, 2021





TITULO: *COMPOSICIÓN FENÓLICA DEL ACEITE DE OLIVA: INFLUENCIA VARIETAL, EFECTOS DE FACTORES TECNOLÓGICOS Y ESTABILIDAD OXIDATIVA*

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UNIVERSIDAD DE CÓRDOBA

DEPARTAMENTOS DE AGRONOMÍA Y QUÍMICA ANALÍTICA

TESIS DOCTORAL

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OLIVE OIL PHENOLIC COMPOSITION: CULTIVAR VARIABILITY, EFFECTS OF TECHNOLOGICAL FACTORS AND OXIDATIVE STABILITY

Los directores,

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Universidad de Córdoba

El doctorando,

Fdo. **Hristofor MIHO**

Trabajo presentado para optar al grado de Doctor con mención internacional en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible

Córdoba, España, 2021

Tesis como compendio de publicaciones

La presente Tesis Doctoral, de acuerdo con el informe correspondiente autorizado por los Directores de Tesis y el Órgano Responsable del Programa de Doctorado, se presenta como un compendio de cuatros artículos (en tres de ellos el doctorando participó como primer autor) publicados en revistas incluidas en el primer decil clasificadas por ámbito de la especialidad, y referenciadas en la última relación publicada por el Journal Citation Reports (SCI y/o SSCI, 2019). Las referencias completas de los artículos que componen esta Tesis Doctoral y en las que el doctorando es el primer autor son las siguientes:

Miho, H., Díez, C. M., Mena-Bravo, A., Sánchez de Medina, V., Moral, J., Melliou, E., … Priego-Capote, F. (2018). Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. *Food Chemistry*, *266*, 192–199. https://doi.org/10.1016/j.foodchem.2018.06.002

Miho, H., Moral, J., Barranco, D., Ledesma-Escobar, C. A., Priego-Capote, F., & Díez, C. M. (2020). Influence of genetic and interannual factors on the phenolic profiles of virgin olive oils. *Food Chemistry*, 342, 128357. https://doi.org/10.1016/j.foodchem.2020.128357

Miho, H., Moral, J., López-González, M. A., Díez, C. M., & Priego-Capote, F. (2020). The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability. *Food Chemistry*, *314*. https://doi.org/10.1016/j.foodchem.2020.126183

Además, se consideró oportuno incluir en la Tesis un artículo en el que el doctorando participó como coautor y que constituyó una revisión del estado del arte de parte de su investigación. Este artículo ha contribuido de manera notable a su formación:

Rallo, L., Díez, C. M., Morales-Sillero, A., **Miho, H**., Priego-Capote, F., & Rallo, P. (2018). Quality of olives: A focus on agricultural preharvest factors. *Scientia Horticulturae*, *233*, 491–509. https://doi.org/10.1016/j.scienta.2017.12.034

Concepción Muñoz Díez, Profesora Contratada Doctora del Departamento de Agronomía, Escuela Técnica Superior de Ingeniería Agronómica y de Montes, Universidad de Córdoba, y **Feliciano Priego Capote**, Profesor Titular del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, en calidad de Directores de la Tesis Doctoral presentada por el doctorando Hristofor Miho**,** con el título "**Composición fenólica del aceite de oliva: influencia varietal, efectos de los factores tecnológicos y estabilidad oxidativa**",

CERTIFICAN:

Que la citada Tesis Doctoral se ha realizado en las instalaciones de los Departamentos de Agronomía y de Química Analítica de la Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, marzo de 2021.

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Fdo. Feliciano Priego Capote

Tesis con mención internacional

Mediante la presentación de esta Memoria se pretende optar a la mención de **Doctorado Internacional**, habida cuenta de que el doctorando reúne los requisitos exigidos para tal mención, a saber:

1. Estancia de tres meses en un centro de investigación de otro país realizando trabajos de investigación relacionados con la Tesis Doctoral:

    - Universidad de Maribor, Eslovenia, Facultad de Química e Ingeniería Química, bajo la supervisión del Prof. Dr. Željko Knez. Fecha de la estancia: de 03 de julio de 2018 a 03 de octubre de 2018.

2. Informes favorables de dos Doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:

    - Prof. Dr. Enzo Perri, Responsable de Consejo de Investigación Agrícola y Análisis de la Economía Agrícola (CREA), Rende, Italia.

    - Prof. Dr. Georgios Kalantzakis, Institute of Olive Tree, Subtropical Plants and Viticulture, Chania, Greece.

3. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de Enseñanza Superior de otro país:

    - Prof. Dr. Georgios Koubouris, Head of the Laboratory for Olive Cultivation, Institute for Olive Tree, Subtropical Crops and Viticulture, Chania, Greece.

4. La exposición y la defensa de parte de esta Tesis se realizarán en una lengua diferente a la materna: inglés.

TÍTULO DE LA TESIS:

Composición fenólica del aceite de oliva: influencia varietal, efectos de factores tecnológicos y estabilidad oxidativa.

Olive oil phenolic composition: cultivar variability, effects of technological factors and oxidative stability.

DOCTORANDO: Hristofor Miho

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se menciona la evolución y desarrollo de la tesis, así como los trabajos y publicaciones derivados de la misma)

El doctorando Hristofor Miho ha mostrado durante el periodo de realización de esta Tesis un gran interés en explorar la influencia varietal sobre la variabilidad fenólica del aceite de oliva. Este trabajo de investigación ha contribuido a caracterizar la gran diversidad fenólica de los aceites de oliva y a clasificarlos en función de esta composición. Asimismo, este trabajo ha evaluado por primera vez la estabilidad del perfil fenólico del aceite de oliva durante tres cosechas consecutivas, poniendo de manifiesto la mayor estabilidad de unos compuestos fenólicos respecto a otros. Por otra parte, en este trabajo se han explorado las posibilidades de ciertos procesos tecnológicos para obtener aceites de oliva adecuados a las necesidades del consumidor.

Fruto de este trabajo han sido los artículos que componen la Memoria de la Tesis y que han despertado el interés de varias empresas agroindustriales que, junto con la Universidad de Córdoba, han diseñado e implementado líneas de mejora genética para

obtener nuevas variedades de olivo que se diferencien por el contenido fenólico de sus aceites.

Uno de los principales avances conseguidos con el desarrollo de la investigación recogida en esta Memoria es la caracterización de la composición fenólica (9 fenoles individuales) del aceite de oliva virgen de 80 variedades de olivo obtenido bajo las mismas condiciones agroambientales y tecnológicas. Esta caracterización permitió clasificar las variedades de acuerdo con su riqueza en fenoles y agruparlas en tres categorías según sus perfiles fenólicos. Posteriormente, gracias a un ensayo trianual, por primera vez fuimos capaces de determinar el peso del factor genético respecto al ambiental en la variabilidad fenólica de los aceites. Este último ensayo permitió consolidar los resultados preliminares que obtuvimos en el primer año del estudio.

Adicionalmente, en un tercer estudio se demostraron los efectos que la aplicación de condiciones del vacío durante el batido y la duración de esta etapa pueden tener sobre la composición fenólica del aceite de oliva. Los resultados revelaron que la implementación del vacío en el proceso de batido contribuye significativamente en la preservación de los compuestos fenólicos, mientras que, un tiempo de batido superior a 30 minutos acelera la degradación de dichos compuestos. Además, los resultados de este ensayo demostraron que los compuestos fenólicos y, particularmente, perfiles y/o fenoles específicos, junto con la composición de ácidos grasos, juegan un papel decisivo en la vida útil de los aceites. Gracias a estos resultados, fuimos capaces de diseñar un modelo matemático que correlaciona el perfil fenólico y el de ácidos grasos de los aceites con la estabilidad oxidativa medida con el método analítico ´Rancimat´, y que nos permite definir la vida útil de los aceites a partir de datos químicos.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 25 de marzo de 2021

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INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES DE LA TESIS

TÍTULO DE LA TESIS: COMPOSICIÓN FENÓLICA DEL ACEITE DE OLIVA: INFLUENCIA VARIETAL, EFECTOS DE LOS FACTORES TECNOLÓGICOS Y ESTABILIDAD OXIDATIVA.

Publicaciones	Factor de Impacto	Decil/Cuartil
Quality of olives: A focus on agricultural preharvest factors. **Scientia Horticulturae** 2018, 233; 491-509.	2.769	D2/Q1 10/84 Horticulture
Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. **Food Chemistry** 2018, 266; 192-199.	6.306	D1/Q1 6/203 Food Science & Technology
The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability. **Food Chemistry** 2020, 314; 126183.	6.306	D1/Q1 6/203 Food Science & Technology
Influence of genetic and interannual factors on the phenolic profiles of virgin olive oils. **Food Chemistry** 2020, 342; 128357.	6.306	D1/Q1 6/203 Food Science & Technology

Otras aportaciones científicas derivadas directamente de la Tesis Doctoral

a) Artículos científicos incluidos como anexos en la Tesis:

Sánchez de Medina, V., **Miho, H.**, Melliou, E., Magiatis, P., Priego-Capote, F., & Luque de Castro, M. D. (2017). Quantitative method for determination of oleocanthal and oleacein in virgin olive oils by liquid chromatography–tandem mass spectrometry. Talanta, 162, 24–31. https://doi.org/10.1016/j.talanta.2016.09.056

b) Comunicaciones a congresos y conferencias:

H. Miho (2016). La variabilidad de los compuestos fenólicos en aceites de oliva vírgenes extra. V Congreso Científico de Investigadores en Formación. Córdoba, España. Presentación oral. https://doi.org/10.5281/zenodo.4415949**.**

H. Miho (2017). La variabilidad fenólica de los aceites y su influencia en la estabilidad oxidativa. VIII Congreso Ibérico de Ciencias Hortícolas. Coimbra, Portugal. Presentación oral. https://doi.org/10.5281/zenodo.4415983.

H. Miho (2018). Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. OLIVEBIOTEQ international conference. Sevilla, España. Comunicación en formato póster. https://doi.org/10.5281/zenodo.4049812.

c) Editoriales en revistas de divulgación:

Luis Rallo y Hristofor Miho (2020). Las variedades de olivo y la diversidad de los AOVEs (p. 34). Grupo Editorial Mercacei: Especial Día Mundial del Olivo 2020. AOVE, el Superalimento del Siglo XXI. http://www.doopaper.com/visor_html5/edimarket/especialdiaolivo#page/34

d) Estancia internacional:

Durante tres meses (03.07.2018 – 03.10.2018) el doctorando realizó una estancia internacional en la Universidad de Maribor, Eslovenia, en la Facultad de Ingeniería Química. El trabajo de investigación que se realizó durante la estancia estuvo centrado en

la determinación de la actividad enzimática en el fruto del olivo durante su maduración para entender mejor las rutas metabólicas de los compuestos fenólicos.

Resultados obtenidos: Se determinó la actividad enzimática de la β-glucosidasa y la esterasa en fruto de 8 cultivares recolectados en 3 diferentes índices de madurez. Los resultaron obtenidos demostraron que la β -glucosidasa tiene una mayor actividad en las primeras fases de la formación del fruto (índice de madurez 1), mientras que con la maduración la actividad de esta enzima se reduce significativamente. Los valores de la actividad enzimática de β-glucosidasa oscilaron entre 16 (fruto verde Empeltre) y 4 (fruto negro Picudo) unidades/ml.

Financiación

Esta Tesis Doctoral ha sido realizada gracias a la ayuda (BECA Nº T6/CO-DOCT 3/16) otorgada al doctorando por el Consejo Oleícola Internacional (COI) durante un periodo de 4 años (2016-2019).

Los costes de los ensayos experimentales y analíticos han sido financiados por los Grupos de Investigación PAIDI AGR-157 y FQM-227 de la Universidad de Córdoba.

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AGRADECIMIENTOS

Ahora que llega el momento final para la presentación de la Tesis Doctoral, tras una carrera de 5 años de investigación profunda me siento muy satisfecho por las metas que he podido alcanzar y por todo lo que he aprendido durante esta época maravillosa de mi vida. Al mismo tiempo, soy consciente que todos estos alcances no hubieran sido posibles sin el apoyo y sin la positividad de muchas personas que me han ayudado y han estado cerca de mí en cada paso.

Por lo tanto, con su permiso, agradezco en primer lugar al Consejo Oleícola Internacional (COI), por darme esta increíble oportunidad, apoyándome y financiando la realización de esta Tesis Doctoral.

A los directores de este trabajo, Dr. Feliciano Priego Capote y Dra. Concepción Muñoz Díez, que, con su profesionalidad, actitud positiva, y su apoyo hicieron mucho más fácil realizar este trabajo. ¡Muchas gracias!

También quiero expresar todo mi respeto y agradecimiento para los profesores Diego Barranco Navero y Luis Rallo Romero, por ofrecerme todo su apoyo y ayuda profesional, dándome más tranquilidad en situaciones difíciles y solucionando cada escollo con su bondad, paciencia y profesionalidad.

Por otro lado, un inmenso agradecimiento va para tod@s los investigadores del grupo UCOLIVO (Agronomía) y FQM-227 (Química Analítica) que siempre habéis estado cerca de mi, ayudándome y compartiendo conmigo muchos momentos bonitos dentro y fuera del trabajo, haciéndome sentir como con mi familia. Son muchísimas las personas con las que he compartido momentos maravillosos durante estos años y que me gustaría mencionar y agradecer, pero este espacio no me permite para tanto… Agradezco en especial a:

Dr. Juan Moral Moral, por estar siempre disponible para resolver cualquier duda y especialmente las cuestiones relacionadas con los análisis estadísticos.

Diego Cabello, por apoyarme en la realización de mi trabajo, ayudándome en cada momento que lo necesitaba, y trasmitiéndome su amable carácter y la amistad característica de los andaluces.

A Pablo y Pedro, por estar disponibles para mí, por darme siempre ánimo para realizar el trabajo y por compartir conmigo los momentos bonitos que nos han acompañado dentro y fuera del ámbito laboral.

A las secretarias incansables Ana y Carmen Vacas y Mª Carmen Cañas, que siempre han estado a mi lado para ayudarme con cualquier trámite.

A los chicos y chicas de Química Analítica, sin ellos sería imposible aprender a manejar los equipos de última generación para analizar los aceites, en especial a Chuck, Vero y Antonio.

Muchas gracias a los profesores que me formaron en Albania, que me hicieron fácil el camino para llegar hasta aquí, como la Profesora Renata Kongoli, el Profesor Abdyl Sinani, la Profesora Anila Kopali, el Profesor Vlash Mara, y muchos más.

Muchas gracias a los Profesores Željko Knez y Mateja Primozic, que me recibieron de la mejor manera en Maribor para realizar mi estancia doctoral en el extranjero y que me hicieron todo fácil para pasar unos meses muy bonitos tanto profesionalmente como socioculturalmente. Asimismo, agradezco a los jóvenes investigadores Gregor Kravanja y Maja Globočnik por compartir conmigo muchos momentos bonitos y por preocuparse de que Maribor fuera mi verdadera casa durante aquellos meses.

Muchas gracias a todos mis amigos y amigas de todo el mundo, de Albania, España, Polonia, Portugal, Grecia, Italia, Eslovenia, América Latina,…, que no han compartido conmigo las instalaciones de la Universidad, pero sí muchísimos momentos de mí vida y que siempre han estado a mi lado apoyándome de diferentes maneras, presencial o virtualmente. Sois muchísimos, y cada uno de vosotr@s tiene su lugar en mi corazón.

Por otro lado, todo este trabajo se lo dedico a mi familia "Miho" y a mi segunda familia "Peçi". Agradezco desde lo más profundo de mi corazón a mis padres, Ahile y Marina, a mis hermanos Perikli y Kristo, a mis primos Elisa y Mivan, que son para mi más que hermanos, y a mis inolvidables Miço y Teta Vangjo, que siempre me ayudaron y apoyaron como si fueran mis padres. ¡Muchísimas gracias por todo lo que habéis hecho para que yo llegase hasta aquí!

Agradecimientos

Finalmente, un inmenso agradecimiento va a la persona más importante, Justyna, mi guía y apoyo en este largo camino. Gracias por ser el pilar que ha ayudado, y ayuda, a mantener todo en su sitio. Gracias por estar a mi lado animándome a aspirar a más y no conformarme con cualquier cosa. Tu forma de ser, de esforzarte y de trabajar para que todas las dificultades salgan adelante ha sido una inspiración continua; por luchar a mi lado día a día, por escucharme pacientemente sin enterarte de lo que te decía; por poner un punto de alegría y de locura en mi vida; por enseñarme un poquitín más de polaco y por compartir conmigo todo para sentirnos cada día mejor. Gracias, porque compartir el día a día de la vida contigo está siendo maravilloso...

A todos y cada uno de ustedes,

¡Muchísimas gracias desde lo más profundo de mi corazón!

"El fin de la ciencia especulativa es la verdad, y el fin de la ciencia práctica es la acción"

Aristóteles

Filósofo griego (384 AC-322 AC)

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INTRODUCCIÓN

INTRODUCTION

1. INTRODUCCIÓN

1.1. El cultivo de olivo y la importancia de los recursos genéticos

El cultivo de olivo (*Olea europaea* L.), con más de 1200 variedades identificadas en todo el mundo, representa un patrimonio inestimable de variabilidad genética seleccionada a lo largo de más de 5500 años. Debido a sus características peculiares, este árbol forma parte de nuestra cultura y mitología (Rallo, Barranco, et al., 2018; Rugini, Baldoni, Muleo, & Sebastiani, 2016). Por otro lado, la elevada variabilidad genética que caracteriza el cultivo de olivo puede derivar en la obtención de productos muy diversos y, especialmente, aceites de oliva con una composición química muy variada.

Hoy en día, los recursos genéticos del olivo se conservan en una red de Bancos de Germoplasma ubicados en distintos países coordinada por el Consejo Oleícola Internacional ('International Olive Council - Germplasm Banks Network', 2020), asegurando así una adecuada conservación y un fácil acceso a los recursos genéticos del olivo para realizar diferentes actividades que mejoren y aseguren la futura existencia del sector olivícola. El Banco Internacional de Germoplasma de Córdoba (España) que ha servido también como punto de partida para el desarrollo de esta Tesis Doctoral, alberga más de 1000 variedades procedentes de 25 países diferentes (Rallo, Barranco, et al., 2018). A pesar de esta gran riqueza varietal, solo un número muy reducido de ellas se utilizan por parte de los productores; por ejemplo, en España solo 3 variedades suponen el 63% de la producción (Inglese et al., 2011). Este fenómeno ocurre porque el consumidor medio no tiene formación y concienciación para diferenciar y valorar adecuadamente las diferencias nutricionales entre los aceites monovarietales. Además, los productores generalmente emplean como criterio de selección de variedades únicamente la producción y la adaptación del cultivo a su sistema de plantación, obviando así el factor ´calidad´ (Rallo, Díez, et al., 2018).

Sin embargo, el consumidor busca cada vez más alimentos con aportaciones saludables con el objetivo de mejorar su calidad de vida y prevenir enfermedades crónicas (Casini, Contini, Marinelli, Romano, & Scozzafava, 2014; Luisa Badenes & Byrne, 2012). Considerando la demanda para la diversificación del Aceite de Oliva Virgen Extra (AOVE) y la obtención de aceites cada vez más destacados por sus propiedades nutricionales, es crucial una profunda caracterización de las variedades existentes en base a sus

componentes bioactivos para poner en valor variedades no explotadas y no demandadas, y para diseñar nuevos programas de mejora genética que ayudarían a obtener nuevos genotipos que se adapten a distintas necesidades y requisitos de los usuarios finales (Byrne, 2012; Rallo, Barranco, et al., 2018).

Los primeros programas de mejora genética, que empezaron en la década de los 60 en Israel e Italia, y posteriormente en los años 90 en Francia y España, estaban principalmente enfocados a obtener variedades de mayor producción y adaptables a la mecanización, dejando así en un segundo plano la mejora en las propiedades nutricionales de los aceites (Moreno-Alías, Rapoport, León, & de la Rosa, 2010; Rallo, 2014). Sin embargo, la concienciación sobre la salud se está convirtiendo en uno de los principales motores del mercado alimentario mundial por lo que urge diseñar y avanzar rápidamente en programas de mejora que consideren la diferenciación nutricional de los aceites de oliva (De la Rosa, Arias-Calderón, Velasco, & León, 2016).

1.2. El aceite de oliva y sus propiedades

El aprecio universal al Aceite de Oliva Virgen (AOV) se debe a sus efectos beneficiosos sobre la salud y a sus características organolépticas que lo hacen tan peculiar comparado con el resto de las grasas vegetales (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Rallo, Díez, et al., 2018; Servili et al., 2014).

Según la Comunidad Europea (Reg. CEE 2568/91 y Reg. CEE 2015/1830) y el Consejo Oleícola Internacional (T.15/NC No 3/Rev. 11, julio de 2016), el AOVes el zumo natural obtenido exclusivamente de los frutos frescos y sanos del olivo (*Olea europaea* L.). El (AOV) se obtiene únicamente por procedimientos mecánicos u otros medios físicos como la aplicación de condiciones térmicas, evitando siempre que ellos conduzcan a alteraciones de la composición del aceite. Además, cualquier tratamiento en el protocolo de extracción que no sea el lavado, la decantación, la centrifugación o la filtración debe ser descartado (European Commission, 2015; International Olive Council, 2016).

El aceite de oliva se compone básicamente de dos fracciones: la saponificable y la insaponificable. La fracción saponificable representa aproximadamente el 98% de la composición del aceite y está formada principalmente por ácidos grasos (esterificados a glicerol) y otros componentes menores, como ácidos grasos libres, fosfolípidos y ceras. Por

otro lado, la fracción insaponificable o minoritaria, que representa aproximadamente el 2% del peso total, abarca un conjunto complejo de compuestos pertenecientes a familias químicas como los fenoles, compuestos volátiles, terpenos, tocoferoles, fitoesteroles, pigmentos, etc (Piroddi et al., 2017; Rallo, Díez, et al., 2018; Servili et al., 2013).

De acuerdo con los estudios científicos y ensayos clínicos, muchos de los compuestos minoritarios y también el perfil de los ácidos grasos, donde destaca el ácido oleico, juegan un papel importante en la dieta aportando múltiples beneficios en la salud. Entre otros, se pueden destacar los efectos antinflamatorios, antioxidantes, anticancerígenos, cardioprotectores, reguladores del colesterol y de sistema endocrino del organismo. Asimismo, dichos compuestos definen los atributos fisicoquímicos del aceite como la estabilidad oxidativa, el color, la viscosidad, etc. Por otro lado, la fracción minoritaria y, específicamente los compuestos fenólicos y volátiles, confieren un sabor genuino al AOV aportando atributos de picor, amargor, astringencia y diversos aromas (Beauchamp et al., 2005; Bendini et al., 2007; Lazzerini & Domenici, 2017; Parkinson & Keast, 2014; Piroddi et al., 2017; Rallo, Díez, et al., 2018; Servili et al., 2016).

1.3. Los compuestos fenólicos

Los fenoles son compuestos que tienen uno o más grupos hidroxilos unidos directamente a una molécula de benceno, conocido también como el anillo aromático. El fenol (**Figura 1**) es la estructura en la que se basa todo el grupo.

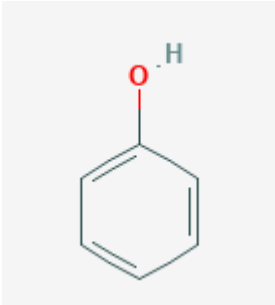

Figura 1. Estructura química del fenol. Fuente: PubChem.

Los polifenoles son compuestos que tienen más de un grupo hidroxilo fenólico unido a uno o más anillos de benceno. El término es algo engañoso, ya que tiende a hacer pensar

en polímeros de moléculas individuales de fenol. Los compuestos fenólicos son característicos de las plantas y, como grupo, suelen encontrarse en forma de ésteres o glucósidos más que como compuestos libres. Dichas características se deben considerar para extraer adecuadamente fenoles de los tejidos vegetales (Vermerris & Nicholson, 2008).

Las familias fenólicas más abundantes encontradas en el AOV son: los secoiridoides (oleuropeína y sus derivados), fenoles simples (tirosol, hidroxitirosol), flavonoides (luteolina, apigenina, rutina, y diosmetina) y lignanos (pinoresinol y derivados). El grupo de los secoiridoides (oleuropeína aglicona, ligstrósido aglicona, oleocantal y oleaceína) representa a los fenoles más abundantes encontrados en el aceite de oliva y está siendo ampliamente estudiado debido a sus prometedoras propiedades saludables (Cirilli et al., 2017; Del Rio, Gutierrez-Casado, Varela-Lopez, & Villalba, 2016; Parkinson & Cicerale, 2016; Rodríguez-López et al., 2020).

Como anteriormente se ha mencionado, los fenoles forman parte de la fracción minoritaria del AOV y juegan un papel clave debido a varios factores como: (I) sus propiedades saludables; (II) su contribución al perfil sensorial; (III) su papel en la definición de la estabilidad oxidativa del aceite; y (IV) su presencia exclusiva y abundante en el AOV en relación con otros aceites y grasas vegetales (Bendini et al., 2007; Carranco, Farrés-Cebrián, Saurina, & Núñez, 2018; Kiritsakis & Shahidi, 2017; Piroddi et al., 2017; Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002; Servili et al., 2016; Silva, Pinto, Carrola, & Paiva-Martins, 2010).

En relación a las propiedades saludables del AOV, diferentes estudios han demostrado las propiedades antioxidantes, antinflamatorias, antimicrobianas, y anticancerígenas de los compuestos fenólicos (Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012; Parkinson & Keast, 2014). En base a estas evidencias, en 2011 la Autoridad Europea de Seguridad Alimentaria (EFSA) aprobó la siguiente alegación: "los fenoles del aceite de oliva contribuyen a la protección de los lípidos sanguíneos frente al estrés oxidativo". Esta alegación se puede incluir en la etiqueta de un aceite siempre y cuando se demuestre que dicho aceite contiene al menos 5 mg de hidroxitirosol y sus derivados (complejos de oleuropeína y tirosol) por cada 20 g de aceite de oliva (EFSA, 2011). Uno de los compuestos fenólicos más comentados y destacado en los últimos años por sus propiedades

nutricionales y saludables ha sido el oleocantal (Beauchamp et al., 2005; Cusimano et al., 2017; Segura Palacios et al., 2019).

Los compuestos fenólicos juegan un papel fundamental en las características sensoriales de los aceites. Concretamente, ellos estimulan los receptores gustativos provocando las percepciones del amargor, el picor y la astringencia. Los isómeros de la oleuropeína y ligstrósido aglicona están principalmente relacionados con el amargor, mientras que el oleocantal y la oleaceina se asocian principalmente a la sensación de picor (Barbieri, Bendini, Valli, & Gallina Toschi, 2015; Bendini et al., 2007; Servili et al., 2004).

Otra característica importante de los compuestos fenólicos es su actividad antioxidante, por lo que, junto con el perfil de los ácidos grasos, contribuyen a la estabilidad oxidativa del aceite y, por consiguiente, a su vida útil. Los fenoles actúan como interruptores de la cadena de oxidación mediante la donación de un hidrógeno a los radicales de peróxido alquílico, que se forman por la oxidación lipídica (Fuentes et al., 2017; Servili et al., 2013; Spatari, De Luca, Ioele, & Ragno, 2017). Sin embargo, hasta el momento el rol de los compuestos fenólicos individuales sobre la estabilidad oxidativa de los aceites ha sido contradictorio (Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007; Kiritsakis & Shahidi, 2017). Para resolver dichas contradicciones, en el Capítulo IV de estas Tesis se analiza en profundidad y se determina el efecto de los compuestos fenólicos individuales y de los perfiles fenólicos sobre la estabilidad oxidativa de los aceites.

1.4. La variabilidad fenólica entre variedades y los factores determinantes.

En los últimos años diferentes estudios han reportado diferencias muy amplias y significativas entre las concentraciones fenólicas de los aceites monovarietales, oscilando entre aproximadamente 10 y 1000 mg/kg (Bajoub et al., 2017; Rodrigues et al., 2019). Dichas variaciones se han atribuido principalmente al factor genético, aunque otros factores como, las condiciones climáticas y agronómicas, los factores edáficos y el método tecnológico aplicado para la extracción del aceite también se han considerado relevantes (Baiano, Terracone, Viggiani, & Nobile, 2013; De la Rosa et al., 2016; El Riachy, Priego-Capote, Rallo, Luque-de Castro, & León, 2012).

a) La influencia del factor genético en la variabilidad fenólica del aceite

Los escasos estudios que se han realizado para determinar el peso del factor genético en la variabilidad fenólica sugieren que este factor podría explicar aproximadamente entre el 60 y el 80% de la varianza (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Inglese et al., 2011). Dicha dependencia genética se asocia a la expresión de las rutas enzimáticas responsables de la metabolización de los compuestos fenólicos. Por ejemplo, la β-glucosidasa de olivo es una enzima altamente específica que cataliza la formación de oleuropeína aglicona y oleaceína a partir de oleuropeína y demetiloleuropeína, respectivamente (Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012). En este contexto, diferentes estudios transcriptómicos y proteómicos han confirmado la existencia de diferentes genes asociados con la regulación de la actividad de las β-glucosidasas de olivo (Alagna et al., 2012; Bianco et al., 2013). Por ejemplo, se ha podido purificar la enzima recombinante (OepGLU) que controla la síntesis de la β-glucosidasa del olivo; asimismo, los resultados han demostrado que la expresión del gen GLU del olivo no sólo está regulada espacial y temporalmente en el fruto, sino que también depende del cultivo, la temperatura, la luz y el régimen hídrico (Velázquez-Palmero et al., 2017).

Sin embargo, todavía falta mucho por entender las rutas de biosíntesis de los compuestos fenólicos y la influencia varietal. A nuestro conocimiento, los estudios realizados en esta temática se han limitado a un número reducido de variedades tradicionales o de nuevos programas de mejora y, por otro lado, estos estudios no se han extendido en el tiempo para generar resultados contundentes sobre la variabilidad fenólica, los factores que la determinan, y la estabilidad fenólica interanual (El Riachy et al., 2011; Rallo, Barranco, et al., 2018; Vinha et al., 2005).

Por lo tanto, resulta crucial caracterizar de forma adecuada un numero representativo y amplio de variedades, para conocer el comportamiento y la variabilidad fenólica y, especialmente, para preparar el terreno para definir nuevos programas de mejora genética que puedan aportar altos valores nutricionales al AOV cumpliendo con las normas definidas por la EFSA (Byrne, 2012; Criado-Navarro, López-Bascón, & Priego-Capote, 2020; Rallo, Barranco, et al., 2018). Dichas cuestiones se abordan en los Capítulos II y III de esta Tesis Doctoral.

b) La influencia de los factores externos en la variabilidad fenólica del aceite

Como anteriormente se ha mencionado, además del factor genético, las condiciones externas asociadas a factores climáticos, agronómicos, fenológicos y tecnológicos afectan de manera directa o indirecta a la concentración fenólica de los aceites de oliva.

- **Factores climáticos, agronómicos y fenológicos**

Los estreses abióticos que sufre la planta, como el déficit hídrico, la salinidad, o la variación de la radiación solar, afectan favorable o desfavorablemente a la síntesis de los fenoles (Caruso et al., 2017; Gucci et al., 2019). Concretamente, el estado hídrico del árbol tiene una relación inversa con el contenido fenólico del aceite y probablemente eso se debe al cambio de la actividad enzimática en el fruto (Cirilli et al., 2017; Servili et al., 2007). Por lo tanto, las condiciones de estrés hídrico estimulan la síntesis de los fenoles y todo parece indicar que esta respuesta es mayor cuando dicho estrés se produce al inicio de desarrollo del fruto, cercano al endurecimiento de hueso (mes de julio) (Ahumada-Orellana, Ortega-Farías, & Searles, 2018; Gómez-del-Campo, 2013; Gucci et al., 2019). Asimismo, otros autores sugieren que una mayor iluminación solar del fruto está asociada con el aumento del contenido fenólico en el aceite (Caruso et al., 2017; Gómez-Del-Campo & García, 2012).

El estado fenológico o la maduración del fruto también afecta al contenido fenólico. Concretamente, durante las primeras fases de desarrollo del fruto (color verde de epidermis) el contenido fenólico se mantiene elevado. Posteriormente, cuando avanza la maduración y el fruto empieza a cambiar su color de verde a negro comienza el descenso de dicho contenido (Gouvinhas, de Almeida, Carvalho, Machado, & Barros, 2015; Peres et al., 2016).

Otro factor agronómico que afecta el contenido fenólico del fruto y del aceite es el estado nutritivo de la planta. La mayoría de los estudios apuntan que el incremento en el abonado nitrogenado reduce el contenido fenólico del aceite (Centeno, García, & Gómez-del-Campo, 2017; Dag et al., 2009; Fernández-Escobar et al., 2006).

- **Factores tecnológicos**

Los métodos y las condiciones tecnológicas de la extracción del aceite se consideran también entre los factores importantes que afectan al contenido fenólico (Kalua, Bedgood, Bishop, & Prenzler, 2006; Stefanoudaki, Koutsaftakis, & Harwood, 2011). Cabe destacar que la composición y concentración fenólica del AOV depende de la composición inicial de los frutos que, posteriormente, se someten a transformaciones enzimáticas durante el proceso de extracción del aceite (Montedoro, Servili, Baldioli, & Miniati, 1992; Taticchi et al., 2013). En la aceituna se encuentra numerosas clases de compuestos fenólicos como ácidos simples, alcoholes fenólicos libres y glicosilados, flavonoides y lignanos; sin embargo, los componentes mayoritarios y típicos de la familia *Oleaceae* son tres glucósidos secoiridoides, concretamente, la oleuropeína, el ligustrósido y la demetiloleuropeína, que comparten una estructura común en la que el ácido elenólico se esterifica con hidroxitirosol o tirosol y se conjuga mediante enlace β-glucosídico con una molécula de glucosa. Dichos glucósidos secoiridoides se encuentran almacenados en las vacuolas del fruto y son los precursores de los componentes fenólicos principales del AOV (Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015). Así, durante el proceso de la extracción del aceite y, principalmente en la molienda y el batido, debido a las roturas celulares los glucósidos secoiridoides se ponen en contacto con diferentes enzimas hidrolíticas y oxidorreductasas que catalizan la formación de compuestos secoiridoides hidrolizados y otros compuestos que migran y acaban en el aceite. Concretamente, los principales compuestos secoiridoides hidrolizados, o también conocidos como derivados secoiridoides del aceite, formados por la actuación de las enzimas β-glucosidasas y esterasas, son las formas aldehídicas de las agliconas de la oleuropeína y el ligustrósido (3,4-DHPEA-EA y p-HPEA-EA) y las formas dialdehídicas de las mismas agliconas demetiladas (3,4-DHPEA-EDA y p-HPEA-EDA), conocidas como oleaceína y oleocantal (Obied, Bedgood, Prenzler, & Robards, 2007; Ryan et al., 2002; Servili et al., 2004).

Además, durante el proceso de extracción del AOV los compuestos fenólicos sufren reacciones de oxidación, bien sea a través de procesos de oxidación química inducidos por radicales libres o por acción de enzimas oxidorreductasas, tales como la polifenoloxidasa (PPO) y la peroxidasa (POX), liberadas durante el proceso de extracción de aceite. La PPO es la principal enzima implicada en la oxidación de los compuestos fenólicos, tanto en

procesos fisiológicos asociados a la maduración del fruto como en cualquier proceso que implique daño o rotura de tejidos. Por otro lado, la contribución de la POX a la oxidación de fenoles está limitada por la disponibilidad de peróxido de hidrógeno. Sin embargo, se ha demostrado un efecto sinérgico de ambas enzimas en la oxidación de fenoles de la aceituna (Colpa, Lončar, Schmidt, & Fraaije, 2017; García-rodríguez, Romero-segura, Sanz, Sánchez-ortiz, & Pérez, 2011).

Como efecto resultante, el contenido fenólico final del AOV es el equilibrio entre los procesos de hidrólisis de los glucósidos fenólicos catalizados por la β-glucosidasa y los procesos de degradación catalizados por las oxidorreductasas, PPO y POX. Las oxidorreductasas pueden oxidar tanto a los glucósidos fenólicos, como a sus derivados hidrolizados obtenidos por la actuación de la β-glucosidasa, reduciendo así el contenido fenólico final en el aceite (García-Rodríguez, Romero-Segura, Sanz, & Pérez, 2015; Romero-Segura et al., 2012). Esta competición enzimática por el mismo sustrato (glucósidos fenólicos) se observa también durante el proceso del batido por la oscilación de la concentración fenólica durante el mismo. Concretamente, aunque en la mayoría de los estudios se hace hincapié en que el contenido fenólico se reduce con el aumento del tiempo de batido (Angerosa, Mostallino, Basti, & Vito, 2001; Jiménez, Sánchez-Ortiz, & Rivas, 2014; Kiritsakis & Shahidi, 2017; Trapani et al., 2017), en varios de ellos se refleja el aumento de varios compuestos fenólicos específicos debido a la predominación de los procesos de hidrolisis. Por supuesto que este aumento de la concentración fenólica se prolonga hasta que se alcanza el punto de inflexión donde los procesos oxidativos se convierten en los predominantes (Germek et al., 2014; Gómez-Rico, Inarejos-García, Salvador, & Fregapane, 2009). Por lo tanto, es crucial definir dicho punto de inflexión para diferentes variedades y compuestos fenólicos específicos con el objetivo de obtener aceites de mayor contenido fenólico y mejor calidad.

Por otro lado, como alternativas para evitar la oxidación de los fenoles durante el proceso de la extracción, diferentes publicaciones sugieren la sustitución del aire atmosférico que se encuentra en la batidora por gases inertes como el nitrógeno o el dióxido de carbono. Este intercambio de gases hace posible la reducción de la actividad enzimática de oxidorreductasas, PPO y POX, las cuales habilitan reacciones oxidativas en cascada impulsadas por el oxígeno (**Figura 2**) (Castagnini, Betoret, Betoret, & Fito, 2015;

Colpa et al., 2017; Mushtaq, 2017). Sin embargo, dicho método no se ha implementado en la industria debido a su alto coste (Servili et al., 2008; Vierhuis et al., 2001). En el Capítulo IV de esta Tesis Doctoral se han tratado aspectos relacionados con la influencia de los factores tecnológicos en la composición fenólica y se han propuesto nuevas alternativas innovadoras para mejorar dichos procesos.

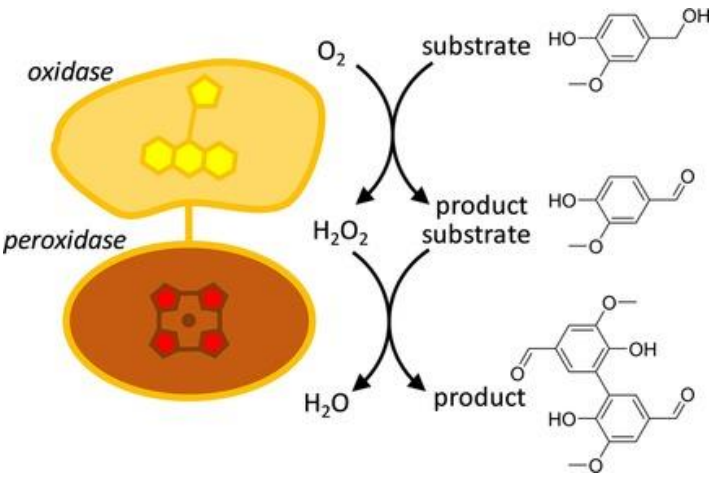

Figura 2. Ejemplo de las reacciones oxidativas en cascada impulsadas por el oxígeno en presencia de oxidasas y peroxidasas. Se muestra como ejemplo la reacción en cascada del alcohol vaníllico a la divanilina. Fuente: Colpa et al. 2017).

1. INTRODUCTION

1.1. The olive tree and the importance of genetic resources

The olive tree (*Olea europaea L.*) represents an invaluable genetic variability heritage with more than 1200 worldwide cultivars selected over more than 5500 years of cultivation. Due to its special characteristics, this crop is an inherent part of the mediterranean culture and mythology (Rallo, Barranco, et al., 2018; Rugini, Baldoni, Muleo, & Sebastiani, 2016). On the other hand, this high genetic variability leads to a wide range of olive products, and especially, olive oils of very different chemical composition.

Nowadays, olive genetic resources are conserved at a network of 23 different countries Germplasm Banks (GBs) coordinated by the International Olive Council - ('International Olive Council - Germplasm Banks Network', 2020). These GBs ensure a proper conservation and easy access to olive genetic resources to perform different activities that improve and ensure the future of the olive sector. The International Germplasm Bank of Cordoba (Spain), which has also served as a starting point for the development of this PhD Thesis, houses more than 1000 cultivars from 25 different countries (Rallo, Barranco, et al., 2018). Despite this great wealth of cultivars, only a very small number of them are used by farmers; for example, in Spain, three cultivars represent 63% of production (Inglese et al., 2011). This trend probably occurs as consumers still lack formation and awareness to adequately distinguish and appreciate the nutritional differences between monovarietal oils. Furthermore, normally producers only consider the oil yield and the adaptation to their planting system as the principal criteria for cultivar selection, and so, they frequently ignore the 'quality' factor (Rallo, Díez, et al., 2018).

On the other hand, consumers are increasingly interested in healthy foods with the aim of improving their life quality and preventing chronic diseases (Casini, Contini, Marinelli, Romano, & Scozzafava, 2014; Luisa Badenes & Byrne, 2012). Considering the high demand for variable and nutritional Extra Virgin Olive Oil (EVOO), it is crucial to carry out a deep characterisation of the existing olive cultivars on their nutritional compounds. This action would promote the valorisation of untapped and not demanded cultivars and the design of new breeding programmes to obtain new genotypes adapted to the different needs and requirements of the end-users (Byrne, 2012; Rallo, Barranco, et al., 2018).

The first breeding programmes, which started in the 1960s in Israel and Italy, and later in the 1990s in France and Spain, were mainly focused on obtaining cultivars with higher production and adaptability to mechanisation, while the nutritional properties of the oils were left in the second place (Moreno-Alías, Rapoport, León, & de la Rosa, 2010; Rallo, 2014). However, health awareness is becoming one of the main drivers of the global food market, making urgent to design and rapidly advance in breeding programmes that consider the nutritional value differentiation of olive oils (De la Rosa, Arias-Calderón, Velasco, & León, 2016).

1.2. Olive oil and its properties

The universal appreciation of Virgin Olive Oil (VOO) comes from its beneficial effects on health and its organoleptic characteristics that make it so unique as compared to other vegetable oils (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Rallo, Díez, et al., 2018; Servili et al., 2014).

According to the European Community (Reg. EEC 2568/91 and Reg. EEC 2015/1830) and the International Olive Oil Council (T.15/NC No 3/Rev. 11, July 2016), VOO is the natural juice obtained exclusively from fresh and healthy fruits of the olive tree (*Olea europaea* L.). VOO is obtained only by mechanical or other physical means such as the application of thermal conditions, always avoiding any alteration in its composition. Furthermore, any treatment in the extraction protocol other than washing, decantation, centrifugation or filtration must be discarded (European Commission, 2015; International Olive Council, 2016).

Olive oil is basically composed out of two fractions: the saponifiable and the unsaponifiable fractions. The saponifiable fraction represents approximately 98% of the VOO composition and is mainly made up of fatty acids (esterified to glycerol) and other minor components such as free fatty acids, phospholipids, and waxes. On the other hand, the unsaponifiable or minor fraction, which represents approximately 2% of the total weight, comprises a complex set of minor compounds belonging to chemical families such as phenolic compounds, volatiles, terpenes, tocopherols, phytosterols, pigments, etc. (Piroddi et al., 2017; Rallo, Díez, et al., 2018; Servili et al., 2013).

According to scientific studies and clinical trials, many of the minor compounds and also the fatty acid profile, where oleic acid stands out, play an important role in the daily diet, providing multiple health benefits. These include anti-inflammatory, antioxidant, anticarcinogenic, cardioprotective, cholesterol-regulating and endocrine-regulating effects. These compounds also define the physico-chemical attributes of the oil, such as oxidative stability, colour, viscosity, etc. On the other hand, the minor fraction and specifically the phenolic and volatile compounds confer a genuine flavour to VOO, providing attributes such as pungency, bitterness, astringency and various aromas (Beauchamp et al., 2005; Bendini et al., 2007; Lazzerini & Domenici, 2017; Parkinson & Keast, 2014; Piroddi et al., 2017; Rallo, Díez, et al., 2018; Servili et al., 2016).

1.3. Phenolic compounds

Phenols are compounds that have one or more hydroxyl groups attached directly to a benzene molecule, also known as the aromatic ring. The phenol (**Figure 1**) is the structure upon which the whole phenol group is based.

Polyphenols are compounds that have more than one phenolic hydroxyl group attached to one or more benzene rings. The term is somewhat misleading, as it tends to suggest polymers of individual phenol molecules. Phenolic compounds are characteristic of plants and are often found in the form of esters or glycosides rather than as free compounds. These characteristics must be considered in order to adequately extract phenols from plant tissues (Vermerris & Nicholson, 2008).

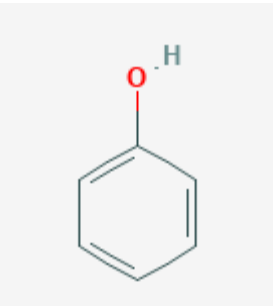

Figure 1. Chemical structure of phenol. Source: PubChem.

The most abundant phenolic families found in VOO are: secoiridoid compounds (oleuropein and its derivatives), simple phenols (tyrosol, hydroxytyrosol), and flavonoids (luteolin, apigenin, rutin, and diosmetin). The secoiridoid group (oleuropein aglycone, ligstroside aglycone, oleocanthal and oleacein) represents the most abundant phenols found in olive oil and is being widely studied due to its promising healthy properties (Cirilli et al., 2017; Del Rio, Gutierrez-Casado, Varela-Lopez, & Villalba, 2016; Parkinson & Cicerale, 2016; Rodríguez-López et al., 2020).

As mentioned above, phenols belong to the minor fraction of VOO and play a key role due to several particular features such as: (I) their healthy properties; (II) their contribution in the sensory profiles; (III) their role in defining the oxidative stability; and (IV) their exclusive and abundant presence in VOO in respect to other vegetable oils (Bendini et al., 2007; Carranco, Farrés-Cebrián, Saurina, & Núñez, 2018; Kiritsakis & Shahidi, 2017; Piroddi et al., 2017; Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002; Servili et al., 2016; Silva, Pinto, Carrola, & Paiva-Martins, 2010).

Specifically, different scientific studies have demonstrated the antioxidant, anti-inflammatory, antimicrobial and anticarcinogenic properties of the phenolic compounds found in VOO (Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012; Parkinson & Keast, 2014). Based on this evidence, in 2011 the European Food Safety Authority (EFSA) approved the following claim: "Consumption of olive oil polyphenols contributes to the protection of blood lipids from oxidative damage". This claim can be included on the label of an olive oil as long as it can be shown that it contains at least 5 mg of hydroxytyrosol and its derivatives (oleuropein and tyrosol complexes) per 20 g of olive oil (EFSA, 2011). One of the most commented and highlighted phenolic compounds in the last few years for its nutritional and health properties has been oleocanthal (Beauchamp et al., 2005; Cusimano et al., 2017; Segura Palacios et al., 2019).

Phenolic compounds play a critical role in the organoleptic characteristics of olive oils. In particular, they stimulate gustative receptors leading to perceptions of bitterness, pungency and astringency. Oleuropein and ligstroside aglycone isomers are mainly related to bitterness, while oleocanthal and oleacein mainly provoke the sensation of pungency (Barbieri, Bendini, Valli, & Gallina Toschi, 2015; Bendini et al., 2007; Servili et al., 2004).

Another important characteristic of phenolic compounds is their antioxidant activity, which, in combination with the fatty acid profile, contributes to the oxidative stability of the olive oil and consequently to its shelf life. Phenols play a role as interrupters of the oxidation chain by donating a hydrogen to the alkyl peroxide radicals, which are formed by lipid oxidation (Fuentes et al., 2017; Servili et al., 2013; Spatari, De Luca, Ioele, & Ragno, 2017). However, the role of individual phenolic compounds on the oxidative stability of VOO has so far been contradictory (Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007; Kiritsakis & Shahidi, 2017). To resolve these contradictions, in Chapter IV of this PhD Thesis it is thoroughly analysed and discussed the role of individual phenolic compounds and phenolic profiles on the olive oil oxidative stability.

1.4. Cultivar phenolic variability and the contributing factors

In recent years different studies have reported large and significant differences between the total phenolic concentrations of monovarietal oils, ranging from approximately 10 to 1000 mg/kg (Bajoub et al., 2017; Rodrigues et al., 2019). These variations have been mainly attributed to the genetic factor, although other factors such as climatic and agronomic conditions, edaphic characteristics and the technological method applied for oil extraction have also been considered relevant (Baiano, Terracone, Viggiani, & Nobile, 2013; De la Rosa et al., 2016; El Riachy, Priego-Capote, Rallo, Luque-de Castro, & León, 2012).

a) The influence of the genetic factor on the olive oil phenolic variability

The few studies that have been carried out to determine the weight of the genetic factor in the phenolic variability among cultivars suggest that the genetic factor could explain approximately 60-80% of the total variance (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Inglese et al., 2011). Such genetic dependence is also evidenced by the expression of the enzymatic pathways responsible for the metabolisation of phenolic compounds. For example, olive β-glucosidase is a highly specific enzyme that catalyses the formation of oleuropein aglycone and oleacein from oleuropein and demethyloleuropein, respectively (Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012); and different transcriptomic and proteomic studies have confirmed the existence of different genes associated with the regulation of olive β-glucosidase activity

(Alagna et al., 2012; Bianco et al., 2013). For example, it has been possible to purify the recombinant enzyme (OepGLU) which regulates the synthesis of β-glucosidase in olive; furthermore, the results have shown that the expression of the olive GLU gene is not only spatially and temporally regulated in the olive fruit, but is also cultivar-dependent and regulated by temperature, light and water regime (Velázquez-Palmero et al., 2017).

However, there is still a long way ahead in understanding the biosynthesis pathways of phenolic compounds and their cultivar variability. To our knowledge, studies on this topic have been limited to a small number of traditional cultivars or new breeding programmes, and have not been extended over time to generate robust results on phenolic variability, the factors that determine it, and inter-annual phenolic stability (El Riachy et al., 2011; Rallo, Barranco, et al., 2018; Vinha et al., 2005).

Hence, it is crucial to carry out broad and in-depth studies to adequately characterise a representative and large number of cultivars, to know the phenolic behaviour and variability and, especially, to pave the way for defining new genetic breeding programmes that can bring high nutritional values to VOO in accordance with the standards defined by the EFSA (Byrne, 2012; Criado-Navarro, López-Bascón, & Priego-Capote, 2020; Rallo, Barranco, et al., 2018). These aspects are evaluated in Chapters II and III of this PhD Thesis.

b) The influence of external factors on olive oil phenolic variability

As previously mentioned, aside from the genetic factor, external conditions associated with climatic, agronomic, phenological and technological factors directly or indirectly affect olive oil phenolic concentration.

- **Climatic, agronomic and phenological factors**

Abiotic stresses experienced by the plant, such as water deficit, salinity or variations in solar radiation, have a favourable or unfavourable effect on phenol synthesis (Caruso et al., 2017; Gucci et al., 2019). Specifically, poor tree hydric conditions have an inverse relationship with the phenolic content of the olive oil and this behaviour is likely due to the changes in the enzymatic activity of the fruit (Cirilli et al., 2017; Servili et al., 2007). Thus, water stress conditions stimulate phenol synthesis and all indications suggest that this response is greater when water stress occurs at the beginning of fruit development, close

to stone hardening stage (July) (Ahumada-Orellana, Ortega-Farías, & Searles, 2018; Gómez-del-Campo, 2013; Gucci et al., 2019). Other authors also suggest that a greater solar illumination of the fruit is associated with an increase in the oil phenolic content (Caruso et al., 2017; Gómez-Del-Campo & García, 2012).

The phenological stage or fruit ripening index also affects the phenolic content. Specifically, during the first stages of fruit development (green skin colour) the phenolic content remains high; then, as ripening progresses and the fruit begins to change its colour from green to black, the phenolic content starts to decrease (Gouvinhas, de Almeida, Carvalho, Machado, & Barros, 2015; Peres et al., 2016).

Another agronomic factor affecting the fruit and oil phenolic content is the nutritional status of the plant. Most of the studies point out that increased nitrogen fertilisation reduces oil phenolic content (Centeno, García, & Gómez-del-Campo, 2017; Dag et al., 2009; Fernández-Escobar et al., 2006).

- **Technological factors**

The methods and technological conditions of oil extraction are also considered among the important factors affecting phenolic content (Kalua, Bedgood, Bishop, & Prenzler, 2006; Stefanoudaki, Koutsaftakis, & Harwood, 2011). It is worth emphasising that VOO phenolic concentration depends on the initial olive fruit composition, which is then subjected to enzymatic transformations during the oil extraction process. (Montedoro, Servili, Baldioli, & Miniati, 1992; Taticchi et al., 2013). Numerous classes of phenolic compounds such as simple acids, free and glycosylated phenolic alcohols, flavonoids and lignans are found in olive fruits; however, the major and characteristic components of the *Oleaceae* family are three secoiridoid glycosides, namely oleuropein, ligstroside and demethyloleuropein, which share a common structure in which the elenolic acid is esterified with hydroxytyrosol or tyrosol and conjugated by a β-glucosidic bond to a glucose molecule. These secoiridoid glycosides are stored in the vacuoles of the fruit and are the precursors of the principal phenolic components of VOO (Cecchi, Migliorini, Cherubini, Innocenti, & Mulinacci, 2015). Therefore, during the oil extraction process, and mainly during milling and malaxation, due to cell ruptures, secoiridoid glycosides of olive fruit get in contact with different hydrolytic and oxidoreductases enzymes that catalyse the

formation of hydrolysed secoiridoids and other compounds which migrate and end up in the olive oil. Specifically, the main hydrolysed secoiridoid compounds, known also as secoiridoid derivatives formed by the action of β-glucosidase and esterase enzymes, are the aldehydic forms of oleuropein and ligstroside aglycones (3, 4-DHPEA-EA and p-HPEA-EA) and the dialdehydic forms of the same demethylated aglycones (3,4-DHPEA-EDA and p-HPEA-EDA), known as oleacein and oleocanthal (Obied, Bedgood, Prenzler, & Robards, 2007; Ryan et al., 2002; Servili et al., 2004).

Furthermore, during the VOO extraction process, phenolic compounds undergo oxidation reactions, either through chemical oxidation processes induced by free radicals or by the action of oxidoreductase enzymes, such as polyphenol oxidase (PPO) and peroxidase (POX), released during the oil extraction process. PPO is the main enzyme involved in the oxidation of phenolic compounds, both in physiological processes associated with fruit ripening and in any process involving tissue damage or rupture. On the other hand, the participation of POX in phenol oxidation is limited by the availability of hydrogen peroxide. However, a synergistic effect of both enzymes in the oxidation of olive phenols has been demonstrated. (Colpa, Lončar, Schmidt, & Fraaije, 2017; García-rodríguez, Romero-segura, Sanz, Sánchez-ortiz, & Pérez, 2011).

As a result, the final phenolic content of VOO is the balance between the hydrolysis processes of the phenolic glycosides catalysed by β-glucosidase and the degradation processes catalysed by the oxidoreductases, PPO and POX. Oxidoreductases can oxidise both phenolic glycosides and their hydrolysed derivatives obtained by the action of β-glucosidase, resulting in a reduction of the final phenolic content in the olive oil. (García-Rodríguez, Romero-Segura, Sanz, & Pérez, 2015; Romero-Segura et al., 2012). This enzymatic competition for the same substrate (phenolic glycosides) is also observed during the malaxation process in which the phenolic concentration fluctuates over time. Although most of studies emphasise that phenolic content decreases with the increase of malaxation time, some of them, show that several specific phenolic compounds increase their concentration because of the predominance of hydrolysis processes (Angerosa, Mostallino, Basti, & Vito, 2001; Jiménez, Sánchez-Ortiz, & Rivas, 2014; Kiritsakis & Shahidi, 2017; Trapani et al., 2017). Of course this increase in phenolic concentration is prolonged until the tipping point is reached and oxidative processes become predominant (Germek

et al., 2014; Gómez-Rico, Inarejos-García, Salvador, & Fregapane, 2009). Therefore, it is crucial to define such a tipping point for different cultivars and specific phenolic compounds in order to obtain oils of higher phenolic content and better quality.

On the other hand, as alternatives to avoid the phenols oxidation during the extraction process, different publications suggest the substitution of the atmospheric air in the mixer by inert gases such as nitrogen or carbon dioxide. This gas exchange makes possible the reduction of the enzymatic activity of oxidoreductases, PPO and POX, which enable oxidative cascade reactions triggered by oxygen (**Figure 2**). (Castagnini, Betoret, Betoret, & Fito, 2015; Colpa et al., 2017; Mushtaq, 2017). However, such a method has not been implemented in industry given its high cost (Servili et al., 2008; Vierhuis et al., 2001). In Chapter IV of this PhD Thesis, we have dealt with aspects related to the influence of technological factors on phenolic composition and we have proposed new innovative alternatives to optimise these processes.

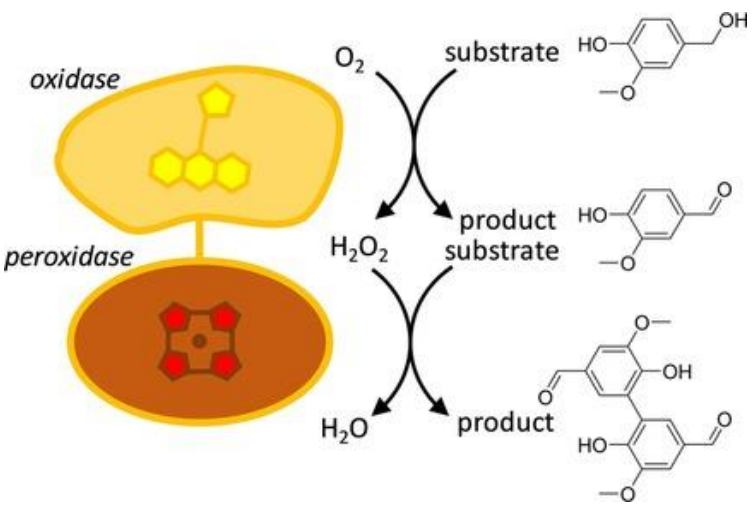

Figure 2. Example of oxidative cascade reactions triggered by oxygen in the presence of oxidases and peroxidases. The cascade reaction of vanillic alcohol to divaniline is shown as an example. Source Colpa et al. 2017).

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OBJETIVOS DE LA TESIS

THESIS OBJECTIVES

2. OBJETIVOS DE LA TESIS

El objetivo general de esta Tesis Doctoral fue estimar la variabilidad de la composición fenólica del aceite de oliva virgen e identificar y evaluar el peso específico de los factores principales que influyen en dicha variabilidad. Para abordar este objetivo general, se plantearon cuatro objetivos principales que se describen a continuación:

1. Definir el estado del arte actual de los factores que afectan a la calidad del aceite de oliva virgen y la importancia que tiene su consumo en la dieta y la salud. Este objetivo fue alcanzado con el artículo de revisión publicado en el Capítulo I: "Rallo, L., Díez, C. M., Morales-Sillero, A., **Miho, H**., Priego-Capote, F., & Rallo, P. (2018). Quality of olives: A focus on agricultural preharvest factors. *Scientia Horticulturae*, 233, 491–509. https://doi.org/10.1016/j.scienta.2017.12.034").

2. Caracterizar el contenido fenólico del aceite de oliva virgen de 80 variedades diferentes que representan la diversidad genética del cultivo de olivo y que se obtuvieron en una sola campaña de cultivo en condiciones agrícolas y tecnológicas homogéneas. Las metas específicas de este objetivo fueron: (i) Realizar una categorización preliminar de las variedades en función del contenido fenólico en el aceite; (ii) determinar el peso del factor genotipo en la varianza total; y (iii) profundizar en el estudio de las rutas metabólicas involucradas en la síntesis de los fenoles para entender su formación. Este objetivo fue desarrollado en la investigación que dio lugar al artículo incluido en el Capítulo II: "**Miho, H.,** Díez, C. M., Mena-Bravo, A., Sánchez de Medina, V., Moral, J., Melliou, E., … Priego-Capote, F. (2018). Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. *Food Chemistry*, 266, 192–199. https://doi.org/10.1016/j.foodchem.2018.06.002").

3. Determinar el contenido fenólico del aceite de oliva virgen obtenido en tres campañas agronómicas consecutivas a partir de 44 variedades en las mismas condiciones agronómicas. Específicamente, a través de este objetivo se plantearon las siguientes metas: (i) Categorizar las variedades en base a su perfil fenólico relativo; (ii) evaluar la varianza interanual para cada compuesto fenólico estableciendo el peso de los factores específicos 'genotipo' y 'campaña de cultivo';

y (iii) agrupar las variedades en función de su perfil fenólico y comprobar la estabilidad de dichos perfiles en las diferentes campañas. Este objetivo fue completado con la investigación publicada en el artículo que recoge el Capítulo III: "**Miho**, H., Moral, J., Barranco, D., Ledesma-Escobar, C. A., Priego-Capote, F., & Díez, C. M. (2020). Influence of genetic and interannual factors on the phenolic profiles of virgin olive oils. *Food Chemistry*, 342, 128357. https://doi.org/10.1016/j.foodchem.2020.128357").

4. Estudiar la formación de los principales compuestos fenólicos encontrados en el aceite de oliva virgen durante el proceso de batido y determinar el rol de los fenoles en la estabilidad oxidativa del aceite. Específicamente se planteó: (i) Determinar la influencia del factor genotipo y de un parámetro tecnológico como es el tiempo de batido sobre la variabilidad fenólica del aceite de oliva virgen; (ii) definir los tiempo de batido óptimos para obtener el perfil fenólico deseado en los aceites; (iii) analizar el efecto de aplicar condiciones de vacío durante el proceso de batido sobre la concentración absoluta y relativa de compuestos fenólicos; (iv) modelar la estabilidad oxidativa del aceite de oliva virgen en función del contenido fenólico y la concentración de los principales ácidos grasos. Este objetivo específico dio como resultado la publicación del artículo recogido en el Capítulo IV: "**Miho, H.,** Moral, J., López-González, M. A., Díez, C. M., & Priego-Capote, F. (2020). The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability. Food Chemistry, 314. https://doi.org/10.1016/j.foodchem.2020.126183").

2. THESIS OBJECTIVES

The general objective of this PhD Thesis was to estimate the variability of the phenolic composition of virgin olive oil and to identify and evaluate the specific weight of the main factors that determine this variability. To address this general objective, four main objectives were set out as described below:

1. To define the current state of the art of the factors that affect the quality of virgin olive oil and the role of olive oil consumption in our diet and health. This objective was achieved with the review article published in Chapter I: "Rallo, L., Díez, C. M., Morales-Sillero, A., **Miho, H**., Priego-Capote, F., & Rallo, P. (2018). Quality of olives: A focus on agricultural preharvest factors. *Scientia Horticulturae*, 233, 491–509. https://doi.org/10.1016/j.scienta.2017.12.034").

2. To characterise the phenolic content of virgin olive oil from 80 different cultivars which represent the genetic diversity of the olive and which were obtained in a single growing season under homogeneous agricultural and technological conditions. The specific goals of this objective were: (i) to perform a preliminary categorisation of the cultivars according to the phenolic content in the oil; (ii) to determine the weight of the genotype factor in the total variance; and (iii) to further study the metabolic pathways involved in the synthesis of phenols in order to understand their formation. This objective was implemented and led to the research article included in Chapter II: "**Miho, H.,** Díez, C. M., Mena-Bravo, A., Sánchez de Medina, V., Moral, J., Melliou, E., … Priego-Capote, F. (2018). Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey. *Food Chemistry*, 266, 192–199. https://doi.org/10.1016/j.foodchem.2018.06.002").

3. To determine the phenolic content of the virgin olive oil obtained in three consecutive agronomic campaigns from 44 cultivars under the same agronomic conditions. Specifically, the following goals were set through this objective: (i) to categorise the cultivars on the basis of their relative phenolic profile; (ii) to evaluate the inter-annual variance for each phenolic compound by establishing the weight of the specific factors 'genotype' and 'crop season'; and (iii) to cluster

the cultivars according to their phenolic profile and to test the stability of these profiles over different seasons. This objective was achieved and addressed in the article included in the Chapter III: "**Miho**, H., Moral, J., Barranco, D., Ledesma-Escobar, C. A., Priego-Capote, F., & Díez, C. M. (2020). Influence of genetic and interannual factors on the phenolic profiles of virgin olive oils. *Food Chemistry*, 342, 128357. https://doi.org/10.1016/j.foodchem.2020.128357").

4. To study the formation of the main phenolic compounds found in virgin olive oil during the malaxation process and to determine the role of phenols in the oil's oxidative stability. Specifically, the following goals were established: (i) to determine the influence of the genotype factor and a technological parameter such as the malaxation time on the phenolic variability of virgin olive oil; (ii) to define the optimal malaxation times to obtain the desired phenolic profile in the oils; (iii) to analyse the effect of malaxation under vacuum conditions on the absolute and relative concentration of olive oil phenolic compounds; (iv) to model the oxidative stability of virgin olive oil as a function of the phenolic and fatty acids content. This specific objective led to the publication of the article reported in Chapter IV: "**Miho, H.,** Moral, J., López-González, M. A., Díez, C. M., & Priego-Capote, F. (2020). The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability. Food Chemistry, 314. https://doi.org/10.1016/j.foodchem.2020.126183").

3. THESIS CHAPTERS

CHAPTER I - Quality of olives: A focus on agricultural preharvest factors.

Scientia Horticulturae, 233; 491-509.

Scientia Horticulturae 233 (2018) 491–509

Contents lists available at ScienceDirect

Scientia Horticulturae

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

Review

Quality of olives: A focus on agricultural preharvest factors

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https://doi.org/10.1016/j.scienta.2017.12.034

Received 14 July 2017; Received in revised form 5 December 2017; Accepted 18 December 2017

Available online 24 February 2018

Abstract

More than 11 million ha of olives (Olea europaea L.) are currently grown worldwide, 98% of which are localized in the Mediterranean Basin, with olives being one of the most important fruit trees in the area. The olive fruit is a very particular drupe since it may not be directly consumed but must instead be processed. Table olives and virgin olive oil are the two main processed products derived from olive fruits. Both are considered staple foods of the Mediterranean Diet and have been produced in the area for centuries, presumably since olive domestication occurred approximately 6.000 years ago. Despite their long history and economic importance, the focus on quality is quite recent. The presence of various and copious amounts of bioactive compounds, some of which are exclusive to olives, is drawing attention to the nutraceutical value of these products. This review aims to integrate the available information regarding the quality of table olives and olive oil with a focus on how preharvest factors may affect quality. The first part of the review describes the main quality attributes considered for each product from different perspectives, including the legal, organoleptic and nutritional points of view, among others. The physiological mechanisms involved in fruit development and ripening, which significantly affect the quality of the fruits, i.e., the raw material for obtaining both products, are also discussed. The review also addresses the potential of both the considerable number of traditional olive cultivars and recent olive breeding programs to obtain products with distinct quality attributes (in terms of sensorial profile and bioactive compounds). Finally, the most recent literature concerning the effect of environmental (soil and climate) and agronomical factors (irrigation, fertilization, canopy management and harvesting) is extensively reviewed.

Keywords*:* Table olives Olive oil, Organoleptic quality, Nutraceutical quality, Volatile compounds, Phenolic compounds.

Abbreviations: DAFB, days after full bloom; EEC, European economic community; EFSA, European food safety authority; EVOO, extra virgin olive oil; FA, free acidity; FAEEs, fatty acid ethyl esters; FATH, fruit abscission threshold; FR, fruit ripening; IOC, International Olive Council; LDL, low-density lipoprotein; MI, maturity index; MUFA, monounsaturated fatty acid; PL, phospholipids; PUFA, polyunsaturated fatty acid; RDI, regulated deficit irrigation; VOO, virgin olive oil.

1. Introduction

Olive growing has been traditionally localized in the Mediterranean Basin for thousands of years. According to the International Olive Council (IOC, http://www.internationaloliveoil.org/), there are more than 11 million ha of olive trees in more than 47 countries. The majority of this surface (97.9%) is localized in the Mediterranean countries. However, new intensive orchards have been planted in the Mediterranean and in new regions, such as Australia, North and South America, over the last 20 years. The mentioned expansion and intensification of olive growing as well as the perception of olive oil and table olives as healthy foods have largely increased both the production and demand of these products.

The olive fruit is a drupe that comprises the exocarp, the fleshy mesocarp (the edible portion) and the stony endocarp. Unlike other well-known drupes (peaches, apricots, cherries, and plums) olive fruit cannot be directly consumed but must instead be processed to eliminate their strong bitter taste, which is caused by the presence of oleuropein, a secoiridoid glucoside (the predominant phenolic compound), in the mesocarp. Other particularities of the olive fruit are its low sugar content (3.5-6%) and the high amount of oil accumulated during maturation (14–30% oil content).

The concept of quality in fruit products is wide, complex and dynamic. It implies a large number of attributes with different significance according to the interest and expectations of the different stakeholders of the chain, from producers to consumers (See Kyriacou and Rouphael, 2017). In the case of the olive, two main products are obtained from olive fruits: virgin olive oil (the juice of the fruit) and table olives; both are staple foods of the Mediterranean Diet. The quality attributes that are considered for each product largely differ from one another; thus, they are addressed separately in the first part of the review. Nevertheless, legal definition according to regulatory standards, chemical and nutritional attributes and organoleptic properties are reviewed in both products.

Preharvest factors directly affect the quality of the “fresh” olive fruits, i.e., the raw material for both table olives and olive oil; therefore, the final quality of both products depends largely on the cultivar, ripening stage and management in the field (olive

orchards). Similarly, the processing required to obtain both products have an important effect on the final quality of table olives and olive oil. Nevertheless, in this review, only preharvest factors, including the physiology of ripening, genetics, and environmental and agronomic conditions, are discussed.

2. Olive quality

2.1. Table olive quality attributes

2.1.1. Legal definition of table olives and types

For commercial trading, olive fruits must conform to obligatory standards that mainly refer to fruit appearance and uniformity in addition to the presence of different defects. Quality standards for table olives were issued by the International Olive Council (IOC, 2004). According to this standard, table olives are defined as the product with the following characteristics:

(a) prepared from the sound fruits of varieties of the cultivated olive tree (Olea europaea L.) that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing.

(b) treated to remove its bitterness and preserved by natural fermentation, or by heat treatment, with or without the addition of preservatives.

(c) packed with or without covering liquid.

Table olive processing procedures are intended to remove oleuropein to reduce the bitterness distinctive to the olive fruit. The methods largely differ among the regions, cultivars and ripening stages of the olives. There are, however, according to the standards (IOC, 2004), four main trade preparations: “treated olives” (fruits undergo an alkaline treatment and are placed in a brine, where fermentation occurs), which includes the well-known Spanish-style green olives; “natural olives” (olive fruits are directly placed in a brine), with Greek-style black olives being the most prevalent preparation within the category; “olives darkened by oxidation” (olives are preserved in a brine and darkened by

oxidation), which are also known as black ripe olives or Californian style olives; and "dehydrated and/or shriveled olives" (fruits are preserved in brine or partially dehydrated in dry salt and/or by heating or any other process).

2.1.2. Fruit traits

The attributes of the olive fruit are key for table olives since the first perception of quality by the consumer relies in many of them: size, shape, color and the absence of damage.

Olive fruit size is expressed as unitary fruit weight and/or volume, although for commercial size grading, it is calculated as the number of fruits per kilogram. Table olives are preferred to be large (over 5 g per fruit) or medium-size (3–5 g per fruit) (Garrido Fernandez et al., 1997). Nevertheless, many table olive cultivars vary considerably in size (Barranco et al., 2000). Fruit shape is usually measured as the ratio between fruit length and width. Many different shapes may be found among table olives, but spherical rounded shapes are often preferred by consumers and by the industry since pitting them is easier. However, very appreciated table olives such as "Kalamata olives" are notably elliptical and asymmetrical (Tsantili, 2014). Having a high flesh-to-stone ratio is essential for table olive acceptance by the consumer. No "legal" minimum value is established, but a 5:1 ratio is acceptable. Stone morphology is also an important trait influencing quality. The surface of the pit should be smooth, and the flesh should be easily detached.

Regarding fruit surface color, chlorophylls and carotenoids are the main pigments responsible for the color of green olives. Consumers prefer the golden-yellow color characteristic of alkali-treated olives to the brownish colors that natural green olives (non-treated with alkali) usually develop (Ramirez et al., 2015). Anthocyanins are involved in the final color of natural black (Greek-style) olives, whereas the color of black ripe (California-style) olives is achieved by oxidation of hydroxytyrosol (Brenes et al., 1992). The dark color of black olives is one of the attributes that is most valued by consumers, but natural black olives do not usually reach the darkness and homogeneity of black-ripe olives (Romero et al., 2015). For green olives, color determination is based on the measured reflectance at wavelengths of 560, 590 and 635 nm (Sánchez Gómez et al., 1985). Alternative methods, primarily the CIE (Commission internationale de l'éclairage) L*

(lightness), a* (redness) and b* (yellowness) parameters, are currently widely used for color determination for both fresh and processed table olives (Ramirez et al., 2015). In ripe and naturally black olives, the parameters proposed are the reflectance of the olive surface at 700 nm (Garrido Fernandez et al., 1997) in addition to the CIE L*, a* b* color space (Marsilio et al., 1990).

As mentioned above, fruit appearance is one of the most decisive factors influencing consumer's choice, and a long list of defects affecting olive fruit surface is included within the International Trade Standard for Table Olives (IOC, 2004). Among these defects, bruising is the most common type of mechanical damage, and its occurrence is mainly related to the impacts suffered by the olive fruit during harvesting. Bruising is generally associated with superficial browning (dark spots) on the fruit exterior, but internal damage within the mesocarp, including ruptured cells and a loss of cell wall thickness, has also been reported (Jimenez et al., 2016). Bruising assessment in commercial regulations (IOC, 2004) and, in most studies, is usually limited to a visual evaluation of external damage (Jimenez-Jimenez et al., 2013; Saracoglu et al., 2011). A recent methodology developed by Jimenez et al. (2016) is being employed to assess and quantify internal damage associated with bruising at the tissue level (Casanova et al., 2017; Jiménez et al., 2017).

2.1.3. Flesh texture

Flesh texture is a quality attribute of great importance for table olives. In fact, "abnormal texture", based on subjective appreciation, is considered a defect within the quality standard (IOC, 2004), and similarly, kinesthetic sensations (directly related to fruit firmness) have been included in the sensory analysis methodology (IOC, 2011). Nevertheless, no average values are specified, and no correlation with instrumental objective methods has yet been established (Sánchez Gómez and García, 2017).

There are no unified standard methodologies to assess the mechanical properties of the olive fruit, although different tests and instruments have been employed for the texture evaluation of table olives. Most methods are based on applying pressure or force to the fruit surface and measuring traits related to fruit consistency, such as fruit deformation (Kilickan and Guner, 2008; Lanza et al., 2010; Mafra et al., 2001), the compression force based on single or continuous measures (Cardoso et al., 2008; Catania et al., 2015), the

puncture force required to penetrate the pulp (Cano-Lamadrid et al., 2015; Cardoso et al., 2008; Fadda et al., 2014; Mafra et al., 2001), and the shear force required to break it (Garcia-Garcia et al., 2014; Rejano Navarro et al., 2008). Instruments for physical measures include pressure testers or durometers, puncture testers or penetrometers and more sophisticated texturometers. Texture is related to the cell structure, the composition of the cell wall, particularly regarding polysaccharides, and the enzymes involved in cell degradation. Thus, indirect methods based on some of these aspects to determine the mechanical properties of the olive drupe have been studied (Fernandez-Bolaños et al., 2001; Mafra et al., 2001; Marsilio et al., 2000).

2.1.4. Chemical composition and nutritional value of table olives

Multiple beneficial health effects have been associated with the consumption of table olives (Accardi et al., 2016). The nutraceutical value of this product largely depends on the chemical composition of the fresh olive fruit. However, during table olive processing, many changes in the chemical composition occur, generally resulting in decreased quality parameters, as will be discussed below.

The olive fruit mesocarp and exocarp, the edible portion, are mainly composed of water (70–75%) and lipids. The oil content in the olive fruit ranges from 14 to 30%, depending on the cultivar and the ripening stage (Bianchi, 2003). Olives are rich in monounsaturated fatty acids (MUFAs), mainly oleic acid (47–84%) and palmitoleic acid (0.3-3.5%) (Servili et al., 2016). High intakes of oleic acid have been widely documented to be associated with reduced LDL cholesterol (Mattson and Grundy, 1985). Moreover, important amounts of polyunsaturated fatty acids (PUFAs), linoleic and linolenic acids are present in the olive fruit. Since they are essential acids, i.e., are not synthesized by humans, they should be consumed as part of one's diet (Servili et al., 2016). The fatty acid profile seems to be only slightly affected by table olive processing (Issaoui et al., 2011; Lopez-Lopez et al., 2015), and thus, all of the mentioned benefits remain in the final product. Their stability has been attributed to their insolubility in the processing medium (Bianchi, 2003).

Sugars in the olive fruit represent up to 3.5-6% (Servili et al., 2016), a small amount compared to other drupes. The major sugars in fresh fruits are glucose, fructose, sucrose, and mannitol (Marsilio et al., 2001), although other sugars, such as galactose, mannose,

sorbitol, xylose and rhamnose, have been reported (Aktas et al., 2014; Issaoui et al., 2011; Lopez-Lopez et al., 2007). Sugars play an important role in the olive fruit: they are related to the textural properties because they are important components of the cell wall, they are precursors of olive oil biosynthesis, and they provide energy for metabolic changes. During table olive processing, sugars are the main source of carbon for microorganism in fermentation and give rise to the secondary metabolites responsible for the distinctive flavor of the final product.

Phenolic compounds are minor constituents of the olive fruit (comprising 1–3% of the fresh pulp weight) but have very important roles in their antioxidant, anti-inflammatory and anticarcinogenic activities (Boskou et al., 2006; Kountouri et al., 2009; Soler-Rivas et al., 2000; Uccella, 2000). They have also been associated with prevention of cardiovascular and degenerative diseases (Bendini et al., 2007). The profile of phenolic compounds in the olive fruits is very complex and depends on factors such as the cultivar, ripening stage or season (see the recent review by Charoenprasert and Mitchell, 2012). The most abundant phenols in fresh olives are oleuropein, demethyloleuropein, hydroxityrosol and verbascoside (Blekas et al., 2002; Romero et al., 2017a; Servili et al., 2016), whereas in processed table olives, almost no oleuropein is found, with hydroxytirosol and tyrosol being the predominant phenolics (Romero et al., 2004). Indeed, table olive processing dramatically reduces the total amount of phenolic compounds and substantially changes the profile. Nevertheless, there are significant differences among the different processing methods. Generally, the total phenolic contents in natural olives and in green treated olives are greater than those in black-ripe olives (Romero et al., 2004).

Fresh olives are rich in triterpenic acids, primarily maslinic and oleanolic acids (1500–3000 mg/kg) (Alexandraki et al., 2014; Medina et al., 2012; Romero et al., 2010), which are mainly concentrated in the skin (Romero et al., 2017a). Although there are significant losses during processing, table olives, especially natural black olives, exhibit greater amounts of these compounds than does olive oil. Many health-promoting effects have been described for the olive triterpenic acids, including anti-cancer, anti-oxidant, anti-microbial and anti-hyperglycemic activities (Horiuchi et al., 2007; Juan et al., 2008; Lozano-Mena et al., 2014; Tsai and Yin, 2012).

Lately, special attention has been paid to phytoprostane (PhytoPs), a bioactive compound with effects on the regulation of immune function and with anti-inflammatory and apoptosis-inducing activity. It has recently been reported in fresh olives and Spanish-style green olives (Collado-González et al., 2015).

The high content of α-tocopherol in table olives (Malheiro et al., 2012; Sakouhi et al., 2008) reinforces the nutritional value of this product because this substance provides protection from free radicals (Cheeseeman and Slater, 1993; Kamal-Eldin and Andersson, 1997) and prevents cancer and arteriosclerosis (Armstrong et al., 1997; Caruso et al., 1997; Nicolaiew et al., 1998).

Table olives are not only an important source of bioactive compounds, as described above; since they are fermented products, they are also potential functional foods as carriers of probiotic lactic acid bacteria (Argyri et al., 2013; Peres et al., 2014a). Although dairy products are still the most common probiotic food products, there is an increasing interest for other food matrices such as fruits and vegetables among which table olives are very promising (Peres et al., 2012).

2.1.5. Organoleptic quality

The first standardized Method for Sensory Analysis of Table Olives is quite recent. It was proposed by the International Olive Council in 2008 and revised in 2011 (IOC, 2011). The attributes assessed in this standard are negative, gustatory and kinesthetic sensations. The negative attributes considered are abnormal fermentation, musty, rancid, cooking effect, metallic, earthy and winey–vinegary. Gustatory attributes are salty, bitter and acid. Kinesthetic sensations are related to the texture of the fruit, and the attributes assessed are hardness, fibrousness and crunchiness.

Trained panel tasters score the intensity of the mentioned attributes on a scale ranging from 1 (no perception) to 11 (extreme). The trade category quality classification is composed of the categories Extra or Fancy, First, Choice or Select, Second or Standard, and Olives that may not be sold as table olives, depending on the intensity of the defect that is predominantly perceived.

This method is being adopted by the table olive industry and by many researchers (Catania et al., 2015; Lanza et al., 2010). Nevertheless, some authors suggest, as an alternative to sensory panels, objective instrumental methodologies, such as the electronic tongue, which has been already used to assess table olive defects (Marx et al., 2017a) and gustatory attributes (Marx et al., 2017b).

2.2. Olive oil quality attributes

2.2.1. Legal definition of olive oil

According to the European Community (EEC Reg. 2568/91 and EEC Reg. 2015/1830) and the International Olive Council (T.15/NC No 3/ Rev. 11, July 2016), olive oil is the natural juice obtained exclusively from the fruits of the olive tree (Olea europaea L.), with exclusion of those oils extracted using solvents or via re-esterification processes (European Communities, 2000; European Commission, 2015; IOC, 2015a). The IOC classification includes virgin olive oil (VOO), refined olive oil, olive oil and olive pomace oil. Virgin olive oil is the oil obtained solely by mechanical or other physical means without application of thermal conditions that lead to alterations in the oil composition. Additionally, any treatment in the extraction protocol other than washing, decantation, centrifugation or filtration must be discarded. Virgin olive oil can be split into the following three categories that can be consumed directly:

Extra virgin olive oil (EVOO), which has a free acidity, expressed as oleic acid, of not more than 0.8 g per 100 g, and the other characteristics of which correspond to those fixed for this category in this standard.

Virgin olive oil, which has a free acidity, expressed as oleic acid, of not more than 2 g per 100 g and the other characteristics of which correspond to those fixed for this category in this standard.

Ordinary virgin olive oil, which has a free acidity, expressed as oleic acid, of not more than 3.3 g per 100 g and the other characteristics of which correspond to those fixed for this category in this standard. This designation may only be sold direct to the consumer if permitted in the country of retail sale. If not permitted, the designation of this product shall comply with the legal provisions of the country concerned.

Another class of olive oil that can be consumed is refined olive oil. The refined olive oil is the olive oil obtained from low quality virgin olive oils by refining methods which do not lead to alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3 g per 100 g and its other physico–chemical and organoleptic characteristics correspond to those fixed for this category in this standard. This product may only be sold direct to the consumer if permitted in the country of retail sale.

Refined olive oil can be mixed with virgin olive oils for consumption. This category, named olive oil has a free acidity, expressed as oleic acid, of not more than 1 g per 100 g and its other physico–chemical and organoleptic characteristics correspond to those fixed for this category in this standard.

The principal quality criteria for those categories according to the IOC documentation (IOC, 2015a) are listed in **Table 1**. This table only includes those categories that are suitable for consumption.

This review is focused on the quality concept, and for this reason, it exclusively considers VOO categories, with a special emphasis on EVOO and VOO.

Table 1. Principal quality criteria for virgin olive oil categories, refined olive oil and olive oil according to the IOC documentation (IOC, 2015a).

	Extra virgin olive oil	Virgin olive oil	Ordinary virgin olive oil	Refined olive oil	Olive Oil (ROO+VOOs)
1- Organoleptic characteristics					
- odour and taste				acceptable	good
- odour and taste (on a continuous scale):					
- median of defect	Me = 0	0 < Me ≤ 3.5	3.5 <Me ≤ 6.0**		
- median of the fruity attribute	Me > 0	Me > 0			
- colour				Light yellow	light, yellow to green
- aspect at 20ºC for 24 hours				limpid	limpid
2 - Free acidity. % m/m expressed in oleic acid	≤ 0.8	≤ 2.0	≤ 3.3	≤ 0.3	≤ 1.0
3 - Peroxide value in milleq. Peroxide oxygen per kg/oil	≤ 20	≤ 20	≤ 20	≤ 5	≤ 15
4 - Absorbance in ultra-violet ($K_{1cm}^{1\%}$)					
270 nm (cyclohexane) / 268 nm (iso-octane)	≤ 0.22	≤ 0.25	≤ 0.30	≤ 1.25	≤ 1.15
- ΔK	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.16	≤ 0.15
- 232 nm*	≤ 2.50**	≤ 2.60**			
5 - Moisture and volatile matter (% m/m)	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.1	≤ 0.1
6 - Insoluble impurities in light petroleum % m/m	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.05	≤ 0.05
7 - Flash point	-	-	-	-	-
8 -Trace metals mg/kg					
Iron	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0
Copper	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1
9 - Fatty acid ethyl esters (FAEEs)	≤ 35 mg/kg				

This determination is solely for application by commercial partners on an optional basis

** Commercial partners in the country of retail sale may require compliance with these limits when the oil is made available to the end consumer.

2.2.2. Chemical composition and bioactivity

Olive oil is basically composed of two fractions: the saponifiable and unsaponifiable fractions. The saponifiable fraction represents approximately 98% of the oil weight. It is mainly formed by fatty acids (esterified to glycerol) and other minor components, such as free fatty acids, phospholipids, waxes, and sterol esters. On the other hand, the unsaponifiable fraction, which represents approximately 2% of the total weight, encompasses a complex set of minor compounds pertaining to chemical families such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, phenols, tocopherols, esters, and pigments and volatile components such as aldehydes, ketones and alcohols (Dabbou et al., 2009; Guillén et al., 2009; Rjiba et al., 2011; Servili et al., 2013).

Table 2. Fatty acids composition limits adopted by the IOC (IOC, 2015a).

Fatty Acid	Formula	Concentration (%)
Myristic acid	C14:0	< 0.03
Palmitic acid	C16:0	7.50 - 20.00
Palmitoleic acid	C16:1	0.30 - 3.50
Heptadecanoic acid	C17:0	< 0.30
Heptadecenoic acid	C17:1	< 0.30
Stearic acid	C18:0	0.50 - 5.00
Oleic acid	C18:1	55.00 - 83.00
Linoleic acid	C18:2	3.50 - 21.00
Linolenic acid	C18:3	< 1.00
Arachidic acid	C20:0	< 0.60
Gadoleic acid	C20:1	< 0.40
Behenic acid	C22:0	< 0.20
Lignoceric acid	C24:0	< 0.20

Because VOO is extracted only via physical methods without increasing its temperature or using chemical solvents, it especially preserves the concentration of minor compounds. **Table 2** lists the fatty acid compositional limits adopted by the International Olive Council (IOC, 2015a). MUFAs are the predominant fatty acids in olive oil, with oleic acid being the most abundant (55–83%) (Al-Bachir and Sahloul, 2016). As previously mentioned, the monounsaturated profile of fatty acids is one of the factors that contribute to explain the healthy benefits of olive oil in the Mediterranean Diet. Apart from oleic acid, it is worth mentioning another concentrated fatty acid, linoleic acid, which is an essential

polyunsaturated fatty acid (PUFA) of nutritional interest (Piroddi et al., 2016; Schwingshackl and Hoffmann, 2014; Vannice and Rasmussen, 2014).

Phospholipids (PLs) are found in small quantities in olive oil (typically < 150 mg/kg), but they play an important role in the syntheses of PUFAs, and they play a key role in membrane cell construction and the transmission of signals between cells (Alves et al., 2016; Boukhchina et al., 2004; Küllenberg de Gaudry et al., 2012). VOO and EVOO do not contain waxes because they are not extracted via mechanical processing. Therefore, the concentration of waxes and wax esters may be an indicator for detection of fraudulent mixtures (Giuffrè, 2013; Mailer et al., 2010). One other chemical family used for fraud detection is phytosterols, which are also nutritionally interesting since they contribute to reducing total cholesterol and LDL-cholesterol in blood (Hassanein et al., 2016; St-Onge et al., 2003; Vivancos and Moreno, 2008). Among olive oil phytosterols, it is worth mentioning sitosterol, campesterol, stigmasterol, avenasterol and stigmastadienol.

Olive oil is also a reliable source of α-tocopherol, a molecule with vitamin E activity. The concentrations of tocopherols found in olive oil range from 10 to approximately 350 mg/kg. α-Tocopherol is one of the most important lipophilic minor compounds found in olive oil owing to its antioxidant activity contributing to health benefits and olive oil shelf-stability. Nevertheless, the presence of this compound is not exclusive to olive oil. Other refined oils, such as sunflower oil, are characterized by higher levels of tocopherols (Ambra et al., 2016; Parveen et al., 2015; Psomiadou and Tsimidou, 1999).

The main triterpenes present in olive oil are oleanolic acid, ursolic acid, maslinic acid, uvaol, and erythrodiol. Several authors have reported that the triterpenes concentration can reach levels greater than 100 mg/kg (Abdallah et al., 2015; Allouche et al., 2009; FernándezHernández et al., 2015). As previously mentioned for table olives, bioactive properties of triterpenes have been reported (Martín et al., 2009; Sánchez-Quesada et al., 2015, 2013). Squalene is a natural polyunsaturated triterpene and the major hydrocarbon found in olive oil, making up more than 90% of this fraction. Squalene is essential for the biosynthesis of steroids and triterpenes and, at the same time, constitutes an intermediate in the biosynthesis of phytosterols. Furthermore, several lines of evidence highlight the numerous benefits of squalene to human health, such as anticancer, antioxidant and cardioprotective activities (Cárdeno et al., 2015; Owen et al., 2000; Popa et al., 2015; Salvo

et al., 2017; Sánchez-Fidalgo et al., 2015). Some authors also suggest that squalene plays an important role in the management of inflammatory conditions (Cárdeno et al., 2015). On the other hand, squalene does directly influence olive oil stability, because its chain-breaking ability contributes to regenerate α-tocopherol (Psomiadou and Tsimidou, 1999; Velasco and Dobarganes, 2002).

The most important classes of pigments found in olive oil are carotenoids and chlorophyll derivatives. Those compounds are responsible for the olive oil color, where chlorophylls are associated with the green color and carotenoids with the yellow/orange color (Portilla et al., 2014). Pigments, especially carotenoids, are associated with immune, endocrine and metabolic benefits owing to their pro-vitamin A activity (Rao and Rao, 2007). The concentration of pigments typically ranges from a few mg/kg to approximately 100 mg/kg, and they can be associated with the age and storage conditions, in addition to the authenticity and quality of the olive oil (Ferruzzi and Blakeslee, 2007; Gandul-Rojas and Minguez-Mosquera, 1996; Lazzerini and Domenici, 2017).

Volatile compounds found in olive oil can be grouped into alcohols, aldehydes, esters, ketones, sulfur compounds and terpenes (Procida et al., 2016). These compounds are synthesized through different pathways, with some of them activated during the growing of the fruit and others during and after the extraction of the olive oil by several enzymes (such as lipoxygenase and alcohol dehydrogenase), and by oxidation reactions during storage. Furthermore, the concentration of specific volatiles reveals the degradation or the authenticity of olive oil (Guclu et al., 2016; Sghaier et al., 2016).

Finally, one of the most relevant chemical families of compounds found in olive oil is that of phenolic compounds owing to the substantial number of research studies dedicated to them. Several authors have reported the importance of phenolic compounds as antioxidants and nutraceutical components (Bennett and Hayes, 2012; Britti et al., 2012; Bulotta et al., 2014; Covas, 2008; Martínez-González et al., 2014). Additionally, they play an important role in protection of olive oil from oxidation, making it more stable and resulting in a longer shelf life (Caponio et al., 2001; Farhoosh and Hoseini-Yazdi, 2013; Silva et al., 2010). Most phenolic compounds identified and quantified in olive oil belong to five different classes: phenolic acids (especially derivatives of benzoic and cinnamic acids), flavonoids (luteolin and apigenin), lignans (pinoresinol and acetoxypinoresinol), phenyl-

ethyl alcohols (hydroxytyrosol, tyrosol) and secoiridoids (aglycone derivatives of oleuropein and ligstroside). Within the great variability of phenolic groups, the role of secoiridoids as conjugated forms of hydroxytyrosol and tyrosol is worth noting. This group of compounds represents the most concentrated phenolic family in olive oil and is being widely studied due to their promising healthy properties (Bendini et al., 2007; Del Rio et al., 2016). This group of compounds, which are specific of the Oleacea family and few others, includes oleuropein and ligstroside aglycone isomers and the decarboxymethylated dialdehyde forms of oleuropein and ligstroside aglycones, which are more frequently referred to as oleacein (3,4-DHPEA-EDA) and oleocanthal (p-HPEA-EDA), respectively.

The most significant interest in phenolic compounds was spurred by the European Food Safety Authority's (EFSA) scientific opinion that led to approving the health claim included in the EU432/2012 Commission Regulation. The health claim state that "olive oil phenols contribute to the protection of blood lipids from oxidative stress". This claim can be included on the label only when the olive oil contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil (Casini et al., 2014; EFSA, 2011; Martín-Peláez et al., 2013).

2.2.3. Organoleptic quality

The international method for the organoleptic assessment of VOO has been proposed by the IOC (T.20/Doc. No 15/Rev. 8, November 2015). This method is only applicable to VOOs and is based on the intensity of the defects and attributes perceived by a group of tasters selected, trained and monitored as a panel. The main positive attributes are fruity, bitter and pungent while defects include fusty, musty, winey, acid, rancid and wet wood, among others (IOC, 2015b).

The sensory attributes of olive oil are ascribed to the strong stimulation of human sensory receptors by both volatile and non-volatile compounds. Non-volatile components, particularly phenols, stimulate the tasting receptors and the free endings of trigeminal nerves, thereby eliciting the bitterness perception, pungency and astringency (Bendini et al., 2007; Servili et al., 2004). These characteristics of olive oil are considered positive attributes (Bendini et al., 2007; Ceci et al., 2017; Peyrot des Gachons et al., 2011).

On the other hand, volatile components stimulate the olfactive receptors and provide positive or negative attributes to olive oil. Complete reviews regarding the volatile composition of olive oil have been presented, and it is possible to find lists of detected volatile compounds and, interestingly, their sensory attributes in the literature (Angerosa et al., 2004; Kalua et al., 2005). The C6 and C5 compounds, with a special emphasis on C6 linear unsaturated and saturated aldehydes (e.g., hexanal, cis/trans-hexenal, hexanol, hexenol, acetate esters, pentenal, pentenol), represent the most important fraction of volatile components, in quantitative terms, of virgin and extra virgin olive oils. The "pungent-sweet-floral", "floral", "cooked-caramel", "greenapple-cut", "grass", "citrus", "paperlike-fatty-sharp-cut" attributes are some of the principal positives attributes associated with these compounds. However, some other pathways are responsible for the negative attributes. These compounds are formed by sugar fermentation (winey), amino acids (leucine, isoleucine and valine) conversion (fusty), enzymatic activities of molds (musty), anaerobic microorganisms (muddy), and auto-oxidative processes (rancid) (Procida et al., 2016).

The contribution of volatile compounds to the overall aroma of virgin olive oil depends not only on their concentration but also on their sensory threshold values (Angerosa et al., 2004; Kalua et al., 2005). In addition, antagonism and/or synergism among different molecules can occur and affect the final flavor of olive oil. Chemical aspects of molecules (volatility, hydrophobic character, size, shape, and conformational structure) and the type and position of functional groups affect the sensory threshold value and, therefore, the odour and taste intensity. These aspects all contribute to favor interaction with receptor proteins and, for this reason, are more important than the concentration levels. Thus, highly concentrated volatile compounds are not necessarily the major contributors of odour (Angerosa et al., 2004; Essid et al., 2016).

2.2.4. Fruit texture and olive oil extraction

Both the quality and extraction efficiency of VOO are directly related with the composition and the texture of olive fruits used mainly for oil production. Huge differences exist in the texture of olive fruits due to several factors such as the cultivar, irrigation, harvesting time, fertilization techniques, pesticide treatments and sanitary status, among others. The oil content expressed in terms of fresh weight varies between

10 and 35% while the moisture content ranges from 40 to 75% (Cruz et al., 2007; Torres-Vila et al., 2003; Vossen, 2005). In the oil extraction process those pastes that are obtained from fruits with high moisture levels (>50%) and low non-fat dry matter (around 25%) are termed "difficult pastes". The negative aspect of these pastes is the formation of emulsions between oil and water that lead to low extractability because part of the oil is lost with residues. Generally, the moisture level in pastes depends essentially on the cultivar and harvesting time (the moisture is higher in low ripeness levels), but also it is worth considering the addition of extra water (Cert et al., 1996; Peres et al., 2014b; Vossen, 2005). Water addition can be minimized by the use of processing aids during malaxation that are able to break the emulsion to enable the separation of the oil. The micronized talc (hydrated magnesium silicate with particle size lower than 40 mm) is commonly added at a concentration of 1% (w/w). Talc absorbs water and reduces the emulsifiers at the surface of oil droplets, thus facilitating the droplet coalescence into a continuous oil phase (Canamasas and Ravetti, 2014; Koprivnjak et al., 2016). The addition of talc as a processing aid has been authorized and regulated by the Spanish Ministry of Health due to its exclusive physical action that does not affect the restrictions of EC regulation (Ambien, 2011). Furthermore, several natural enzymes, namely, hemicellulases, cellulases and pectinases, can also be used to avoid the formation of emulsions by degrading the walls of the oil bearing cells. However, the addition of adjuvants with biochemical action is not currently allowed by the EU legislation (European Commission, 2004; Ranalli et al., 2003).

2.2.5. Storage process of olive fruits and olive oil

The quality of olive oil is related to not only the treatment and storage of the olive fruit before processing (see Section 5.8.) but also with the storage conditions of the olive oil before consumption. Olive oil extracted from degraded fruits is usually characterized by high acidity, low stability and undesirable sensorial attributes (Gutie et al., 1996; Clodoveo et al., 2007).

The principal causes for the deterioration of olive oil during storage are oxidation and hydrolysis reactions and the products of these reactions. Oxidation of lipids is promoted by several factors, such as light, temperature, metals, concentration of pigments, unsaturated fatty acid composition, levels and types of natural antioxidants and

amount of sterols. Lipid oxidation produces hydroperoxide molecules to form volatile compounds that contribute to the typical undesirable oil defects "rancid", "cucumber" and "muddy sediment" (Angerosa et al., 2004; Méndez and Falqué, 2007). Additionally, the levels of physicochemical quality parameters of olive oils, such as the ultra-violet light absorption extinction coefficients (K_{232} and K_{270}), peroxide value (PV) and free acidity (FA), may increase significantly during the storage of oil (Abbadi et al., 2014; Jabeur et al., 2015); in contrast, the oxidative stability (OS) decreases (Stefanoudaki et al., 2010).

The physicochemical traits of olive oils that are going to be packed or stored are directly related to the subsequent quality changes. First, non-filtered oil is more unstable than a filtered oil, which is justified because non-filtered oils contain solids (fruit pulp particles) and water in suspension, which promote the fermentation process and enzymatic reactions and are responsible for unpleasant odors (Lozano-Sánchez et al., 2010). In contrast, the main factors contributing to the oxidative stability of the olive oil are the ratio of MUFAs to PUFAs (M/P ratio), tocopherol content and phenolic levels. The M/P ratio ranges from 4.0 to 9.2 and can be an appropriate measure of the tendency of olive oil to undergo autoxidation. Higher ratios correspond to a higher oxidative stability of the olive oil. Tocopherols and phenolics, with a synergic activity, are the most important antioxidant compounds found in olive oils. During the storage time, a noticeable decrease in the levels of secoiridoid derivatives, which are considered the most relevant antioxidant phenols in olive oil (Hachicha Hbaieb et al., 2016, 2015), is observed.

3. Physiological mechanisms affecting quality

3.1. Ripening physiology and quality: general overview

Ripening is defined as the final transformation of physiologically mature fruit. The completion of fruit, seed and embryo growth is related to fruit maturation and precedes ripening. Ripening changes also trigger natural fruit abscission. Maturity indices (MI) try to relate ripening stages to the time of harvest maximizing fruit size, oil yield and quality in the context of efficient orchard management. **Fig. 1** represents the evolution of fruit size, oil content, sugar content and natural fruit abscission during olive fruit growth and ripening.

Table 3. Changes in physicochemical other traits and sensorial characteristics during fruit ripening.

Characteristics	Changes	References
Color	MI* from green (MI 0) to veraison (MI 2-3) to black MI >4). Differences between cultivars and crop.	Beltrán et al. (2017)
Pigments	Chlorophyll and carotenoids disappear. Anthocyanins appear.	Beltrán et al. (2017), Tombesi (2003)
Firmness	Associated to peptic compounds. Anhydrous-galacturonic acid disappear with the advance of ripening. Changes associated to increase in softness starting at veraison (MI 2-3).	Tombesi (2003)
Respiration	Minimum value at veraison (MI 2-3)	(Beltrán et al., 2017)
Oil content	Sigmoid asymptotic accumulation from pit hardening (45 DAFB) to initial veraison (MI 2), i.e. 150 - 180 DAFB depending on cultivar and cropping. Then oil content stabilizes.	Beltrán et al. (2017), Tombesi (2003), Trapani et al. (2016)
Time form Full Bloom to Veraison (MI 2-3)	Dependent from cultivar, temperature, water status and cropping. Thermal time may be a predictor between years.	Beltrán et al. (2017), Tombesi (2003)
Natural fruit abscission	Fruit retention force (FRF) avoids abscission before version (MI 2-3) then percentage of FRF decreases and fruit abscission progresses. Harvest should start before a threshold of fruit abscission (FATH) between 5-10%.	Beltrán et al. (2017), Humanes (1975)
Sensorial attributes	Bitter and pungency associated to oleuropein and total phenols. Peaks of volatiles appear at different times during ripening promoting different sensorial perceptions (See section 1biii). For instance, to trans-2-hexanal peak is associate to MI 1 early harvest).	Tombesi (2003)
Autoxidation and shelf life	See 2.2.4.	

* Maturity Index (MI): 0. Skin intense green; 1. Skin green to yellow; 2. Skin with reddish-purple spots; 3. Skin mostly purple; 4. Skin black and flesh white; 5. Skin black and flesh partially purple; 6. Skin black and flesh mostly purple; 7. Skin black and flesh fully purple (Uceda et al. 1980).

Fruit ripening is associated with deep changes in color, firmness and composition of the fruits and therefore affects quality. The main changes in physicochemical and sensorial properties (**Table 3**) and in virgin olive oil compounds (**Table 4**) that take place during olive fruit ripening are summarized in this section.

3.2. Defining the optimal time of harvesting to obtain quality.

From the horticultural point of view, the changes associated with ripening determine the optimum time of harvesting, which depends on the final marketable products. Generally, in olive growing, maturity indices (MI) and the oil content expressed in dry and fresh weight are the most widely used variables.

The time of harvest of table olives is related to the processing methods according to the standards procedures established by the International Olive Council (IOC, 2004) (see Section 2.1.). For Spanish style green olives, the time of harvesting is determined by the change of external color from intense green to yellow green (MI = 1, **Fig. 1**) which usually occur at the time of maximum seed and embryo size (approximately 150 days after full bloom (DAFB)) (Rapoport and Moreno-Alías, 2017). Fruits reach maximum size at this stage, and the pulp is separated easily from the stone, which avoid an excessive bruising damage. Harvesting of black ripe or Californian-style can be delayed until the change of color in the skin, but the proportion of fruits in this state should not exceed 20%. Naturally black or Greek style olives can be harvested later, although it's recommended not to delay it much avoiding the loss of texture (Sánchez Gómez and García, 2017).

For oil-processed olives, harvesting starts after a purple external color is achieved (MI = 2-3, **Fig. 1**) and extends for several months in traditional manually harvested depending on the availability of manpower. Since the beginning of mechanical harvesting, the harvest period has been drastically shortened, which allows advancing the time of harvest. The following criteria determine the optimal harvest period: maximum oil accumulation; a threshold for fruit retention force associated with the efficiency of the harvester; and a maximum threshold for natural fruit abscission, all of which are related to the process of ripening and the cultivar (Beltrán et al., 2017; Tombesi, 2003; Trapani et al., 2016). Furthermore, the capacity to harvest olive mechanically in a short time has allowed paying attention to the quality of the oil, which has become a new criterion for determining the time of harvest.

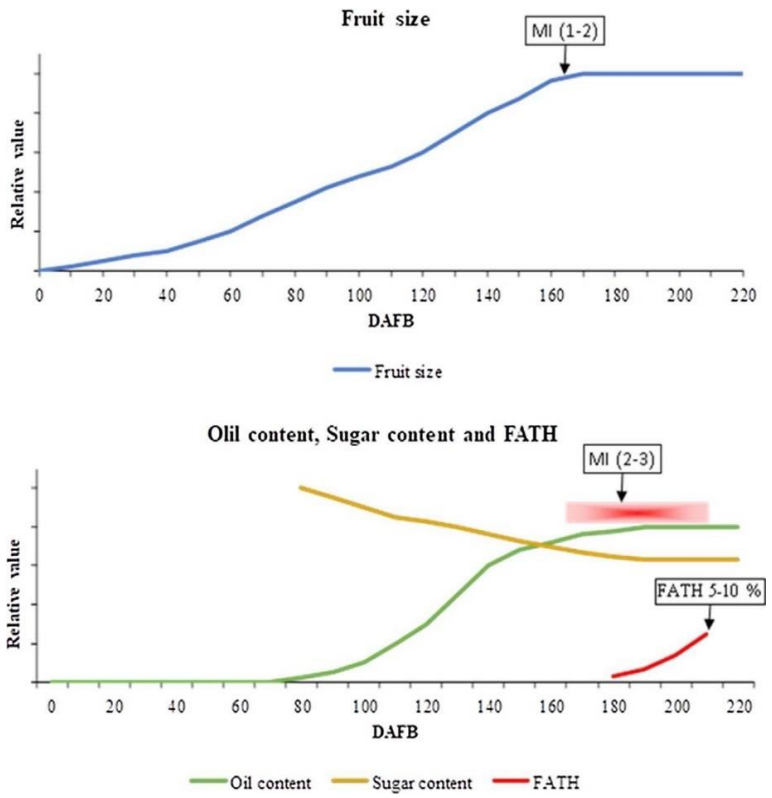

Fig. 1. Relative size, relative oil and sugar content, and natural fruit abscission (%) related to the optimum harvest period (Bar). Elaborated from data from Beltrán et al., 2017; Humanes, 1975; Tombesi, 2003; Trapani et al., 2016.
DAFB – Days after Full Bloom, MI – Maturity Index (Uceda et al., 1980),
FATH – Fruit Abscission Threshold: Maximum value 5–10%. Bar – Harvest Period.

Many experimental works have been done in different countries trying to determine the optimal period for harvesting. The reported values in these works changed according to cultivar, site, year and growing practices (Ben Youssef et al., 2010; Bodoira et al., 2015; Dag et al., 2014, 2011; Jimenez et al., 2014; Lazzez et al., 2011; Romero et al., 2002; Salvador et al., 2001; Trapani et al., 2016). For instance, the MI recommended ranges for 'Picudo' and 'Hojiblanca' in Cordoba (Spain) are 2.8–3.1 and 2.7–4.2, respectively (Jimenez et al., 2014); for 'Arauco' in Rioja (Argentina), the range is 1–2 (Bodoira et al., 2015); for 'Barnea', 'Coratina' and 'Picual' in the Jordan Valley (Israel), the ranges are 2.1–2.5, 2.3 and 3.2, respectively (Dag et al., 2011); for 'Chemlali' in Sfax (Tunisia), the range is 2.5–3.5 (Lazzez et al., 2011); and for 'Cornicabra' in Toledo (Spain), the range is 3.0–4.5 (Salvador et al., 2006).

Within the period of harvesting, the physicochemical and sensorial attributes that enable specific organoleptic and nutraceutical profiles of virgin olive oils (**Tables 3 and 4**) are analyzed to advance or delay the date of harvesting within the harvest period (**Fig. 1**). As a rule, the farmer first guarantees the maximum olive oil yield and then specific virgin olive oil profiles. The oil content expressed on a dry weight basis is generally stable after veraison (MI 2–3) whereas the oil content expressed in fresh weight changes due to differences in water content. Fatty acid composition profiles exhibit little changes, but minor compounds may change within the cultivar in a different manner; i.e., there is an interaction between the cultivar and MI for many minor compounds of the virgin olive oil. Usually, late harvesting results in oils with the lowest hydrophilic phenols concentration and aromatic profile due to minor LOX activity produced from overripe olives (Servili et al., 2015). Increase of free acidity and decrease of the chlrorophyll content, the M/P and saturated/unsaturated fatty acid ratios, and the fruitiness perception in sensorial analyses, have also been reported (Dag et al., 2011; Salvador et al., 2001). In contrast, early harvesting, when oil content is still increasing, usually results in oils with higher phenols content, which contributes to the level of bitterness and pungency and the stability to oxidation, although the level of these positive attributes may be in some cases excessive and unacceptable by consumers (Dag et al., 2011).Therefore, the optimal MI score for harvest should be complemented with analytical data of the compounds determining the farmer's target quality for their virgin olive oil. In fact, all of the

experiments described above represent empirical approaches to establishing a safe farming strategy that balances the oil quantity and desired quality.

Table 4. Changes in chemical compounds during fruit ripening.

Compound group	Observations	References
Fatty acids	During fruit growth, the amount of all fatty acids increases. After veraison no significant changes in oil content and in fatty acids occur. Eventually small changes in oleic and linoleic are observed. Differences in oil content, fatty acid and triacylglycerol profiles are genotype dependent. Standardizing the time of maturity for sampling allows the selection of genotypes in breeding programs.	Beltrán et al. (2017), De la Rosa et al. (2013), Famiani et al. 2002), Sánchez de Medina et al. (2015a), Vekiari et al. (2010), Yorulmaz et al. (2013)
Tocopherol's	α-Tocopherol is associated with antioxidant capacity and both variables decrease during fruit ripening (FR). γ-Tocopherol increases during FR	Baccouri et al. (2008), Beltrán et al. (2010), Georgiadou et al. (2016)
Phytosterols	β-Sitosterol decreases from September to November while δ-5-avenasterol increases with the maturation. The most important variables for differentiating fresh oils according to degree of ripening were δ-7-campesterol/β-sitosterol, uvaol/stigmasterol, clerosterol/δ-5-avenasterol and sitostanol/uvaol.	Fernandez-Cuesta et al. (2013), Gutiérrez et al. (2000), Lukić et al. (2013), Yorulmaz et al. (2013)
Triterpenes	Triterpenoids represent the major triterpenic compounds of the fruit. Triterpenic diols were replaced by triterpenic acids during ripening. Maslinic acid is the main triterpenoid, only accompanied by oleanolic acid in the fruit and decreased during ripening Squalene, a polyinsaurated triterpene, increased from fruit green to veraison and then stabilizes in correspondence to the maximum oil content	Fernandez-Cuesta et al. (2013), Guinda et al. (2010), Stiti et al. (2007)
Volatiles	Volatile compounds appear during ripening and are associated with different fruit sensorial attributes. The volatile profile is cultivar dependent and has been used for cultivar discrimination.	Angerosa and Basti (2001), Aparicio and Morales (1998), Cevik et al. (2014), Dhifi et al. (2005), Gomez-Rico

Compound group	Observations	References
Phenols	Hexanal and hexyl acetate-both produced from linoleic acid-are major contributors to the ripeness characterization. The most important contributors to olive oil aroma are C-6 aldehydes, alcohols, and esters. Specifically, the concentration of total esters, carbonyl, C6 and C5 increases or decreases with the ripening degree according to the cultivars, site and available water in the orchard.	et al. (2006), Hbaieb et al. (2017), Kalua et al. (2005), Toker et al. (2016), Tombesi (2003)
	During ripening the profile of phenolic compounds of different cultivars undergo wide modifications that strongly influence sensorial attributes, shelf life and nutritional value if olive and olive oil.	Alagna et al. (2012), Amiot et al. (1989), El Riachy et al. (2011), Gomez- Rico et al. (2006), Servili, (2014), Servili et al. (2016), Tombesi (2003), Tombesi et al. (2009)
	Total phenolic concentration decreased during fruit development and maturation.	
	Very large differences and peculiar trend between cultivars in the content of oleuropein, demthyloleuropein, ligstroside, tyrosol, hydroxityrosol, verbascoside and lignans have been reported. Oleuropein is the major component of many cultivars and decline from green maturation to black ripen fruit. Degradation of oleuropein during ripening is concomitant with the accumulation of dimethyl oleuropein and total phenols.	
Pigments	Chlorophyll and carotenoids disappear. Anthocyanins appear at veraison.and increses afterwards	Tombesi (2003)

3.3. Secondary metabolic pathways associated with sensory or bioactive quality attributes.

As previously explained, the detection of quality attributes in olive fruit and oil is directly associated with molecules involved in secondary metabolic pathways. Therefore, it is key to understand the biosynthetic pathways involved in the formation of phenols and volatile compounds, in addition to the main factors contributing to regulate them. Two main pathways have been proposed for the biosynthesis of secoiridoids in olive fruits, both ending in the synthesis of oleuropein, even though this glycoside phenol is rarely detected

in olive oil. These two pathways are mechanistically diverse. The first pathway, proposed in 1993 by Damtoft et al. (1993), is initiated by mevalonic acid for the formation of iridoids and then to ligstroside, which is the precursor of oleuropein. An alternative pathway was proposed in 2002 by Ryan et al. (2002), with tyrosol as a precursor produced via phenylpropanoid biosynthesis. In this second mechanism, tyrosol is the substrate of two synthetic schemes that led to a common final product, oleuropein. The first scheme deals with ligstroside as intermediate, whereas the second one goes through oleacein and oleuropein aglycone before its final conversion into oleuropein. Both synthetic pathways would occur at the first ripening stages to accumulate oleuropein in the fruit.

At advanced maturation and especially during olive oil extraction, enzymatic and non-enzymatic bio-transformations are produced to form the secoiridoid derivatives present in olive oil. The most complete biotransformation pathway was proposed by Obied et al. (2008), who explained the appearance of secoiridoid derivatives from oleuropein as a precursor. In this pathway, oleuropein is converted into several derivatives according to the involvement of two enzymes, esterases and β-glucosidases. Depending on the reaction mechanism, oleuropein can be mainly converted to oleuropein aglycone isomers or to oleacein via the following two main routes. The most important difference between the routes is the typology of the enzymes involved. Thus, the formation of oleuropein aglycone isomers is performed by β-glucosidase, which is activated during crushing and malaxation. The second route is characterized by the involvement of methylesterases to cleave the methyl group part of the elenolic acid. This second route would be responsible for the formation of oleacein. The same conversions would occur for ligstroside to preferentially produce ligstroside aglycone isomers and oleocanthal. The genotype and agronomical and technical factors are critical to obtain a particular phenolic profile since a variation in them could favor the kinetics of certain enzymatic processes.

Volatile compounds are rapidly formed during VOO extraction because of enzymatic activities included in the lipoxygenase (LOX) pathway. The LOX pathway encompasses a set of endogenous enzymes that use lipids as substrates to activate a series of events that lead to volatile compounds responsible for the positive attributes detected in olive oil (e.g., fruity, mature, apple, almond). As previously mentioned, the positive attributes are strongly associated with the C6 and C5 compounds, especially with C6 linear unsaturated

and saturated aldehydes. In contrast, volatile components such as C7-C11 monounsaturated aldehydes, C6-C10 dienals, C5 branched aldehydes and alcohols or some C8 ketones can reach relatively high concentrations in olive oils that are characterized by organoleptic defects or off-flavors.

As **Fig. 2** shows, C6 and C5 volatiles are enzymatically produced from PUFAs through the LOX pathway, in which the activity of the involved enzymes influences the concentrations of compounds. The pathway consists of the formation of 9 and 13-hydroperoxides from linoleic and linolenic acids with subsequent cleavage by specific hydroperoxide lyases to produce C6 aldehydes, namely, hexanal, cishexenal and trans-hexenal. Alcohols, primarily hexanol and hexenol isomers, are synthesized via the reduction of C6 aldehydes by alcohol dehydrogenase enzyme. In the same manner, alcohols can lead to acetate esters by alcohol acetyl transferases.

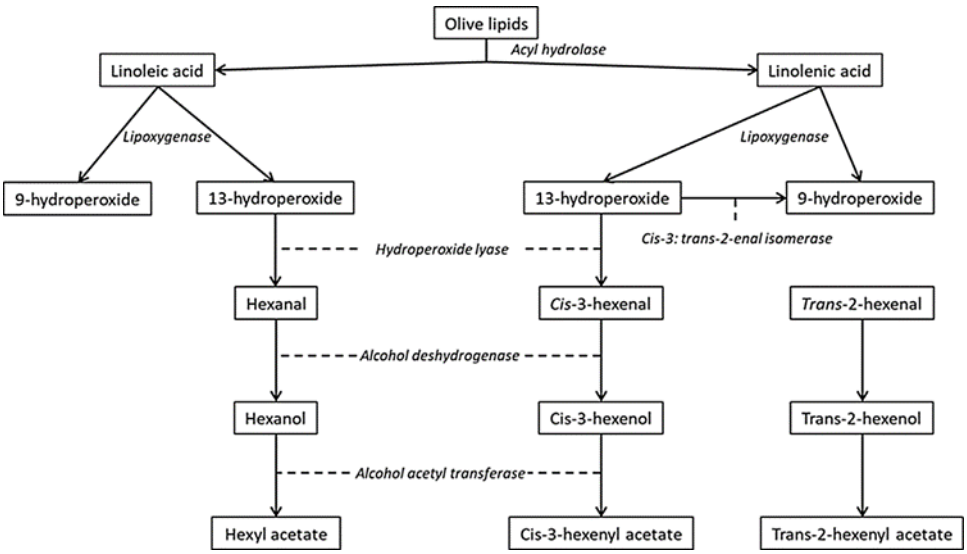

Fig. 2. Pathway for the formation of major volatile compounds in virgin olive oil and enzymes involved in the production (adapted from Kalua et al., 2005, with permission of Elsevier).

An additional pathway for linolenic acid as substrate is the formation of 1,3-pentene radicals that can dimerize to C10 hydrocarbons or react with hydroxy radicals to form C5 alcohols, which can be oxidized enzymatically into C5 carbonyl compounds such as 2-pentenal or 1penten-3-one (Angerosa et al., 2004).

As an example, water stress conditions stimulate the synthesis of phenolic compounds in olive fruits but exert a negative influence on volatile compounds related to the LOX pathway (Servili et al., 2007).

4. Breeding for olive quality: the genotypic influence

Cultivated olive still mostly consists of a broad diversity of traditional and clonally propagated cultivars, which are presumably very old and of uncertain pedigree (Diez et al., 2015; Trujillo et al., 2014). These traditional cultivars have been the foundation for the solid and extensive rainfed olive growing system that has developed over centuries.

However, lately, olive cultivation has been changing radically. The yield has risen owing to increasing irrigation and high-density planting. Accordingly, most new olive orchards are designed for mechanical harvesting and early fruit-bearing. This new paradigm of olive growing imposes significant changes that affect, among others, the characteristics of successful cultivars. However, only a handful of traditional cultivars fit the requirements needed for high-density systems. For instance, few alternatives to the use of 'Arbequina', 'Arbosana' and 'Koroneiki' are found for the new narrow hedgerow (> 1500 olive/ha) plantation system (Diez et al., 2016; Rallo et al., 2013; Rosati et al., 2013). In addition, irrigated high-density plantation systems have been related to a higher incidence of certain diseases compared with standard rainfed orchards (Rallo et al., 2013). These included airborne defoliating fungus diseases and *Verticillium* wilt caused by the fungus *Verticillium dahliae Kleb*. Despite the recent outbreak of *Xylella fastidiosa* in the south of Italy, *Verticillium* wilt is currently the most important diseases affecting this tree in the majority of olive-growing countries (Jiménez-Díaz et al., 2012; López-Escudero and Mercado-Blanco, 2011). Unfortunately, most of the olive cultivars evaluated to date are susceptible or extremely susceptible to *V. dahliae* (Garcia-Ruiz et al. 2014; López-Escudero et al., 2004; Trapero et al., 2015, 2013).

Thus, breeding of new cultivars that maintain the inherent genetic diversity of the traditional cultivars but also address the requirements of intensive plantation systems and resistance to airborne fungus diseases and *Verticillium* wilt is required. In addition, global warming may modify the adaptation of the autochthonous olive cultivars producing irregular and asynchronous flowering or even its complete absence (Ramos et

al., 2018). Therefore, given the forecast for climate change in the Mediterranean Basin and its possible effects on olive growth (Ponti et al., 2014), breeding olive cultivars with different chilling requirements might also be crucial for the sustainability of olive growing in the future.

Olive breeding programs are relatively new compared with other fruit species and they are principally based on crossbreeding techniques. Non-conventional breeding approaches leading, for example, to the generation of stable diploid and tetraploid dwarfing olive genotypes (Rugini et al., 2016a) or transgenic olive plants with improved agronomical characteristics, have been applied with variable level of success (Rugini et al., 2016b for a review). Despite the possible applicability of these new approaches in the future, up to date, olive breeding programs are mostly based on the application of conventional breeding methods.

The first olive breeding programs using cross-breeding were initiated between 1960 and 1971 in Israel (Lavee, 1990) and Italy (Bellini et al., 2002), respectively. Since the mid-1980s, new breeding programs, mostly focused on obtaining cultivars for olive oil production, have been developed in France (1986), Spain (1991), Morocco (1994), Tunisia (1994), Turkey (1994), Greece (1996), Australia (1997), Iran (1999), and Portugal (2002) (De la Rosa and León., 2009). Most of these programs have been focused on obtaining cultivars for olive oil, and only a few have focused on table olive or both (Rallo et al., 2011). To date, only the programs from Israel, Italy, and Spain have released new cultivars, producing a total of 14 new cultivars (De la Rosa and León., 2009).

A procedure consisting of three basic steps is generally performed to select outstanding individuals. This procedure starts with directed crosses between cultivars, followed by selection within the progenies and finally the cloning of outstanding individuals (advanced selections) exhibiting high oil production and eventually early bearing, in addition to adaptation to mechanical harvesting and high-density plantations (Rallo, 2014). To date, in most olive oil breeding programs, the total oil content is still the main trait that is selected for. Normally, only advanced selections are further characterized in terms of their oil fatty acid profiles and minor compounds content due to the high labor cost of these processes (Leon et al., 2008; León et al., 2011). Similarly, in the case of table olives, the size of the fruit, shape and flesh-to-stone ratio are the main selection criteria in

the first stages of large progenies evaluation, whereas the analysis of table olive compounds, such as sugars, phenolics and triterpenic acids, has been limited to a few selected genotypes (Medina et al., 2012; Rallo et al., 2012). However, health concerns are becoming one of the major driving forces of the world food market (Byrne, 2012); therefore, obtaining new cultivars with health-enhancing properties is progressively becoming the focus of olive breeding programs (De la Rosa et al., 2016).

As has been described in previous sections, both virgin olive oil and table olives are staple products that contribute to the health benefits of the Mediterranean Diet. This is mainly due to their unsaturated fatty acid profile and the presence of other compounds with proven bioactive properties, such as tocopherols, squalene, pigments, triterpenic acids and phenolic compounds. The increasing awareness of the health benefits of food and the willingness of consumers to pay a premium for them is creating a new market for fruit crops cultivars with health enhanced properties (see Byrne, 2012 and Wargovich et al., 2012 for a complete review). Thus, products from olives (virgin olive oil and table olives) are perfect candidates to be considered "superfoods", a marketing term that can boost the consumption of the target product, which is touted to have exceptional health benefits. For instance, blueberries and pomegranate have enjoyed significantly increased consumption in only a few years as a result of their high anthocyanin content and consideration as "superfruits".

The central requirement to start a breeding program with the goal of increasing the content of certain compounds is the availability of genotypic variation for these compounds among the cultivars to be used as genitors. Several studies have noted the remarkable genetic diversity and heterozygosity of traditional olive cultivars (Diez et al., 2015; Trujillo et al., 2014), and others have already proven how this variability translates in terms of oil fatty acid composition (Aguilera et al., 2017) and content of some bioactive compounds (Beltrán et al., 2016, 2010; Kyçyk et al., 2016; Sánchez de Medina et al., 2015a, 2015b). Most of these studies have also shown that the effect of the genotype is the main factor responsible of this variation, although it is modulated by yearly variation and edaphoclimatic differences between orchard locations. Nevertheless, the influence of the cultivars in many of the quality traits mentioned in Sections 2.1. and 2.2. is still unexplored (Rallo et al., 2011).

On the other hand, this high variability and heterozygosity make necessary the evaluation of a vast number of individuals within progenies. The influences of genitors and crosses on olive oil composition (De la Rosa et al., 2016, 2008; El Riachy et al., 2012; Leon et al., 2008, 2004b) and on other characteristics, such as the time to reach flowering (Moral et al., 2013), the tree vigor (Santos-Antunes et al., 2005), and the resistance to *Verticillium* wilt (Trapero et al., 2015), have been demonstrated. However, the variability observed within olive progenies has always been greater than that between progenies and genitors, with individuals frequently exhibiting transgressive segregation (De la Rosa et al., 2016; Leon et al., 2004a; Trapero et al., 2015).

The need to evaluate large progenies requires the application of clear selection criteria based on easily measurable traits in addition to the optimization of tools to simplify and automatize the selection process. For instance, the application of near infrared reflectance spectroscopy (NIRS) to evaluate characters such as the oil content, moisture, and fatty acid composition in intact olive fruits has provided reliable, fast and accurate determination of these characteristics (Leon et al., 2003, 2004c; Morales-Sillero et al., 2011). Additionally, optimized protocols, based on the application of chromatography techniques, have revealed a high correlation between the compositions of olive fruit and olive oil in terms of fatty acids (Garces and Mancha, 1993), tocopherol, sterol and squalene (Velasco et al., 2014), allowing the evaluation of olive progenies at the initial steps of selection (De la Rosa et al., 2016).

Indirect selection, based on the linkage between different traits, has also simplified and accelerated the screening process. This has been the case for the relationship between the high seedling vigor (height and trunk diameter) and its early flowering in olive (De la Rosa et al., 2006; Moreno-Alías et al., 2010). This relationship has allowed more than 40% of olive seedlings with long juvenile phases to be discarded just a few months after their germination (Rallo et al., 2008).

The recent publication of the reference genome of the cultivar 'Farga' (Cruz et al., 2016) and the wild olive (Unver et al., 2017) is likely to accelerate the development of early markers for selection and shorten the long breeding cycles of this crop. The primary use of genomics in breeding is marker-assisted selection (MAS) for traits controlled by major genes or quantitative trait loci (QTLs). Via MAS, genetic markers that are either known to

cause a phenotype or are strongly linked to the causal genetic variant can be genotyped at the seedling stage, thus enabling prediction of the phenotype of the adult plant (Rallo et al., 2016). Since the cost of phenotyping is likely to increase in the future, whereas the cost of genotyping has decreased considerably over time, and this trend is expected to continue (McClure et al., 2014), generalization of genomic tools for the characterization of genetic resources and the selection of candidate genotypes is expected to become a general trend in plant breeding. These new technologies will also increase our knowledge about the genetic control and expression of bioactive compounds in fruit crops and specifically in olive, thereby opening new possibilities for breeding olive cultivars with specific bioactive profiles.

5. Environmental and agronomic factors affecting olive quality

5.1. Soil

Olive trees may be cultivated in a great diversity of soils, but some of them limit tree development and fruit and oil quality. Soil properties affect water availability, and this, in turn, may affect fruit ripeness and weight and oil quality. Bucelli et al. (2011) found that the quality of the fruits and oils from 'Frantoio' and 'Moraiolo' cultivars, both growing under rainfed conditions, differed depending on the soil properties, in particular, the soil water availability. The soil inducing a relatively more intense and longer water deficit in summer (Skeleti Calcaric Regosol) favored an earlier ripening of the olives and an increase in phenols content in the oils that had superior sensorial properties when compared to a Haplic Calcisol. In soils with low clay content or low moisture content, potassium deficiency symptoms are often found, and fruit may exhibit smaller sizes and even wrinkling. In contrast, high pH and calcareous soils favor iron chlorosis, which decreases the value of table olives with a smaller size and chlorotic color of the skin. In these soils, boron deficiencies are also frequent and may induce fruit malformations. In contrast, in low-pH and thus acidic soils, the lack of calcium may affect table olive quality by the decrease of pulp firmness (Fernández-Escobar, 2017) and favor the higher incidence of Anthracnose disease, which decreases the quality of both fruits and oil (Moral et al., 2014). Magnesium deficiencies can also be found in acids in addition to sandy soils, in which fruits also acquire chlorotic appearance.

5.2. Climate

Olive is usually grown in warm areas characterized by cool-to-mild winters and warm-to-hot summers, with most rainfall events occurring from autumn to spring. Frost damage occurs when the temperature decreases below 0 °C. Fruits at -1.7 °C may exhibit surface blisters and spots, and they may have aqueous consistency and drop or remain wrinkled until harvest. At -3 °C or lower temperature, fruit freezing occurs, negatively affecting table olive quality as a consequence of cell dehydration and the destruction of the cells by ice crystals provoking oxidative processes (Sanzani et al., 2012). The oil quality also decreases by losses in the stability to oxidation, which are related to the decreases in the concentration of most phenolic compounds, particularly secoiridoid derivatives. Sensorial analysis of these oils reveals less pungent taste and absence of bitter taste (Morelló et al., 2003). In contrast, high summer temperatures may induce the presence of redness or dried spots on the surface of olives and over-maturation of the olive fruit, whereas in the oil, increases in free acidity and the content of palmitoleic and linoleic fatty acids and a decrease in oleic acid content have been reported (Orlandi et al., 2012). Moreover, a high temperature during the ripening period negatively affects fruit weight, oil content and even fatty acid composition since oleic acid decreases, a common fact in EVOO from new warm cultivation areas (García-Inza et al., 2014; Inglese et al., 2011; Rondanini et al., 2014). For phenols, the effect of high temperature is not yet clear. Whereas Ripa et al. (2008) found that total phenol content decreases when high temperatures accumulate from fruit set to harvesting, Tura et al. (2008) found that in cool areas, the phenol content increases with degree-days accumulation in the same period. Moreover, a warmer spring with sufficient rain in spring and autumn has a positive effect on the oil's volatile composition (Tura et al., 2009).

The altitude at which olives are grown has also been demonstrated to have an influence on oil fatty acid composition and, to a lesser extent, on phenols content. Oils from orchards cultivated at higher altitudes usually have greater contents of unsaturated fatty acids, particularly oleic acid. Different studies also show increases of phenols content, which positively affect the sensorial properties. Decreases in fruit weight and a delay in the maturation have also been reported (Di Vaio et al., 2013; Merchaket al., 2017).

Other abiotic factors may reduce both olive and oil quality, particularly wind and hail. Winds may contribute, in combination with drought/high temperature conditions, to the shriveling of the olives and favor bruising damage due to the beating of the fruits. In addition, injuries caused by winds can induce entry points for pathogens. Blemishes and malformations caused by hailstorms may also reduce table olive quality. They can also induce injuries that favor internal tissue oxidation and consequently increase the free acidity in the oil (Sanzani et al., 2012).

Studies concerning climate change and its impact on olive and oil quality are still really scarce. Simulations made on 'Arbequina' cultivar in Córdoba (Spain) based on an increase of air temperature of 4 °C and increases of CO2 concentration from 380 up to 740 ppm, show very small effects on oil yield because of the compensation of the negative and positive effects of both parameters on phtosynthesis and respiration, but possible changes in olive and oil quality have not yet been studied (Morales et al., 2016). However, global warming is expected to force farmers to cultivate under extreme climatic conditions, i.e higher temperature and lower rainfall, which causes fruits to ripen faster negatively affecting oil quality (Dag et al., 2014). Changes in the weather patterns have been related to the higher incidence of oil sensory defects that were uncommon a few decades ago. Indeed, the 'frostbitten olives' defect has been associated to low temperatures before harvesting, in particular to several freeze-thaw cycles accounting in warm autumns and in the beginning of winters (Romero et al., 2017b).

5.3. Cultivation system

For agronomic practices, the available literature demonstrates the importance of all of them in terms of the production of quality. In the design of a new olive orchard, the choice of tree density and the row orientation must be performed while considering the soil and climate conditions and the water availability and quality. An excessive tree density and/or vigor may imply competition for light among trees reducing fruit size, oil quantity and quality. Particular attention should be given to olive hedgerow orchards, which have been developed since the 1990s, because the choices of row spacing and row orientation determine the illumination of the walls, which can affect the mesocarp size and composition (water and oil) (Trentacoste et al., 2016). Furthermore, high-density

conditions may create ventilation problems that favor diseases development (Moral et al., 2014).

5.4. Canopy management

Canopy management (pruning, fertilization and water in the case of irrigated orchards) is key for the control of vigor, interception of solar radiation and fruit load regulation. An appropriate canopy management strategy, adapted to the cultivation system, is necessary to favor solar radiation interception and porosity to maximize olive yield and the efficiency of mechanical harvesting. The light environment around the canopy of tree is not uniform. Olive fruits located in zones of the canopy that are significantly exposed to solar radiation, such as the top, are larger and tend to have oblong shapes; they ripen faster and produce more oil than fruits located in shadow zones, such as the lower parts and inner zones of the trees (Bartolini et al., 2014; Benelli et al., 2014; Connor et al., 2014). Furthermore, the oil quality varies according to the fruit position in the canopy, and the oils from fruits that are highly exposed to light, usually from the middle-outer and upper canopy, exhibit higher phenol content (Castillo-Ruiz et al., 2015), greater stability to oxidation, high palmitic and linoleic acid contents and lower oleic acid content compared with oils from lower-canopy fruit (Gómez del campo and García, 2012). The influence of crop load should also be considered. Olive is a biennial bearing species in which developing fruits reduce shoot growth and bloom return in nearby distal shoots. The sequence of high cropping (ON) and low cropping (OFF) years is a well-known habit of olive (De Almeida, 1940; Lavee, 2007; Rallo and Cuevas, 2017). From the horticultural point of view, several aspects of the biennial bearing represent serious shortcomings for both the table olive and olive oil sectors. ON cropping years are associated with small fruit size, low flesh-to-stone ratio and late ripening (Gucci et al., 2007; Trentacoste et al., 2010). For table olives, size is a major attribute for quality as mentioned in Section 2.1 (e.g., considering 'Manzanilla de Sevilla' olives, the main cultivar for Spanish-style green olives, those fruits with weight < 2.5 g are unmarketable for processing table olive, and, thus, are processed for oil). Additionally, small-sized fruits in ON years impede the attainment of the maximum oil content in any cultivar because the flesh-to-stone ratio and the percent of oil decrease concomitantly at any time of harvest. However, the total amount of oil per tree and hectare is always greater in ON than in OFF years. This biennial habit represents a

logistic constrain to fix the total storage capacity of the olive oil mills. Some empirical and experimental approaches to partially modulate the imbalance between cropping and quality have been developed. Besides girdling and fruit thinning for table olive production (Lavee et al., 1983; Rallo and Cuevas, 2017), adequate pruning in the ON years and highly bearing cultivars should trend to equilibrate the fruit load. Furthermore, mechanical pruning has been recently developed for intensive and super-high-density orchards as a way to decrease the production costs. Appropriate management is needed to avoid the excessive development of new branches after cutting of young branches, which may decrease solar radiation interception in the canopy (Gucci and Cantini, 2000).

5.5. Irrigation

Olive tree responds positively to irrigation, a common practice in intensive and super-high-density orchards that may also alleviate the inconvenient of olive biennial bearing (Ben-Gal et al., 2011a). Strong drought in summer can lead to fruit ripening and negatively influence the fruit size and flesh-to-stone ratio, even causing shriveling of the fruit, although it does not affect the accumulation of oil (Bartolini et al., 2014). Irrigation therefore increases the water content, fruit size and flesh-to-stone ratio. Nevertheless, firmness, total phenols and sugar content can decrease with increased irrigation, although no differences are observed in sensory characteristics after preservation in brine (Gucci et al., 2007; Patumi et al., 2002; Proietti and Antognozzi, 1996).

Concerning oil quality, water stress conditions stimulate the synthesis of phenolic compounds in olive fruits but exert a negative influence on volatile compounds related to the LOX pathway (Servili et al., 2007, Bucelli et al., 2011). However, oils are occasionally characterized as excessively bitter (Servili et al., 2007). An increased water content in the fruit can make it difficult to extract the oil (Grattan et al., 2006). In addition, although the criteria for classifying in commercial categories have not been modified, oil quality usually decreases with the loss of minor compounds, mainly phenols, and with changes in their profiles (Patumi et al., 2002; Tovar et al., 2002). This may be particularly negative for cultivars as 'Arbequina', whose oils are characterized by low stability to oxidation (Romero et al., 2002). In contrast, irrigation offers an opportunity to modulate the excessive bitterness of the oils of other cultivars, as 'Cornicabra' (Gomez-Rico et al., 2007) and

'Manzanilla de Sevilla' (García et al., 1996). However, abundant precipitation events can override the effects of irrigation on phenols (García et al., 2017; Stefanoudaki et al., 2009a).

Not only phenols but also volatile content and composition change with irrigation. Thus, irrigation may favor the concentration of compounds responsible for green-fruity sensory perceptions, such as (E)hex-2-enal and (Z)-hex-2-enal, hexanal and hexan-1-ol (Gomez-Rico et al., 2006; Servili et al., 2009), but panels do not always identify differences in flavor description of the VOOs (Morales-Sillero et al., 2013).

Recent studies regarding regulated deficit irrigation (RDI) have shown that an appropriate strategy for controlling the moment and intensity of water restriction may result in increased fruit weight, size, skin hardness and linoleic acid content (D'Andria et al., 2004; CanoLamadrid et al., 2015; Grattan et al., 2006). It also favors saltiness, bitterness, green olive note, aftertaste and hardness, after green processing of 'Manzanilla de Sevilla' olives, thereby increasing consumer global acceptance compared with other irrigation strategies (CanoLamadrid et al., 2015). An appropriate RDI strategy can also decrease bruising damage (Casanova et al., 2017) and may increase the content of phytoprostane (Collado-González et al., 2015). Regarding the oil quality, different studies are also now showing that the application of deficit irrigation in summer is not only an imperative option in many orchards but also the best for EVOO quality production, in particular, by increasing the content of total phenols and sensory quality (García et al., 2017; Gomez-Rico et al., 2007; Gómez del Campo and García, 2013). However, increases in the phenol content with irrigation dose or no effect of this practice on oil quality have also been reported (Dabbou et al., 2011; Tognetti et al., 2007), which means that VOO quality depends on the cultivar and on the environment and management of the orchard. Controversial or inconsistent results regarding oil content, fatty acid composition and other traits, such as tocopherols, acidity, peroxide index or extinction coefficients, can also be found (GomezRico et al., 2007; Tovar et al., 2002; Stefanoudaki et al., 2009a). An appropriate RDI strategy should be chosen considering the stress level and fruit load (Ben-Gal et al., 2011b; Martín-Vertedor et al., 2011). The quality of the water should also be considered. Irrigation with saline water decreases fruit weight and oil content and modifies the fatty acid composition, although it can also increase the contents of total phenols and major phenols (Ben Ahmed et al., 2009; Stefanoudaki et al., 2009b).

5.6. Fertilization

Fertilization is especially necessary when trees are subject to deficiency conditions. However, the influence of this practice on olives and oil quality has not been sufficiently studied. Nitrogen is commonly employed in many orchards owing to its importance on olive nutrition. Foliar applications of different fertilizers with or without N produce decreased total phenol and tocopherol contents in addition to changes in the profiles of minerals, sugars, phenols and tocopherols (Tekaya et al., 2014). High doses of nitrogen applied by fertigation in combination with phosphorus and potassium increase the fruit weight, fleshto-stone ratio, potassium and water contents but also degrade the pulp texture and decrease the total sugar content (Morales-Sillero et al., 2008). However, the extent to which all of these changes observed in table olives influence sensory properties or antioxidant activity is unknown. Regarding oil, there is a negative effect of an excess of N fertilization on oil quality because the phenol content and, thus, the stability to oxidation and bitterness decrease with an increased concentration of this element in the fruit (Fernández-Escobar et al., 2006). High N levels in the fruit also decrease the oleic acid content. The effects of P and K fertilization on oil quality are minor and negligible, respectively (Erel et al., 2013).

5.7. Phytosanitary control

Climate and agronomic practices as canopy management determine the sanitary status of olive orchards. Phytosanitary control should prevent those plagues and diseases, such as olive fly and Anthracnose, respectively, which have clear influences on the quality of olives and the extracted oils. The olive fly (*Bactrocera oleae*) is the most important plague, and its abundance and distribution is favored by arid locations with hot summer temperature. Larvae consume the pulp of fruits, thereby destroying tissues, which leads to a severe fruit drop. Moreover, fruits are prone to fruit contamination. Large-size and green fruits attract adult flies more than smaller and ripened fruits. Moreover, the wax composition of the epicarp and volatile compounds emitted by fruits and leaves are relevant for oviposition. Infested table olives exhibit browning spots, deformities or mutilations, and a negative attribute, mustiness, is perceived in sensorial analyses. Kinesthetic sensations are also drastically affected as a consequence of pulp consumption

and larvae development. Thus, fruits infested by B. oleae are not processed for table olive. Concerning oil production, infested fruits are destined to industry extraction, but the oils exhibit considerable increases in free acidity and extinction coefficients (K_{232} and K_{270}) that often prevent them from being classified as EVOO. Furthermore, stability to oxidation and antioxidant capacity decrease due to reductions in phenol compounds (even up to 80%) and in chlorophylls and carotenoids pigments, with the oil becoming lighter in color and more golden-yellow than green. Sensory attributes, such as green, fruity and cut grass, decrease with the loss of volatiles compounds, such as (E)-2hexenal (Malherio et al., 2015). Integrated pest management, including culture practices, such as the removal of the fruits that remain in trees after harvest, are recommended to eradicate or reduce infestations (Yokoyama, 2015).

Anthracnose, which it is caused by two species of *Colletotrichum*, is the most important disease form the point of view of oil quality deterioration. The infection of the olives increases after the first autumn rains and when the temperature is in the range of 17–20 °C. Mature fruits of highly susceptible cultivars are usually the most affected, showing typical depressed spots of ocher or brown color and developing rot. The extracted oils show a characteristic reddish color and increases in the free acidity, peroxide index, extinction coefficients (K_{232} and K_{270}), phenol compounds and alkyl esters. The literature regarding the effects on oil quality, however, is scarce (Moral et al., 2014).

5.8. Harvesting

Harvesting olives as intact and healthy as possible at the right time and proper subsequent transport to the industry should guarantee production of table olives and oil of superior quality.

Harvest timing is key to obtain quality in both table olives and olive oil as it has been extensively discussed in Section 3.2. Besides, early harvesting can also reduce the negative effects of irrigation on oil quality and prevent damages from frost (Gracia et al., 2012), olive fly adult activity (Rojnic× et al., 2015) and Anthracnose (Moral et al., 2014). Moreover, harvesting at night to avoid the effects of high temperature at harvest seems to improve the oil's sensory qualities (Di Serio et al., 2014).

Manual harvesting is the best option to maximize the quality of table olive and oil. However, mechanical harvesting is a necessary method to decrease cost in many orchards. Trunk shakers are frequently used in olive orchards for oil production, but in the case of table olive, they are mainly used for cultivars characterized by low susceptibility to fruit damage, such as 'Hojiblanca'. Other cultivars, such as 'Manzanilla de Sevilla', exhibit significant damage, such as bruising and cutting of the fruit, after trunk shaker harvesting. The level of bruising is 12 times higher than when olives are hand-picked, and most bruising occurs in the first hour after harvesting and exhibits an exponential increase after it (Jimenez-Jimenez et al., 2013). Post-harvest field immersion in a dilute solution of NaOH and transportation in liquid to industry have been proposed to reduce bruising (Rejano Navarro et al., 2008; Zipori et al., 2014). Strategies such as adaptation of tree pruning to reduce the canopy volume and to facilitate the transmission of the vibration, the adjustment of the vibration parameters of the trunk shakers, and postharvest treatments have also been proposed to facilitate table olive harvesting using trunk shakers, in particular when the fruit is intended for green-style processing (Ferguson et al., 2010; Castro-García et al., 2015). Recent studies about table olives have opened new interesting alternatives for mechanical harvesting via the use of canopy contact harvesters (Ferguson et al., 2010), including those adapted from the grape straddle machines used in super-high-density hedgerows (Morales-Sillero et al., 2014).

Despite the fact that mechanical harvesting of the olives for oil mill extraction is now a reality in many countries, studies of the effect of this method on oil quality are very scarce, probably because the production of EVOO is usually feasible in these conditions. However, losses of natural antioxidants and flavor components, and in some cases, increased free acidity, have been observed with different types of harvesters, either hand-vibrating combs (Dag et al., 2008) or grape straddle harvesters (Yousfi et al., 2013). This is probably a response to fruit internal damage caused by harvesters (Jiménez et al., 2017) and to physiological alterations to the fruit, such as increases in respiration rate and, mainly, ethylene production (Morales-Sillero and García, 2015; Yousfi et al., 2013). Therefore, to minimize fruit and oil quality losses, it is important to search for a compromise between harvesting efficiency and fruit damage (Connor et al., 2014). Furthermore, to maintain the olives' quality, harvested fruits should not be mixed with fruits picked from the soil and the transport of the fruit must be performed as quickly as

possible to minimize the time elapsed between harvesting and processing. Production of high-quality table olives and EVOO will be facilitated if fruits are transported separated by quality and in trailers or containers with limited cargo to avoid cramping and anaerobic conditions. Storage of olive fruits in adverse conditions, such as sacks or piles, negatively affects the sensorial quality of the oil. Under such conditions, diverse types of microorganisms proliferate, producing different undesired metabolites, such as branched aldehydes, alcohols and the corresponding carboxylic acids that contribute to negative attributes (typically the "fusty" defect). When the temperature is increased due to storage of fruits, yeasts produce ethanol and ethyl acetate, which are detected as the "winey" defect. In contrast, under low-temperature conditions, proliferation of fungi and yeast leads to the appearance of the "musty-humid" defect. To avoid these defects, it is recommended to process the fruits as soon as possible after harvest (Angerosa et al., 2004; Kalua et al., 2005).

6. Conclusions

Quality itself is becoming a fundamental goal for most horticultural products, including table olives and olive oil, the two main products obtained from olive fruits. The concept of quality in olives is complex, and a large number of traits might be considered. Different definitions may apply according to the point of view and final goal of producers, traders, consumers and/or nutritionists. For example, legal quality refers to the standards to which both products must conform for trading purposes but is usually insufficient to define the holistic quality of table olives and olive oil. For table olives, traits related to fruit appearance, such as the size, shape, color or absence of damage in the surface are, among others, the most common quality criteria, whereas for olive oil, the quality is largely determined by its physico-chemical properties (e.g., free acidity, peroxide value, UV absorbency) and its composition.

Consumers are becoming especially aware of the influence of food on human health, and, in this sense, a market niche for food products with preventive and therapeutic properties is rapidly emerging. Table olives and virgin olive oil may be considered as nutraceutical products since they are rich in many compounds with beneficial health effects. A significant body of literature about this matter has been published in medical and chemical journals, especially for olive oil, but table olives are also being studied from this

point of view. In addition to the well-known benefits of the consumption of olive products derived from their fatty acid profile, which is rich in MUFAs and linoleic acid (PUFA), and from the high content of α-tocopherol (vitamin E), the roles of other compounds with bioactive activity are also being highlighted. Among them, triterpenic acids (maslinic and oleanolic), squalene and phenolic compounds (oleuropein complex, tyrosol and hydroxytyrosol) have been reported to exhibit antioxidant, anti-inflammatory, anticancer and cardioprotective activities, among others. The fact that olive products are among the top food sources containing these compounds enhances the nutritional interest in olive oil and table olives. In fact, the EFSA has approved a health claim label for olive oil exhibiting high contents of phenolic compounds.

The particular sensorial attributes of olive oil and table olives are also gaining much interest among consumers. Non-volatile components, particularly phenols, and volatile compounds (especially the C6 and C5 compounds) determine the gustatory and olfactory perceptions of olive oil, respectively. For both products, standardized methods for sensory analysis are available.

Although processing has very important effects on the quality of both products, and these effects have been studied extensively, the determination of olive quality begins in the field with decisions such as what cultivars should be used, where to plant, how to design and manage the orchard, and when and how to harvest the fruits to achieve the best possible quality. Unfortunately, less work has focused on these issues.

Regarding plant material, olive cultivar diversity is extremely rich, and many of these traditional cultivars may produce table olives and EVOOs with high and distinct quality attributes. Nevertheless, most of these varieties remain unexplored in terms of some of the aforementioned quality traits, and further efforts should be made to evaluate them. Similarly, olive breeding programs are still few and relatively recent, but the focus on specific quality attributes in some of these programs, along with the development of genomic tools to accelerate the selection of interesting genotypes, will probably help to yield, in the midterm, new olive cultivars with enhanced quality.

Regarding the influence of environmental conditions, soil properties (particularly the pH, the limestone content and deficiencies in certain elements) and climatic factors (low

temperatures that may cause frost damage or high temperatures in summer) have been reported to affect the quality of olive fruits and, hence, the quality of their products. Among agronomical factors, particularly interesting is the choice of tree density and row orientation when establishing an orchard because these choices affect the interception of solar radiation by the fruits, which ultimately influences traits such as the fruit size, shape and oil content. Furthermore, increases in phenols, palmitic and linoleic content have been reported in fruits with greater exposure to light. In this sense, pruning is essential to manage the light environment in the canopy and to regulate the fruit load. Irrigation and fertilization are becoming common practices in recent intensive and superintensive olive orchards. Olive trees positively respond to irrigation, as many recent works have noted. Nevertheless, an appropriate strategy should be implemented because losses of minor components, particularly phenols, due to irrigation have been reported. Similarly, good control of fertilization should be performed to avoid the negative effects on olive quality (reduced firmness and decreases in the sugar and phenol contents) observed when applying excess nitrogen. Controlling pest and diseases in the olive orchard is also key to achieve high-quality products. The olive fly (*Bactrocera oleae*) and Anthracnose (*Colletotrichum spp*) represent the most important sanitary problems from the point of view of quality deterioration.

Finally, the harvesting timing and procedure play decisive roles in determining the final product quality. Maturity indices (MIs) are commonly used to decide when to harvest. In the case of table olives, fruits are usually picked at MI 1 for green or black-ripe processing, although the latter can be delayed until the beginning of the skin color change. Natural black olives are harvested once the fruit color has changed to black on the tree. In the case of olive oils, harvesting at MI 3 (veraison) is often recommended to ensure the maximum oil content. Nevertheless, ripening dramatically modifies the physico-chemical traits and compounds of olive fruit (see **Tables 3 and 4**); thus, these changes should be considered to achieve specific organoleptic and nutraceutical profiles of olive products by advancing or delaying the date of harvest. Regarding the harvesting method, manual harvesting is the best option to obtain the highest quality for both products, but it is economically inviable in most olive orchards. Mechanical harvesting is a common practice for olive oil production, but although EVOO may be obtained, decreases in certain quality attributes have been reported. In the case of table olives, the main constrain for mechanical

harvesting is the high proportion of damaged fruits obtained. Nevertheless, strategies that include new machinery designs, adapting the tree canopy and postharvest treatments will help improve the quality of mechanically harvested olives.

Conflicts of interest

The authors declare no conflicts of interest.

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CHAPTER II - Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey.

Food Chemistry 2018, 266; 192-199.

Food Chemistry 266 (2018) 192–199

Contents lists available at ScienceDirect

Food Chemistry

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

Cultivar influence on variability in olive oil phenolic profiles determined through an extensive germplasm survey

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https://doi.org/10.1016/j.foodchem.2018.06.002

Received 8 February 2018; Received in revised form 25 May 2018; Accepted 3 June 2018

Available online 04 June 2018

Abstract

Despite the evident influence of the cultivar on olive oil composition, few studies have been devoted to exploring the variability of phenols in a representative number of monovarietal olive oils. In this study, oil samples from 80 cultivars selected for their impact on worldwide oil production were analyzed to compare their phenolic composition by using a method based on LC–MS/MS. Secoiridoid derivatives were the most concentrated phenols in virgin olive oil, showing high variability that was significantly due to the cultivar. Multivariate analysis allowed discrimination between four groups of cultivars through their phenolic profiles: (i) richer in aglycone isomers of oleuropein and ligstroside; (ii) richer in oleocanthal and oleacein; (iii) richer in flavonoids; and (iv) oils with balanced but reduced phenolic concentrations. Additionally, correlation analysis showed no linkage among aglycone isomers and oleocanthal/oleacein, which can be explained by the enzymatic pathways involved in the metabolism of both oleuropein and ligstroside.

Keywords: Phenolic compounds; cultivar; germplasm; oleocanthal; oleacein; oleuropein aglycone; ligstroside aglycone; LC-MS/MS.

Chemical compounds studied in this article

Hydroxytyrosol (PubChem CID: 82755); Oleacein (3,4-DHPEA-EDA) (PubChem CID: 18684078); Oleocanthal (p-HPEA-EDA) (PubChem CID: 16681728); Oleuropein aglycone (3,4-DHPEA-EA) (PubChem CID: 124202093); Luteolin (PubChem CID: 5280445); Apigenin (PubChem CID: 5280443).

1. Introduction

The olive tree, *Olea europaea* subsp. *europaea* var. *sativa*, was likely domesticated approximately 6000 years ago in the Middle East from its wild ancestor *Olea europaea* subsp. *europaea* var. *sylvestris*, (Besnard et al., 2013). Currently, more than 11 million hectares of olives are grown in 47 countries worldwide (International Olive Council, 2015). However, olive growing is still based on a vast number of traditional olive cultivars, with complex genetic relationships among them (Diez et al., 2015).

Virgin olive oil (VOO) is demonstrated to be endowed with healthy properties, thanks to its monounsaturated fatty acids profile and a plethora of multiple minor components with biological properties (Piroddi et al., 2017). VOO composition is characterized by saponifiable and unsaponifiable fractions. The saponifiable fraction represents approximately 98% of the olive components and includes triglycerides, fatty acids, phospholipids, waxes and sterol esters. The unsaponifiable fraction, approximately 2% of the total composition, encompasses a complex set of minor compounds (approximately 230 compounds) pertaining to various chemical families including aliphatic and triterpenic alcohols, sterols, hydrocarbons, phenols, tocopherols, esters, pigments, and volatile components such as aldehydes, ketones and alcohols (Servili et al., 2013). The preservation of this composition is guaranteed by the extraction process performed using physical methods at relatively low temperatures (approximately 28 °C) and without the addition of chemical solvents.

Among the minor components of VOO, phenols are worthy of attention due to their (i) health properties (Piroddi et al., 2017); (ii) association with organoleptic attributes such as oil pungency and bitterness (Bendini et al., 2007); (iii) contribution to VOO shelf-life (Silva, Pinto, Carrola, & Paiva-Martins, 2010); (iv) uniqueness, supported by the fact that some families are exclusive of the Oleaceae family and few other dicotyledonous families (Carranco, Farrés-Cebrián, Saurina, & Núñez, 2018; Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002; Servili et al., 2016); and (v) high concentration in VOO (Servili et al., 2016). Given the great variability of phenolic families, the role of secoiridoids, conjugated forms of hydroxytyrosol and tyrosol, is notable. This group of compounds is the most concentrated in olive oil and is widely studied due to evidence of its healthy properties (Piroddi et al., 2017). The group includes oleuropein and ligstroside aglycone

isomers and the decarboxymethylated dialdehyde forms of oleuropein and ligstroside aglycons, better known as oleacein (3,4-DHPEA-EDA) and oleocanthal (*p*-HPEA-EDA), respectively. Beauchamp and colleagues reported the natural non-steroidal anti-inflammatory activity of oleocanthal due to its ibuprofen-like cyclooxygenase (COX-1 and COX-2) inhibiting capacity (Beauchamp et al., 2005). Oleacein has also shown antioxidant activity similar to oleocanthal (Czerwińska, Kiss, & Naruszewicz, 2014). It appears that the healthy properties of VOO phenols are attributed individually and not to the total phenolic content (Agrawal et al., 2014; Yakhlef et al., 2018).

There is increasing social interest in VOO as a functional food; for instance, consumers are willing to pay up to 6.02 €/L more for VOO labelled with functional health claims compared to the unlabelled product (Casini, Contini, Marinelli, Romano, & Scozzafava, 2014). Numerous studies supporting the health properties of olive oil have recently pushed the European Food Safety Authority (EFSA) to approve several health claims on the commercial label of VOOs that meet specific quality requirements. The claim ‘‘olive oil phenols contribute to the protection of blood lipids from oxidative stress’’ can be included on the label when the VOO contains at least 5 mg as the sum of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil (EFSA, 2011).

As mentioned elsewhere, phenols also contribute to the organoleptic properties of VOO (Angerosa et al., 2004). The bitter taste is particularly related to the aglycone forms (Bendini et al., 2007), whereas the presence of oleocanthal and oleacein has been linked to the pungency of VOO, which might be described as biting tactile sensations, which are characteristic of some VOOs (Barbieri, Bendini, Valli, & Gallina Toschi, 2015).

Several studies have noted that the main factors that influence the qualitative and quantitative variability of phenolic compounds in olive oil are genotype (cultivar), climatic and agronomic conditions, edaphic factors, and the technological method applied for oil extraction. Among these factors, genotype has a preponderant influence on phenolic composition (Baiano, Terracone, Viggiani, & Nobile, 2013; De la Rosa, Arias-Calderón, Velasco, & León, 2016). Previous researches have been focused on a limited number of genotypes, either traditional cultivars with regional importance in terms of VOO production or new cultivars from breeding programs (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Vinha et al., 2005). Therefore, an exhaustive evaluation and

classification of an extensive panel of olive cultivars according to the phenolic profiles of monovarietal oils has never been published.

In this context, this study was aimed to a) characterize the phenolic profile of a representative panel of monovarietal VOOs; b) evaluate the influence of cultivar on phenolic variability, and c) determine association patterns among cultivars according to the phenolic composition of their VOO. To accomplish these goals, we selected a set of 80 olive cultivars from 15 countries representing the main VOO producing areas worldwide. The olive cultivars were grown under the same agroclimatic conditions, and their oils were extracted by application of the same protocol to allow an unbiased characterization of the influence of genotype on the VOO phenolic profiles.

2. Material and methods

2.1. Experimental location and vegetal material

Vegetal material was collected from the World Olive Germplasm Bank of Cordoba (WOGB) (CAP-UCO-IFAPA), specifically in the collection located at the University of Cordoba (Cordoba, Spain, 37°55'56.5" N, 4°43'13.3" W and 173 m a.s.l.). The olive trees were planted in 2011 in a North-South orientation with 7 m between rows and 6 m between trees (238 trees ha^{-1}). This collection includes 368 olive cultivars from 22 countries, which were identified and authenticated by morphological and molecular methods, so all them are true to type (Trujillo, Ojeda, Urdiroz, & Potter, 2014).

The climate of the area where the WOGB is located is typically Mediterranean; the average annual precipitation from 2001–2016 was 635.6 mm, with a summer drought with less than 30 mm of precipitation from June to September. The precipitation in 2014 and 2015 was 635.3 and 770 mm, respectively. The average potential evapotranspiration (ETP) from 2001 to 2016 was 1261.9 mm, while the average annual, maximum and minimum temperatures for the same period were 18.2, 47.2, and 0.0 ˚C, respectively (Villalobos & Testi, 2017). The collection area is characterized by vertisol soil with a texture of 41% sand, 6% silt, and 53% clay. The soil was approximately 40 cm deep, with 0.6% organic matter content. The collection was irrigated from May to September, applying 100 m^3 per ha per week (2000 m^3 of water per year) using drip irrigation. Foliar

fertilization (2% potassium nitrate) was applied four times per year during November (after harvesting), March, May and September.

A set of 80 olive cultivars were selected during the 2015–2016 crop season according to their importance for the worldwide olive oil production, their geographical origin, and fruit availability (**Table 1**). We also studied 25 same cultivars during two consecutive seasons, 2014–2015 and 2015–2016, to estimate the reproducibility of the results. Fruits were independently collected from two olive trees per cultivar, and the VOO was extracted in each case. Therefore, each cultivar provided two independent biological samples yielding a total number of 160 samples (80 cultivars $\times$ 2 trees = 160 samples).

Table 1. Cultivars selected for the analysis of phenolic compounds in monovarietal VOOs.

2014/2015 crop season	**2015/2016 crop season**			
Alameño de Montilla	Abou Choki	Empeltre	Mission Moojeski	Verdale
Alfafara	Alameño de	Enagua de	Mixani	Verde Verdelho
Arbequina	Alfafara	Farga	Mollar de Cieza	Verdial de Huévar
Arbosana	Amygdalolia	Frantoio	Moraiolo	Villalonga
Blanqueta	Arbequina	Galega Vulgar	Morisca	Zaity
Bosana	Arbosana	Gemlik	Morona	
Caballo	Ascolana	Gordal de	Morrut	
Cerezuela	Azapa	Hojiblanca	Nasuhi	
Coratina	Barnea	Jabaluna	Negrillo de la Carlota	
Farga	Blanqueta	Joanenca	Ojo de Liebre	
Gordal de Granada	Bodoquera	Kalamon	Palomar	
Joanenca	Bosana	Koroneiki	Pendolino	
Kalamon	Bouteillan	Kotruvsi	Picholine Marocaine	
Koroneiki	Caballo	Kusha	Picual	
Loaime	Carolea	Lastovka	Picual de Almería	
Mastoidis	Carrasqueño	Leccino	Picudo	
Mollar de Cieza	Cerezuela	Lechín de	Plementa Bjelica	
Morona	Changlot Real	Levantinka	Rapasayo	
Negrillo de la Carlota	Chemlal de	Loaime	Royal de Calatayud	
Ojo de Liebre	Chetoui	Lucio	Royal de Cazorla	
Picual de Almería	Çobrancosa	Manzanilla	Sabatera	
Plementa Bjelica	Coratina	Manzanilla de	Sandalio	
Royal de Cazorla	Cordovil de	Manzanilla	Sikitita	
Sabatera	Cornicabra	Mastoidis	Tanche	
Sikitita	Cornicabra de	Megaritiki	Ulliri i Bardhe i Tiranes	

2.2. Sampling and VOO extraction

We manually harvested 2 kg of olive fruits from each tree by sampling all orientations within the canopy. The trees were sampled from October to December when the fruits were at a ripening index (RI) of 2.0 (yellowish-red color) according to the method proposed by the International Olive Oil Council (International Olive Council, 2011).

Monovarietal VOOs were obtained using an Abencor extraction system (MC2 Ingeniería y Sistemas, Sevilla, Spain) under optimized conditions (Peres, Martins, & Ferreira-Dias, 2014). The olives were crushed with a hammer mill equipped with a 4-mm sieve at 3000 rpm. Malaxation of olive pomace was performed at 28 ℃ for 30 min, and then, the biphasic system was centrifuged at 3500 rpm for 2 min. No water was added to the olive pomace at any step of the process. The VOO was decanted in graduated cylinders for approximately 8 h. Water traces were removed by filtering the samples through a cellulose filter. The samples were stored in amber glass bottles at −20 ℃ until analysis.

2.3. Reagents and standards

The solvents used for the analysis of phenols in VOOs were mass spectrometry (MS) grade methanol (MeOH) and *n*-hexane, both from Scharlab (Barcelona, Spain). MS-grade formic acid, also from Scharlab, was used as an ionization agent in the chromatographic mobile phases. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare both the aqueous mobile phase and the hydroalcoholic mixture used as extractant.

The evaluated phenols were hydroxytyrosol, oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycone (3,4-DHPEA-EA), ligstroside aglycone (p-HPEA-EA), luteolin and apigenin. The aglycone forms of oleuropein and ligstroside were discriminated according to their structures. Thus, it was possible to discriminate between the aldehyde open forms of oleuropein aglycone (AOleAgly, the sum of stereoisomers) and the monoaldehyde closed form of the oleuropein aglycone (MAOleAgly). By analogy, it was possible to discriminate between the aldehyde open forms of ligstroside aglycone (ALigAgly, the sum of stereoisomers) and the monoaldehyde closed form of ligstroside aglycone (MALigAgly). Standards for hydroxytyrosol, apigenin and luteolin were

purchased from Extrasynthese (Genay, France). Oleacein, oleocanthal, and the aldehydic open forms of oleuropein aglycone and ligstroside aglycone were provided by Prof. Magiatis of the University of Athens (Greece). The monoaldehyde forms were quantified using the corresponding standards for the aldehyde open forms. Standard solutions of non-secoiridoid phenols were prepared in methanol (1 mg/mL), while secoiridoids were prepared at the same concentration in pure acetonitrile to preserve their stability and avoid undesired conversion to acetal and hemiacetal derivatives.

2.4. Sample preparation for analysis of phenolic compounds

Phenolic compounds were isolated by liquid-liquid extraction following previously published protocols (Verónica Sánchez de Medina, Priego-Capote, & Luque de Castro, 2015). For this purpose, 1 g of VOO was mixed with 2 mL *n*-hexane; then, 1 mL of 60:40 (v/v) methanol-water was added and shaken for 2 min, and the hydroalcoholic phase was separated by centrifugation. The extraction was repeated to enhance the extraction efficiency (V. Sánchez de Medina et al., 2017). The resulting phenolic extracts were analyzed by LC–QqQ MS/MS with three different dilution factors (1:2, 1:50 and 1:200 v/v) to encompass the concentration variability.

2.5. LC-MS/MS analysis of phenolic compounds

Analyses were performed by reversed-phase liquid chromatography followed by electrospray ionization (ESI) in negative mode and tandem mass spectrometry (MS/MS) detection. Ten µL of extract was injected in triplicate into the LC system for chromatographic separation of the target compounds using a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 µm particle size) from Varian (Walnut Creek, CA, USA). The column compartment was kept at 30 ˚C. Mobile phase A was 0.1% formic acid in water, while phase B was 0.1% formic acid in MeOH. The gradient program, at a 0.4 mL/min constant flow rate, was as follows: initially, 50% phase A and 50% phase B were maintained for 0.5 min; from 0.5 to 2 min, mobile phase A was from 50 to 20%; and from min 2 to 4, mobile phase A was from 20 to 0%. This last composition was maintained for 1 min. After each analysis, the column was equilibrated for 5 min to the initial conditions.

The entire eluate was electrosprayed and monitored by MS/MS in Multiple Reaction Monitoring (MRM) mode for selective transitions from precursor to product ions for each analyte. The MRM parameters for the analysis of target phenols are listed in **Supplementary Table 1**. The flow rate and temperature of the drying gas (N_2) were 10 L/min and 300 ℃, respectively. The nebulizer pressure was 50 psi, and the capillary voltage was 3000 V. The dwell time was set at 200 μs.

2.6. Quantitation of the target compounds and statistical analysis

Absolute quantitative analysis was performed by calibration curves obtained using refined sunflower oil spiked with the target phenols. The absence of quantifiable levels of phenols in the refined oil was checked by direct analysis with the developed method. Nine phenolic concentrations from 0.1 ng/mL to 5 μg/mL were injected in triplicate to obtain the calibration curves. The concentration of phenols in the monovarietal VOOs was determined with these models, using three replicates per sample.

ANOVA factorial analysis ($P < 0.05$) was performed to determine the influence of the independent variable (genotype) on the phenolic composition of VOO in the two crop seasons. Box-Cox transformation of the data to fit normality was applied when appropriate. Furthermore, Principal Component Analysis (PCA) was applied to identify groups of cultivars with similar phenolic profiles. The existence of significant pairwise differences among the groups formed in the PCA was evaluated with a Bonferroni post-hoc test, while Pearson correlation was used to find associations between the quantified phenols. These analyses were performed using XLSTAT software (v.2014.5.03, Addinsoft, Paris, France).

3. Results and Discussion

3.1. Evaluation of the phenolic variability in monovarietal VOOs

The phenolic composition of VOO strongly depends on numerous factors, among which the cultivar (genotype) plays a key role (Baiano et al., 2013; El Riachy et al., 2011). This evidence and the absence of studies analyzing a significant number of cultivars prompted us to analyze the phenolic composition of the VOOs in a complete cultivar panel.

We selected 80 olive cultivars **(Table 1)** according to the following criteria: a) importance in terms of VOO production, b) geographical origin, and c) fruit availability in the WOGB. Furthermore, basing on the same criteria, a subset of 25 cultivars was considered for two harvest seasons (2014/2015 and 2015/2016) to test the reproducibility of the results.

The sum of the individual concentration of phenols in the VOOs ranged from hundreds to thousands of mg/kg, as shown in **Table 2**, which lists the concentrations found in the two crop seasons. **Supplementary Tables 2** and **3** show the concentration of each phenolic compound of monovarietal oils analyzed during two crop seasons (two individual trees for each cultivar were analyzed). The high variability in the phenolic levels of VOO can also be visualized in **Supplementary Fig. 1** and **2**, which illustrate the distribution of monovarietal VOOs according to the concentration of each phenol. The genetic variability of the cohort was the main factor responsible for the high variation in the concentration of phenolic compounds, given that the olive trees were grown under the same agronomical conditions and that samples were extracted by the same protocol.

The high concentration of phenolic compounds found in our monovarietal VOOs compared to other studies (Fuentes et al., 2017; Karkoula, Skantzari, Melliou, & Magiatis, 2012) might be explained by the extraction protocol. In large-scale olive oil production, water is normally added to enhance the separation of oil from olive paste. Due to the hydrophilic character of phenolic compounds, this addition can result in phenolic loses. In contrast, during the extraction of the oil samples in the Abencor system, which was used for us in the present study, no water was added so it could have preserved the phenolic compounds in the oil phase. However, our phenolic concentration levels looks to be similar with a recent research (García-Rodríguez, Belaj, Romero-Segura, Sanz, & Pérez, 2017). In agreement with previous studies (García-Rodríguez et al., 2017; Karkoula, Skantzari, Melliou, & Magiatis, 2014), secoiridoid derivatives were the most abundant phenols in all evaluated monovarietal VOOs. Secoiridoid derivatives are aglycone forms of the secoiridoid glucosides formed during oil extraction by β-glucosidase enzymatic hydrolysis of oleuropein, demethyloleuropein, and ligstroside (Servili et al., 2004). The concentration of oleocanthal, one of the most recognized phenols in VOO due to its anti-inflammatory and antioxidant properties, showed an almost 100-fold variation in the cultivar set, ranging from 17 to 1600 mg/kg (**Table 2**). While most cultivars showed an oleocanthal concentration close to their average value (274 mg/kg) several cultivars, such as

'Kalamon', 'Plementa Bjelica', 'Alfafara', 'Pendolino', 'Kotruvsi', 'Enagua de Arenas', 'Caballo', and 'Koroneiki', showed more than 750 mg/kg, and other 14 cultivars showed levels < 50 mg/kg (**Supplementary Tables 2** and **3**; **Supplementary Figs. 1** and **2**).

Oleacein is structurally similar to oleocanthal and is also considered to have similar pharmacological properties (Paiva-Martins et al., 2009). In this study, oleacein also showed high concentrations, with a maximum of 903 mg/kg, but they were lower than those of oleocanthal. Oleacein was found at high levels (366-900 mg/kg) in 'Pendolino', 'Blanqueta', 'Arbequina', 'Cerezuela', 'Kalamon', 'Alfafara', 'Caballo', and 'Koroneiki' and at low levels (7-50 mg/kg) in cultivars such as 'Jabaluna', 'Picual', and 'Morisca', with an average value of 364 mg/kg in the whole set (**Table 2; Supplementary Tables 2** and **3; Supplementary Figs. 1** and **2**). Previous studies have reported oleacein levels ranging from 100 to 400 mg/kg in monovarietal oils, while for oleocanthal, the measured levels were below 350 mg/kg (García-González, Tena, & Aparicio, 2010; Karkoula et al., 2012). Conversely, other authors provided particularly low levels of both phenols that did not exceed 30 mg/kg (Baiano et al., 2013; Ramos-Escudero, Morales, & G. Asuero, 2015). The variability in the concentration ranges for both phenolic compounds among studies might exist due to differences in analytical methodology, such as detection technique, quantitation strategy, or absolute or relative quantitation using non-specific standards for calibrations of the analytical equipment.

The aglycone isomers of oleuropein and ligstroside were also among the most concentrated phenols in VOO. These compounds are associated with the bitter and pungent taste of olive oil (Barbieri et al., 2015). Aglycone isomers are characterized by high antioxidant activity (Taticchi, Esposto, & Servili, 2013). The concentration of aldehyde open forms of oleuropein aglycone ranged from 8 to 918 mg/kg, with an average value of 222 mg/kg. The aldehyde open forms of ligstroside aglycone were less concentrated than the analogous oleuropein isomers, ranging from 2 to 133 mg/kg, with an average value of 31 mg/kg. The monoaldehyde closed forms of oleuropein and ligstroside were quantified in a relative manner because the aldehyde forms were used as quantitation standards. Remarkably, 'Chetoui', 'Villalonga', 'Coratina', 'Zaity', and 'Cornicabra' were among the top five cultivars, with the highest content in the sum of the aglycone isomers.

Three minor phenols (hydroxytyrosol, apigenin and luteolin) were also included in the list of quantified phenols since they have been frequently analyzed in VOO for their beneficial health properties (Tuck & Hayball, 2002). Hydroxytyrosol is a simple alcohol conjugated to form oleuropein derivatives, while luteolin and apigenin are the two most representative flavonoids found in VOO. The concentration of hydroxytyrosol in the analyzed cohort ranged from 0 to 9 mg/kg, while apigenin and luteolin ranged from 0 to 20 mg/kg and from 0 to 11 mg/kg, respectively (**Table 2; Supplementary Tables 2** and **3; Supplementary Figs. 1** and **2**).

Table 2. Mean, minimum and maximum concentration (expressed as mg/kg) of phenolic compounds found in VOO from the cultivars selected in the two crop seasons.

2014/2015 crop season (25 cultivars)				
Phenol	Minimum	Maximum	Mean	SD*
Hydroxytyrosol	0.46	4.89	1.68	1.08
Oleacein	59.7	866.7	364.3	201.7
AOleAgly	10.2	1545.8	283.7	425.1
MAOleAgly	39.0	1136.3	273.2	246.2
Oleocanthal	53.0	2931.1	730.9	739.1
ALigAgly	1.75	1049.7	225.1	299.0
MALigAgly	3.98	326.5	66.2	77.0
Luteolin	0.45	6.25	3.31	1.82
Apigenin	0.04	11.53	1.87	2.34
2015/2016 crop season (80 cultivars)				
Phenol	Minimum	Maximum	Mean	SD*
Hydroxytyrosol	0.28	7.57	2.04	1.51
Oleacein	7.1	903.0	159.6	151.5
AOleAgly	4.10	3501.3	577.4	753.9
MAOleAgly	8.6	918.3	222.2	188.0
Oleocanthal	17.3	1602.3	274.1	332.0
ALigAgly	1.19	1718.2	226.1	304.5
MALigAgly	2.26	133.6	31.7	26.6
Luteolin	0.52	11.35	3.72	2.49
Apigenin	0.18	19.79	2.98	2.88

*SD: Standard deviation.

3.2. Influence of cultivar on phenolic profile variability of olive oil

As mentioned above, the cultivar (genotype) plays a key role in the diversity and concentration of phenolic compounds present in VOO (Baiano et al., 2013). However, neither the phenolic diversity present in the VOO nor the magnitude of the genotypic effect

driving this variability has been extensively explored by analyzing a large, geographically representative set of olive cultivars. Our study provides an outstanding opportunity to shed light on these topics. To do so, we analyzed the phenolic profiles of monovarietal VOOs extracted from 80 selected cultivars growing in the same climatic conditions and subjected to the same agronomical practices. An ANOVA test was applied to test the influence of the cultivar on the phenolic compound variability. The goodness of fit statistics revealed that the percentage of the variability (R^2) explained by the genotype was highly significant (p-value < 0.001) for the nine phenolic compounds and the two consecutive crop seasons. For the first crop season (2014/2015), the percentage of variability explained by the genotype was between 75 and 96%, while for the second crop season (2015/2016), it was between 83 and 97%, respectively, for the hydroxytyrosol and aldehydic open forms of ligstroside aglycone (ALigAgly) (**Table 3)**. Therefore, in agreement with previous studies (De la Rosa et al., 2016; Perez et al., 2014), the genotype was the main factor responsible for the variability found in the phenols analyzed in this set of cultivars.

The reproducibility of these results was further corroborated by the correlation test of phenolic concentrations between two consecutive growing seasons (**Supplementary Table 4**). The highest correlation was observed for apigenin and oleocanthal (p-value < 0.0001; R = 0.90 and R = 0.83, respectively), followed by MALigAgly and the sum of monitored phenols (p-value = 0.0001; R = 0.78 and R = 0.77, respectively). Finally, luteolin (p-value = 0.002; R = 0.75), oleacein (p-value = 0.001; R = 0.72), MAOleAgly (p-value < 0.005; R = 0.40), and ALigAgly (p-value < 0.005; R = 0.39) also provided a significant correlation coefficient between the two seasons. Although only 25 cultivars were included in this consistency test, the high correlation observed for most phenols highlighted the preponderance and stable weight of the genotype in the phenolic variability of VOOs.

Table 3. ANOVA analysis results show the influence of genotype on the concentration of the nine phenolic compounds.

Phenol	R^2	F	*p*-value
2014/2015 crop season (25 cultivars)			
Hydroxytyrosol	0,916	11,360	< 0,0001
Apigenin	0,965	28,487	< 0,0001
Luteolin	0,962	26,065	< 0,0001
Oleocanthal	0,889	8,370	< 0,0001
Oleacein	0,754	3,185	0,003
MALigAgly	0,960	25,215	< 0,0001
ALigAgly	0,925	12,844	< 0,0001
MAOleAgly	0,894	8,764	< 0,0001
AOleAgly	0,887	8,211	< 0,0001
Phenol	R^2	F	*p*-value
2015/2016 crop season (80 cultivars)			
Hydroxytyrosol	0,831	4,984	< 0,0001
Apigenin	0,948	18,585	< 0,0001
Luteolin	0,887	7,961	< 0,0001
Oleocanthal	0,924	12,270	< 0,0001
Oleacein	0,908	9,943	< 0,0001
MALigAgly	0,930	13,529	< 0,0001
ALigAgly	0,973	36,917	< 0,0001
MAOleAgly	0,957	22,405	< 0,0001
AOleAgly	0,956	21,759	< 0,0001

R^2 (determination coefficient): percentage of variability explained by the genotype in the total variance.
F ratio: variation between samples/variation within the samples.
p-value: significance level.

3.3. Classification of olive cultivars attending to their VOO phenolic profiles.

Once the contribution of the cultivar to the phenolic composition of monovarietal VOO was elucidated, the next step was to determine distinctive patterns in the set of cultivars according to their phenolic profiles. First, PCA was applied using the concentrations of individual phenols determined in the 80 monovarietal oils. The first three principal components (PC1, PC2 and PC3) explained 74.1% of the cumulative variability and allowed clustering of the cultivars into four main groups (G1, G2, G3 and G4), characterized by their distinctive phenolic compositions (**Fig. 1).** The G1 group included 18 cultivars characterized by the high concentration of oleuropein and ligstroside aglycone isomers; G2 grouped 16 cultivars with high levels of oleocanthal and oleacein; G3 clustered 10 cultivars with a high concentration of apigenin and luteolin; and finally, G4 included 36 cultivars that showed a balanced composition, with no remarkable concentration in any of

the studied phenolic compounds (**Supplementary Table 5). Fig. 2** illustrates differences in the concentration of these phenolic compounds in the four groups of cultivars differentiated according to the PCA.

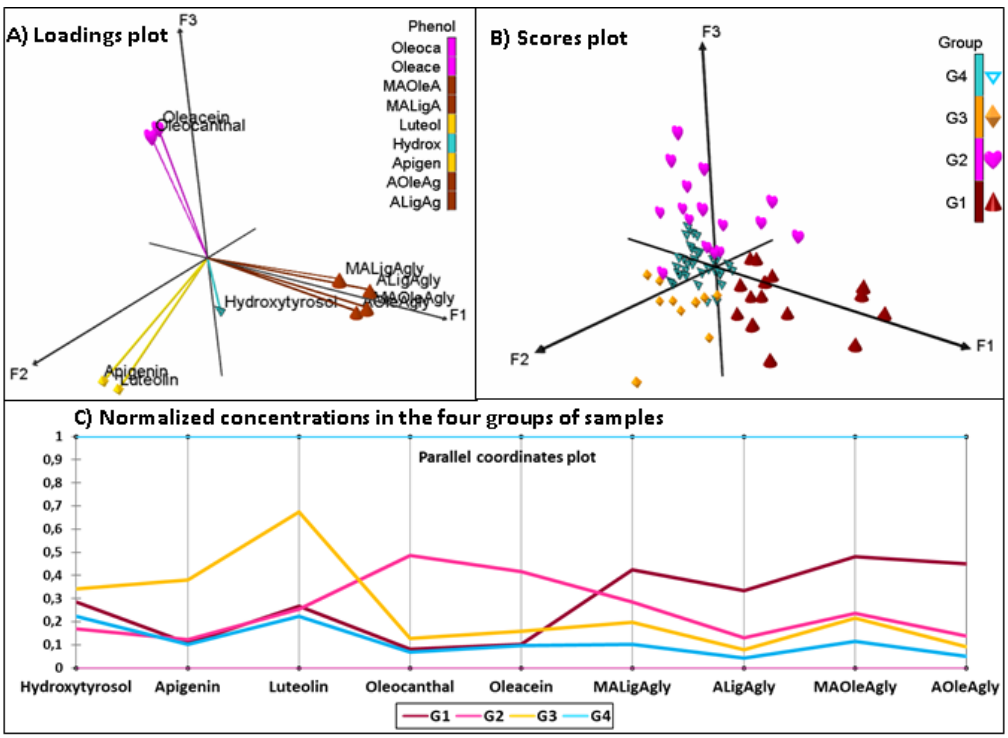

Fig. 1. Principal component analysis for the phenolic profiles of the 80 monovarietal VOOs. (A) Loadings plot. (B) Scores plot. (C) Normalized concentration profiles of the four groups of cultivars classified attending to their phenolic profile.

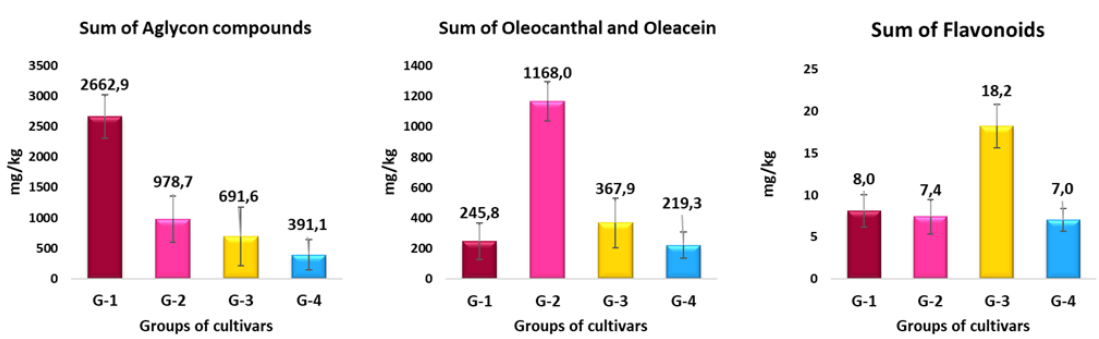

Fig. 2. Differences in the concentration of aglycon compounds, olecanthal and oleacein and flavonoids found in the four groups of monovarietal VOO classified according to the PCA.

A clear difference in the concentration of the aglycone isomers of oleuropein and ligstroside and that of oleocanthal and oleacein was observed between groups G1 and G2. This difference was visualized in the MRM chromatograms obtained by analyzing monovarietal VOO from two cultivars assigned to G1 and G2 (**Fig. 3)**. The presence of the oleuropein aglycone and ligstroside aglycone isomers within the same phenolic profile was justified because they are synthesized through the same pathway. In fact, a strong significant correlation in concentration (p-value < 0.0001 and R = 0.87) was found between both pairs of isomeric forms (**Supplementary Fig. 3**).

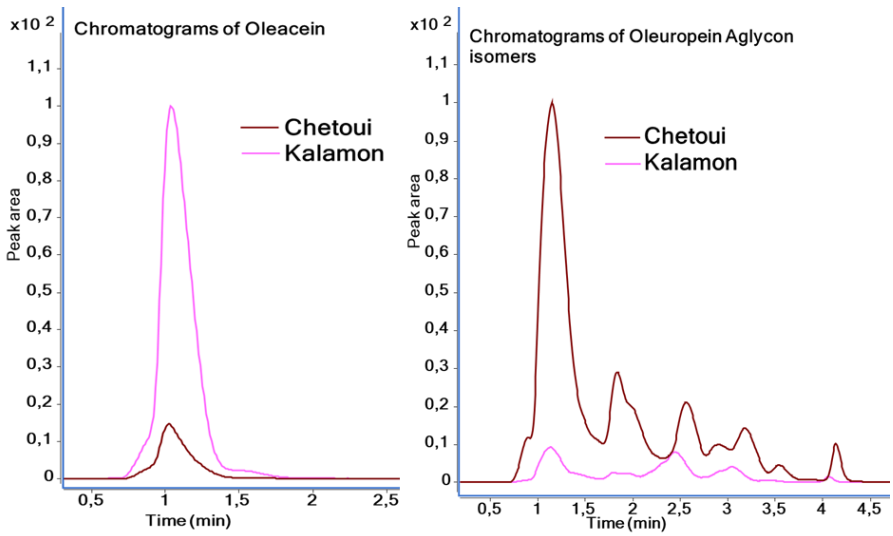

Fig. 3. Chromatograms obtained by the analysis of the VOO from two cultivars 'Chetoui' and 'Kalamon') showing differences in the concentration of oleacein and algycon isomers of oleuropein.

An ANOVA test was used to further evaluate differences between groups of cultivars according to their phenolic profiles. The test revealed highly significant differences (p < 0.0001) among the four groups of cultivars (**Supplementary Table 6.A**). Bonferroni post-hoc test detected significant differences (p < 0.0001) among the groups. The results of ANOVA and post-hoc tests reinforced the results of the PCA analysis (**Supplementary Tables 6.B**).

The consistency of this classification between agronomic years was tested by applying the analyses described above to the subset of 25 cultivars in two consecutive seasons. As a result, despite climatic differences between years that might significantly affect the phenolic concentration of VOOs, more than 80% of cultivars were consistently

assigned to the same PCA group in both years (**Supplementary Table 7**). Therefore, genotype crucially influences VOO phenolic profiles and especially the concentration of secoiridoid derivatives, as they are the most concentrated phenols in olive oil.

3.4. Influence of pathways on synthesis of secoiridoids that differentiate olive oils.

Understanding the pathways leading to the biosynthesis of phenolic compounds is necessary to decipher the genetic basis of their variation. It is especially important to understand the biochemical pathways responsible for the synthesis of secoiridoid derivatives due to their high contribution to phenolic differences in monovarietal VOOs. The two main pathways proposed for the synthesis of secoiridoids in olive fruits end in the synthesis of oleuropein, although this glycoside phenol is rarely detected in VOO. The proposed pathways are mechanistically diverse and differ in the precursor of oleuropein. The first pathway, proposed by Damtoft et al (Damtoft, Franzyk, & Jensen, 1993), is initiated with mevalonic acid with the formation of iridoids, and then to ligstroside, which is the precursor of oleuropein. An alternative pathway was proposed in 2002 by Ryan et al., with tyrosol as a precursor produced through the phenylpropanoid biosynthesis (Ryan, Antolovich, Herlt, et al., 2002). In this second mechanism, tyrosol is the substrate of two synthetic pathways that led to a common final product: oleuropein. The first pathway addresses ligstroside as an intermediate, while the second transforms to the oleacein and oleuropein aglycone before final conversion into oleuropein. Both synthetic pathways occur in the first ripening stages as a common strategy to accumulate oleuropein in the fruit.

At advanced maturation and especially during olive oil extraction, enzymatic and non-enzymatic biotransformations are produced to form the secoiridoid derivatives found in VOO. The most complete biotransformation pathway was proposed by (Obied, Bedgood, Prenzler, & Robards, 2007), who explained the appearance of secoiridoid derivatives from oleuropein as a precursor. In this pathway, oleuropein is converted into several derivatives according to the involvement of esterases and β-glucosidases enzymes. Depending on the enzymatic activity, oleuropein can mainly be converted to oleuropein aglycone isomers or to oleacein via two main pathways (**Supplementary Fig. 4**). Thus, the formation of oleuropein aglycone isomers is catalyzed by β-glucosidase, activated during crushing and malaxation, which leads to stable isomers discriminated by functional groups: the

aldehyde and monoaldehyde forms of oleuropein aglycone, the latter with the closed heterocyclic ring. The second pathway is characterized by the involvement of methylesterases to cleave the methyl group in the elenolic acid. This second pathway would be responsible for the formation of oleacein. A similar situation would occur for ligstroside to preferentially produce ligstroside aglycone isomers and oleocanthal. The technical factors of VOO extraction are critical to obtain a phenolic profile, since variation in technological factors could favor the kinetics of certain enzymatic processes.

A Pearson correlation analysis was used to find associations between the monitored phenols in the complete set of monovarietal VOOs. Several strong correlations (p-value < 0.0001 and R > 0.69) were found between pairs of phenolic compounds. The most interesting result was the detection of significant correlations between the isomers of oleuropein aglycone and ligstroside aglycone, and between the decarboxymethylated dialdehydic compounds (oleocanthal and oleacein) (**Supplementary Table 8**). No statistical association was observed between the two groups of secoiridoid derivatives, which supports the fact that aglycons and decarboxymethylated dialdehyde aglycons are produced following two independent pathways from the same initial substrates, oleuropein and ligstroside. The group of cultivars with a high content of oleocanthal and oleacein would be characterized by increased activity of demethylesterases as the key step towards the production of these two dialdehydes. Therefore, this study suggests that it is possible to breed new olive cultivars to obtain monovarietal oils enriched with certain phenols, despite the influence of agronomic and technological factors—essentially, ripening index, grinding and malaxation time and temperature. It would be promising to study the interaction between these other factors and genotype to evaluate how they can modulate variability in the phenolic profiles of VOO.

4. Conclusions

In this study, remarkable variability was found for nine phenolic compounds in the largest set of monovarietal VOOs analyzed to date. Genotype was the main factor contributing to this variability for all phenolic compounds with a percentage of total variance between 83% and 97%. The secoiridoid derivatives were the most abundant phenols of all monovarietal VOOs evaluated in this study. Various previously

undistinguished olive cultivars were revealed to be very rich, interesting cultivars for certain phenolic compounds.

Multivariate analysis allowed detection of four groups of cultivars (G1, G2, G3 and G4) via their phenolic profile. G1 was characterized by a high concentration of oleuropein and ligstroside aglycone isomers and G2 by a high concentration of oleocanthal and oleacein; G3 was rich in two flavonoids (apigenin and luteolin). The last group, G4, included cultivars for VOOs that did not stand out in terms of the monitored phenols. The differences in the phenolic profiles of VOOs pertaining to G1 and G2 groups allowed detection of two independent pathways in the metabolism of oleuropein and ligstroside, through the involvement of demethylesterases and β-glucosidases.

The extensive and accurate characterization of phenolic compounds in VOO is necessary for the production of high-quality VOOs. This study opens new avenues in this research area, for example, studying the phenolic contents and their routes of production or the influence of the phenolic profiles on human health, the organoleptic features and olive oil shelf-life.

Acknowledgments

This research was jointly financed by the Spanish Ministerio de Economía y Competitividad and the Interreg-Med Program through the projects CTQ2015-68813-R and MED-1033. Both projects are co-funded by the European Regional Development Fund/European Social Fund ("Investing in your future"). H. Miho thanks the International Olive Council (IOOC) for a doctoral fellowship).

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Supplementary data

SUPPLEMENTAL TABLES

Supplementary Table 1. Multiple Reaction Monitoring (MRM) parameters for quantitative analysis of phenolic compounds by LC–MS/MS.

Phenol		Retention time (min)	Q1 voltage (V)	Precursor ion (m/z)	Collision energy (eV)	Quantitative transition (m/z)	Product ion confirmation (m/z)
Hydroxytyrosol		2.1	110	153.1	10	153-123	108
3.4-DHPEA-EDA (Oleacein)		4.3	110	319.1	12	319-59	139
3.4-DHPEA-EA	AOleAgly	4.6	110	377	12	377-275	307
	MAOleAgly	5.9	110	377	12	377-275	307
p-HPEA-EDA (Oleocanthal)		5.4	110	303.1	12	303-59	137
p-HPEA-EA	ALigAgly	5.5	110	361.1	12	361-291	101
	MALigAgly	6.2	110	361.1	12	361-291	101
Luteolin		6.3	170	285	35	285-133	175
Apigenin		6.6	170	269	35	269-117	151

AOleAgly – Aldehydic open forms of Oleuropein Aglycone; **MAOleAgly** – Monoaldehydic closed form of Oleuropein Aglycone.
ALigAgly – Aldehydic open forms of Ligstroside Aglycone; **MALigAgly** – Monoaldehydic closed form of Ligstroside Aglycone.

Supplementary Table 2. Concentration of phenolic compounds (expressed as mg/kg) found in the selected 25 monovarietal VOOs in the 2014–2015 season.

Cultivar	Hydroxytyros	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Alameño de Montilla	1.57 ± 0.02	1.47 ± 0.47	4.78 ± 1.23	703 ± 35	453 ± 47	30 ± 3	14.05 ± 1.77	137 ± 22	19 ± 1
Alfafara	0.93 ± 0.23	0.79 ± 0.03	1.39 ± 0.10	2931 ± 26	572 ± 266	174 ± 52	285 ± 254	251 ± 151	127 ± 132
Arbequina	0.73 ± 0.01	2.77 ± 0.96	6.25 ± 0.61	303 ± 15	405 ± 29	4.22 ± 0.39	5.53 ± 0.94	128 ± 16	14.15 ± 2.36
Arbosana	0.68 ± 0.25	11.53 ± 0.46	6.01 ± 1.01	238 ± 5	285 ± 35	6.56 ± 1.06	52 ± 19	141 ± 28	147 ± 61
Blanqueta	2.31 ± 0.62	0.12 ± 0.04	2.15 ± 0.03	539 ± 206	485 ± 283	12.83 ± 2.39	3.00 ± 1.72	102 ± 51	10.19 ± 6.72
Bosana	2.25 ± 0.54	1.97 ± 0.25	2.86 ± 0.23	568 ± 298	392 ± 94	66 ± 15	280 ± 225	352 ± 136	469 ± 455
Caballo	2.66 ± 0.71	0.04 ± 0.17	0.81 ± 0.40	592 ± 41	269 ± 123	34 ± 7	37 ± 18	130 ± 33	30 ± 3
Cerezuela	1.59 ± 0.04	0.11 ± 0.03	0.85 ± 0.09	1232 ± 221	775 ± 60	76 ± 2	201 ± 71	356 ± 16	210 ± 92
Coratina	2.37 ± 0.59	0.18 ± 0.23	0.45 ± 0.49	932 ± 299	366 ± 30	140 ± 14	1049 ± 70	524 ± 15	1399 ± 24
Farga	1.27 ± 0.01	1.44 ± 0.25	1.87 ± 0.20	149 ± 0.2	209 ± 36	15.93 ± 0.67	30 ± 1	200 ± 24	83 ± 20
Gordal de Granada	1.03 ± 0.44	1.87 ± 0.12	4.92 ± 0.34	480 ± 190	344 ± 4	43 ± 18	117 ± 34	229 ± 2	156 ± 11
Joanenca	4.23 ± 3.26	0.32 ± 0.09	2.26 ± 0.32	769 ± 10	474 ± 620	37 ± 36	24 ± 34	167 ± 165	40 ± 56
Kalamon	1.06 ± 0.20	0.46 ± 0.07	2.26 ± 0.09	2776 ± 277	866 ± 285	202 ± 32	636 ± 69	410 ± 79	250 ± 5
Koroneiki	2.04 ± 0.29	2.72 ± 0.83	3.53 ± 0.76	442 ± 38	324 ± 53	71 ± 10	363 ± 66	348 ± 31	478 ± 59
Loaime	1.10 ± 0.06	3.17 ± 0.12	4.91 ± 0.33	393 ± 93	217 ± 69	43 ± 22	142 ± 101	176 ± 82	134 ± 98
Mastoidis	4.89 ± 0.60	1.53 ± 0.48	2.68 ± 0.67	823 ± 61	363 ± 76	326 ± 70	613 ± 61	1136 ± 103	605 ± 140
Mollar de Cieza	1.68 ± 0.04	1.40 ± 0.20	3.64 ± 0.52	684 ± 36	486 ± 24	26 ± 6	40 ± 23	169 ± 32	48 ± 22
Morona	0.90 ± 0.12	4.63 ± 0.41	5.97 ± 0.71	130 ± 35	132 ± 46	12 ± 0.34	65 ± 6	148 ± 22	152 ± 21
Negrillo de la	1.44 ± 0.14	1.57 ± 0.35	4.90 ± 0.72	897 ± 137	348 ± 37	27 ± 14	16.87 ± 16.82	79 ± 24	14.12 ± 9.22
Ojo de Liebre	0.46 ± 0.00	0.81 ± 0.19	3.35 ± 0.68	593 ± 109	254 ± 51	31 ± 12	36 ± 4.96	97 ± 22	29 ± 0.41
Picual de Almería	0.78 ± 0.03	0.07 ± 0.05	1.13 ± 0.28	75 ± 6	60 ± 9	108 ± 12	822 ± 215	712 ± 13	1545 ± 404
Plementa Bjelica	2.63 ± 0.14	3.45 ± 1.06	4.38 ± 0.16	1644 ± 308	649 ± 77	119 ± 4	597 ± 317	489 ± 41	743 ± 421
Royal de Cazorla	0.55 ± 0.14	0.68 ± 0.17	1.60 ± 0.09	53 ± 11	59 ± 5	3.98 ± 0.62	1.75 ± 0.82	38 ± 2	13.36 ± 3.35
Sabatera	1.39 ± 0.03	1.36 ± 0.08	4.16 ± 0.36	289 ± 13	195 ± 33	17.88 ± 0.84	12.17 ± 0.82	124 ± 37	21 ± 0.86
Sikitita	1.79 ± 0.35	2.24 ± 0.09	5.75 ± 0.37	245 ± 15	177 ± 66	8.37 ± 0.30	16.22 ± 12.58	76 ± 30	23 ± 16

Supplementary Table 3. Concentration of phenolic compounds (expressed as mg/kg) found in the selected 80 monovarietal VOOs in the 2015–2016 season.

Cultivar	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Abou Choki	0.89 ± 0.09	1.42 ± 0.13	2.12 ± 0.23	112 ± 15	55 ± 17	89 ± 4.03	653 ± 113	285 ± 42	482 ± 16
Alameño de Montilla	1.52 ± 0.23	2.96 ± 0.35	4.87 ± 0.08	219 ± 61	199 ± 90	11.09 ± 0.16	14.77 ± 0.20	92 ± 22	21 ± 10
Alfafara	1.73 ± 0.06	1.56 ± 0.19	1.90 ± 0.36	1324 ± 252	403 ± 107	60 ± 13	224 ± 40	145 ± 23	127 ± 26
Amygdalolia Nana	1.83 ± 0.45	0.64 ± 0.01	1.35 ± 0.10	37 ± 0	84 ± 8	9.81 ± 1.38	92 ± 8	329 ± 46	640 ± 186
Arbequina	0.65 ± 0.06	3.34 ± 0.01	4.53 ± 0.07	325 ± 107	540 ± 41	2.53 ± 0.61	3.43 ± 2.28	37 ± 16	50 ± 1.61
Arbosana	1.28 ± 0.93	9.10 ± 2.07	3.74 ± 1.36	137 ± 5	130 ± 5	2.81 ± 0.11	3.79 ± 0.70	39 ± 4.23	25 ± 4.18
Ascolana Tenera	4.40 ± 3.63	2.16 ± 0.74	3.14 ± 1.17	89 ± 53	95 ± 12	26 ± 21	67 ± 29	389 ± 111	218 ± 69
Azapa	3.46 ± 0.33	8.09 ± 0.01	7.77 ± 0.13	339 ± 3	292 ± 8	38 ± 0.53	250 ± 18	361 ± 20	589 ± 9
Barnea	1.31 ± 0.34	2.30 ± 0.11	3.98 ± 0.10	85 ± 16	45 ± 11	71 ± 9	336 ± 15	384 ± 80	1152 ± 225
Blanqueta	4.26 ± 4.28	0.87 ± 0.14	1.60 ± 0.61	627 ± 124	618 ± 202	13.39 ± 2.70	10.96 ± 0.81	165 ± 50	117 ± 98
Bodoquera	0.57 ± 0.05	0.35 ± 0.22	2.12 ± 0.72	172 ± 37	197 ± 29	10.13 ± 3.32	16.13 ± 2.26	110 ± 12	64 ± 11
Bosana	1.07 ± 0.64	2.78 ± 1.84	5.96 ± 4.65	282 ± 351	164 ± 130	54 ± 52	345 ± 52	304 ± 17	1305 ± 796
Bouteillan	0.61 ± 0.22	6.96 ± 0.23	5.52 ± 0.78	58 ± 18	47 ± 9	57 ± 13	614 ± 15	432 ± 93	1140 ± 237
Caballo	0.65 ± 0.10	0.64 ± 0.17	0.94 ± 0.04	769 ± 117	402 ± 67	75 ± 16	413 ± 89	511 ± 162	805 ± 536
Carolea	5.45 ± 0.19	2.54 ± 0.00	3.90 ± 0.00	21 ± 2	22 ± 0	30 ± 2.90	102 ± 12	198 ± 6	542 ± 108
Carrasqueño de Elvas	0.69 ± 0.24	5.76 ± 0.24	8.64 ± 0.02	154 ± 21	70 ± 18	15.28 ± 2.23	130 ± 12	118 ± 13	268 ± 25
Cerezuela	0.50 ± 0.21	1.51 ± 0.90	2.36 ± 1.52	436 ± 393	504 ± 298	26 ± 33	479 ± 25	484 ± 25	1651 ± 422
Changlot Real	5.19 ± 0.25	1.09 ± 0.03	1.30 ± 0.18	383 ± 38	217 ± 19	57 ± 8	499 ± 27	292 ± 67	629 ± 142
Chemlal de Kabilye	0.92 ± 0.15	1.18 ± 0.09	1.62 ± 0.37	190 ± 36	121 ± 9	69 ± 3.13	287 ± 60	306 ± 15	504 ± 311
Chetoui	1.85 ± 0.60	3.76 ± 0.47	3.55 ± 0.64	40 ± 9	52 ± 13	64 ± 11	936 ± 476	723 ± 154	3501 ± 387
Çobrancosa	1.31 ± 0.29	2.54 ± 0.01	3.38 ± 0.59	122 ± 42	93 ± 29	25 ± 11	214 ± 192	193 ± 134	785 ± 831
Coratina	1.76 ± 0.06	1.66 ± 0.00	1.99 ± 0.06	202 ± 37	156 ± 25	55 ± 28	1356 ± 587	918 ± 306	2747 ± 189
Cordovil de Serpa	4.10 ± 0.07	4.35 ± 1.17	6.14 ± 1.44	165 ± 5	131 ± 3	43 ± 9	135 ± 32	243 ± 38	300 ± 138
Cornicabra	2.02 ± 1.11	0.58 ± 0.01	1.93 ± 0.09	354 ± 25	121 ± 7	100 ± 21	1718 ± 62	309 ± 53	2022 ± 586

Cultivar	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Cornicabra de Mérida	1.21 ± 0.21	1.12 ± 0.02	1.53 ± 0.51	132 ± 7	75 ± 48	2.26 ± 0.97	3.99 ± 1.94	29 ± 8	25 ± 12
Empeltre	1.42 ± 0.00	0.69 ± 0.00	1.34 ± 0.00	94 ± 77	152 ± 123	10.69 ± 1.46	70 ± 36	110 ± 61	280 ± 207
Enagua de Arenas	0.28 ± 0.02	1.16 ± 0.06	2.21 ± 0.25	798 ± 325	190 ± 120	13.77 ± 6	16.18 ± 9	55 ± 22	18.82 ± 0.63
Farga	1.38 ± 0.92	2.30 ± 0.55	2.25 ± 0.69	78 ± 25	94 ± 14	8.26 ± 3.50	24 ± 10	89 ± 8	163 ± 49
Frantoio	3.71 ± 2.34	1.29 ± 0.48	2.05 ± 1.26	256 ± 34	94 ± 12	45 ± 26	272 ± 9	185 ± 61	293 ± 51
Galega Vulgar	2.26 ± 0.08	1.24 ± 0.59	0.83 ± 0.42	173 ± 78	294 ± 191	2.63 ± 1.21	2.17 ± 1.11	29 ± 15	12.85 ± 0.75
Gemlik	1.15 ± 0.03	4.62 ± 0.67	3.79 ± 1.26	45 ± 5	22 ± 3	25 ± 1.98	116 ± 1.93	146 ± 7	174 ± 77
Gordal de Granada	1.23 ± 0.09	0.67 ± 0.05	0.77 ± 0.02	47 ± 8	28 ± 3	8.49 ± 1.04	7.48 ± 1.69	34 ± 3.24	19.24 ± 5
Hojiblanca	1.33 ± 0.01	6.76 ± 1.81	8.14 ± 3.21	92 ± 43	84 ± 43	8.57 ± 4.17	26 ± 8	64 ± 29	74 ± 23
Jabaluna	1.13 ± 0.29	0.55 ± 0.01	1.04 ± 0.46	17 ± 2	7.10 ± 4	11.55 ± 0.38	47 ± 7	163 ± 42	198 ± 95
Joanenca	1.01 ± 0.49	1.16 ± 0.62	1.31 ± 1.08	621 ± 91	149 ± 7	16.50 ± 11	3.37 ± 0.84	48 ± 20	10.11 ± 0.37
Kalamon	1.16 ± 0.13	1.80 ± 0.18	4.82 ± 0.60	1602 ± 688	435 ± 116	44 ± 1.06	99 ± 26	101 ± 9	108 ± 27
Koroneiki	0.96 ± 0.15	2.14 ± 0.00	3.14 ± 0.26	752 ± 8	366 ± 131	69 ± 17	941 ± 413	470 ± 160	1638 ± 431
Kotruvsi	0.92 ± 0.03	10.56 ± 1.23	7.64 ± 0.99	876 ± 141	228 ± 33	55 ± 12	230 ± 42	153 ± 35	184 ± 30
Kusha	2.13 ± 0.28	5.27 ± 0.63	10.41 ± 2.81	24 ± 1	30 ± 2	41 ± 17	212 ± 85	433 ± 191	491 ± 105
Lastovka	2.98 ± 0.53	3.35 ± 0.22	6.65 ± 0.95	96 ± 10	197 ± 18	32 ± 1.58	137 ± 8	397 ± 15	701 ± 62
Leccino	2.44 ± 0.03	4.88 ± 1.29	5.35 ± 1.51	113 ± 18	85 ± 19	8.25 ± 2.30	21 ± 11	41 ± 22	49 ± 6
Lechín de Sevilla	3.71 ± 3.46	0.41 ± 0.02	0.52 ± 0.11	30 ± 4	35 ± 3	19.67 ± 4.05	180 ± 69	188 ± 20	658 ± 350
Levantinka	5.24 ± 0.89	2.58 ± 0.03	3.23 ± 0.17	569 ± 203	254 ± 45	40 ± 15	200 ± 75	220 ± 70	460 ± 74
Loaime	1.09 ± 0.17	0.38 ± 0.15	0.90 ± 0.06	302 ± 70	109 ± 40	3.15 ± 0.25	35 ± 0.32	8.60 ± 0.80	45 ± 5
Lucio	2.34 ± 0.36	1.71 ± 0.50	2.10 ± 0.49	43 ± 4	75 ± 14	12.73 ± 3.05	76 ± 44	229 ± 111	433 ± 385
Manzanilla Cacereña	0.80 ± 0.13	2.70 ± 0.51	2.28 ± 0.71	28 ± 5	21 ± 3	3.91 ± 0.17	9.45 ± 0.97	11.12 ± 1.64	22 ± 4.98
Manzanilla de Sevilla	5.18 ± 4.78	2.40 ± 0.54	2.45 ± 0.74	136 ± 50	125 ± 48	16.18 ± 10	50 ± 50	138 ± 79	201 ± 144
Manzanilla Prieta	4.04 ± 3.82	0.89 ± 0.24	1.19 ± 0.61	234 ± 38	154 ± 46	134 ± 17	954 ± 297	709 ± 52	1294 ± 543
Mastoidis	2.87 ± 0.22	3.11 ± 1.03	3.69 ± 1.04	300 ± 67	111 ± 0	54 ± 16	256 ± 52	211 ± 42	233 ± 86

Cultivar	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Megaritiki	1.86 ± 0.02	0.97 ± 0.12	1.51 ± 0.76	413 ± 21	303 ± 29	51 ± 1.31	114 ± 40	248 ± 6	270 ± 162
Mission Moojeski	6.62 ± 0.01	19.79 ± 1.97	11.35 ± 0.93	617 ± 20	227 ± 17	54 ± 15	360 ± 18	210 ± 4.44	511 ± 18
Mixani	2.29 ± 0.33	3.59 ± 0.15	6.76 ± 0.96	76 ± 5	115 ± 65	27 ± 0.15	246 ± 63	425 ± 12	1571 ± 206
Mollar de Cieza	2.19 ± 1.48	2.72 ± 0.71	3.31 ± 0.44	195 ± 21	140 ± 73	5.67 ± 1.68	16.92 ± 17	61 ± 42	43 ± 14
Moraiolo	2.56 ± 0.37	1.95 ± 0.01	3.56 ± 0.25	274 ± 241	294 ± 197	42 ± 0.44	134 ± 10	295 ± 13	479 ± 42
Morisca	1.37 ± 1.01	1.66 ± 1.28	3.98 ± 3.78	33 ± 14	19 ± 26	3.80 ± 0.30	2.35 ± 1.24	23 ± 1.08	4.10 ± 3.54
Morona	2.65 ± 0.06	6.51 ± 0.84	7.78 ± 1.40	82 ± 5	80 ± 3	36 ± 11	92 ± 23	129 ± 27	240 ± 102
Morrut	3.46 ± 1.01	0.18 ± 0.15	0.76 ± 0.06	160 ± 74	188 ± 91	42 ± 6	323 ± 81	374 ± 9	791 ± 133
Nasuhi	2.03 ± 0.23	2.39 ± 0.13	3.97 ± 0.15	80 ± 1	52 ± 9	48 ± 4.05	389 ± 73	658 ± 149	1887 ± 289
Negrillo de la Carlota	1.04 ± 0.04	2.68 ± 0.59	5.01 ± 1.79	564 ± 113	204 ± 0	54 ± 7	268 ± 29	142 ± 6	299 ± 18
Ojo de Liebre	0.50 ± 0.05	1.74 ± 0.29	3.26 ± 0.30	199 ± 76	108 ± 34	39 ± 15	182 ± 77	212 ± 58	221 ± 113
Palomar	1.48 ± 1.38	4.43 ± 0.38	4.07 ± 1.01	151 ± 77	60 ± 44	16.55 ± 2.08	128 ± 8	54 ± 36	70 ± 64
Pendolino	1.01 ± 0.45	3.16 ± 1.12	3.33 ± 1.36	1079 ± 820	903 ± 394	28 ± 3.44	171 ± 8	238 ± 44	377 ± 128
Picholine Marocaine	7.57 ± 2.77	0.43 ± 0.24	1.60 ± 0.41	82 ± 23	69 ± 29	17.68 ± 7	203 ± 40	346 ± 142	1303 ± 126
Picual	0.50 ± 0.01	4.43 ± 2.75	6.27 ± 2.74	29 ± 1	10.14 ± 5	25 ± 27	318 ± 365	159 ± 141	609 ± 640
Picual de Almería	3.41 ± 1.72	0.87 ± 0.13	2.10 ± 0.41	117 ± 5	86 ± 7	59 ± 24	496 ± 126	317 ± 28	924 ± 238
Picudo	2.57 ± 0.78	7.56 ± 0.02	7.58 ± 1.08	490 ± 319	242 ± 67	10.91 ± 7	14.53 ± 14	57 ± 7	21 ± 13
Plementa Bjelica	0.35 ± 0.08	5.73 ± 2.04	5.38 ± 2.06	1564 ± 692	302 ± 19	46 ± 24	292 ± 49	274 ± 1.07	1244 ± 948
Rapasayo	0.62 ± 0.06	2.15 ± 0.19	2.49 ± 0.00	358 ± 53	43 ± 2	53 ± 9	244 ± 68	58 ± 0.95	61 ± 4.92
Royal de Calatayud	2.25 ± 0.38	4.46 ± 0.38	10.97 ± 0.43	101 ± 15	56 ± 5	75 ± 3.53	541 ± 31	630 ± 6	1545 ± 0.79
Royal de Cazorla	2.03 ± 0.06	2.53 ± 0.16	1.76 ± 0.16	43 ± 13	54 ± 12	2.95 ± 0.47	3.86 ± 0.87	73 ± 18	48 ± 16
Sabatera	0.82 ± 0.04	2.26 ± 0.97	2.21 ± 1.39	130 ± 35	131 ± 66	12.43 ± 2.71	24 ± 16	139 ± 68	127 ± 77
Sandalio	0.99 ± 0.11	1.66 ± 0.19	2.20 ± 0.41	287 ± 105	205 ± 86	4.56 ± 0.51	3.24 ± 0.02	54 ± 20	126 ± 37
Sikitita	1.55 ± 0.47	2.31 ± 0.82	5.59 ± 0.07	131 ± 4	132 ± 24	6.33 ± 3.29	11.05 ± 6	39 ± 20	40 ± 21
Tanche	1.66 ± 0.43	4.76 ± 0.48	5.57 ± 0.60	70 ± 17	99 ± 20	4.14 ± 0.42	8.61 ± 1.14	54 ± 19	29 ± 0.35

Cultivar	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Ulliri i Bardhe i Tiranes	1.71 ± 0.23	3.01 ± 0.28	5.41 ± 0.17	72 ± 11	120 ± 20	5.17 ± 0.86	17.27 ± 10	71 ± 2.13	43 ± 3.95
Verdale	1.44 ± 0.05	3.17 ± 1.38	5.08 ± 1.72	57 ± 4	96 ± 16	4.74 ± 1.95	11.60 ± 8	75 ± 19	57 ± 50
Verde Verdelho	1.18 ± 0.29	0.80 ± 0.27	1.73 ± 1.17	148 ± 19	86 ± 1	2.30 ± 0.14	1.19 ± 0.43	24 ± 16	6.78 ± 3.27
Verdial de Huévar	3.45 ± 0.14	1.73 ± 0.14	2.81 ± 0.35	149 ± 46	71 ± 1	42 ± 22	103 ± 53	77 ± 13	104 ± 56
Villalonga	1.52 ± 1.23	6.04 ± 0.85	4.55 ± 0.41	42 ± 30	49 ± 26	14.87 ± 4.78	293 ± 107	263 ± 22	3212 ± 3070
Zaity	0.33 ± 0.01	0.98 ± 0.03	1.49 ± 0.04	35 ± 8	48 ± 22	13.88 ± 0.36	186 ± 22	364 ± 40	2461 ± 599

Supplementary Table 4. Pearson correlation analysis between two crop seasons (2014-2015 and 2015–2016) for each phenolic compound monitored in 25 same cultivars.

Phenol	**R**	**R^2**	***p*-value**
Hydroxytyrosol	0.018	0.0003	0.931
Apigenin	0.907	0.824	< 0.0001
Luteolin	0.752	0.566	< 0.0001
Oleocanthal	0.838	0.702	< 0.0001
Oleacein	0.723	0.524	< 0.0001
MALigAgly	0.780	0.609	< 0.0001
ALigAgly	0.717	0.514	< 0.0001
MAOleAgly	0.408	0.166	0.047
AOleAgly	0.399	0.159	0.047
Sum of phenols	0.772	0.596	< 0.0001

Supplementary Table 5. Classification of the 80 olive cultivars into the four groups established by the PCA according to their phenolic profiles.

G1	G2	G3	G4	
Abou Choki	Alfafara	Arbosana	Alameño de Montilla	Manzanilla Cacereña
Barnea	Arbequina	Azapa	Amygdalolia Nana	Manzanilla de Sevilla
Bosana	Blanqueta	Carrasqueño de Elvas	Ascolana Tenera	Mastoidis
Bouteillan	Caballo	Cordovil de Serpa	Bodoquera	Mollar de Cieza
Changlot Real	Cerezuela	Hojiblanca	Carolea	Morisca
Chemlal de Kabilye	Enagua de Arenas	Kusha	Çobrancosa	Ojo de Liebre
Chetoui	Joanenca	Lastovka	Cornicabra de Mérida	Palomar
Coratina	Kalamon	Mission Moojeski	Empeltre	Picual
Cornicabra	Koroneiki	Morona	Farga	Rapasayo
Manzanilla Prieta	Kotruvsi	Picudo	Frantoio	Royal de Cazorla
Mixani	Levantinka		Galega Vulgar	Sabatera
Morrut	Megaritiki		Gemlik	Sandalio
Nasuhi	Moraiolo		Gordal de Granada	Sikitita
Picholine Marocaine	Negrillo de la Carlota		Jabaluna	Tanche
Picual de Almería	Pendolino		Leccino	Ulliri i Bardhe i Tiranes
Royal de Calatayud	Plementa Bjelica		Lechín de Sevilla	Verdale
Villalonga			Loaime	Verde Verdelho
Zaity			Lucio	Verdial de Huévar

Supplementary Table 6. Results from multiple comparisons analysis between the groups of cultivars considering the three phenolic profiles as quantitative variables: Sum of oleuropein and ligstroside aglycone isomers; sum of oleocanthal and oleacein, sum of apigenin and luteolin.

(A) ANOVA test

Parameter	Sum of Aglycone compounds	Sum of Oleocanthal & Oleacein	Sum of Flavonoids
R^2	0,508	0,525	0,326
F	26,116	28,005	12,230
p-value	< 0,0001	< 0,0001	< 0,0001

(B) Bonferroni: Analysis of the differences between the categories with a confidence interval of 95%

Contrast	Difference[1]	Standardized difference	Critical value	Pr > Diff	Significant
Sum of Aglycone compounds					
G1 vs G4	2271,774	8,824	2,709	< 0,0001	Yes
G1 vs G3	1971,255	4,755	2,709	< 0,0001	Yes
G1 vs G2	1684,153	4,645	2,709	< 0,0001	Yes
G2 vs G4	587,621	3,167	2,709	0,002	Yes
G2 vs G3	287,102	0,693	2,709	0,490	No
G3 vs G4	300,519	1,880	2,709	0,064	No
Sum of Oleocanthal and Oleacein					
G2 vs G4	948,660	8,914	2,709	< 0,0001	Yes
G2 vs G1	922,173	7,092	2,709	< 0,0001	Yes
G2 vs G3	800,138	4,880	2,709	< 0,0001	Yes
G3 vs G4	148,522	1,990	2,709	0,050	No
G3 vs G1	122,035	1,191	2,709	0,237	No
G1 vs G4	26,487	0,836	2,709	0,406	No
Sum of Flavonoids					
G3 vs G4	11,243	5,885	2,709	< 0,0001	Yes
G3 vs G2	10,853	5,095	2,709	< 0,0001	Yes
G3 vs G1	10,177	4,635	2,709	< 0,0001	Yes
G1 vs G4	1,066	0,955	2,709	0,343	No
G1 vs G2	0,676	0,657	2,709	0,513	No
G2 vs G4	0,390	0,167	2,709	0,868	No

Modified significance level: 0,008

[1]Non transformad data.

Supplementary Table 7. Categorization of the 25 cultivars selected from the two crop seasons study according to the phenolic profile in the four groups differentiated by PCA.

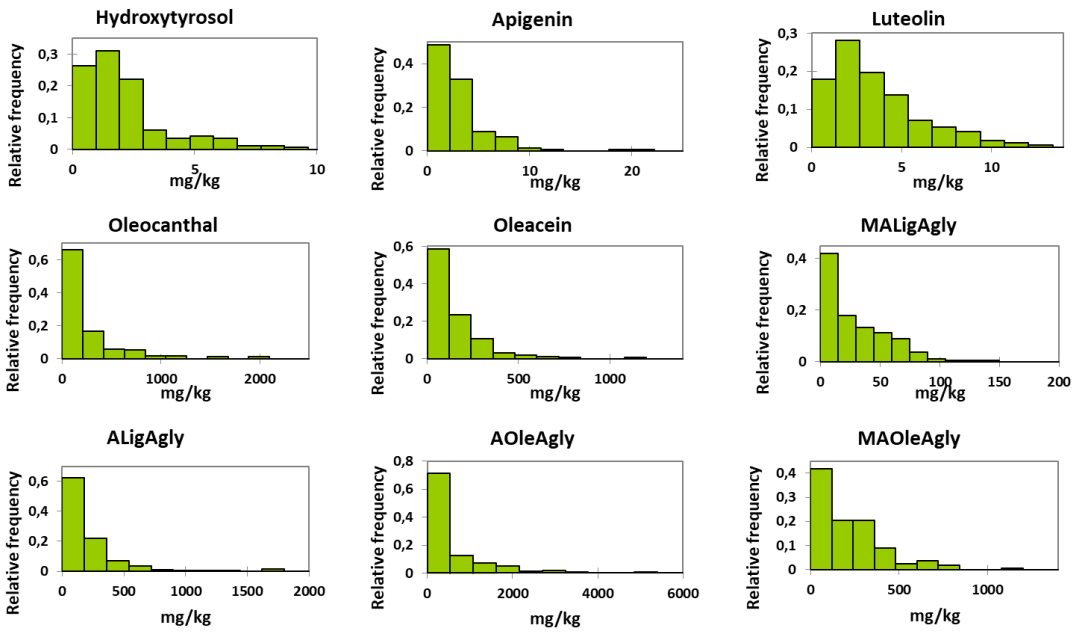
2014/2015 crop season			
G1	G2	G3	G4
Cerezuela	Alfafara	Arbosana	Alameño de Montilla
Coratina	Blanqueta	Arbequina	Farga
Mastoidis	Caballo	Morona	Gordal de Granada
Picual de Almería	Joanenca		Loaime
Plementa Bjelica	Kalamon		Mollar de Cieza
Bosana	Koroneiki		Negrillo de la Carlota
			Ojo de Liebre
			Royal de Cazorla
			Sabatera
			Sikitita
2015/2016 crop season			
G1	G2	G3	G4
Cerezuela	Alfafara	Arbequina	Alameño de Montilla
Coratina	Blanqueta	Arbosana	Farga
Picual de Almería	Caballo	Morona	Gordal de Granada
Plementa Bjelica	Joanenca		Loaime
Bosana	Kalamon		Mastoidis
	Koroneiki		Mollar de Cieza
			Negrillo de la Carlota
			Ojo de Liebre
			Royal de Cazorla
			Sabatera
			Sikitita
Similarity percentage between the assignations of the two consecutive crop seasons			
G1	**G2**	**G3**	**G4**
83,3%	100%	100%	90,9%

Supplementary Table 8. Pearson correlation analysis between the concentration of monitored phenols in monovarietal VOOs from the 80 cultivars selected from the 2015–2016 crop season.

Correlation matrix (Pearson):									
Phenol	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Hydroxytyrosol	**1**	0,002	0,011	-0,099	0,016	0,154	0,107	0,202	0,131
Apigenin	0,002	**1**	**0,870**	0,025	-0,009	0,037	0,025	-0,017	-0,002
Luteolin	0,011	**0,870**	**1**	0,018	-0,006	0,116	0,094	0,075	0,069
Oleocanthal	-0,099	0,025	0,018	**1**	**0,843**	**0,318**	0,143	0,009	-0,055
Oleacein	0,016	-0,009	-0,006	**0,843**	**1**	0,132	0,024	0,094	0,004
MALigAgly	0,154	0,037	0,116	**0,318**	0,132	**1**	**0,895**	**0,771**	**0,699**
ALigAgly	0,107	0,025	0,094	0,143	0,024	**0,895**	**1**	**0,839**	**0,878**
MAOleAgly	0,202	-0,017	0,075	0,009	0,094	**0,771**	**0,839**	**1**	**0,907**
AOleAgly	0,131	-0,002	0,069	-0,055	0,004	**0,699**	**0,878**	**0,907**	**1**
***p*-values**									
Phenol	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly
Hydroxytyrosol	**0**	0,987	0,924	0,383	0,886	0,172	0,344	0,072	0,248
Apigenin	0,987	**0**	**< 0,0001**	0,824	0,939	0,743	0,824	0,884	0,987
Luteolin	0,924	**< 0,0001**	**0**	0,871	0,955	0,305	0,407	0,507	0,542
Oleocanthal	0,383	0,824	0,871	**0**	**< 0,0001**	**0,004**	0,206	0,934	0,629
Oleacein	0,886	0,939	0,955	**< 0,0001**	**0**	0,243	0,830	0,409	0,974
MALigAgly	0,172	0,743	0,305	**0,004**	0,243	**0**	**< 0,0001**	**< 0,0001**	**< 0,0001**
ALigAgly	0,344	0,824	0,407	0,206	0,830	**< 0,0001**	**0**	**< 0,0001**	**< 0,0001**
MAOleAgly	0,072	0,884	0,507	0,934	0,409	**< 0,0001**	**< 0,0001**	**0**	**< 0,0001**
AOleAgly	0,248	0,987	0,542	0,629	0,974	**< 0,0001**	**< 0,0001**	**< 0,0001**	**0**

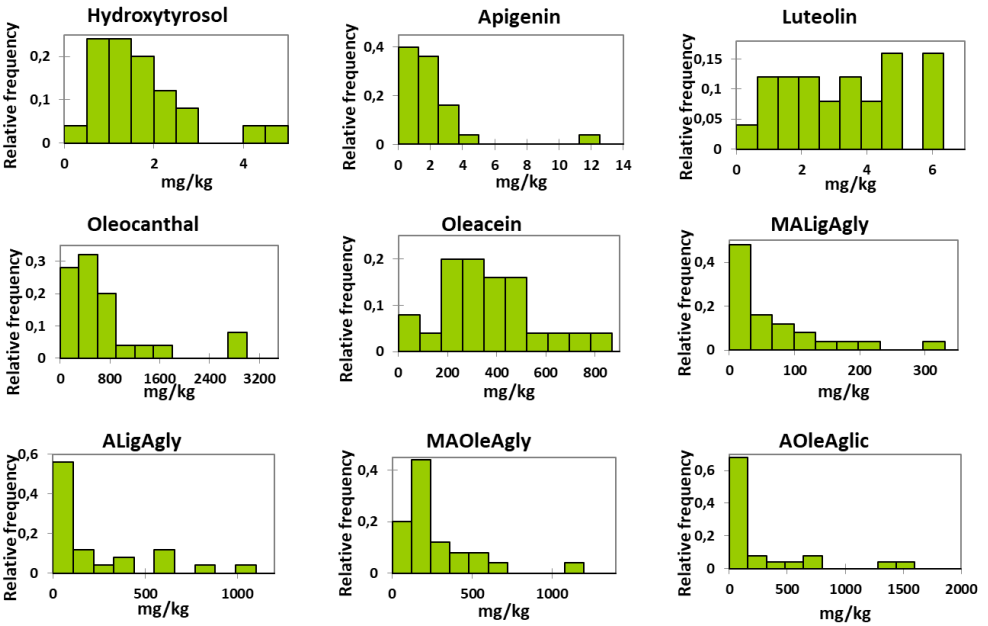
Values in bold are different from 0 with a significance level α = 0,05

SUPPLEMENTAL FIGURES

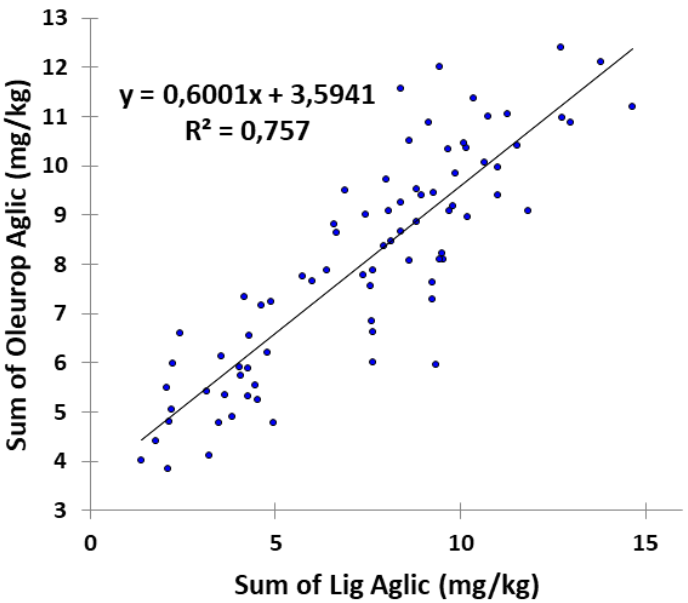


Supplementary Figure 1. Histograms showing the distribution of the concentration of the nine phenolic compounds evaluated in the 24 monovarietal VOOs (2015/2016 crop season).
AOleAgly – Aldehydic open forms of Oleuropein Aglycone; **MAOleAgly** – Monoaldehydic closed form of Oleuropein Aglycone.

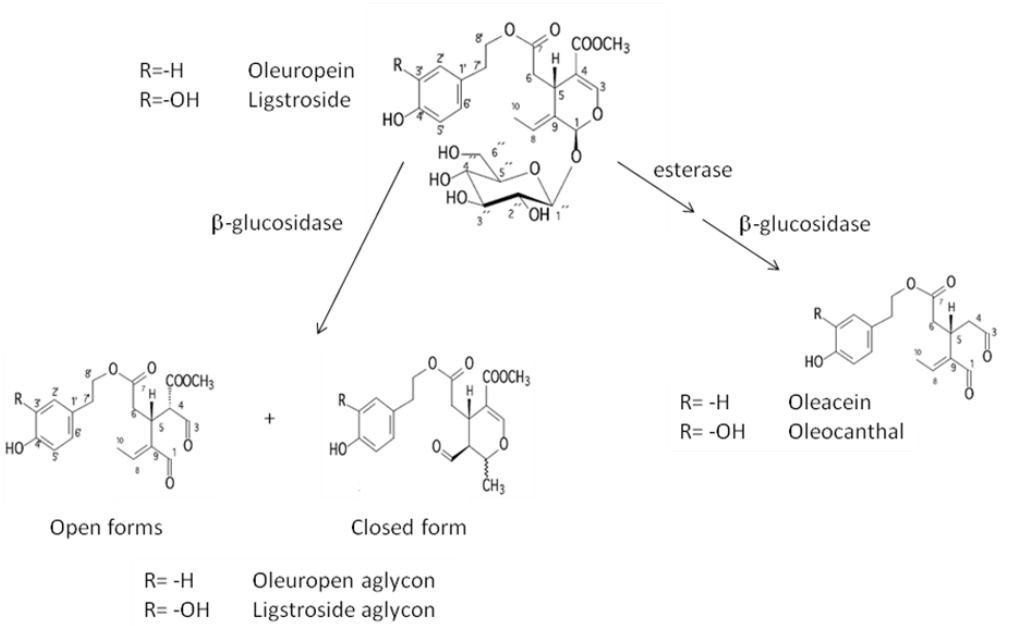
ALigAgly – Aldehydic open forms of Ligstroside Aglycone; **MALigAgly** – Monoaldehydic closed form of Ligstroside Aglycone.



Supplementary Figure 2. Histograms showing the distribution of the concentration of the nine phenolic compounds evaluated in the 24 monovarietal VOOs (2014/2015 crop season).



Supplementary Figure 3. Pearson correlation between the concentrations of the sum of ligstroside aglycone isomers and oleuropein aglycone isomers determined in the set of 80 cultivars studied in the 2015–2016 crop season.



Supplementary Figure 4. Metabolic pathways for hydrolysis of oleuropein/ligstroside by enzymatic action.

CHAPTER III - Influence of genetic and interannual factors on the phenolic profiles of virgin olive oils.

Food Chemistry 2020; 342; 128357.

Food Chemistry 342 (2021) 128357

Contents lists available at ScienceDirect

Food Chemistry

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

Influence of genetic and interannual factors in the phenolic profiles of virgin olive oils

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https://doi.org/10.1016/j.foodchem.2020.128357

Received 13 August 2020; Received in revised form 5 October 2020; Accepted 7 October 2020

Abstract

Phenolic compounds in virgin olive oil (VOO) contribute to its health properties, organoleptic features and oxidative stability. In this study, a total of 44 olive tree cultivars categorized by the International Olive Council to be among the most internationally widespread varieties were exhaustively and homogenously evaluated by analysis of the VOO phenolic profile during three consecutive crop seasons. Differences among cultivars resulted in up to 15-fold variations in the total phenol concentration. The 'cultivar' factor contributed the most to the variance (66.8% for total phenolic concentration) for almost all the phenols. However, the 'interannual variability' factor and the interaction 'cultivar × interannual variability' exhibited significant influences on specific phenols. According to the phenolic profile of the VOOs, we determined the presence of three groups of cultivars marked by the predominance of secoiridoid derivatives, which supports the phenolic profile as a criterion to be considered in olive breeding programs.

Keywords: *Olea europaea*; phenols; crop season; cultivar; genotype; virgin olive oil; clustering; classification.

Chemical compounds studied in this article

Hydroxytyrosol (PubChem CID: 82755); Oleacein (3,4-DHPEA-EDA) (PubChem CID: 18684078); Oleocanthal (p-HPEA-EDA) (PubChem CID: 16681728); Oleuropein aglycone (3,4-DHPEA-EA) (PubChem CID: 124202093); Ligstroside Aglycone (p-HPEA-EA) (PubChem CID: 11652416); Luteolin (PubChem CID: 5280445); Apigenin (PubChem CID: 5280443).

1. Introduction

Olive tree (*Olea europaea* L.) is characterized by a vast number of cultivars that represent an invaluable heritage of genetic variability selected over more than 5500 years of cultivation in Mediterranean countries (Rallo, Barranco, et al., 2018). Based on the FAO Olive Germplasm Plant Production and Protection Division data, the world olive germplasm comprises over 2.600 different olive cultivars (Muzzalupo, Vendramin, & Chiappetta, 2014).

Virgin olive oil (VOO), the most valuable product obtained from the olive tree, is one of the supporting pillars of the health properties associated with the Mediterranean diet, which considerably contributes to the prevention of chronic diseases (EFSA, 2011). Chemically, VOO is composed of major components (approximately 98% of the total weight), mainly fatty acids such as acylglycerides, and minor components (2%) that include a diversity of chemical families such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, phenols, tocopherols, esters, pigments and volatile components (Rallo, Díez, et al., 2018). The high presence of monounsaturated oleic acid (55–83%) is one of the main contributors to the health benefits of olive oil (Piroddi et al., 2017). The phenolic fraction also contributes significantly to these benefits due to its antioxidant, antimicrobial and anti-inflammatory properties (Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012; Parkinson & Keast, 2014). For instance, the phenolic compound oleocanthal has demonstrated activity in reducing inflammatory-related diseases and specific cancers (Parkinson & Keast, 2014), acting through similar mechanisms to that of the nonsteroidal anti-inflammatory drug (NSAID) ibuprofen (Beauchamp et al., 2005). Additionally, the same study suggested that the long-term consumption of 50 g of extra-VOO (containing 200 mg/kg oleocanthal) per day corresponded to approximately 10% of the ibuprofen dosage recommendation for adult pain relief (Beauchamp et al., 2005). In 2011, the European Food Safety Authority (EFSA) approved the claim: ‘‘olive oil phenols contribute to the protection of blood lipids from oxidative stress’’. This claim may be added to a product label when the VOO contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil (EFSA, 2011).

Phenolic compounds also contribute to the organoleptic properties of VOO. They stimulate the tasting receptors in the mouth and free endings of trigeminal nerves, eliciting

bitterness, pungency and astringency perception (Rallo, Díez, et al., 2018). Additionally, phenols protect VOO against oxidation because they scavenge free radicals, which provoke the oxidative chain reaction and dramatically reduce the quality of olive oil (Kiritsakis & Shahidi, 2017).

In VOO, different groups of phenolic compounds, such as phenolic acids, phenolic alcohols, hydroxy-isochromans, flavonoids, lignans, and secoiridoids, are found. However, secoiridoids, such as the aldehydic forms of oleuropein and ligstroside aglycone, oleocanthal and oleacein, are the predominant phenolic compounds of VOO. These compounds are also the most important regarding health properties, oil oxidative stability and organoleptic contribution (Del Rio, Gutierrez-Casado, Varela-Lopez, & Villalba, 2016; Servili et al., 2013).

The presence of phenolic compounds in VOO depends on numerous variables such as the genotype, the climatic conditions, agronomical and phytosanitary factors, the extraction technology and the post-processing (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Rugini, Baldoni, Muleo, & Sebastiani, 2016). Genotype (cultivar) significantly contributes to the composition and concentration of most important phenols found in VOO when agronomic conditions are controlled (El Riachy, Priego-Capote, León, Luque de Castro, & Rallo, 2012; Miho et al., 2018). However, differential climatic variables such as water availability or annual temperatures, rainfall, light exposure, and fertilization also play important roles in the phenolic variability in VOOs (Rugini et al., 2016). For instance, an increase in water availability involves a reduction in the total phenolic concentration in fresh olive fruit and in the extracted oil (Caruso et al., 2017). Likewise, dry summers and autumns seem to increase the phenolic content (Rugini et al., 2016). Additionally, olive fruits in shaded areas of the canopy have higher phenolic contents than those of fruits in well-illuminated zones (Gómez-Del-Campo & García, 2012). However, these studies present several limitations, such as the scarce number of cultivars evaluated and the absence of interannual crop replicates.

The effect of interannual variation has been evaluated in other relevant VOO fractions, such as fatty acids. Several studies have concluded that the relative influence of climatic and environmental conditions on fatty acid composition is relatively low, with cultivar being the most relevant factor impacting variability in the fatty acid profile (De la Rosa,

Talhaoui, Rouis, Velasco, & León, 2013; León et al., 2008). This result has allowed for the selection of new olive cultivars with specific fatty acid profiles considering only a single year of evaluation (León et al., 2008).

To our knowledge, there are no studies evaluating the relative effect of the cultivar and interannual variability sources on the VOO phenolic profile in a representative number of cultivars. Estimating this phenolic variation is crucial to assess a) the interannual consistency of the attributes related to VOO quality, such as health benefits and organoleptic properties; b) the effect of specific interannual variables, such as climatic factors, average temperature or pluviometry, on VOO phenolic profiles; c) the possible specific interactions between cultivar and interannual variables; and d) possible new breeding lines to obtain new cultivars enriched in specific phenolic compounds. In this respect, it is necessary to estimate the minimum number of necessary years to perform a consistent evaluation of phenolic compounds, taking into account their interannual variability, which will depend not only on the heritability but also on the stability of the phenolic composition. In this study, we focused on evaluating the phenolic profiles of 44 olive cultivars growing under the same conditions during three consecutive crop seasons. The selected cultivars covered the remarkable genetic diversity and heterozygosity of traditional olives (Diez et al., 2015; Trujillo, Ojeda, Urdiroz, & Potter, 2014).

2. Material and methods

2.1. Experimental location and vegetal material

Vegetal material was collected from the World Olive Germplasm Bank of Cordoba (WOGB) (CAP-UCO-IFAPA), specifically in the collection located at the University of Cordoba (Cordoba, Spain, 37°55'56.5" N, 4°43'13.3" W and 173 m a.s.l.). The olives were planted in 2011 in a north-south orientation with 7 m between rows and 6 m between trees (238 trees ha^{-1}). This collection includes 368 different cultivars from 22 different countries that were correctly identified and authenticated by morphological and DNA molecular markers (Trujillo et al., 2014).

The climate of the WOGB-UCO area is typically Mediterranean; the average annual precipitation and potential evapotranspiration from 2001 to 2017 were 549.5 and 1317.6

mm, respectively. The accumulated rainfall in 2015, 2016 and 2017 was 352.1, 735.8, and 463.5 mm, respectively (Villalobos & Testi, 2017). The experimental area is characterized by vertisol soil with a texture of 41% sand, 6% silt, and 53% clay. The soil was approximately 40 cm deep, with a 0.6% organic matter content. The collection was irrigated from May to September, applying 100 m^3 of water per ha per week (2000 m^3 of water per year) using drip irrigation. Foliar fertilization (2% potassium nitrate) was applied four times per year in November (after harvesting), March, May, and September.

During three consecutive crop seasons (2015/16, 2016/17, and 2017/18), we extracted and analyzed the VOOs of 44 olive cultivars. These cultivars were selected according to their share of olive oil production worldwide, geographical origin, and genetic diversity (**Table 1**). Two fruit samples per cultivar were independently collected from two olives. Therefore, we analyzed a total of 264 samples of VOO during three consecutive years (44 cultivars × 2 replicates × 3 crop seasons = 264 samples).

2.2. Sampling and VOO extraction

Olive fruit samples (2 kg) were manually harvested from each tree by sampling all orientations within the canopy. The olives were sampled from October to December when the fruits were at a ripening index (RI) of 2.0 (yellowish-red color) according to the method proposed by the International Olive Oil Council (International Olive Council, 2020) to standardize the conditions. With this strategy, 70% of the cultivars were harvested in October, 25% in November and 5% in December.

The extraction of VOOs was conducted by an Abencor system (MC2 Ingeniería y Sistemas, Sevilla, Spain) under optimized conditions (Peres, Martins, & Ferreira-Dias, 2014). The olive fruits were crushed with a hammer mill equipped with a 4-mm sieve at 3000 rpm. Malaxation of olive pomace was performed at 28 °C for 30 min; then, the biphasic system was centrifuged at 3500 rpm for 2 min. No water was added to the olive pomace at any step of the process. The VOO was decanted in graduated cylinders for approximately 2 h and the water traces were removed by filtering the samples through a cellulose filter for approximately 15 minutes at room temperature (20 °C) and avoiding the exposition to sunlight. VOO samples were stored in amber glass bottles and the analyses were carried out in approximately 30 days after the extraction.

2.3. Reagents and standards

Mass spectrometry (MS)-grade methanol (MeOH) and *n*-hexane, both from Scharlab (Barcelona, Spain), were used for the determination and quantification of the phenolic compounds in the VOOs. MS-grade formic acid, also from Scharlab, was used as an ionization agent in the chromatographic mobile phases. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare both the aqueous and organic mobile phases, and a hydroalcoholic mixture was used as the sample extractant.

The quantified phenolic compounds were hydroxytyrosol, oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycone (3,4-DHPEA-EA), ligstroside aglycone (p-HPEA-EA), luteolin, and apigenin. Two isomers of oleuropein and ligstroside aglycones were distinguished according to their retention times (Sánchez de Medina et al., 2017). Thus, it was possible to discriminate between the open aldehyde form of oleuropein aglycone (AOleAgly) and the closed monoaldehyde form of the oleuropein aglycone (MAOleAgly). In the same way, it was possible to differentiate among the open aldehyde form of ligstroside aglycone (ALigAgly) and the closed monoaldehyde form of ligstroside aglycone (MALigAgly). Standards for hydroxytyrosol, apigenin and luteolin were purchased from Extrasynthese (Genay, France). Oleacein, oleocanthal, and the closed monoaldehyde isomers of oleuropein aglycone and ligstroside aglycone were provided by Professor Prokopios Magiatis (University of Athens, Greece). The open aldehyde forms were quantified using the corresponding closed monoaldehyde standards. Standard solutions of nonsecoiridoid phenols were prepared in methanol (1 mg/mL), while secoiridoids were prepared at the same concentration in pure acetonitrile to preserve their stability and avoid undesired conversion to their acetal and hemiacetal derivatives.

2.4. Sample preparation for analysis of phenolic compounds

We implemented a liquid-liquid extraction method for the isolation of phenols according to a previous protocol (Sánchez de Medina, Priego-Capote, & Luque de Castro, 2015). For this purpose, 1 g of VOO was mixed with 2 mL *n*-hexane; then, 1 mL of 60:40 (v/v) methanol-water was added and shaken for 2 min, and the hydroalcoholic phase was separated by centrifugation. The extraction was repeated to enhance the extraction

efficiency (Sánchez de Medina et al., 2017), and the two resulting extracts were combined for each sample. The extracts were analyzed by LC–QqQ-MS/MS with two different dilution factors (1:2 and 1:50 v/v) to encompass the concentration variability.

2.5. LC–MS/MS analysis of phenolic compounds

Analyses were performed by reversed-phase liquid chromatography followed by electrospray ionization (ESI) in negative mode with tandem mass spectrometry (MS/MS) detection. Ten microliters of the extract were injected in triplicate into the LC system for chromatographic separation of the target compounds using a C18 Pursuit XRs Ultra column (50 mm×2.0 mm i.d., 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The column compartment was kept at 30 °C. Mobile phase A was 0.1% (v/v) formic acid in water, while phase B was 0.1% (v/v) formic acid in MeOH. The gradient program, at a constant flow rate of 0.4 mL/min, was as follows: initially, 50% phase A and 50% phase B were maintained for 0.5 min; from 0.5 to 2 min, mobile phase A decreased from 50 to 20%; and from min 2 to 4, mobile phase A decreased from 20 to 0%. This last composition was maintained for 1 min. After each analysis, the column was equilibrated for 5 min at the initial conditions.

The entire eluate was ionized via electrospray ionization and monitored by MS/MS in multiple reaction monitoring (MRM) mode for selective transitions from the most favored precursor ion to product ion of each analyte. The MRM parameters for the target phenols are listed in **Supplementary Table 1**. The flow rate and temperature of the drying gas (N_2) were 10 L/min and 300 °C, respectively. The nebulizer pressure was 50 psi, and the capillary voltage was 3000 V. The dwell time was set at 200 μs.

2.6. Quantitation of the target compounds and statistical analysis

Calibration curves were prepared by using refined sunflower oil spiked with variable concentrations of the phenolic standards (nine concentrations from 0.1 ng/mL to 5 μg/mL). Each concentration level, prepared in triplicate, was analyzed after the complete procedure including sample preparation. The absence of quantifiable levels of phenols in the refined oil was checked by direct analysis. The calibration equations were used to calculate the absolute concentration of target phenols in the VOO samples and the "Sum of

phenols" parameter by the addition of the individual concentrations of the different phenols. Three replicates of each VOO were also analyzed to obtain the mean concentration.

Friedman nonparametric two-way analysis of variance was performed to evaluate the effect of the cultivar on the concentrations of the nine phenolic compounds. This test was used because the data did not satisfy the requirements of parametric tests regarding normality, homogeneity of variance, or sphericity. The means were compared using Dunn's test with a Bonferroni adjustment (p = 0.05) (Demšar, 2006).

To estimate the stability of the VOO phenolic composition for each cultivar during the three studied periods, we calculated the proportion of variability explained by the cultivar, interannual variability sources, and the cultivar × interannual variability interaction. For this purpose, analysis of variance (ANOVA) was performed on the phenolic composition of VOOs produced by the cultivars during the three consecutive crop seasons with a representative number of repetitions (two olive trees of the same cultivar). Subsequently, we calculated the effect size (eta squared [η^2]) of each independent variable (cultivar and season) and its interaction as the ratio between the variability associated with a factor and the total variability of our analysis ($\eta 2 = \frac{SS_{factor}}{SS_{total}}$). Friedman and ANOVA tests were performed using 269 Statistix software (Version 10; Statistix, Tallahassee, FL). Furthermore, principal component analysis (PCA) followed by discriminant analysis (DA) was used to identify groups of cultivars with similar phenolic profiles. The existence of significant pairwise differences between the groups depicted by PCA was evaluated with a Bonferroni post hoc test, while Pearson correlation was used to find relationships between the concentrations of studied phenols. These analyses were performed using XLSTAT software (v.2014.5.03, Addinsoft, Paris, France).

Table 1. Average concentration of phenolic compounds (expressed as mg/kg) for the 44 monovarietal VOOs during the three evaluated crop seasons. The letters indicate the homogeneous groups according to Friedman nonparametric two-way analysis of variance ($P < 0.05$). Letters indicate significant differences according to the Friedman test.

Cultivar	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Sum of phenols
'Abou Choki'	1.6 a-d	3.2 a-f	8.0 a-e	189 a-g	252 b-f	122 a-e	396 a-d	305 a-h	262 a-g	1538 a-g
'Alameño de Montilla'	1.5 a-d	3.3 a-f	9.3 a-e	339 a-g	589 a-f	46.3 a-g	51.9 b-h	108 a-h	75.4 c-g	1224 a-g
'Alfafara'	1.5 a-d	3.7 a-f	5.9 a-e	852 ab	632 a-f	151 a-c	333 a-e	170 a-h	162 a-g	2311 a-e
'Arbequina'	0.8 d	5.4 a-f	12.2 a-e	308 a-g	650 a-e	15.1 g	10.5 gh	80.7 d-h	65.6 e-g	1148 a-g
'Arbosana'	0.9 b-d	15.4 ab	8.8 a-e	190 a-g	256 a-f	13.9 g	12.0 h	37.1 h	48.6 g	583 fg
'Blanqueta'	2.3 a-d	10.0 a-f	9.9 a-e	477 a-g	606 a-e	27.8 d-g	55.1 d-h	157 a-h	104 a-g	1450 a-g
'Caballo'	1.4 a-d	2.8 a-f	7.3 a-e	465 a-g	774 a-f	64.6 a-g	83.6 a-h	291 a-h	166 a-g	1856 a-g
'Cerezuela'	1.7 a-d	2.6 b-f	14.1 a-e	694 a-e	1333 a	90.3 a-g	261 a-h	442 a-f	370 a-e	3208 a
'Chetoui'	2.8 a-c	5.3 a-f	12.2 a-e	122 a-g	357 a-f	108 a-g	344 a-e	612 a	558 a	2122 a-f
'Coratina'	2.0 a-d	6.1 a-f	8.8 a-e	300 a-g	427 a-f	130 a-f	486 a-d	512 ab	553 ab	2425 a-d
'Cornicabra'	1.8 a-d	1.0 ef	3.7 de	412 a-g	645 a-f	224 a	701 a	452 a-d	481 a-c	2923 ab
'Empeltre'	1.8 a-d	1.6 d-f	7.2 a-e	143 b-g	277 a-f	84.1 a-g	228 a-h	330 a-f	368 a-e	1441 a-g
'Farga'	1.5 a-d	4.0 a-f	6.6 a-e	183 a-g	339 a-f	62.6 a-g	161 a-h	162 a-h	185 a-g	1106 a-g
'Frantoio'	2.1 a-d	2.7 b-f	6.3 a-e	218 a-g	261 b-f	233 ab	568 a-c	317 a-h	336 a-f	1944 a-g
'Gemlik'	1.7 a-d	7.8 a-f	10.8 a-e	97.6 d-g	165 c-f	94.0 a-g	261 a-h	247 a-h	233 a-g	1119 a-g
'Gordal de Granada'	1.1 a-d	3.7 a-f	9.0 a-e	186 a-g	160 b-f	39.1b-g	50.7 c-h	58.3 f-h	71.3 d-g	579 e-g
'Hojiblanca'	1.7 a-d	9.7 a-c	20.7 ab	224 a-g	189 a-f	46.3 a-g	48.9 b-h	151 a-h	120 a-g	812 c-g
'Jabaluna'	1.1 a-d	2.0 c-f	5.5 a-e	298 b-g	124 c-f	39.2b-g	84.6 b-h	140 a-h	102 a-g	795 b-g
'Kalamon'	2.1 a-d	2.6 b-f	7.2 a-e	1186 a	875 a-c	108 a-g	124 a-h	105 a-h	104 a-g	2514 a-c
'Koroneiki'	2.1 a-d	5.3 a-f	10.5 a-e	358 a-g	541 a-f	107 a-g	297 a-f	405 a-g	405 a-g	2132 a-g
'Kotruvsi'	1.1 a-d	15.4 a-c	17.1 a-e	558 a-f	533 a-f	81.0 a-g	120 a-h	131 a-h	115 a-g	1572 a-g
'Kusha'	2.2 a-d	8.4 a-d	23.4 a	21.9 g	105 f	86.7 a-g	245 a-h	345 a-e	306 a-g	1145 a-g
'Lechín de Sevilla'	2.6 a-d	1.1 f	1.7 e	94.1 fg	165 b-f	117 a-g	359 a-h	279 a-h	288 a-g	1309 a-g
'Loaime'	0.8 cd	4.6 a-f	7.4 a-e	405 a-g	215 a-f	42.2b-g	33.6 e-h	56.8 gh	43.1 fg	808 b-g

Cultivar	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Sum of phenols
'Manzanilla Cazareña'	0.9 a-d	5.1 a-f	8.1 a-e	102 d-g	94.0 c-f	56.2 a-g	98.0 a-h	72.6 c-h	84.8 c-g	522 fg
'Manzanilla de Sevilla'	5.5 a	2.8 a-f	2.5 de	274 a-g	184 a-f	99.3 a-g	309 a-h	229 a-h	251 a-g	1357 a-g
'Manzanilla Prieta'	3.7 a-d	2.3 b-f	5.3 b-e	225 a-g	320 a-f	208 a	545 ab	472 a-c	391 a-c	2173 a-f
'Mastoidis'	2.3 a-d	10.2 a-c	17.0 a-d	190 a-g	149 b-f	131 a-f	274 a-h	207 a-h	227 a-g	1208 a-g
'Mision Moojeski'	3.3 a-d	19.8 a	17.3 a-c	730 a-c	904 a-d	160 a-d	327 a-h	188 a-h	189 a-g	2538 a-d
'Mixani'	1.6 a-d	6.6 a-e	21.0 ab	80.1 fg	259 b-f	53.1 a-g	124 a-h	305 a-h	352 a-g	1203 a-g
'Mollar de Cieza'	1.6 a-d	3.5 a-f	6.7 a-e	232 a-g	250 a-f	22.6 fg	21.3 e-h	77.4 d-h	64.3 e-g	680 fg
'Morona'	1.5 a-d	9.5 a-c	16.0 a-d	155 a-g	206 b-f	42.6 a-g	52.1 b-h	134 a-h	106 a-g	723 d-g
'Negrillo de la Carlota'	1.5 a-d	4.3 a-f	15.5 a-d	614 a-d	774 a-d	93.3 a-g	86.9 a-h	158 a-h	86.8 a-g	1835 a-g
'Ojo de Liebre'	0.8 d	2.7 a-f	9.1 a-e	311 a-g	411 a-f	105 a-g	285 a-h	246 a-h	195 a-g	1566 a-g
'Pendolino'	0.9 b-d	3.6 a-f	5.7 a-e	580 a-f	616 a-f	93.9 a-g	169 a-h	225 a-h	211 a-g	1906 a-g
'Picual'	3.5 a-d	2.4 b-f	6.2 a-e	67.1 g	97.3 d-f	84.5 a-g	201 a-h	190 a-h	179 a-g	831 b-g
'Picual de Almería'	1.9 a-d	1.7 d-f	4.4 b-e	92.5 c-g	188 b-f	111 a-g	279 a-h	344 a-e	379 a-d	1401 a-g
'Picudo'	2.9 ab	10.7 a-c	16.8 a-d	370 a-g	589 a-f	40.0 b-g	31.9 d-h	126 a-h	67.2 d-g	1255 a-g
'Plementa Bjelica'	1.2 a-d	11.8 ab	15.3 a-d	936 ab	828 ab	94.6 a-g	268 a-h	328 a-h	358 a-g	2841 ab
'Royal de Cazorla'	1.2 a-d	4.9 a-f	4.5 c-e	29.7 g	103 ef	14.7 g	16.7 f-h	62.8 e-h	52.5 g	290 g
'Sabatera'	1.9 a-d	4.7 a-f	9.6 a-e	208 a-g	411 a-f	108 a-g	249 a-h	283 a-h	241 a-g	1515 a-g
'Sikitita'	1.6 a-d	7.1 a-f	14.9 a-d	233 a-g	262 a-f	22.1 e-g	42.6 d-h	76.3 c-h	88.4 b-g	748 d-g
'Ulliri i Bardhe i Tiranes'	1.8 a-d	4.9 a-f	12.6 a-e	204 a-g	483 a-f	36.2 c-g	26.2 e-h	88.2 b-h	141 a-g	998 a-g
'Villalonga'	3.0 a-d	7.9 a-d	10.4 a-e	68.9 e-g	322 b-f	107 a-g	319 a-g	396 a-c	370 a-d	1603 a-g

3. Results and discussion

3.1. Variability in the phenolic profile of VOOs

We measured the individual concentrations of nine phenolic compounds and the "Sum of phenols" in the VOOs of 44 olive cultivars during three consecutive crop seasons. The mean values of phenolic concentrations are presented in **Table 1**. Similar to previous studies (Bajoub et al., 2017; El Riachy et al., 2011), we found significant differences ($p < 0.05$) in phenolic content among cultivars. For instance, the total phenolic content ("Sum of phenols") was 9-fold higher in VOO from the 'Cerezuela' cultivar than in that from the 'Royal de Cazorla' cultivar (3208 *versus* 290 mg/kg, respectively).

In agreement with previous studies (Kalogeropoulos & Tsimidou, 2014; Rodrigues et al., 2019), the frequency distributions of cultivars according to their phenolic concentration were nonsymmetric (**Fig. 1**), and almost all phenols reported right skew parameters explaining the predominance of cultivars with relatively low contents of these compounds. Homogeneous cultivar groups according to the concentration of the nine phenolic compounds were identified by the Friedman test (**Table 1**). However, we determined the best cultivars according to the total and average concentrations of each phenol. Regarding the Sum of phenols, 'Cerezuela', 'Cornicabra', and 'Plementa Bjelica' samples reported maximum phenolic contents of 3208, 2923, and 2841 mg/kg, respectively. The richest cultivars in oleocanthal were 'Kalamon', 'Plementa Bjelica' and 'Alfafara', with 1186, 936 and 852 mg/kg, respectively, while 'Cerezuela', 'Mision Moojeski' and 'Kalamon' were the richest in oleacein, containing 1333, 904 and 875 mg/kg, respectively. Complementarily, the cultivars that stood out in concentration of oleuropein aglycone isomers were 'Chetoui', 'Coratina' and 'Cornicabra', with 1170, 1065 and 933 mg/kg, respectively, and, for ligstroside aglycone isomers, these were 'Cornicabra', 'Frantoio' and 'Manzanilla Prieta' with 925, 801 and 753 mg/kg, respectively.

The most abundant individual phenols (mean values) found in this three-year study were the oleuropein aglycone isomers (447 mg/kg), oleacein (407 mg/kg), oleocanthal (319 mg/kg) and ligstroside aglycone isomers (292 mg/kg) (**Table 2**). These results are in qualitative and quantitative concordance with experimental results previously reported by other authors (Miho et al., 2018; Rodrigues et al., 2019).

Table 2. Statistical summary by analysis of the phenolic concentration (expressed as mg/kg) in VOO from 44 cultivars during three consecutive crop seasons.

Crop season	Variable	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Sum of phenols
2015-2016	Minimum	0.50	0.52	0.54	15.8	12.8	4.4	4.1	39.7	9.9	110
	Maximum	8.2	13.1	13.1	1474	870	157	368	834	903	3139
	Mean	**2.3**	**3.0**	**3.8**	**417**	**276**	**44.2**	**80.9**	**292**	**242**	**1360**
	SD*	1.7	2.5	2.8	393	219	35.4	76.6	221	216	799
2016-2017	Minimum	0.24	1.1	1.4	1.2	17.9	16.7	8.8	5.8	13.1	324
	Maximum	1.6	25.6	31.7	1441	598	287	778	363	380	2959
	Mean	**0.73**	**8.2**	**11.2**	**356**	**225**	**110**	**292**	**147**	**218**	**1370**
	SD	0.34	5.7	6.5	286	154	65.1	223	109	87.8	567
2017-2018	Minimum	0.91	0.41	2.3	8.3	48.7	12.9	10.8	49.9	19.6	260
	Maximum	7.5	28.1	38.5	763	2575	380	1048	707	726	4497
	Mean	**2.7**	**6.1**	**15.9**	**184**	**721**	**106**	**242**	**249**	**192**	**1719**
	SD	1.4	6.4	9.3	165	559	78.1	257	155	181	1030
Average of three crop seasons	Minimum	0.55	0.69	1.4	8.4	26.5	11.4	7.9	31.8	14.2	231
	Maximum	5.7	22.3	27.8	1226	1347	275	731	635	670	3532
	Mean	**1.9**	**5.8**	**10.3**	**319**	**407**	**86.7**	**205**	**230**	**217**	**1483**
	SD	1.1	4.9	6.2	281	311	59.5	186	162	162	799

* SD: Standard deviation.

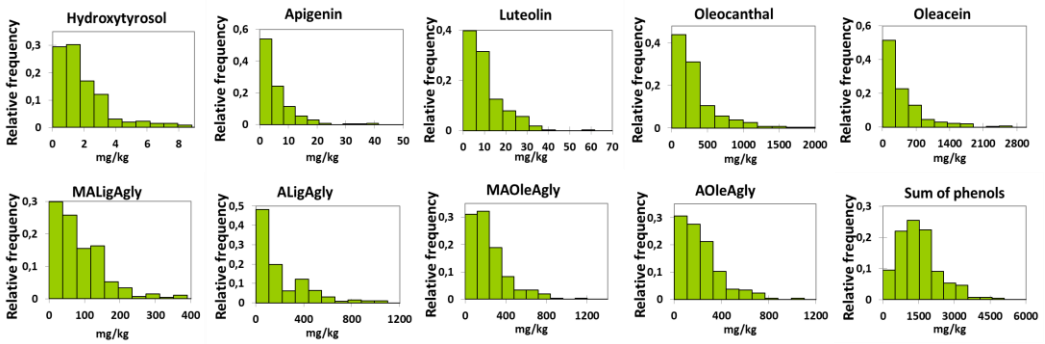

Figure 1. Distribution of the concentration of phenolic compounds evaluated in the olive oil of 44 cultivars during three consecutive crop seasons represented as histogram charts. **AOleAgly** – aldehydic open form of oleuropein aglycone; **MAOleAgly** – monoaldehydic closed form of oleuropein aglycone. **ALigAgly** – aldehydic open form of ligstroside aglycone; **MALigAgly** – monoaldehydic closed form of ligstroside aglycone.

3.2. Factors contributing to VOO phenolic variability.

The phenolic profile of VOOs is affected by several factors, such as the cultivar (genotype), fruit phenological stage (ripening), alternate bearing, location and climatic conditions, and agronomic practices. However, the magnitude and relative influence of these factors on the chemical composition of VOO and, specifically, in the present phenols, have been poorly explored. In this study, we evaluated for the first time the effect of the cultivar and the interannual variation as well as the interaction between both factors on VOO phenolic variability. The rest of the mentioned factors hardly affected our results because the evaluated cultivars were the same age, in the same orchard and under the same agronomic management; thus, we considered them fixed factors. A previous study has pointed out that malaxation has a significant effect on the phenolic content of VOO and, importantly, this effect is not equal for all cultivars at each ripening stage (Diamantakos, Giannara, Skarkou, Melliou, & Magiatis, 2020). In our research, we selected the ripening index 2.0 as harvesting period following the recommendations of the International Olive Oil Council (International Olive Council, 2020) to increase the VOO quality without decreasing the production yield, and consequently, to enhance the probability of VOO production (Rallo, Díez, et al., 2018). Also, to guarantee quality and efficiency of the extraction process we applied the conditions recommended by the Abencor manufacturer and accepted by the scientific community (Peres et al., 2014). Variation in the average concentration of the nine evaluated phenolic compounds during the three consecutive

agronomic seasons is summarized in **Table 2**. We observed some compounds with high between-year variability; for example, oleacein exhibited mean levels of 276 and 225 mg/kg in the first two seasons, while in the third season, its concentration increased up to 721 mg/kg. On the other hand, the concentrations of aglycone isomers of oleuropein and ligstroside were relatively stable over time (**Table 2**).

ANOVA confirmed that the cultivar, the interannual variation and the interaction between both factors (cultivar × interannual variation) significantly ($p < 0.001$) affected the concentration of VOO phenols (**Table 3**). According to the eta squared (η^2) value, the cultivar was the principal factor explaining up to 66.8% of the total phenolic (Sum of phenols) variability. The interannual variation explained just 3.7% of the variability, while the cultivar × interannual variation interaction accounted for 24.5% of the total variance. Similarly, the cultivar was the factor impacting the concentration of individual phenols the most. However, not all individual phenols were equally affected by cultivar. **Fig. 2** shows that oleocanthal and aglycone isomers of oleuropein and ligstroside were mainly influenced by the cultivar factor, with over 50% of the total variability explained by the cultivar (above 60% for oleocanthal and AOleAgly). Furthermore, the cultivar was the secondary factor in explaining the variability of oleacein, hydroxytyrosol and the two flavonoids apigenin and luteolin.

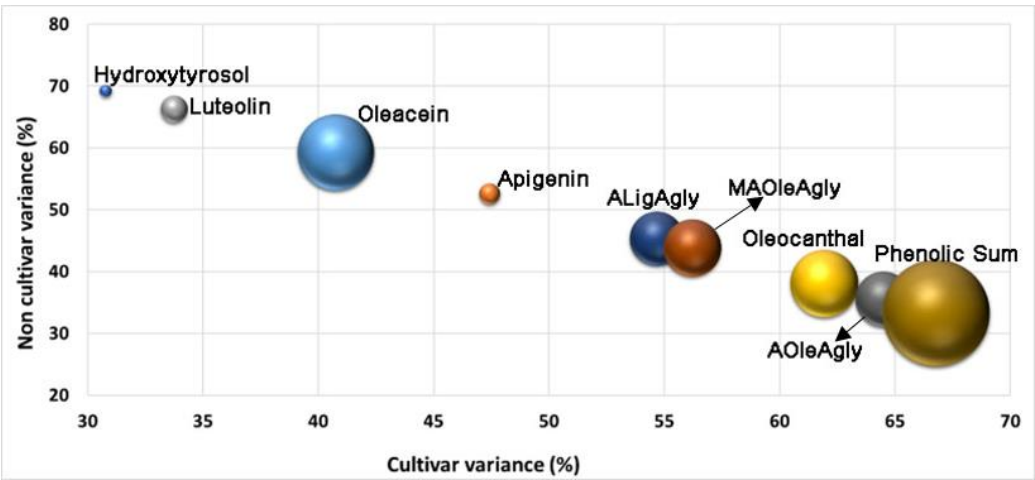

Figure 2. Representation of the variance explained by cultivar (genotype) and noncultivar factors (i.e., crop season, crop season × cultivar, and error) for each phenolic compound. The bubble size explains the relative concentration of each phenol in the evaluated VOOs.

Our results are in agreement with those of previous studies that evaluated the phenolic variability in olives and other crop species, highlighting the high impact of the genotype on phenolic profiles (El Riachy et al., 2011; Luisa Badenes & Byrne, 2012). Therefore, the genotype is the most relevant factor in the breeding of new olive cultivars providing high phenolic VOOs. Similarly, according to previous studies, the genetic factor is the most relevant in defining the fatty acid profile of VOOs, contributing between 40 and 90% of the total fatty acid variance (De la Rosa et al., 2013; Rallo, Díez, et al., 2018).

Climatic variables, such as accumulated rainfall, temperature variation, and light interception, are the key factors that affect the phenolic content of olive fruits during oil accumulation, normally from July (pit hardening) to November, which corresponds with fruit harvesting (Cirilli et al., 2017; Gucci et al., 2019; Rugini et al., 2016). Given the restricted number of seasons monitored in this research, it was not possible to establish any strong relationship between these variables and the annual variability that we observed in the VOO phenolic profile. However, we observed some general trends regarding precipitation values and the overall phenolic concentration of the VOOs that were in line with those of previous studies (Cirilli et al., 2017; Gucci et al., 2019; Rugini et al., 2016; Vidal et al., 2019). We observed that the year with the lowest accumulated rainfall during the oil accumulation period (2017) produced fruits with the highest phenolic contents (**Supplementary Fig. 1)**. On the other hand, no remarkable relationship was observed between the mean temperatures and the mean phenolic concentrations of VOOs. This fact may be explained by the lack of significant differences among the mean temperatures in the analyzed crop seasons **(S**upplementary Fig. 1).

Table 3. ANOVA analysis that points out the proportion of variance in phenolic content explained by each factor: cultivar, interannual variability and the resulting interaction.

Factors		Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Phenolic Sum
Cultivar	η^2(%)	30.78	47.43	33.75	61.94	40.76	56.00	54.68	56.21	64.50	66.79
	F	6.76	10.02	9.51	25.92	43.48	38.15	38.7	20.44	36.45	39.8
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Interannual	η^2(%)	25.19	11.02	29.51	9.70	27.18	16.24	14.99	11.11	1.62	3.67
	F	118.9	50.06	178.75	87.24	623.35	237.85	228.08	86.87	19.72	47.09
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cultivar x Interannual	η^2(%)	30.05	27.02	25.84	21.01	29.22	23.26	25.96	24.24	28.45	24.44
	F	3.3	2.85	3.64	4.4	15.59	7.92	9.19	4.41	8.04	7.28
	p	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Error	η^2(%)	13.98	14.53	10.90	7.34	2.88	4.51	4.34	8.44	5.43	5.15

Eta squared (η^2): percentage of phenolic variability explained by each factor and the interaction.
F ratio: variation between samples/variation within the samples.
***p*-value**: significance level.

3.3. Clustering of olive cultivars based on the VOO phenolic profile.

A previous study based on data from one agronomic season (Miho et al., 2018), defined four tentative groups of cultivars according to their VOO phenolic profiles: (i) oils rich in aglycone isomers of oleuropein and ligstroside, (ii) oils rich in oleocanthal and oleacein, (iii) oils rich in flavonoids, and (iv) oils with balanced but reduced phenolic concentrations. The present research extended this evaluation during three consecutive agronomic seasons to evaluate interannual variability effects, mainly due to climatological variables. For this purpose, PCA based on the average phenolic concentrations in VOOs obtained from three agronomic seasons was carried out to determine clusters of cultivars based on their phenolic profiles. **Fig. 3** shows the PCA biplot with the two principal components (PC1 and PC2) explaining 63.7% of the total variability. The 44 cultivars were grouped into three groups: *G1* (19 cultivars), rich in the aglycone isomers of oleuropein and ligstroside; *G2* (13 cultivars), enriched with oleocanthal and oleacein compounds; and *G3* (12 cultivars), with balanced and no remarkable content of any specific phenol (**Supplementary Table 2**). In general, this classification agreed with the results proposed by Miho *et al.* in one agronomic season (Miho et al., 2018). The only difference was that a fourth group of cultivars leading to VOOs with high concentrations of flavonoids was not identified in the extended three-season research probably because the concentration of flavonoids was not stable enough over time in this group, which was formed by only 10 cultivars.

ANOVA with a Bonferroni post hoc test was performed to find statistically significant differences in phenolic concentrations among these three groups of cultivars defined by PCA. The results supported this classification since *G1* and *G2* cultivars were characterized by VOOs significantly rich ($p<0.0001$) in the aglycone isomers of oleuropein and ligstroside and the sum of oleocanthal and oleacein, respectively (**Supplementary Fig. 2)**. In contrast, the *G3* group included VOOs that reported a low phenolic content without significant differences ($p>0.05$) between the concentration of aglycone isomers of oleuropein and ligstroside and the sum of oleocanthal and oleacein. The *G1* group exhibited a mean concentration of aglycone isomers of oleuropein and ligstroside of 1166 mg/kg, and the *G2* group exhibited a corresponding concentration of 536 mg/kg. On the other hand, the *G2 group* provided a mean concentration of oleocanthal + oleacein of 1275 mg/kg *versus* 491

mg/kg for the *G1* group. The G3 group provided mean concentrations of 318 and 362 mg/kg for aglycone isomers and oleocanthal + oleacein, respectively. These results highlight the relevance of the secoiridoid derivatives for the classification of olive cultivars. These secoiridoids are produced by enzymatic action from the precursors oleuropein and ligstroside. The aglycone isomers of oleuropein and ligstroside are synthesized by β–glucosidases through hydrolysis reaction, whereas oleocanthal and oleacein are formed by the combined action of β–glucosidases and methylesterases enzymes (Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002; Servili et al., 2004). Thus, the enzymatic conversion of oleuropein and ligstroside precursors is a phenotypic characteristic that can be measured by determining the concentration of secoiridoids in VOO.

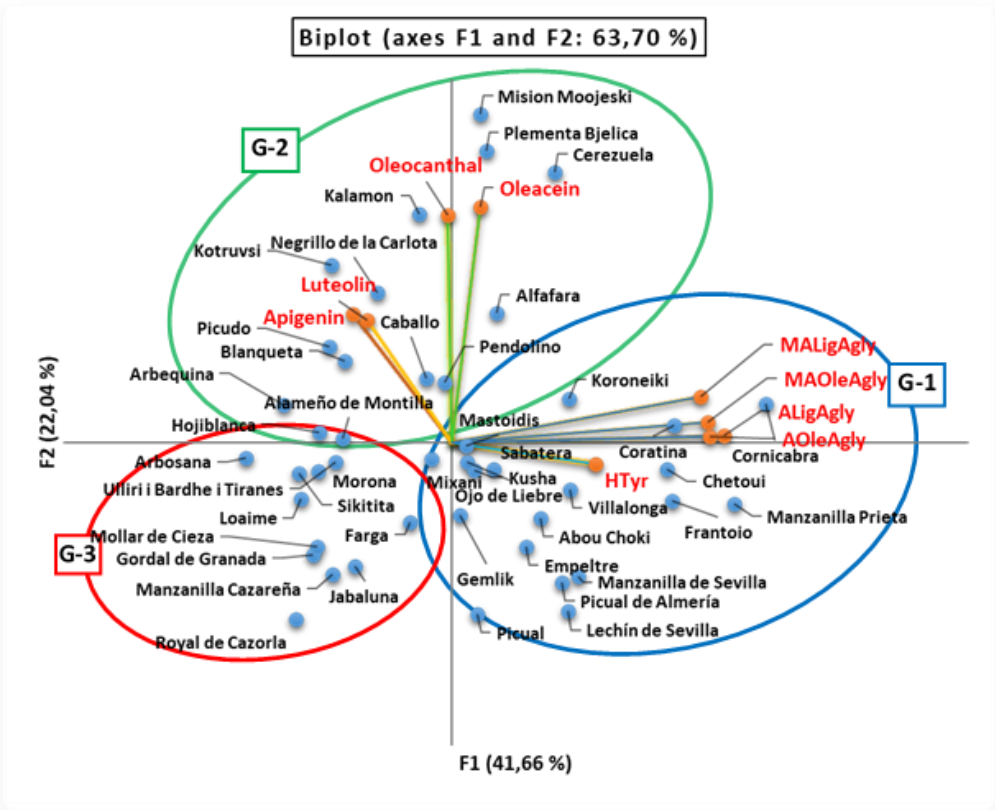

Figure 3. PCA showing the distribution of the cultivars based on the phenolic concentrations in VOOs obtained in three consecutive crop seasons. G-1, cultivars rich in aldehydic forms of oleuropein and ligstroside aglycone; G-2, cultivars rich in oleocanthal and oleacein compounds; and G-3, cultivars with no remarkable content of any specific phenolic compound.

To confirm that the olive cultivars were correctly classified by PCA, discriminant analysis (DA) considering the total olive sample replications was carried out (**Supplementary Fig. 3**). Likewise, DA corroborated that the phenolic profiles of most cultivars did not change with increasing crop year. In particular 85.2% of cultivars were classified during three consecutive seasons in the same PCA group, whereas only 14.8% changed their group assignment over the seasons (**Supplementary Table 3**). Most likely, these last cultivars presented increased sensitivity to interannual factor variability, or levels of certain phenols were relatively close to the threshold for classification into the G3 group. Indeed, cultivar allocation changes occurred from *G1* and *G2* groups to G3 or *vice versa*, but no changes were observed between *G1* and *G2*. This result indicates that the *G3* cultivar group may be considered an intermediate group, while G1 and G2 groups include cultivars providing VOOs with contrasting and relatively stable phenolic profiles.

Our results highlight for the first time the relative stability of the VOO phenolic profile over several cultivation years in an extended set of olive cultivars. This main finding, along with previous breeding experiences in other fruit species such as blueberry, raspberry, and peach (Cantín, Moreno, & Gogorcena, 2009; Connor, Luby, & Tong, 2002), indicates the possibility of developing health-enhanced olive cultivars able to produce VOOs enriched in specific phenols. The selection of olive cultivars to be used as progenitors in a breeding program and the estimation of their genetic contribution to the biotransformation of phenols are imperative (Rallo, Barranco, et al., 2018). Consumers are increasingly willing to pay for food quality, especially for healthy fruits and vegetables (Luisa Badenes & Byrne, 2012). Given that olive breeding is a lengthy process, breeders of perennial species should expect future market trends at least 10 years ahead (Byrne, 2005). This study opens new venues to obtaining healthy olive cultivars to satisfy consumer requirements.

4. Conclusions

These three years of study revealed that the phenolic content and composition enormously differ among the olive cultivars, where the richest cultivar resulted to be fifteen time more concentrated that the purest one. This significant variability ($p<0.005$) is attributed to the 'genotype' and 'interannual' factors. According to the variability of the sum of phenolic content, the genotype explained the biggest proportion (66.79 %) of variance, while the interannual factor explained just 3,67 % of variance, however, the

interaction between factors resulted to be much (24.44 %). The aforenamed factors differently affected each individual phenolic compound variability, highlighting that the 'genotype' factor mostly affected the AOleAgly compound explaining 64,50% of its total variance, while the 'interannual' factor mostly affected the oleacein compound explaining 21.01% of total variance. Our results suggested that the drought conditions foster the phenolic accumulation in the olive oil, but more studies are needed.

The most abundant phenolic compounds found in olive were the aldehydic forms of oleuropein aglycone isomers, oleacein, oleocanthal, and the aldehydic forms of ligstroside aglycone isomers, while hydroxytyrosol, apigenin and luteolin resulted to be the scarcest phenols.

Regarding the top olive cultivars: 'Kalamon', 'Plementa Bjelica' 'Cerezuela', 'Mision Moojeski' and 'Alfafara' were richest one in oleocanthal and oleacein; while 'Frantoio', 'Cornicabra', 'Manzanilla Prieta', 'Chetoui', and 'Coratina' were richest ones in the aldehydic form of oleuropein and ligstroside aglycones.

Furthermore, the total set of 44 analyzed cultivars during three consecutive crop seasons were clustered in base of their phenolic profile. The PCA followed by a DA analysis clustered the cultivars in three consistent groups: G1 (19 cultivars) - rich in aldehydic forms of oleuropein and ligstroside aglycone; G2 (13 cultivars) – cultivars rich in oleocanthal and oleacein compounds, and G3 (12 cultivars). – intermediate, balanced and no remarkable cultivars to any specific phenolic profile.

These results give rise to future olive breeding programs to obtain new health-enhanced olive oils rich in specific phenolic compounds and to design new coupage strategies to obtain tastier and longer shelf-life olive oil.

Funding Sources

This research was jointly financed by the Interreg-Med Program through the project MED-1033 and the European Regional Development Fund/European Social Fund ("Investing in your future"). H. Miho is grateful to the International Olive Council (IOOC) for a doctoral fellowship awarded. J. Moral received a Marie Skłodowska Curie fellowship launched by the European Union's H2020 (contract number 658579).

CRediT authorship contribution statement

H. Miho: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **J. Moral**: Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing. **D. Barranco**: Funding acquisition, Investigation, Writing - original draft, Writing - review & editing. **C.A. Ledesma-Escobar**: Investigation, Methodology, Writing - review & editing. **F. Priego-Capote**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing. **C.M. Díez**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary data

Supplementary tables

Supplementary Table 1. Multiple Reaction Monitoring (MRM) parameters for quantitative analysis of phenolic compounds by LC–MS/MS.

Phenolic compouds		Retention time (min)	Q1 voltage (V)	Precursor ion (m/z)	Collision energy (eV)	Quantitative transition (m/z)	Product ion confirmation (m/z)
Hydroxytyrosol		2.1	110	153.1	10	153-123	108
3.4-DHPEA-EDA (Oleacein)		4.3	110	319.1	12	319-59	139
3.4-DHPEA-EA	AOleAgly	4.6	110	377	12	377-275	307
	MAOleAgly	5.9	110	377	12	377-275	307
p-HPEA-EDA (Oleocanthal)		5.4	110	303.1	12	303-59	137
p-HPEA-EA	ALigAgly	5.5	110	361.1	12	361-291	101
	MALigAgly	6.2	110	361.1	12	361-291	101
Luteolin		6.3	170	285	35	285-133	175
Apigenin		6.6	170	269	35	269-117	151

AOleAgly – Aldehydic open form of oleuropein aglycone; **MAOleAgly** – Monoaldehydic closed form of oleuropein aglycone.

ALigAgly – Aldehydic open form of ligstroside aglycone; **MALigAgly** – Monoaldehydic closed form of ligstroside aglycone.

Supplementary Table 2. Olive cultivars classified into the three groups defined by the phenolic profile of VOO.

"G-1"	"G-2"	"G-3"
Abou Choki	Alameño de Montilla	Arbosana
Chetoui	Alfafara	Farga
Coratina	Arbequina	Gordal de Granada
Cornicabra	Blanqueta	Hojiblanca
Empeltre	Caballo	Jabaluna
Frantoio	Cerezuela	Loaime
Gemlik	Kalamon	Manzanilla Cazareña
Koroneiki	Kotruvsi	Mollar de Cieza
Kusha	Mision Moojeski	Morona
Lechín de Sevilla	Negrillo de la Carlota	Royal de Cazorla
Manzanilla de Sevilla	Pendolino	Sikitita
Manzanilla Prieta	Picudo	Ulliri i Bardhe i Tiranes
Mastoidis	Plementa Bjelica	
Mixani		
Ojo de Liebre		
Picual		
Picual de Almería		
Sabatera		
Villalonga		

G-1: rich in aldehydic form of oleuropein and ligstroside aglycone, **G-2** rich in oleocanthal and oleacein, and **G-3** balanced and no remarkable cultivars to any specific phenolic profile.

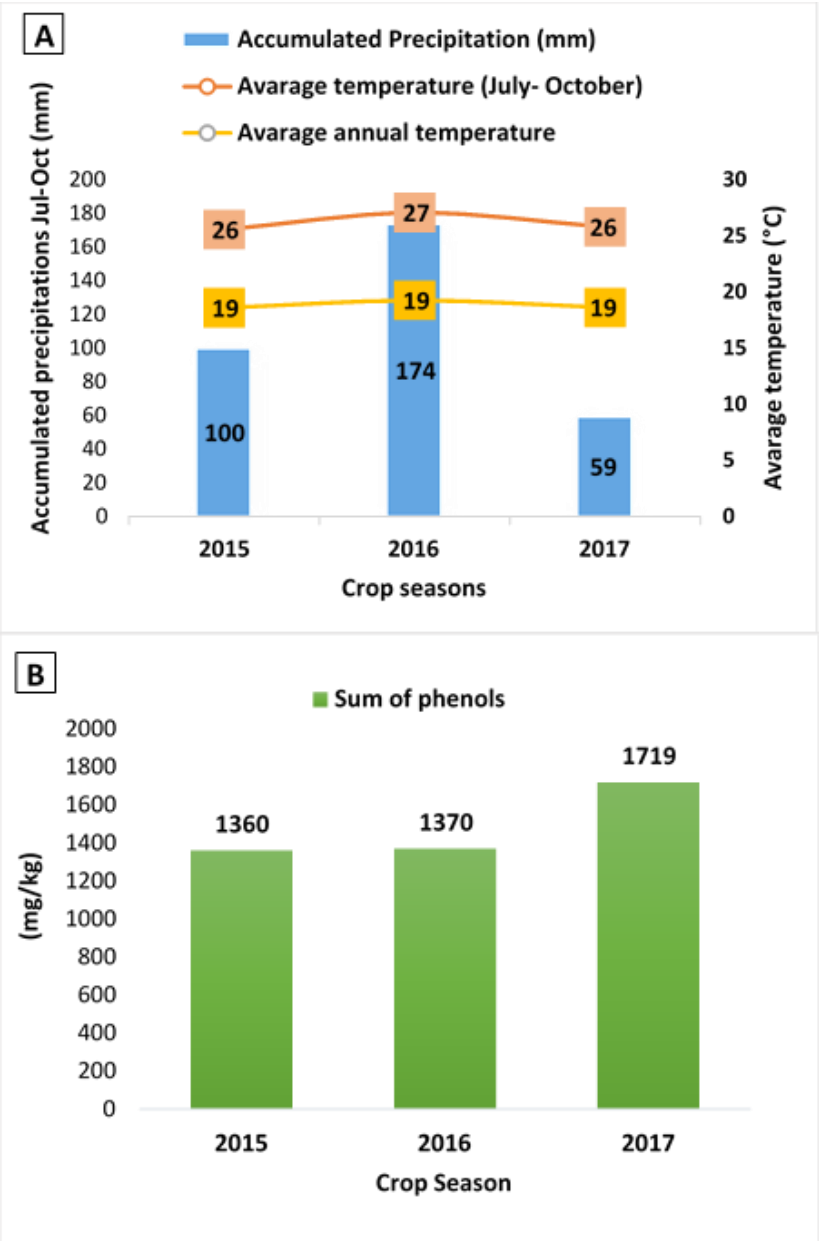
Supplementary Table 3. Discriminant Analysis error matrix: total samples reclassification summary. The "% correct" variable indicates the number of olive samples that have been consistently classified over the total number of observations into the same PCA group G1, G2 and G3.

		DA reclassification[2]				
	From ↓ \ To→	G1	G2	G3	Total	% correct
PCA classification[1]	G1	99	0	15	114	86.84%
	G2	2	58	12	72	80.56%
	G3	4	6	68	78	87.18%
Total		**105**	**64**	**95**	**264**	**85.23%**

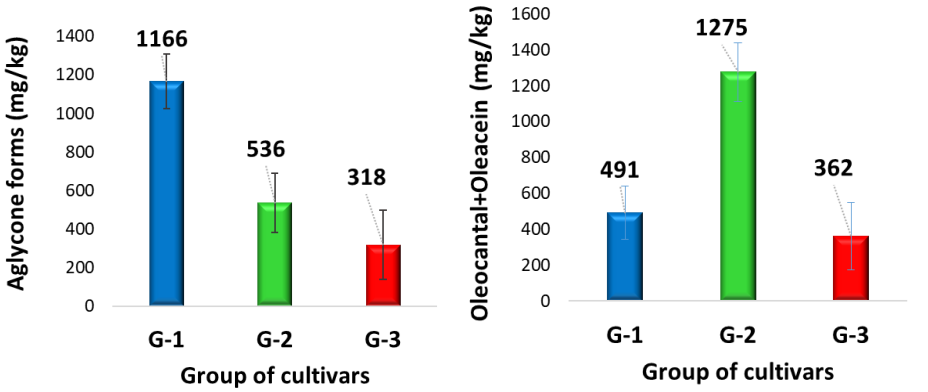
[1]Cultivars classification (clustering) using the mean phenolic concentration.

[2]Cultivars reclassification using the total observations (samples replication).

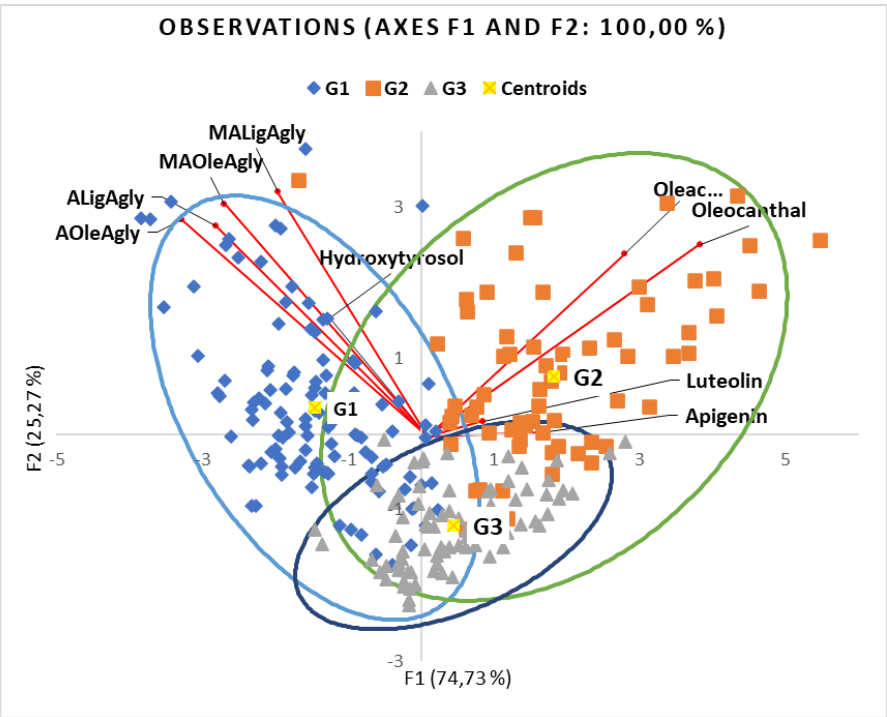
Supplementary figures



Supplementary Fig. 1. A- Accumulated precipitations (mm) and mean temperature (Celsius degrees) in the three crop seasons. **B-** Mean concentration of 'Sum of phenols' in the VOOs obtained in the three consecutively evaluated crop seasons.



Supplementary Figure 2. Concentration of the aglycone forms (mg/kg, left) and oleocantal+oleacein (mg/kg, right) in olive oils from different cultivars, which were grouped according to their phenolic profile: G-1 (19 cultivars), G-2 (13 cultivars), and G3 (12 cultivars). Columns shows the mean concentration and bars represent the standard error. Significant differences according to Bonferroni post-host test were observed among the three groups for both variables.



Supplementary Figure 3. Linear Discriminant Analysis (F1: 74.73%, and F2: 25.27%): 2D plot showing the discrimination of 264 VOO samples according to the predefined PCA groups (G1, G2, and G3).

CHAPTER IV - The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability

Food Chemistry 2020, 314; 126183.

Food Chemistry 314 (2020) 126183

Contents lists available at ScienceDirect

Food Chemistry

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

The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability

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https://doi.org/10.1016/j.foodchem.2020.126183

Received 19 August 2019; Received in revised form 8 January 2020; Accepted 8 January 2020

Available online 11 January 2020

Abstract

Phenolic compounds largely contribute to the nutraceutical properties of virgin olive oil (VOO), the organoleptic attributes and the shelf life due to their antioxidant capabilities. Due to the relevance of malaxation in the oil extraction process, we tested the effects of malaxation time on the concentrations of relevant phenolic compounds in VOO, and we evaluated the influence of performing malaxation under vacuum. An increase in malaxation time significantly decreased the concentrations of aglycone isomers of oleuropein and ligstroside but, conversely, increased the oleocanthal and oleacein contents. Additionally, malaxation under vacuum led to an increase in phenolic contents compared to standard conditions carried out at atmospheric pressure. Finally, we explored the possibility of predicting the VOO oxidative stability on the basis of the phenolic profile, and a model (R^2 = 0.923; p < 0.0001) was obtained by combining the concentration of the VOO phenolic compounds and the main fatty acids.

Keywords: *Olea europaea*; phenols; antioxidants; fatty acids; vacuum; Rancimat; predictive model; shelf life, malaxation, olive oil.

Chemical compounds studied in this article

Hydroxytyrosol (PubChem CID: 82755); Oleacein (3,4-DHPEA-EDA) (PubChem CID: 18684078); Oleocanthal (p-HPEA-EDA) (PubChem CID: 16681728); Oleuropein aglycone (3,4-DHPEA-EA) (PubChem CID: 124202093); Luteolin (PubChem CID: 5280445); Apigenin (PubChem CID: 5280443).

1. Introduction

Virgin olive oil (VOO) is the oil produced from healthy olive fruits using only mechanical methods at low temperature (typically below 28 °C) without the addition of chemical solvents. Most olive oil is produced and consumed in the Mediterranean Basin, but VOO is gaining popularity worldwide due to its excellent organoleptic and nutraceutical properties (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Servili et al., 2014).

The olive oil is composed by the saponifiable and unsaponifiable fractions representing respectively about 98% and 2% of the total weight. The saponifiable fraction is mainly composed of the fatty acids, while the unsaponifiable fraction contains a heterogeneous complex pool of minor compounds (Servili et al., 2014). Several chemical families are included in this heterogeneous pool such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, phenols, tocopherols, esters, pigments and volatile components, among others (Rallo et al., 2018).

Phenolic compounds play a significant role in the VOO nutritional value and organoleptic properties, and they greatly contribute to the shelf life of the product, improving its oxidative stability (Bendini et al., 2007; Piroddi et al., 2017; Silva, Pinto, Carrola, & Paiva-Martins, 2010). The VOO phenolic profile highly depends on the genotype (Miho, Díez, Medina, et al., 2018), but it is also influenced by the oil extraction process. Malaxation, consisting of mixing olive paste to induce the coalescence of tiny oil droplets into large droplets, leading to a continuous lipid phase, crucially affects the VOO phenolic composition (Kalua, Bedgood, Bishop, & Prenzler, 2006; Stefanoudaki, Koutsaftakis, & Harwood, 2011). This phenomenon is because malaxation induces the activation of several endogenous fruit enzymes, such as β-glucosidases and esterases, which hydrolyze precursors of oleuropein and ligstroside to produce secoiridoid derivatives (Clodoveo, 2012).

During the whole extraction process, other endogenous fruit enzymes such as phenoloxidases and peroxidases become active and catalyze the oxidation of phenolic compounds, resulting in a reduced phenolic concentration in VOO (Taticchi et al., 2013). Therefore, as a general trend, the malaxation time (MT) negatively affects the

concentration of major phenolic compounds found in VOO (Angerosa, Mostallino, Basti, & Vito, 2001; Jiménez, Sánchez-Ortiz, & Rivas, 2014; Kiritsakis & Shahidi, 2017; Trapani et al., 2017). However, some studies have shown that the concentrations of specific phenolic compounds, such as hydroxytyrosol (3,4-DHPEA), tyrosol (p-HPEA), oleocanthal (p-HPEA-EDA), ligstroside-aglycone (p-HPEA-EA) and vanillin, are positively affected by MT (Germek et al., 2014; Gómez-Rico, Inarejos-García, Salvador, & Fregapane, 2009). The reason for these opposite patterns remains unclear, although VOO resistance to oxidation consistently decreases with MT, and this fact seems to be related to the degradation of the main phenols (Kiritsakis & Shahidi, 2017; Stefanoudaki et al., 2011).

Few studies have evaluated the effect of oxygen (O_2) on phenolic compounds during the malaxation process. The replacement of atmospheric air with inert gases such as nitrogen or carbon dioxide seems to decrease enzymatic activity, and preserve the VOO phenolic content, but these alternatives are not considered cost-effective (Servili et al., 2008; Vierhuis et al., 2001). The vacuum method, which removes oxygen traces, is applied to different food industrial processes with the aim of preventing oxidation of the valuable natural chemical compounds (such as phenols) (Castagnini, Betoret, Betoret, & Fito, 2015; Mushtaq, 2017). Although some VOO extraction companies have started to apply an "under vacuum extraction technology" (ApolloOliveOil, 2018), no scientific studies have evaluated the significance of this approach concerning the VOO phenolic profile.

The resistance of VOO to oxidative deterioration has been principally attributed to its fatty acid composition and phenolic profile (Arcoleo et al., 1999; Bruscatto et al., 2017; Rallo et al., 2018; Velasco, 2002). Concerning fatty acids, oils with a high oleic acid/linoleic acid ratio tend to be more stable against oxidation than oils with a low ratio between these two acids (Aparicio, Roda, Albi, & Gutiérrez, 1999; Spatari, De Luca, Ioele, & Ragno, 2017). While the relationship between the total phenolic concentration of VOO and the oxidative stability is well supported, the effects of individual phenolic compounds in this regard remain controversial. For instance, VOOs coming from different cultivars, with different phenolic profiles and concentrations, have exhibited similar oxidation rates (Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007; Kiritsakis & Shahidi, 2017). Additionally, molecules with catechol moieties, such as oleuropein aglycone (3,4-DHPEA-EA) and oleacein (3,4-DHPEA-EDA), have shown a remarkable resistance to oxidation

(Baldioli, Servili, Perretti, & Montedoro, 1996; Romani et al., 2007); but the presence of simple phenols such as hydroxytyrosol has shown the opposite effect, contributing to low oxidative stability of the oils (Paiva-Martins, Santos, Mangericao, & Gordon, 2006). Therefore, it seems that the distribution of phenolic compounds plays an important role in VOO oxidative stability. However, there are no conclusive results as to whether the varying phenolic composition of VOO contributes to determining its resistance to oxidation (Kiritsakis & Shahidi, 2017).

In this study, we evaluated the effect of MT and the absence of oxygen during malaxation in the phenolic profiles of eight monovarietal VOOs with three goals: first, to evaluate the effect of MT in the VOO phenolic profiles under the recommended malaxation temperature, set at 28 ºC; second, to evaluate the effect of vacuum conditions during malaxation on the VOO composition by reducing the occurrence of undesired enzymatic and non-enzymatic processes; and finally, to evaluate the extent to which the phenolic and fatty acid profiles of VOO might be associated to the oxidative stability of VOO.

2. Materials and methods

2.1. Olive cultivars and experimental design

Olive fruit samples were collected during the 2017/2018 crop season from an experimental orchard located at the World Olive Germplasm Bank of Cordoba (WOGB) (CAP-UCO-IFAPA), specifically in the cultivar collection located at the University of Cordoba (Cordoba, Spain, 37°55'56.5" N, 4°43'13.3" W and 173 m above sea level) (Trujillo, Ojeda, Urdiroz, & Potter, 2014).

Three experiments were designed to accomplish the proposed goals of this study: the first and second experiments were aimed at evaluating the influence of malaxation time and malaxation atmospheric conditions (vacuum) on the phenolic profile. The third experiment evaluated the association of the VOO oxidative stability to its phenolic and fatty acid composition.

For the first experiment, we selected six cultivars showing remarkable diversity in their phenolic profiles according to Miho et al. (2018): 'Arbosana', 'Bosana', 'Blanqueta', 'Coratina', 'Frantoio' and 'Mixani'. The VOO of these cultivars was extracted by applying

three different MTs: 10, 30 and 60 min, where 30 min is the standard time used in the olive oil extraction process. The experiment was conducted in triplicate for a total of 54 VOO analyzed samples (6 cultivars × 3 MTs × 3 replicates = 54 samples).

The second experiment was focused on the analysis of the VOO samples from six different cultivars: 'Bosana', 'Blanqueta', 'Frantoio', 'Levantinka', 'Mixani' and 'Picual'. The selection of these cultivars followed the same criteria as the first experiment, but it was also subject to the availability of fruits to conduct both experiments. For this reason, four cultivars were shared by experiments 1 and 2, but the other two cultivars were unique to each experiment. In experiment 2, we evaluated the effect of VOO extraction under vacuum conditions versus standard conditions (atmospheric pressure) with three replicates. In total, 36 VOO samples were analyzed (6 cultivars × 2 extraction conditions × 3 replicates = 36 samples).

Finally, the third experiment combined the 90 VOO samples used in the previous experiments (1 and 2) to exhaustively characterize the predictive capacity and the effect of specific phenolic and fatty acid profiles on the oxidative stability of the product, previously determined by the Rancimat method.

2.2. Olive fruit sampling and VOO extraction

Two olive trees per cultivar were manually harvested by sampling all orientations within the canopy. A total of 3 kg of fruit per tree was collected at ripening index (RI) 2.0 (yellowish-red in color) according to the method proposed by the International Olive Oil Council (International Olive Council, 2011).

Monovarietal VOOs were obtained using an Abencor extraction system (MC2 Ingeniería y Sistemas, Sevilla, Spain) under the conditions recommended by the manufacturer (Peres, Martins, & Ferreira-Dias, 2014). The olives were crushed with a hammer mill equipped with a 4-mm sieve at 3000 rpm. The malaxation temperature was kept constant at 28 °C for all the treatments to comply with the methodology optimized by Miho et al. (2018). Meanwhile, three different malaxation times (10, 30 and 60 min) were applied in the first experiment to evaluate its influence on VOO phenolic profile.

Additionally, performing malaxation under vacuum was tested through the Abencor system with a technical modification designed by the authors (**Fig. S1**). This modification was based on coupling an external pump that allowed for operation under pressurized conditions (12 psi) to minimize the presence of oxygen during malaxation. The malaxation time was programmed for 30 minutes.

Afterwards, the olive pomace of all treatments was centrifuged at 3500 rpm for 2 min. No water was added to the pomace at any step of the process. The VOO was decanted into graduated cylinders for 30 min and immediately stored in amber glass bottles at –20 °C until analysis.

2.3. Quantitative analysis of phenolic compounds in olive oil

Nine individual phenolic compounds were determined in olive oil samples by following the method developed by Miho et al. (2018). The method is described in Supplementary Information. The determined phenols were: hydroxytyrosol, apigenin; luteolin; oleocanthal, oleacein, aldehydic open forms of oleuropein aglycone (AOleAgly), monoaldehydic closed form of oleuropein aglycone (MAOleAgly), aldehydic open forms of ligstroside aglycone (ALigAgly); monoaldehydic closed form of ligstroside aglycone (MALigAgly). Absolute quantitative analysis was performed by calibration curves obtained using refined high oleic sunflower oil spiked with multistandard solutions of the target phenols. The absence of quantifiable levels of phenols in the refined oil was checked by direct analysis with the developed method. Nine phenolic concentrations from 0.1 ng/mL to 5 μg/mL were injected in triplicate to obtain the calibration curves. The concentrations of phenols in the monovarietal VOOs were determined with these calibration curves using three replicates per sample.

2.4. Determination of the oxidation stability index in VOO samples

The oxidation stability index was measured by the Rancimat Method using a 743 Rancimat System from Metrohm (Herisau, Switzerland). The analysis was performed by heating 3.2 g of each VOO sample to 100 °C with a 10 L/h air flow rate. Rancimat analyses were carried out in the Agricultural and Food Laboratory of Cordoba (Cordoba, Spain), which is the reference laboratory of the Andalusia Regional Government.

2.5. VOO fatty acid composition analysis

Determination of the fatty acid composition of VOO samples was carried out by GC–FID analysis after derivatization by transesterification. The protocol was initiated by taking 0.1 g of VOO that was mixed with 2 mL of n-hexane. Then, 1 mL of methanolic potassium hydroxide (0.5 mol/L was added as the derivatization agent and the mixture was agitated for 1 min at 1500 × g. The processed samples were allowed to rest for 10 min to permit the separation of the phases. Next, the organic phase, enriched with the fatty acid methyl esters, was diluted 1:10 (v/v) prior to injection into the GC–FID system (Agilent 7820A GC System). For chromatographic separation, an SPTM-2560 fused silica column (100m x 0.25mm x 0.2 μm film thickness) from Supelco (Bellefonte, PA, USA) was used as the stationary phase. The injection volume was 1 μL in splitless injection mode. Helium was used as the carrier gas with a 1 mL/min flow rate. The injector and detector temperatures were set at 250 ˚C. The EZ Chrom Elite Compact (Version 3.3.2, Agilent Technologies) was employed as the acquisition and data treatment software. The results were expressed in relative terms as percentages.

2.6. Statistical analysis

The effects of the cultivar, MT and vacuum conditions on the phenolic composition were evaluated by factorial analysis of variance (ANOVA) ($p < 0.05$). Box-Cox transformation of the data was applied to fit normality when appropriate. The existence of significant pairwise differences among the different processing conditions for the same cultivar was evaluated by a Bonferroni post hoc test. The relationship between VOO composition and oxidative stability was studied by multiple linear regression analysis. To test the contribution of subsets of the independent variable to the regression model, a stepwise analysis of variance of the individual variables was used (Norman & Streiner, 2008). Collinearity among independent variables of the model was studied using variance inflation factors (VIFs). Subsequently, the simplest model was selected, and the contribution of a subset of the independent variables to the overall model was selected using an F-test of the difference between the cumulative sums of squares for the model with/without each variable (O'Brien, 2007). To validate the regression model, the entire data set was split into regression (70%) and validation (30%) by a randomly process using

MS Excel (Microsoft, Redmond, WA). The regression/validation process was conducted 15 times (i.e., 15 randomizations of the data). A regression analysis was used to validate the model output against laboratory data according to the coefficient of determination (R^2), R^2 adjusted for degrees of freedom (Ra^2), and the pattern of residuals over predicted and independent variables.

Furthermore, principal component analysis (PCA) was applied to evaluate potential association patterns between individual phenols and the VOO oxidative stability measured by the Rancimat test. The statistical analyses were performed using SPSS and XLSTAT software (IBM SPSS Statistics, version 23; XLSTAT v.2014.5.03, Addinsoft, Paris, France).

3. Results and Discussion

3.1. Influence of malaxation time on the VOO phenolic profiles

Malaxation variables (temperature and time) significantly influence the VOO phenolic profile, eliciting enzymatic activity with special emphasis on β-glucosidases and esterases. β-glucosidases catalyze the hydrolysis of oleuropein and ligstroside to form their aglycone forms but also contribute to the formation of oleacein and oleocanthal in combination with esterases. On the other hand, an excessive malaxation time negatively affects the VOO phenolic concentrations by triggering undesired reactions catalyzed by oxidases and phenoloxidases (Kalua et al., 2006; Stefanoudaki et al., 2011; Taticchi et al., 2013).

The six selected cultivars ('Arbosana', 'Blanqueta', 'Bosana', 'Coratina', 'Frantoio' and 'Mixani') were characterized by diverse and contrasting VOO phenolic profiles (**Table 1**) (Miho, Díez, Medina, et al., 2018). The phenolic profiles of VOOs extracted from 'Frantoio' and 'Mixani' showed a predisposition for the aglycone isomers to dominate, while 'Arbosana', 'Blanqueta', 'Bosana' and 'Coratina' VOOs reported high concentrations of oleocanthal and oleacein. In line with these results, it is evident that there is an interaction between cultivar and malaxation time.

According to the ANOVA statistical analyses, MT significantly (p-value < 0.0001) affected the VOO phenolic concentration (**Table S2** and **Fig. S2)**. As a general trend, the concentration of aglycone forms decreased with MT, while the concentration of oleocanthal and oleacein increased, mostly in the interval from 10 to 30 min.

Table 1. Phenolic concentrations (expressed as mg/kg) determined in virgin olive oil from six cultivars made by using the Abencor system at three malaxation times.

Cultivar	MT*	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Phenolic Sum
Arbosana	10	0.71 ± 0.10	9.7 ± 0.28	8.6 ± 0.83	93.9 ± 4.0	380 ± 41.2	25.7 ± 1.5	53.5 ± 9.2	95.8 ± 12.1	52.7 ± 11.3	721 ± 67.2
Arbosana	30	0.88 ± 0.23	8.5 ± 0.1	7.0 ± 0.24	116 ± 6.8	470 ± 111	22.1 ± 1.6	25.9 ± 3.0	89.0 ± 5.6	25.3 ± 4.1	765 ± 119
Arbosana	60	1.1 ± 0.22	7.8 ± 0.16	7.0 ± 0.09	104 ± 3.2	439 ± 85.2	20.2 ± 0.28	7.2 ± 0.06	74.9 ± 6.3	14.8 ± 2.3	675 ± 96.3
Bosana	10	0.85 ± 0.19	3.0 ± 0.28	5.6 ± 0.31	140 ± 31.8	394 ± 93.6	73.5 ± 15.6	207 ± 29.3	163 ± 20.5	81.8 ± 12.7	1069 ± 162
Bosana	30	1.4 ± 0.34	2.7 ± 0.24	5.1 ± 0.66	201 ± 14.4	545 ± 9.8	73.2 ± 6.5	66.0 ± 11.9	143 ± 5.5	40.2 ± 2.7	1077 ± 15.2
Bosana	60	2.3 ± 0.63	2.3 ± 0.04	4.4 ± 0.37	184 ± 5.3	472 ± 22.1	63.6 ± 0.92	26.3 ± 4.9	119 ± 14.5	22.3 ± 2.9	896 ± 31.8
Blanqueta	10	2.3 ± 0.35	0.97 ± 0.13	4.0 ± 0.28	187 ± 11.7	1314 ± 165	36.7 ± 4.0	66.2 ± 6.7	167 ± 5.6	83.1 ± 8.4	1861 ± 147
Blanqueta	30	2.7 ± 0.24	0.87 ± 0.16	3.7 ± 0.42	199 ± 6.2	1354 ± 293	37.4 ± 1.3	24.3 ± 2.0	155 ± 7.4	38.4 ± 3.3	1815 ± 293
Blanqueta	60	2.1 ± 0.36	0.78 ± 0.11	3.3 ± 0.17	183 ± 10.5	1009 ± 227	36.1 ± 4.4	6.1 ± 0.8	125 ± 17.5	17.7 ± 3.1	1383 ± 246
Coratina	10	1.8 ± 0.20	2.2 ± 0.05	4.7 ± 0.14	109 ± 23.7	545 ± 27.8	84.3 ± 11.5	334 ± 37.7	220 ± 34.5	244 ± 24.9	1544 ± 143
Coratina	30	1.4 ± 0.14	1.7 ± 0.08	3.6 ± 0.31	206 ± 30.4	1062 ± 69.7	79.5 ± 9.9	137 ± 19.2	167 ± 36.7	101 ± 4.4	1759 ± 75.7
Coratina	60	1.5 ± 0.11	1.4 ± 0.14	2.8 ± 0.29	236 ± 28.2	1021 ± 55.7	71.8 ± 10.9	51.9 ± 7.5	140 ± 29.2	35.1 ± 5.2	1562 ± 117
Frantoio	10	1.3 ± 0.24	0.66 ± 0.07	1.6 ± 0.28	63.5 ± 11.8	321 ± 29.6	260 ± 5.3	832 ± 62.7	479 ± 74.8	762 ± 205	2720 ± 340
Frantoio	30	1 ± 0.15	0.43 ± 0.07	1.0 ± 0.09	87.3 ± 11.9	390 ± 54.3	288 ± 27.7	665 ± 116.1	417 ± 52.5	557 ± 98.7	2407 ± 329
Frantoio	60	0.91 ± 0.13	0.39 ± 0.09	0.82 ± 0.11	121 ± 24.0	415 ± 89.7	306 ± 29.4	600 ± 78.9	415 ± 80.6	431 ± 74.9	2291 ± 307
Mixani	10	0.9 ± 0.22	4.3 ± 0.36	12.2 ± 1.2	26.6 ± 5.8	218 ± 47.7	67.4 ± 2.1	301 ± 31.2	235 ± 31.8	330 ± 54.8	1195 ± 117
Mixani	30	1.2 ± 0.15	3.6 ± 0.53	10.0 ± 2.1	55.4 ± 9.3	382 ± 50.2	63.3 ± 6.1	196 ± 36.0	198 ± 18.1	226 ± 54.5	1135 ± 103
Mixani	60	0.83 ± 0.05	3.0 ± 0.26	8.1 ± 1.1	72.0 ± 9.8	327 ± 12.0	56.5 ± 4.3	114 ± 9.8	128 ± 7.0	110 ± 14.8	820 ± 37.3

*Malaxation time (min).

Afterwards, from 30 to 60 min, the oleocanthal and oleacein concentrations in VOOs from all cultivars did not significantly change (**Fig. 1, Table S3**). The observed reduction of oleuropein and ligstroside aglycone isomers during malaxation is in agreement with a previous report by Germerk and Gómez-Rico (Germek et al., 2014; Gómez-Rico et al., 2009). However, our results further highlighted a positive effect of MT on the concentrations of oleocanthal and oleacein. Thus, an increase in malaxation time from 10 to 60 min increased the oleocanthal concentration in VOOs obtained from 'Coratina', 'Frantoio' and 'Mixani' cultivars by 2.2-, 1.9- and 2.7-fold, respectively.

The sum of phenolic compounds (or total phenolic compound concentration) was also affected by MT (p-value < 0.002). The maximum total phenolic concentration was achieved for MTs of 10 and 30 min, with no significant differences between the treatments (**Fig. 1, Table S3**). In contrast, the lowest total amount of phenolic compounds was observed with the longest MT (60 min) (**Fig. 1**). This result is in agreement with previous studies (Kiritsakis & Shahidi, 2017; Trapani et al., 2017). 'Arbosana' cultivar was the exception to this trend since the phenolic content did not show any significant difference (p-value > 0.05) with MT. In fact, the total phenolic concentration in this cultivar did not significantly change from 10 to 60 min of MT.

ANOVA analysis showed that the variability of the nine target phenolic compounds was significant ($p < 0.0001$) and highly explained by the model with R^2 values ranging from 0.670 to 0.991 (**Table S2**). According to the eta-squared value (η^2), which explains the contribution of each factor to the observed variability, the cultivar (genotype) was the most relevant factor in explaining the variability of the monitored phenols. This value, η^2, for the cultivar factor, ranged from 50.6% for hydroxytyrosol to 96.7% for apigenin. On the other hand, the MT factor significantly accounted for a proportion of variance ranging from 1% for MALigAgly to 19.7% for ALigAgly and AOleAgly ($p < 0.05$). The interaction of *cultivar* $\times$ *MT* was only significant for oleocanthal and ALigAgly compounds, and the η^2 value was always below 8%. The different transformation dynamics of phenolic compounds in different cultivars during the MT justified this occasionally significant interaction. For example, the oleocanthal concentration increased constantly from 10 to 60 min of MT for half of the cultivars (Coratina, Frantoio, Mixani), while for the other half

(Arbosana, Blanqueta, Bosana), the concentration of this phenol increased from 10 to 30 min of MT but then decreased from 30 to 60 min.

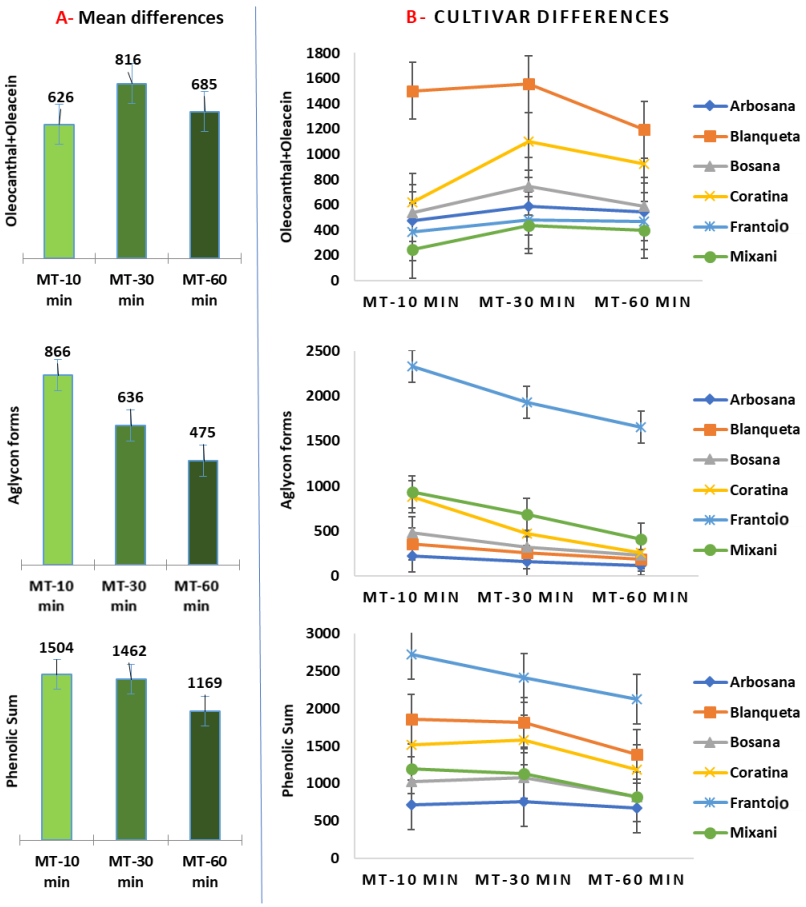


Figure 1. (A) Mean differences for the main groups of phenolic compounds found in olive oil from six cultivars processed at three different malaxation times. (B) Phenolic concentrations for each cultivar processed at three different malaxation times. Phenolic concentrations are expressed in mg/kg.

3.2. Effect of malaxation under vacuum conditions on the VOO phenolic profiles

Different strategies have been proposed to minimize the oxygen content during the malaxation process to preserve minor compounds that are degraded by enzymatic activity. The most common approach, but not cost-effective, is the injection of inert gasses, mainly nitrogen or carbon dioxide, to replace the oxygen (Servili et al., 2008; Vierhuis et al., 2001; Yorulmaz, Yıldırım, Duran, Kula, & Kıvrak, 2017). A potential less expensive method is to

apply vacuum conditions during malaxation. In this research study, we compared the effect of malaxation under vacuum and atmospheric (standard) conditions in the VOO phenolic profiles of six cultivars (**Table 2**). 'Blanqueta', 'Bosana', and 'Levantinka' VOOs were characterized by the predominance of oleocanthal and oleacein, while 'Frantoio', 'Mixani' and 'Picual' provided VOOs enriched in aglycone forms (**Fig. S4)**. When VOO was extracted under vacuum conditions, the concentration of oleocanthal and oleacein was increased by 19.5%, the concentration of oleuropein and ligstroside aglycone forms was increased by 23.3%, and the total phenolic content was increased by 21.4% (**Fig. S4**). On the other hand, the oil extraction yield was not significantly higher under vacuum as compared to standard conditions.

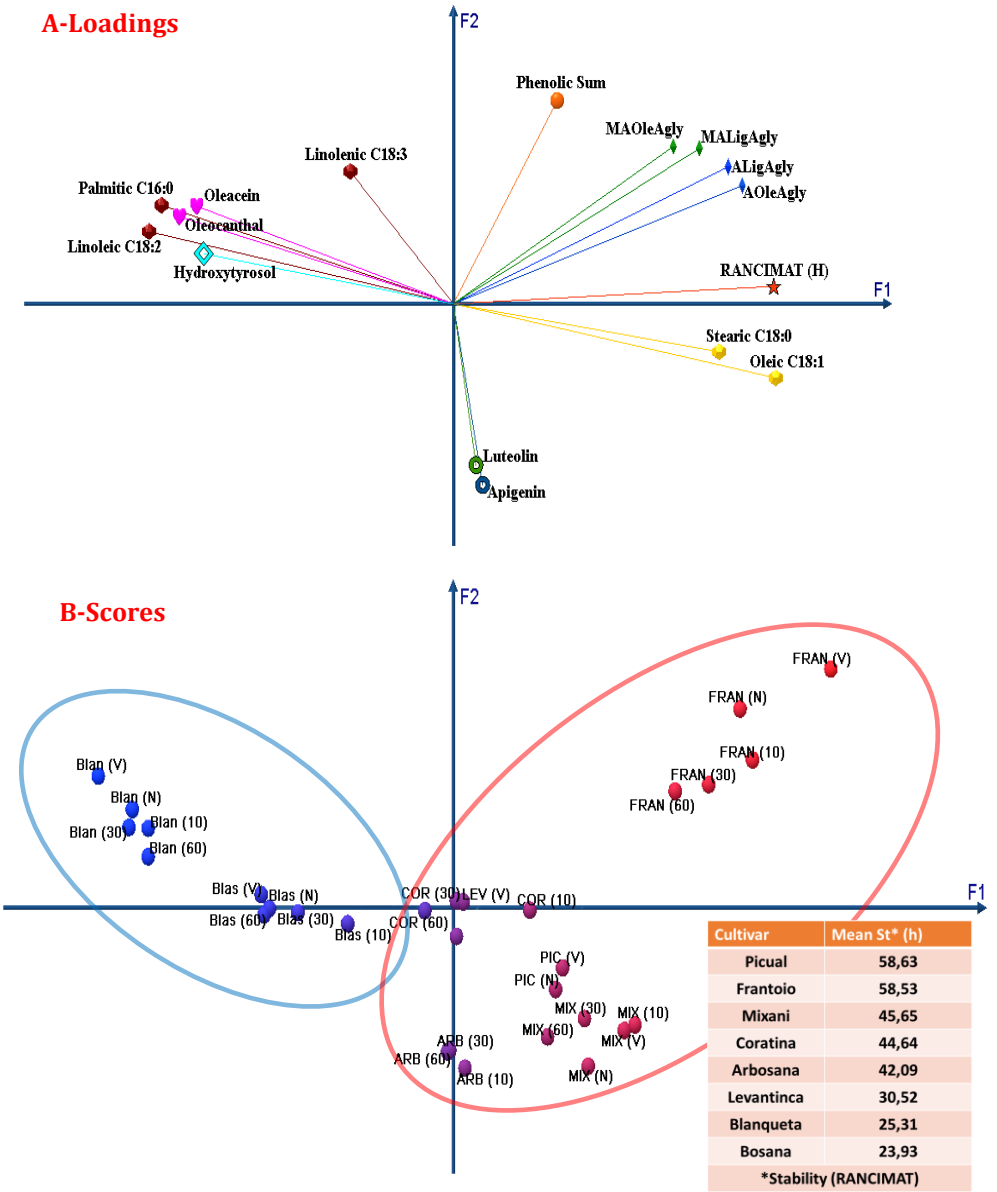
A factorial ANOVA was executed to determine the relative influence of the involved factors on the VOO phenolic profile: (1) malaxation conditions (vacuum versus standard conditions); (2) cultivar (genotype); and (3) the interaction between both factors. All the phenolic compounds analysed were highly and significantly ($p < 0.005$) affected by the three evaluated factors with R^2 ranging from 0.832 to 0.988 (**Table S4, Fig. S3**)**.** Similarly to previous studies (Baiano, Terracone, Viggiani, & Nobile, 2013; Dagdelen, Tümen, Özcan, & Dündar, 2013; Miho, Díez, Mena-Bravo, et al., 2018), the cultivar contributed to explaining the highest phenolic variability, with η^2 ranging from 77.4% in case of hydroxytyrosol to 98.2% for luteolin, and MALigAgly ($p < 0.0001$). The malaxation conditions (vacuum/standard) also contributed significantly to explain the differences found in VOO phenolic profiles (η^2 from 0.3 to 5.6%; $p < 0.05$). In this last case, the concentration of most phenolic compounds was significantly increased with the application of vacuum condition during the malaxation process. The exceptions to this trend were oleocanthal, luteolin and MALigAgly, which increased their concentration but not significantly **(Table S5, Fig. S4)**. The interaction between factors (cultivar × malaxation conditions) was significant only for MaOleAgly and the Phenolic Sum, with η^2 = 2.3% ($p = 0.012$) and η^2 = 1.1% ($p = 0.041$), respectively. This interaction between factors occurs because the phenolic compounds are not transformed in the same way for all cultivars during the extraction process. Thus, 'Blanqueta', 'Frantoio' and 'Mixani' cultivars resulted in being significantly ($p < 0.05$) richer in Phenolic Sum concentration when extracted under vacuum condition, while the concentration of the rest of cultivars were not significantly increased ($p > 0.05$) (**Table S5)**.

Table 2. Phenolic concentrations (expressed as mg/kg) determined in virgin olive oil from six cultivars made by using the Abencor system under vacuum and in standard conditions (atmospheric pressure).

Cultivar	(N-V)*	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Phenolic Sum
Blanqueta	N	2.1 ± 0.22	1.3 ± 0.07	5.8 ± 0.01	247 ± 17.8	1621 ± 239	48.5 ± 0.9	30.1 ± 3.1	224 ± 15.9	49.6 ± 5.4	2230 ± 264
Blanqueta	V	2.7 ± 0.21	1.3 ± 0.01	5.7 ± 0.25	263 ± 3.0	1834 ± 110	49.2 ± 4.7	39.0 ± 5.0	247 ± 28.6	56.5 ± 12.0	2498 ± 145
Bosana	N	2.3 ± 0.20	4.6 ± 0.60	8.8 ± 0.97	238 ± 17.0	616 ± 69.6	85.5 ± 8.0	85.9 ± 22.1	160 ± 14.3	70.1 ± 10.0	1272 ± 112
Bosana	V	2.5 ± 0.36	4.8 ± 0.54	9.2 ± 0.73	258 ± 14.2	747 ± 83.7	89.5 ± 4.7	103 ± 37.0	176 ± 11.1	81.9 ± 19.1	1472 ± 157
Frantoio	N	0.81 ± 0.03	1.1 ± 0.19	2.4 ± 0.39	112 ± 9.1	419 ± 33.8	364 ± 25.1	1149 ± 96.2	422 ± 53.0	579 ± 92.3	3049 ± 270
Frantoio	V	1.0 ± 0.05	1.2 ± 0.12	2.7 ± 0.34	106 ± 14.8	635 ± 119	341 ± 16.8	1280 ± 55.0	598 ± 49.3	916 ± 80.4	3881 ± 246
Levantinka	N	2.0 ± 0.47	2.0 ± 0.29	10.2 ± 1.4	85.0 ± 17.5	493 ± 130	100 ± 3.9	200 ± 18.6	329 ± 16.7	177 ± 31.0	1398 ± 123
Levantinka	V	2.1 ± 0.65	2.5 ± 0.17	9.9 ± 0.83	83.7 ± 22.4	526 ± 80.0	101 ± 11.8	249 ± 26.7	361 ± 58.6	251 ± 30.8	1586 ± 144
Mixani	N	1.1 ± 0.02	7.4 ± 0.34	17.7 ± 0.89	67.1 ± 17.7	365 ± 32.0	79.4 ± 3.5	211 ± 22.5	185 ± 7.4	225 ± 32.4	1159 ± 10.7
Mixani	V	1.4 ± 0.06	7.8 ± 0.37	18.6 ± 0.77	69.2 ± 16.2	583 ± 90.2	83.9 ± 5.5	250 ± 31.1	276 ± 8.0	378 ± 50.4	1668 ± 29.6
Picual	N	2.0 ± 0.46	3.3 ± 0.26	7.8 ± 0.48	26.9 ± 5.5	110 ± 16.6	85.6 ± 6.5	271 ± 12.6	147 ± 9.1	172 ± 8.6	826 ± 45.7
Picual	V	2.3 ± 0.53	3.0 ± 0.32	7.0 ± 0.45	29.1 ± 1.3	124 ± 11.3	90.9 ± 2.0	313 ± 15.8	171 ± 5.9	214 ± 17.6	955 ± 50.1

3.3. Contribution of the individual phenolic compounds to the VOO oxidative stability

The preventive action of the main individual phenols against the oxidative deterioration of VOO has not been thoroughly investigated. Most of the previous studies were focused on the influence of the fatty acid composition and total phenolic content, considering in some cases a few individual phenols (Gómez-Alonso et al., 2007; Kiritsakis & Shahidi, 2017; Paiva-Martins et al., 2006; Romani et al., 2007). To address this point, the VOO samples evaluated in both previous experiments (a total of 90 VOO samples from eight olive cultivars, 'Arbosana', 'Blanqueta', 'Bosana', 'Coratina', 'Frantoio', 'Levantinka', 'Mixani' and 'Picual') were merged into a unique data set (**Tables 1 and 2**). The characterization of these samples was completed by analyzing the oxidative stability (Rancimat hours) and the fatty acid content **(Tables S6 and S7)**. Differences can be found between reported data for these genotypes and those found in other studies of the literature. These variations are explained by non-genetic factors such as the crop season, agronomic management, analytical methods and others external factors such as climatological conditions, among others (Aguilera et al., 2005; Beltrán, Del Rio, Sánchez, & Martínez, 2004; Deiana et al., 2019; Miho, Díez, Medina, et al., 2018; Servili et al., 2004; Stefanoudaki et al., 2011). A PCA was performed with our data set to identify clusterings of samples and the relationships between the explanatory variables: concentration of phenols and fatty acids versus the oxidative stability (Rancimat hours). The first two principal components (PC1 and PC2) explained 85.3% of the variability. **Fig. 2** and **Tables S8 and S9** show that oxidative stability, expressed in Rancimat hours, reported a high positive correlation with the aglycone isomers, oleic acid and moderate positive correlation with stearic acid, in agreement with previous studies (Aparicio et al., 1999; Baldioli et al., 1996; Romani et al., 2007; Velasco & Dobarganes, 2002). Conversely, the oxidative stability was negatively correlated with the concentrations of oleocanthal, oleacein, and hydroxytyrosol as well as linoleic, linolenic, and palmitic acids. These negative correlations seem to be spurious correlations due to the secondary negative correlations that exist among several phenolic compounds and fatty acids (**Tables S8 and S9**). For example, the Rancimat variable is highly positively correlated with the ALigAgly compound and highly negatively correlated with oleocanthal; on the other hand, ALigAgly and oleocanthal are also highly negatively correlated.



Cultivar	Mean St* (h)
Picual	58,63
Frantoio	58,53
Mixani	45,65
Coratina	44,64
Arbosana	42,09
Levantinca	30,52
Blanqueta	25,31
Bosana	23,93
*Stability (RANCIMAT)	

Figure 2. The loading plot (A) and scores plot (B) of the principal component analysis of olive oil samples obtained from eight cultivars. PCA was done using the concentrations of phenolic compounds, the fatty acid profile and oxidative stability measured by the Rancimat method as input components. The color scale of scores (cultivars) represents their oxidative stability classification. The violet-red cultivars tend to be much more stable than the blue cultivars. **RANCIMAT** – olive oil oxidative stability expressed in hours; **Phenolic sum** – the sum of all phenolic compounds analyzed; **AOleAgly** – Aldehydic open forms of oleuropein aglycone; **MAOleAgly** – Monoaldehydic closed form of oleuropein aglycone; **ALigAgly** – Aldehydic open forms of ligstroside aglycone; **MALigAgly** – Monoaldehydic closed form of ligstroside aglycone. **Pic** – Picual, **Mix** – Mixani, **Lev** – Levantinka, **Fran** – Frantoio, **Cor** – Coratina, **Bos** – Bosana, **Blan** – Blanqueta, **Arb** – Arbosana.

Furthermore, the variable "Phenolic Sum" (or total phenolic content) showed a low and non-significant ($p > 0.05$) correlation with the oxidative stability variable (Rancimat), which illustrates that the oxidative stability is related to the concentration and composition of individual phenolic compounds rather than to the total phenolic content. **Fig. 2** also shows the distribution of VOO samples according to their chemical profile. Clearly, the cultivars marked in a red-violet color were characterized by higher oxidative stability than the cultivars marked in a blue color. Therefore, the cultivars were clustered into two main groups according to their chemical composition. The samples of the first group (red) were characterized by high concentrations of oleuropein and ligstroside aglycone isomers and/or high concentrations of stearic and oleic acid; the second group samples (blue) were rich in oleocanthal, oleacein, and hydroxytyrosol as well as linoleic, linolenic, and palmitic acids.

According to these results, we might conclude that VOO oxidative stability highly depends on the fatty acid and individual phenolic composition. To better demonstrate this effect, we defined the "*f*" ratio as an indicator of the main phenols found in VOO:

$$f = \frac{[aglycone\ forms]}{[oleocanthal]+[oleacein]}$$

where *[aglycone forms]* is the sum of the concentrations of all aglycone isomers of oleuropein and ligstroside, and [*oleocanthal*] and [*oleacein*] are the concentrations of these phenols in the VOO samples. A high *f* index value indicates that the VOO phenolic profile is predominantly composed of aglycone isomers, while a low *f* index value indicates that VOO is predominantly composed of oleocanthal and oleacein. Both aglycone isomers and oleocanthal-oleacein are produced from the same substrates, oleuropein and ligstroside. However, aglycone isomers are produced by β-glucosidase hydrolysis, while oleocanthal and oleacein are formed by the action of β-glucosidase and esterase enzymes (Kalua et al., 2006; Stefanoudaki et al., 2011; Taticchi et al., 2013). The *f* ratio value of the analyzed samples revealed a significant correlation between this parameter and VOO oxidative stability ($R^2 = 0.6824$; $p < 0.05$). This association is also demonstrated in **Fig. 3** as a proof of concept by dividing the total set of samples (90 samples) into two groups obtained by PCA (red and blue groups). The groups were nominally defined as "less stable oils" (33 samples with Rancimat response < 31 h) and "very stable oils" (57 samples with Rancimat

response > 44 h). Then, a relative and descriptive comparison of the parameters that might be related to the oil oxidative stability was carried out between both groups. These descriptive comparisons demonstrated that the total phenolic content was similar for both groups of VOOs, but the individual phenolic profile was completely different, owing to the varied concentrations of secoiridoids. The *f* ratio clearly shows that the individual phenolic profile of the "less stable" group was completely different as compared to the "very stable" group. The "less stable" group shows a low *f* ratio (rich in oleocanthal and oleacein, and poor in aglyconeaglycone forms), while the contrary was observed for the "very stable" group. Complementarily, the oleic acid/linoleic acid ratio had higher values for highly stable VOOs compared to less stable VOOs.

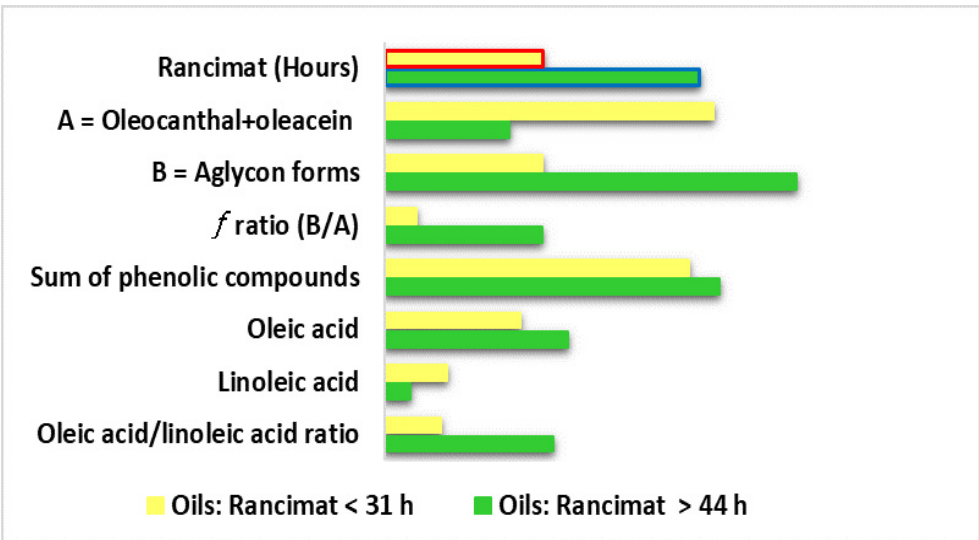


Figure 3. Influence of the VOO chemical composition on its oxidative stability.

3.4. Oxidative stability predictive model by multiple regression analysis

Subsequently, we performed a multiple linear regression analysis to determine a predictive mathematical model for VOO stability (expressed in Rancimat hours) based on the phenolic and fatty acid compositions. After using the stepwise selection process and checking the VIF values (cutoff < 1.7) to avoid collinearity among the predictive variables, a first linear regression including three phenolic compounds (hydroxytyrosol, oleacein, and AOleAgly) and one fatty acid (linoleic acid) as explanatory variables was designed. This first regression model explained 95.4% (R^2) of the total variance with the individual and

general *p*-values being highly significant ($p < 0.0001$). According to the F-test, the contribution of oleacein to the sum of squares was not significant at $p = 0.08$. Subsequently, four different multiple linear regressions were conducted using only three of the four independent selected variables (hydroxytyrosol, AOleAgly, and linoleic acid). The multiple linear regression using hydroxytyrosol, AOleAgly, and linoleic acid as variables explained 92.3% (R^2) of the total variance with the individual and general *p*-values being highly significant ($p < 0.0001$). After the iteration process, the selected model was **(Table 3)**:

Y (hours) = 49.6+5.34(hydroxytyrosol)+0.02(AOleAgly)-1.6(linoleic acid)

The 15 regression lines (output against analytical data) used for the validation of the multiple regression model showed an R^2 value > 0.909 with highly significant *p*-values ($p < 0.0001$). These results highlight that individual phenols and fatty acids determine the oxidative stability of olive oil, introducing the possibility of prediction based on the concentration of these compounds.

Table 3. Multiple linear regression analysis results used to develop a predictive model using the concentrations of phenolic compounds as explanatory variables to determine the oxidative stability measured by the Rancimat test.

Model Summary[a]

R	R^2	Adjusted R^2	Estimation standard error	Change Statistics					Durbin-Watson
				R^2 Change	F Change	DF regression	Total DF	Sig. F Change	
0.9609[b]	0.9234	0.9207	2.2660	0.9234	345.64	3	89	0.000	1.368

Coefficients[b]

Explanatory variables	Coefficient	Std. Error	T	Significance (*p*)	VIF	Correlations with the dependent variable		
						Pearson Correlation (R)	Partial	Part
(Constant)	49.603	1.44	34.46	0.0000	5			
Hydroxytyrosol	5.348	0.77	6.93	0.0000	1.5	-0.340	0.515	0.093
AOleAgly	0.029	0.00	14.89	0.0000	1.2	0.753	0.636	0.127
Linoleic C18:2	-1.625	0.08	-19.53	0.0000	1.7	-0.831	-0.670	-0.139

Mathematic model to estimate the oxidative stability (OS) expressed as Rancimat hours

OS (Hours) = (49.603) + (5.348•hydroxytyrosol) + (0.029•AOleAgly) + (−1.625•Linoleic acid)

[a]Dependent Variable: Oxitative stability (OS) expressed in RANCIMAT hours.
[b]Predictors (explanatory variables): Hydroxytyrosol, AOleAgly, Linoleic C18:2.

4. Conclusions

The influence of malaxation conditions has been evaluated in the phenolic profile of VOO extracted from different cultivars. The total phenolic content and the concentration of most relevant phenols was decreased with malaxation time, except for oleocanthal and oleacein that were increased. On the other hand, malaxation under vacuum conditions significantly increased the total phenolic content by 22% as compared to the process at atmospheric pressure.

Furthermore, the statistical analysis demonstrated that the relative phenolic profile highly explained the VOO oxidative stability. Thus, VOOs with high relative content in oleuropein and ligstroside aglycone forms tend to be more stable than those with low content in these phenols. In fact, a preliminary study revealed that it is possible to predict the VOO oxidative stability with a regression model based on hydroxytyrosol, AOleAgly and linoleic acid as explanatory variables. These promising results might strongly aid to enhance the VOO quality and shelf life, and to design strategies for preparation of coupages with better features. According to this preliminary study, the effect of vacuum in the extraction of VOO should be evaluated for other chemical families such as tocopherols, carotenoids or pigments.

CRediT authorship contribution statement

H. Miho: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. J. Moral: Methodology, Formal analysis, Writing - original draft, Writing - review & editing. M.A. López-González: Methodology, Investigation. C.M. Díez: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration. F. Priego-Capote: Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This research was jointly financed by the Spanish Ministerio de Economía y Competitividad and the Interreg-Med Program through the projects CTQ2015-68813-R and MED-1033. Both projects are co-funded by the European Regional Development Fund/European Social Fund ("Investing in your future"). H. Miho is grateful to the International Olive Council (IOOC) for a doctoral fellowship awarded. J. Moral received a Marie Skłodowska Curie fellowship launched by the European Union's H2020 (contract number 658579).

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Supplementary data

SUPPLEMENTARY TABLES

Supplementary table 1. Multiple Reaction Monitoring (MRM) parameters for quantitative analysis of phenolic compounds by LC–MS/MS.

Phenolic compouds		Retention time (min)	Q1 voltage (V)	Precursor ion (*m/z*)	Collision energy (eV)	Quantitative transition (*m/z*)	Product ion confirmation (*m/z*)
Hydroxytyrosol		2.1	110	153.1	10	153-123	108
3.4-DHPEA-EDA (Oleacein)		4.3	110	319.1	12	319-59	139
3.4-DHPEA-EA	AOleAgly	4.6	110	377	12	377-275	307
	MAOleAgly	5.9	110	377	12	377-275	307
p-HPEA-EDA (Oleocanthal)		5.4	110	303.1	12	303-59	137
p-HPEA-EA	ALigAgly	5.5	110	361.1	12	361-291	101
	MALigAgly	6.2	110	361.1	12	361-291	101
Luteolin		6.3	170	285	35	285-133	175
Apigenin		6.6	170	269	35	269-117	151

AOleAgly – Aldehydic open forms of Oleuropein Aglycone; **MAOleAgly** – Monoaldehydic closed form of Oleuropein Aglycone.

ALigAgly – Aldehydic open forms of Ligstroside Aglycone; **MALigAgly** – Monoaldehydic closed form of Ligstroside Aglycone.

Supplementary table 2. ANOVA results for the evaluation of the influence of malaxation time and genotype on the phenolic profile of virgin olive oil. The results include the R2 coefficient (proportion of variability explained by the studied factors), F-value and type III SS (type III sum of squares) for the general model, while the F-value, η2 (contribution of each factor to the observed variability) and p-value are listed for each factor (cultivar and malaxation time) as well as for the interaction between both factors.

Parameter		**Hydroxytyrosol**	**Apigenin**	**Luteolin**	**Oleocanthal**	**Oleacein**	**MALigAgly**	**ALigAgly**	**MAOleAgly**	**AOleAgly**	**Phenolic Sum**
R^2		0.691	0.991	0.979	0.955	0.670	0.963	0.988	0.832	0.966	0.830
F		4.730	226.385	98.877	45.123	4.300	55.025	174.758	10.523	60.347	10.320
Type III SS		6.339	50.876	92.935	10558.825	33.259	1.817	264.02	19.058	20.769	5.702
p-value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cultivar	*F*	11.790	750.996	317.006	121.978	12.626	184.255	456.124	29.846	154.918	31.378
	$\eta^2(\%)$	**50.6**	**96.7**	**92.3**	**75.9**	**57.9**	**94.8**	**75.8**	**69.4**	**72.9**	**74.2**
	p-value	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Malaxation time	*F*	0.892	40.333	39.946	47.516	2.903	4.857	296.056	9.505	104.593	7.775
	$\eta^2(\%)$	1.5	**2.1**	**4.7**	**11.8**	5.3	**1.0**	**19.7**	**8.8**	**19.7**	**7.4**
	p-value	0.419	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.068	**0.014**	**< 0.0001**	**0.0009**	**< 0.0001**	*0.002*
Cultivar× MT	*F*	1.968	1.290	1.599	6.217	0.417	0.443	9.816	1.065	4.212	0.300
	$\eta^2(\%)$	16.9	0.3	0.9	**7.7**	3.8	0.5	**3.3**	5.0	**4.0**	1.4
	p-value	0.067	0.272	0.147	**< 0.0001**	0.929	0.915	**< 0.0001**	0.413	**0.001**	0.977
Error	$\eta^2(\%)$	30.9	0.9	2.1	4.5	33.0	3.7	1.2	16.8	3.4	17.0
	Type III SS	2.838	0.476	1.990	495.535	16.378	0.070	3.199	3.835	0.729	1.170

Supplementary table 3. Pairwise comparisons (Bonferroni test) to analyze the differences in phenolic concentrations of VOO for each cultivar at different malaxation times (10, 30 and 60 min) with 95% confidence level.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Upper Bound
Hydroxytyrosol	Arbosana	10.0	30.0	-0,173	0,294	1,000	-0,911	0,565
			60.0	-0,344	0,294	0,749	-1,082	0,394
		30.0	10.0	0,173	0,294	1,000	-0,565	0,911
			60.0	-0,171	0,294	1,000	-0,908	0,567
		60.0	10.0	0,344	0,294	0,749	-0,394	1,082
			30.0	0,171	0,294	1,000	-0,567	0,908
	Blanqueta	10.0	30.0	-0,376	0,294	0,627	-1,114	0,362
			60.0	0,243	0,294	1,000	-0,495	0,981
		30.0	10.0	0,376	0,294	0,627	-0,362	1,114
			60.0	0,619	0,294	0,126	-0,119	1,357
		60.0	10.0	-0,243	0,294	1,000	-0,981	0,495
			30.0	-0,619	0,294	0,126	-1,357	0,119
	Bosana	10.0	30.0	-0,546	0,294	0,213	-1,284	0,192
			60.0	**-0,824***	0,294	0,024	-1,562	-0,086
		30.0	10.0	0,546	0,294	0,213	-0,192	1,284
			60.0	-0,278	0,294	1,000	-1,016	0,460
		60.0	10.0	**0,824***	0,294	0,024	0,086	1,562
			30.0	0,278	0,294	1,000	-0,460	1,016
	Coratina	10.0	30.0	0,398	0,294	0,552	-0,340	1,136
			60.0	0,602	0,294	0,143	-0,136	1,340
		30.0	10.0	-0,398	0,294	0,552	-1,136	0,340
			60.0	0,204	0,294	1,000	-0,534	0,942

		60.0	10.0	-0,602	0,294	0,143	-1,340	0,136
			30.0	-0,204	0,294	1,000	-0,942	0,534
	Frantoio	10.0	30.0	0,299	0,294	0,947	-0,439	1,037
			60.0	0,387	0,294	0,589	-0,351	1,125
		30.0	10.0	-0,299	0,294	0,947	-1,037	0,439
			60.0	0,088	0,294	1,000	-0,650	0,826
		60.0	10.0	-0,387	0,294	0,589	-1,125	0,351
			30.0	-0,088	0,294	1,000	-0,826	0,650
	Mixani	10.0	30.0	0,076	0,294	1,000	-0,662	0,814
			60.0	0,412	0,294	0,509	-0,326	1,150
		30.0	10.0	-0,076	0,294	1,000	-0,814	0,662
			60.0	0,336	0,294	0,781	-0,402	1,074
		60.0	10.0	-0,412	0,294	0,509	-1,150	0,326
			30.0	-0,336	0,294	0,781	-1,074	0,402

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
Luteolin	Arbosana	10.0	30.0	**1,629***	0,577	0,023	0,180	3,078
			60.0	**1,609***	0,577	0,025	0,160	3,058
		30.0	10.0	**-1,629***	0,577	0,023	-3,078	-0,180
			60.0	-0,020	0,577	1,000	-1,469	1,429
		60.0	10.0	**-1,609***	0,577	0,025	-3,058	-0,160
			30.0	0,020	0,577	1,000	-1,429	1,469
	Blanqueta	10.0	30.0	0,297	0,577	1,000	-1,152	1,746
			60.0	0,688	0,577	0,723	-0,761	2,137
		30.0	10.0	-0,297	0,577	1,000	-1,746	1,152
			60.0	0,390	0,577	1,000	-1,058	1,839
		60.0	10.0	-0,688	0,577	0,723	-2,137	0,761
			30.0	-0,390	0,577	1,000	-1,839	1,058
	Bosana	10.0	30.0	0,502	0,577	1,000	-0,946	1,951
			60.0	1,187	0,577	0,141	-0,262	2,636
		30.0	10.0	-0,502	0,577	1,000	-1,951	0,946
			60.0	0,684	0,577	0,730	-0,765	2,133
		60.0	10.0	-1,187	0,577	0,141	-2,636	0,262
			30.0	-0,684	0,577	0,730	-2,133	0,765
	Coratina	10.0	30.0	1,087	0,577	0,203	-0,362	2,536
			60.0	**1,829***	0,577	0,009	0,380	3,278
		30.0	10.0	-1,087	0,577	0,203	-2,536	0,362
			60.0	0,742	0,577	0,620	-0,707	2,191
		60.0	10.0	**-1,829***	0,577	0,009	-3,278	-0,380
			30.0	-0,742	0,577	0,620	-2,191	0,707
	Frantoio	10.0	30.0	0,566	0,577	1,000	-0,883	2,015
			60.0	0,742	0,577	0,621	-0,707	2,190
		30.0	10.0	-0,566	0,577	1,000	-2,015	0,883
			60.0	0,176	0,577	1,000	-1,273	1,624
		60.0	10.0	-0,742	0,577	0,621	-2,190	0,707
			30.0	-0,176	0,577	1,000	-1,624	1,273
	Mixani	10.0	30.0	**2,287***	0,577	0,001	0,838	3,736

			60.0	**4,151***	0,577	0,000	2,702	5,600
		30.0	10.0	**-2,287***	0,577	0,001	-3,736	-0,838
			60.0	**1,864***	0,577	0,008	0,415	3,313
		60.0	10.0	**-4,151***	0,577	0,000	-5,600	-2,702
			30.0	**-1,864***	0,577	0,008	-3,313	-0,415

*Significant at 95% confidence level. a-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
Oleocanthal	Arbosana	10.0	30.0	-22,125	13,553	0,334	-56,158	11,908
			60.0	-9,661	13,553	1,000	-43,694	24,372
		30.0	10.0	22,125	13,553	0,334	-11,908	56,158
			60.0	12,464	13,553	1,000	-21,569	46,497
		60.0	10.0	9,661	13,553	1,000	-24,372	43,694
			30.0	-12,464	13,553	1,000	-46,497	21,569
	Blanqueta	10.0	30.0	-11,797	13,553	1,000	-45,830	22,237
			60.0	4,533	13,553	1,000	-29,500	38,566
		30.0	10.0	11,797	13,553	1,000	-22,237	45,830
			60.0	16,330	13,553	0,708	-17,704	50,363
		60.0	10.0	-4,533	13,553	1,000	-38,566	29,500
			30.0	-16,330	13,553	0,708	-50,363	17,704
	Bosana	10.0	30.0	**-61,163***	13,553	0,000	-95,196	-27,130
			60.0	**-44,355***	13,553	0,007	-78,389	-10,322
		30.0	10.0	**61,163***	13,553	0,000	27,130	95,196
			60.0	16,807	13,553	0,669	-17,226	50,841
		60.0	10.0	**44,355***	13,553	0,007	10,322	78,389
			30.0	-16,807	13,553	0,669	-50,841	17,226
	Coratina	10.0	30.0	**-96,787***	13,553	0,000	-130,821	-62,754
			60.0	**-127,132***	13,553	0,000	-161,165	-93,098
		30.0	10.0	**96,787***	13,553	0,000	62,754	130,821
			60.0	-30,344	13,553	0,094	-64,378	3,689
		60.0	10.0	**127,132***	13,553	0,000	93,098	161,165
			30.0	30,344	13,553	0,094	-3,689	64,378
	Frantoio	10.0	30.0	-23,776	13,553	0,264	-57,809	10,258
			60.0	**-57,064***	13,553	0,000	-91,097	-23,031
		30.0	10.0	23,776	13,553	0,264	-10,258	57,809
			60.0	-33,288	13,553	0,057	-67,321	0,745
		60.0	10.0	**57,064***	13,553	0,000	23,031	91,097
			30.0	33,288	13,553	0,057	-0,745	67,321
	Mixani	10.0	30.0	-28,799	13,553	0,122	-62,832	5,235
			60.0	**-45,371***	13,553	0,006	-79,404	-11,337

		30.0	10.0	28,799	13,553	0,122	-5,235	62,832
			60.0	-16,572	13,553	0,688	-50,605	17,461
		60.0	10.0	**45,371***	13,553	0,006	11,337	79,404
			30.0	16,572	13,553	0,688	-17,461	50,605

*Significant at 95% confidence level. [a]Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
Oleacein	Arbosana	10.0	30.0	-90,072	156,248	1,000	-482,417	302,273
			60.0	-58,671	156,248	1,000	-451,016	333,674
		30.0	10.0	90,072	156,248	1,000	-302,273	482,417
			60.0	31,401	156,248	1,000	-360,944	423,746
		60.0	10.0	58,671	156,248	1,000	-333,674	451,016
			30.0	-31,401	156,248	1,000	-423,746	360,944
	Blanqueta	10.0	30.0	-40,120	156,248	1,000	-432,465	352,225
			60.0	304,870	156,248	0,177	-87,475	697,215
		30.0	10.0	40,120	156,248	1,000	-352,225	432,465
			60.0	344,990	156,248	0,101	-47,355	737,335
		60.0	10.0	-304,870	156,248	0,177	-697,215	87,475
			30.0	-344,990	156,248	0,101	-737,335	47,355
	Bosana	10.0	30.0	-150,443	156,248	1,000	-542,788	241,902
			60.0	-11,034	156,248	1,000	-403,379	381,311
		30.0	10.0	150,443	156,248	1,000	-241,902	542,788
			60.0	139,409	156,248	1,000	-252,936	531,754
		60.0	10.0	11,034	156,248	1,000	-381,311	403,379
			30.0	-139,409	156,248	1,000	-531,754	252,936
	Coratina	10.0	30.0	-383,359	156,248	0,057	-775,704	8,986
			60.0	-175,869	156,248	0,803	-568,214	216,477
		30.0	10.0	383,359	156,248	0,057	-8,986	775,704
			60.0	207,490	156,248	0,578	-184,855	599,836
		60.0	10.0	175,869	156,248	0,803	-216,477	568,214
			30.0	-207,490	156,248	0,578	-599,836	184,855
	Frantoio	10.0	30.0	-69,524	156,248	1,000	-461,870	322,821
			60.0	-28,170	156,248	1,000	-420,515	364,175
		30.0	10.0	69,524	156,248	1,000	-322,821	461,870
			60.0	41,355	156,248	1,000	-350,990	433,700
		60.0	10.0	28,170	156,248	1,000	-364,175	420,515
			30.0	-41,355	156,248	1,000	-433,700	350,990
	Mixani	10.0	30.0	-164,020	156,248	0,903	-556,365	228,325

			60.0	-109,510	156,248	1,000	-501,855	282,835
		30.0	10.0	164,020	156,248	0,903	-228,325	556,365
			60.0	54,510	156,248	1,000	-337,835	446,855
		60.0	10.0	109,510	156,248	1,000	-282,835	501,855
			30.0	-54,510	156,248	1,000	-446,855	337,835

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
MALigAgly	Arbosana	10.0	30.0	3,599	27,778	1,000	-66,153	73,350
			60.0	5,449	27,778	1,000	-64,302	75,200
		30.0	10.0	-3,599	27,778	1,000	-73,350	66,153
			60.0	1,850	27,778	1,000	-67,901	71,602
		60.0	10.0	-5,449	27,778	1,000	-75,200	64,302
			30.0	-1,850	27,778	1,000	-71,602	67,901
	Blanqueta	10.0	30.0	-0,703	27,778	1,000	-70,454	69,048
			60.0	0,552	27,778	1,000	-69,199	70,303
		30.0	10.0	0,703	27,778	1,000	-69,048	70,454
			60.0	1,255	27,778	1,000	-68,496	71,006
		60.0	10.0	-0,552	27,778	1,000	-70,303	69,199
			30.0	-1,255	27,778	1,000	-71,006	68,496
	Bosana	10.0	30.0	0,302	27,778	1,000	-69,449	70,053
			60.0	9,975	27,778	1,000	-59,776	79,727
		30.0	10.0	-0,302	27,778	1,000	-70,053	69,449
			60.0	9,674	27,778	1,000	-60,078	79,425
		60.0	10.0	-9,975	27,778	1,000	-79,727	59,776
			30.0	-9,674	27,778	1,000	-79,425	60,078
	Coratina	10.0	30.0	4,823	27,778	1,000	-64,929	74,574
			60.0	12,426	27,778	1,000	-57,325	82,177
		30.0	10.0	-4,823	27,778	1,000	-74,574	64,929
			60.0	7,603	27,778	1,000	-62,148	77,354
		60.0	10.0	-12,426	27,778	1,000	-82,177	57,325
			30.0	-7,603	27,778	1,000	-77,354	62,148
	Frantoio	10.0	30.0	-28,594	27,778	0,930	-98,345	41,157
			60.0	20,558	27,778	1,000	-49,194	90,309
		30.0	10.0	28,594	27,778	0,930	-41,157	98,345
			60.0	49,152	27,778	0,256	-20,599	118,903
		60.0	10.0	-20,558	27,778	1,000	-90,309	49,194
			30.0	-49,152	27,778	0,256	-118,903	20,599
	Mixani	10.0	30.0	4,065	27,778	1,000	-65,686	73,816

			60.0	10,896	27,778	1,000	-58,855	80,647
		30.0	10.0	-4,065	27,778	1,000	-73,816	65,686
			60.0	6,831	27,778	1,000	-62,920	76,582
		60.0	10.0	-10,896	27,778	1,000	-80,647	58,855
			30.0	-6,831	27,778	1,000	-76,582	62,920

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
ALigAgly	Arbosana	10.0	30.0	30,952	32,812	1,000	-51,440	113,344
			60.0	47,705	32,812	0,464	-34,688	130,097
		30.0	10.0	-30,952	32,812	1,000	-113,344	51,440
			60.0	16,753	32,812	1,000	-65,640	99,145
		60.0	10.0	-47,705	32,812	0,464	-130,097	34,688
			30.0	-16,753	32,812	1,000	-99,145	65,640
	Blanqueta	10.0	30.0	41,857	32,812	0,631	-40,536	124,249
			60.0	58,457	32,812	0,250	-23,935	140,850
		30.0	10.0	-41,857	32,812	0,631	-124,249	40,536
			60.0	16,601	32,812	1,000	-65,792	98,993
		60.0	10.0	-58,457	32,812	0,250	-140,850	23,935
			30.0	-16,601	32,812	1,000	-98,993	65,792
	Bosana	10.0	30.0	**140,563***	32,812	0,000	58,171	222,956
			60.0	**183,628***	32,812	0,000	101,236	266,021
		30.0	10.0	**-140,563***	32,812	0,000	-222,956	-58,171
			60.0	43,065	32,812	0,593	-39,328	125,457
		60.0	10.0	**-183,628***	32,812	0,000	-266,021	-101,236
			30.0	-43,065	32,812	0,593	-125,457	39,328
	Coratina	10.0	30.0	**196,389***	32,812	0,000	113,997	278,782
			60.0	**281,677***	32,812	0,000	199,284	364,069
		30.0	10.0	**-196,389***	32,812	0,000	-278,782	-113,997
			60.0	**85,287***	32,812	0,040	2,895	167,680
		60.0	10.0	**-281,677***	32,812	0,000	-364,069	-199,284
			30.0	**-85,287***	32,812	0,040	-167,680	-2,895
	Frantoio	10.0	30.0	**166,332***	32,812	0,000	83,940	248,725
			60.0	**231,406***	32,812	0,000	149,013	313,798
		30.0	10.0	**-166,332***	32,812	0,000	-248,725	-83,940
			60.0	65,074	32,812	0,165	-17,319	147,466
		60.0	10.0	**-231,406***	32,812	0,000	-313,798	-149,013
			30.0	-65,074	32,812	0,165	-147,466	17,319
	Mixani	10.0	30.0	**105,507***	32,812	0,008	23,115	187,900

			60.0	**187,822***	32,812	0,000	105,429	270,214
		30.0	10.0	**-105,507***	32,812	0,008	-187,900	-23,115
			60.0	82,314	32,812	0,050	-0,078	164,707
		60.0	10.0	**-187,822***	32,812	0,000	-270,214	-105,429
			30.0	-82,314	32,812	0,050	-164,707	0,078

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
MAOleAgly	Arbosana	10.0	30.0	6,860	36,768	1,000	-85,465	99,186
			60.0	20,937	36,768	1,000	-71,389	113,263
		30.0	10.0	-6,860	36,768	1,000	-99,186	85,465
			60.0	14,077	36,768	1,000	-78,249	106,402
		60.0	10.0	-20,937	36,768	1,000	-113,263	71,389
			30.0	-14,077	36,768	1,000	-106,402	78,249
	Blanqueta	10.0	30.0	12,195	36,768	1,000	-80,131	104,521
			60.0	41,876	36,768	0,787	-50,449	134,202
		30.0	10.0	-12,195	36,768	1,000	-104,521	80,131
			60.0	29,681	36,768	1,000	-62,644	122,007
		60.0	10.0	-41,876	36,768	0,787	-134,202	50,449
			30.0	-29,681	36,768	1,000	-122,007	62,644
	Bosana	10.0	30.0	-12,919	36,768	1,000	-105,245	79,407
			60.0	10,680	36,768	1,000	-81,645	103,006
		30.0	10.0	12,919	36,768	1,000	-79,407	105,245
			60.0	23,600	36,768	1,000	-68,726	115,925
		60.0	10.0	-10,680	36,768	1,000	-103,006	81,645
			30.0	-23,600	36,768	1,000	-115,925	68,726
	Coratina	10.0	30.0	52,484	36,768	0,486	-39,841	144,810
			60.0	**112,870***	36,768	0,012	20,545	205,196
		30.0	10.0	-52,484	36,768	0,486	-144,810	39,841
			60.0	60,386	36,768	0,328	-31,940	152,712
		60.0	10.0	**-112,870***	36,768	0,012	-205,196	-20,545
			30.0	-60,386	36,768	0,328	-152,712	31,940
	Frantoio	10.0	30.0	62,355	36,768	0,296	-29,971	154,680
			60.0	**97,251***	36,768	0,036	4,925	189,576
		30.0	10.0	-62,355	36,768	0,296	-154,680	29,971
			60.0	34,896	36,768	1,000	-57,430	127,222
		60.0	10.0	**-97,251***	36,768	0,036	-189,576	-4,925
			30.0	-34,896	36,768	1,000	-127,222	57,430
	Mixani	10.0	30.0	36,093	36,768	0,998	-56,233	128,419

			60.0	**106,017***	36,768	0,020	13,691	198,343
		30.0	10.0	-36,093	36,768	0,998	-128,419	56,233
			60.0	69,924	36,768	0,196	-22,402	162,250
		60.0	10.0	**-106,017***	36,768	0,020	-198,343	-13,691
			30.0	-69,924	36,768	0,196	-162,250	22,402

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
AOleAgly	Arbosana	10.0	30.0	20,812	49,504	1,000	-103,495	145,120
			60.0	31,251	49,504	1,000	-93,056	155,559
		30.0	10.0	-20,812	49,504	1,000	-145,120	103,495
			60.0	10,439	49,504	1,000	-113,868	134,746
		60.0	10.0	-31,251	49,504	1,000	-155,559	93,056
			30.0	-10,439	49,504	1,000	-134,746	113,868
	Blanqueta	10.0	30.0	44,732	49,504	1,000	-79,575	169,040
			60.0	65,404	49,504	0,584	-58,904	189,711
		30.0	10.0	-44,732	49,504	1,000	-169,040	79,575
			60.0	20,671	49,504	1,000	-103,636	144,979
		60.0	10.0	-65,404	49,504	0,584	-189,711	58,904
			30.0	-20,671	49,504	1,000	-144,979	103,636
	Bosana	10.0	30.0	28,680	49,504	1,000	-95,627	152,988
			60.0	46,485	49,504	1,000	-77,822	170,793
		30.0	10.0	-28,680	49,504	1,000	-152,988	95,627
			60.0	17,805	49,504	1,000	-106,502	142,112
		60.0	10.0	-46,485	49,504	1,000	-170,793	77,822
			30.0	-17,805	49,504	1,000	-142,112	106,502
	Coratina	10.0	30.0	**156,525***	49,504	0,010	32,217	280,832
			60.0	**215,391***	49,504	0,000	91,084	339,699
		30.0	10.0	**-156,525***	49,504	0,010	-280,832	-32,217
			60.0	58,867	49,504	0,727	-65,441	183,174
		60.0	10.0	**-215,391***	49,504	0,000	-339,699	-91,084
			30.0	-58,867	49,504	0,727	-183,174	65,441
	Frantoio	10.0	30.0	**204,704***	49,504	0,001	80,397	329,011
			60.0	**330,354***	49,504	0,000	206,046	454,661
		30.0	10.0	**-204,704***	49,504	0,001	-329,011	-80,397
			60.0	**125,649***	49,504	0,047	1,342	249,957
		60.0	10.0	**-330,354***	49,504	0,000	-454,661	-206,046
			30.0	**-125,649***	49,504	0,047	-249,957	-1,342

	Mixani	10.0	30.0	104,652	49,504	0,125	-19,655	228,960
			60.0	**220,265***	49,504	0,000	95,958	344,572
		30.0	10.0	-104,652	49,504	0,125	-228,960	19,655
			60.0	115,613	49,504	0,076	-8,695	239,920
		60.0	10.0	**-220,265***	49,504	0,000	-344,572	-95,958
			30.0	-115,613	49,504	0,076	-239,920	8,695

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Dependent Variable	Cultivar	(I) MT	(J) MT	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Lower Bound
Phenolic Sum	Arbosana	10.0	30.0	-47,306	230,318	1,000	-625,644	531,032
			60.0	40,140	230,318	1,000	-538,198	618,478
		30.0	10.0	47,306	230,318	1,000	-531,032	625,644
			60.0	87,446	230,318	1,000	-490,892	665,784
		60.0	10.0	-40,140	230,318	1,000	-618,478	538,198
			30.0	-87,446	230,318	1,000	-665,784	490,892
	Blanqueta	10.0	30.0	46,183	230,318	1,000	-532,155	624,521
			60.0	476,808	230,318	,137	-101,531	1055,146
		30.0	10.0	-46,183	230,318	1,000	-624,521	532,155
			60.0	430,625	230,318	,209	-147,713	1008,963
		60.0	10.0	-476,808	230,318	,137	-1055,146	101,531
			30.0	-430,625	230,318	,209	-1008,963	147,713
	Bosana	10.0	30.0	-54,660	230,318	1,000	-632,999	523,678
			60.0	196,462	230,318	1,000	-381,876	774,800
		30.0	10.0	54,660	230,318	1,000	-523,678	632,999
			60.0	251,122	230,318	,848	-327,216	829,461
		60.0	10.0	-196,462	230,318	1,000	-774,800	381,876
			30.0	-251,122	230,318	,848	-829,461	327,216
	Coratina	10.0	30.0	-67,970	230,318	1,000	-646,308	510,369
			60.0	322,556	230,318	,510	-255,782	900,894
		30.0	10.0	67,970	230,318	1,000	-510,369	646,308
			60.0	390,526	230,318	,296	-187,812	968,864
		60.0	10.0	-322,556	230,318	,510	-900,894	255,782
			30.0	-390,526	230,318	,296	-968,864	187,812
	Frantoio	10.0	30.0	312,598	230,318	,549	-265,740	890,936
			60.0	**595,735***	230,318	,042	17,397	1174,074
		30.0	10.0	-312,598	230,318	,549	-890,936	265,740
			60.0	283,137	230,318	,681	-295,201	861,476
		60.0	10.0	**-595,735***	230,318	,042	-1174,074	-17,397
			30.0	-283,137	230,318	,681	-861,476	295,201
	Mixani	10.0	30.0	60,515	230,318	1,000	-517,823	638,854

			60.0	375,923	230,318	,334	-202,415	954,261
		30.0	10.0	-60,515	230,318	1,000	-638,854	517,823
			60.0	315,407	230,318	,538	-262,931	893,746
		60.0	10.0	-375,923	230,318	,334	-954,261	202,415
			30.0	-315,407	230,318	,538	-893,746	262,931

Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Supplementary table 4. ANOVA results for the evaluation of the influence of malaxation under vacuum conditions and genotype on the phenolic profile of virgin olive oil. The results include the R2 coefficient (proportion of variability explained by the studied factors), F-value and type III SS (type III sum of squares) for the general model, while the F-value, η2 (contribution of each factor to the observed variability) and p-value are listed for each factor (cultivar and malaxation time) as well as for the interaction between both factors.

Parameter		**Hydroxytyrosol**	**Apigenin**	**Luteolin**	**Oleocanthal**	**Oleacein**	**MALigAgly**	**ALigAgly**	**MAOleAgly**	**AOleAgly**	**Phenolic Sum**
R^2		0.832	0.985	0.985	0.974	0.978	0.984	0.988	0.970	0.980	0.981
F		10.822	140.528	145.978	80.609	96.599	137.897	172.619	71.632	107.447	110.140
Type III SS		10.405	13.012	42.319	153.953	218.978	0.005	39.687	0.001	12.48	0.017
p-value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cultivar	*F*	22.135	306.440	320.126	177.022	206.016	302.764	377.123	144.739	228.272	227.976
	η^2*(%)*	**77.4**	**97.6**	**98.2**	**97.2**	**94.8**	**98.2**	**98.1**	**89.1**	**94.6**	**92.3**
	p-value	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Vacuum	*F*	6.419	4.440	0.127	0.390	23.293	1.371	12.233	45.535	33.301	57.812
	η^2*(%)*	**4.5**	**0.3**	0.0	0.0	**2.1**	0.1	**0.6**	**5.6**	**2.8**	**4.7**
	p-value	**0.018**	**0.046**	0.724	0.538	**< 0.0001**	0.253	**0.002**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Cultivar× vacuum	*F*	0.389	1.833	1.000	0.240	1.843	0.336	0.193	3.744	1.451	2.769
	η^2*(%)*	1.4	0.6	0.3	0.1	0.8	0.1	0.1	**2.3**	0.6	**1.1**
	p-value	0.851	0.144	0.439	0.941	0.142	0.886	0.962	**0.012**	0.242	**0.041**
Error	η^2*(%)*	16.8	1.5	1.5	2.6	2.2	1.6	1.2	3.0	2.0	1.9
	Type III SS	2.098	0.202	0.633	4.167	4.946	0.000	0.502	0.000	0.253	0.000

Supplementary table 5. Pairwise comparisons (Bonferroni test) to analyse the differences in phenolic concentrations of VOO for each cultivar at two different malaxation conditions (under vacuum or standard conditions) with 95% confidence level.

Dependent variable	Cultivar	(I) Condition	(J) Condition	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Upper Bound
Hydroxytyrosol	Blanqueta	V	N	**0.592***	0.280	0.045	0.014	1.169
	Bosana	V	N	0.157	0.280	0.579	-0.420	0.735
	Frantoio	V	N	0.193	0.280	0.498	-0.385	0.770
	Levantinka	V	N	0.098	0.280	0.729	-0.479	0.676
	Mixani	V	N	0.311	0.280	0.277	-0.266	0.889
	Picual	V	N	0.329	0.280	0.251	-0.248	0.907
Apigenin	Blanqueta	V	N	0.059	0.263	0.824	-0.484	0.602
	Bosana	V	N	0.174	0.263	0.514	-0.369	0.717
	Frantoio	V	N	0.132	0.263	0.619	-0.411	0.675
	Levantinka	V	N	0.541	0.263	0.051	-0.002	1.084
	Mixani	V	N	0.389	0.263	0.153	-0.154	0.932
	Picual	V	N	-0.279	0.263	0.300	-0.822	0.264
Luteolin	Blanqueta	V	N	-0.104	0.594	0.862	-1.331	1.123
	Bosana	V	N	0.391	0.594	0.517	-0.836	1.618
	Frantoio	V	N	0.334	0.594	0.579	-0.893	1.561
	Levantinka	V	N	-0.292	0.594	0.628	-1.519	0.935
	Mixani	V	N	0.878	0.594	0.152	-0.348	2.105
	Picual	V	N	-0.730	0.594	0.231	-1.957	0.497
Oleocanthal	Blanqueta	V	N	15.707	11.857	0.198	-8.766	40.179
	Bosana	V	N	19.665	11.857	0.110	-4.808	44.137
	Frantoio	V	N	-5.846	11.857	0.626	-30.318	18.626
	Levantinka	V	N	-1.365	11.857	0.909	-25.837	23.108
	Mixani	V	N	2.118	11.857	0.860	-22.355	26.590
	Picual	V	N	2.229	11.857	0.852	-22.244	26.701
Oleacein	Blanqueta	V	N	**213.545***	84.631	0.019	38.875	388.215
	Bosana	V	N	131.389	84.631	0.134	-43.281	306.059
	Frantoio	V	N	**215.625***	84.631	0.018	40.955	390.295
	Levantinka	V	N	33.047	84.631	0.700	-141.623	207.718
	Mixani	V	N	**217.938***	84.631	0.017	43.268	392.609
	Picual	V	N	14.196	84.631	0.868	-160.474	188.867
MALigAgly	Blanqueta	V	N	0.749	8.392	0.930	-16.571	18.069
	Bosana	V	N	3.949	8.392	0.642	-13.371	21.270
	Frantoio	V	N	**-22.444***	8.392	0.013	-39.764	-5.124
	Levantinka	V	N	0.845	8.392	0.921	-16.475	18.165

Dependent variable	Cultivar	(I) Condition	(J) Condition	Mean Difference (I-J)	Standard error	Sig.*	95% Confidence interval[a]	
							Lower Bound	Upper Bound
	Mixani	V	N	4.501	8.392	0.597	-12.819	21.821
	Picual	V	N	5.292	8.392	0.534	-12.028	22.612
ALigAgly	Blanqueta	V	N	8.834	30.835	0.777	-54.807	72.474
	Bosana	V	N	16.664	30.835	0.594	-46.977	80.305
	Frantoio	V	N	**130.672***	30.835	0.000	67.031	194.313
	Levantinka	V	N	48.859	30.835	0.126	-14.781	112.500
	Mixani	V	N	39.596	30.835	0.211	-24.045	103.237
	Picual	V	N	41.762	30.835	0.188	-21.879	105.403
MAOleAgly	Blanqueta	V	N	22.391	24.244	0.365	-27.646	72.429
	Bosana	V	N	16.185	24.244	0.511	-33.853	66.222
	Frantoio	V	N	**176.595***	24.244	0.000	126.557	226.632
	Levantinka	V	N	31.889	24.244	0.201	-18.148	81.927
	Mixani	V	N	**90.770***	24.244	0.001	40.733	140.808
	Picual	V	N	23.658	24.244	0.339	-26.379	73.696
AOleAgly	Blanqueta	V	N	6.941	34.567	0.843	-64.401	78.283
	Bosana	V	N	11.791	34.567	0.736	-59.551	83.133
	Frantoio	V	N	**336.840***	34.567	0.000	265.497	408.182
	Levantinka	V	N	**74.133***	34.567	0.042	2.791	145.475
	Mixani	V	N	**152.877***	34.567	0.000	81.535	224.219
	Picual	V	N	42.288	34.567	0.233	-29.054	113.630
Phenolic Sum	Blanqueta	V	N	**268.713***	129.687	0.049	1.053	536.373
	Bosana	V	N	200.365	129.687	0.135	-67.295	468.025
	Frantoio	V	N	**832.101***	129.687	0.000	564.441	1099.761
	Levantinka	V	N	187.757	129.687	0.161	-79.903	455.417
	Mixani	V	N	**509.379***	129.687	0.001	241.719	777.038
	Picual	V	N	128.746	129.687	0.331	-138.914	396.406

*Significant at 95% confidence level. [a]-Adjustment for multiple comparisons by Bonferroni test.

Supplementary table 6. Concentration of the main fatty acids (expressed as %) found in virgin olive oil samples for the experiment evaluating the influence of malaxation time in the phenolic profile. The oxidative stability measured by the Rancimat test (expressed in hours) is also listed.

Cultivar	MT*	Palmitic acid C16:0	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3	Racimat test
Arbosana	10 min	14.4 ± 0.07	2.1 ± 0.01	66.5 ± 0.32	8.9 ± 0.27	0.81 ± 0.01	38.4 ± 2.0
Arbosana	30 min	14.5 ± 0.05	2.1 ± 0.03	66.5 ± 0.32	8.8 ± 0.14	0.8 ± 0.02	43.0 ± 4.4
Arbosana	60 min	14.4 ± 0.13	2.1 ± 0.01	66.5 ± 0.20	8.9 ± 0.22	0.8 ± 0.02	44.9 ± 2.4
Bosana	10 min	15.3 ± 0.06	2.2 ± 0.01	51.9 ± 0.45	19.4 ± 0.27	1.0 ± 0.01	19.7 ± 1.8
Bosana	30 min	15.4 ± 0.2	2.3 ± 0.01	52.3 ± 0.43	19.6 ± 0.28	1.0 ± 0.01	23.2 ± 2.4
Bosana	60 min	15.5 ± 0.09	2.2 ± 0.01	51.9 ± 0.26	19.7 ± 0.22	1.0 ± 0.02	23.8 ± 3.4
Blanqueta	10 min	19.2 ± 0.12	1.8 ± 0.01	42.2 ± 0.39	25.8 ± 0.34	0.89 ± 0.01	24.3 ± 0.51
Blanqueta	30 min	19.1 ± 0.11	1.8 ± 0.02	42.6 ± 0.27	26.0 ± 0.23	0.89 ± 0.01	26.9 ± 1.1
Blanqueta	60 min	19.1 ± 0.10	1.8 ± 0.01	42.2 ± 0.89	26.2 ± 0.62	0.91 ± 0.03	24.0 ± 1.8
Coratina	10 min	12.9 ± 0.18	3.0 ± 0.04	64.0 ± 0.15	11.1 ± 0.12	0.95 ± 0.02	45.9 ± 1.0
Coratina	30 min	12.8 ± 0.04	3.0 ± 0.03	64.2 ± 0.13	11.1 ± 0.15	0.94 ± 0.01	44.2 ± 5.1
Coratina	60 min	12.7 ± 0.11	3.0 ± 0.03	64.0 ± 0.45	11.0 ± 0.28	0.93 ± 0.01	43.9 ± 1.3
Frantoio	10 min	13.7 ± 0.18	2.7 ± 0.08	66.5 ± 0.63	9.8 ± 0.36	0.89 ± 0.01	61.0 ± 6.0
Frantoio	30 min	13.6 ± 0.17	2.7 ± 0.05	66.6 ± 0.57	9.8 ± 0.42	0.89 ± 0.01	57.3 ± 4.6
Frantoio	60 min	13.6 ± 0.33	2.7 ± 0.02	66.4 ± 0.56	9.7 ± 0.35	0.88 ± 0.02	56.3 ± 3.2
Mixani	10 min	11.9 ± 0.08	3.0 ± 0.04	65.2 ± 0.12	11.2 ± 0.16	0.63 ± 0.01	45.5 ± 4.0
Mixani	30 min	11.8 ± 0.08	3.0 ± 0.06	64.7 ± 0.70	11.2 ± 0.13	0.63 ± 0.01	45.1 ± 1.8
Mixani	60 min	12.1 ± 0.10	3.0 ± 0.02	65.2 ± 0.08	11.4 ± 0.06	0.64 ± 0.01	39.2 ± 2.0

*Malaxation time

Supplementary table 7. Concentration of the main fatty acids (expressed as %) found in virgin olive oil samples for the experiment evaluating the influence of vacuum during malaxation in the phenolic profile. The oxidative stability measured by the Rancimat test (expressed in hours) is also listed.

Cultivar	(N-V)*	Palmitic acid C16:0	Stearic acid C18:0	Oleic acid C18:1	Linoleic acid C18:2	Linolenic acid C18:3	Rancimat test
Blanqueta	N	19.6 ± 0.08	1.8 ± 0.02	42.4 ± 0.45	25.9 ± 0.37	0.85 ± 0.02	25.6 ± 1.1
Blanqueta	V	19.8 ± 0.02	1.7 ± 0.04	42.4 ± 0.64	26.0 ± 0.15	0.94 ± 0.02	25.8 ± 0.35
Bosana	N	15.7 ± 0.03	2.2 ± 0.02	52.5 ± 0.44	19.6 ± 0.21	1.1 ± 0.01	26.0 ± 1.3
Bosana	V	15.9 ± 0.21	2.2 ± 0.01	52.8 ± 0.31	19.6 ± 0.35	1.1 ± 0.02	26.8 ± 1.7
Frantoio	N	14.0 ± 0.14	2.7 ± 0.05	66.0 ± 0.17	9.7 ± 0.24	1.17 ± 0.29	56.3 ± 3.3
Frantoio	V	14.0 ± 0.06	2.7 ± 0.03	66.3 ± 0.31	10.0 ± 0.14	0.87 ± 0.02	66.7 ± 1.6
Levantinka	N	13.0 ± 0.10	2.8 ± 0.06	55.6 ± 0.10	20.3 ± 0.21	0.71 ± 0.03	29.6 ± 3.8
Levantinka	V	12.8 ± 0.06	2.8 ± 0.18	55.5 ± 0.38	20.1 ± 0.18	0.92 ± 0.21	31.5 ± 3.3
Mixani	N	12.5 ± 0.08	3.1 ± 0.06	65.9 ± 0.13	11.5 ± 0.08	0.69 ± 0.01	45.1 ± 1.4
Mixani	V	12.3 ± 0.09	3.1 ± 0.02	65.6 ± 0.28	11.4 ± 0.11	0.67 ± 0.01	53.4 ± 0.89
Picual	N	13.5 ± 0.36	2.3 ± 0.02	66.3 ± 0.78	5.8 ± 0.07	0.87 ± 0.02	56.2 ± 2.2
Picual	V	13.6 ± 0.15	2.3 ± 0.01	66.2 ± 0.14	5.8 ± 0.15	0.87 ± 0.01	61.0 ± 1.5

*Malaxation under vacuum (V) or standard conditions (N).

Supplementary table 8. Correlation matrix of RANCIMAT and phenolic compounds (Pearson).

Variables	RANCIMAT (h)	Hydroxytyrosol	Apigenin	Luteolin	Oleocanthal	Oleacein	MALigAgly	ALigAgly	MAOleAgly	AOleAgly	Phenolic Sum
RANCIMAT (h)	**1**	**-0,485**	0,037	-0,047	**-0,658**	**-0,507**	**0,616**	**0,711**	**0,529**	**0,750**	0,352
Hydroxytyrosol	**-0,485**	**1**	-0,315	-0,025	**0,515**	**0,554**	-0,361	**-0,409**	-0,209	**-0,396**	-0,016
Apigenin	0,037	-0,315	**1**	**0,697**	-0,270	-0,354	**-0,439**	-0,344	**-0,461**	-0,283	**-0,587**
Luteolin	-0,047	-0,025	**0,697**	**1**	-0,338	-0,244	**-0,427**	-0,318	-0,255	-0,180	**-0,451**
Oleocanthal	**-0,658**	**0,515**	-0,270	-0,338	**1**	**0,789**	-0,230	**-0,384**	-0,253	**-0,467**	0,160
Oleacein	**-0,507**	**0,554**	-0,354	-0,244	**0,789**	**1**	-0,268	-0,330	-0,106	-0,338	0,341
MALigAgly	**0,616**	-0,361	**-0,439**	**-0,427**	-0,230	-0,268	**1**	**0,943**	**0,884**	**0,883**	**0,774**
ALigAgly	**0,711**	**-0,409**	-0,344	-0,318	**-0,384**	-0,330	**0,943**	**1**	**0,883**	**0,949**	**0,762**
MAOleAgly	**0,529**	-0,209	**-0,461**	-0,255	-0,253	-0,106	**0,884**	**0,883**	**1**	**0,918**	**0,854**
AOleAgly	**0,750**	**-0,396**	-0,283	-0,180	**-0,467**	-0,338	**0,883**	**0,949**	**0,918**	**1**	**0,743**
Phenolic Sum	0,352	-0,016	**-0,587**	**-0,451**	0,160	0,341	**0,774**	**0,762**	**0,854**	**0,743**	**1**

Values in bold are different from 0 with a significance level alpha=0,05

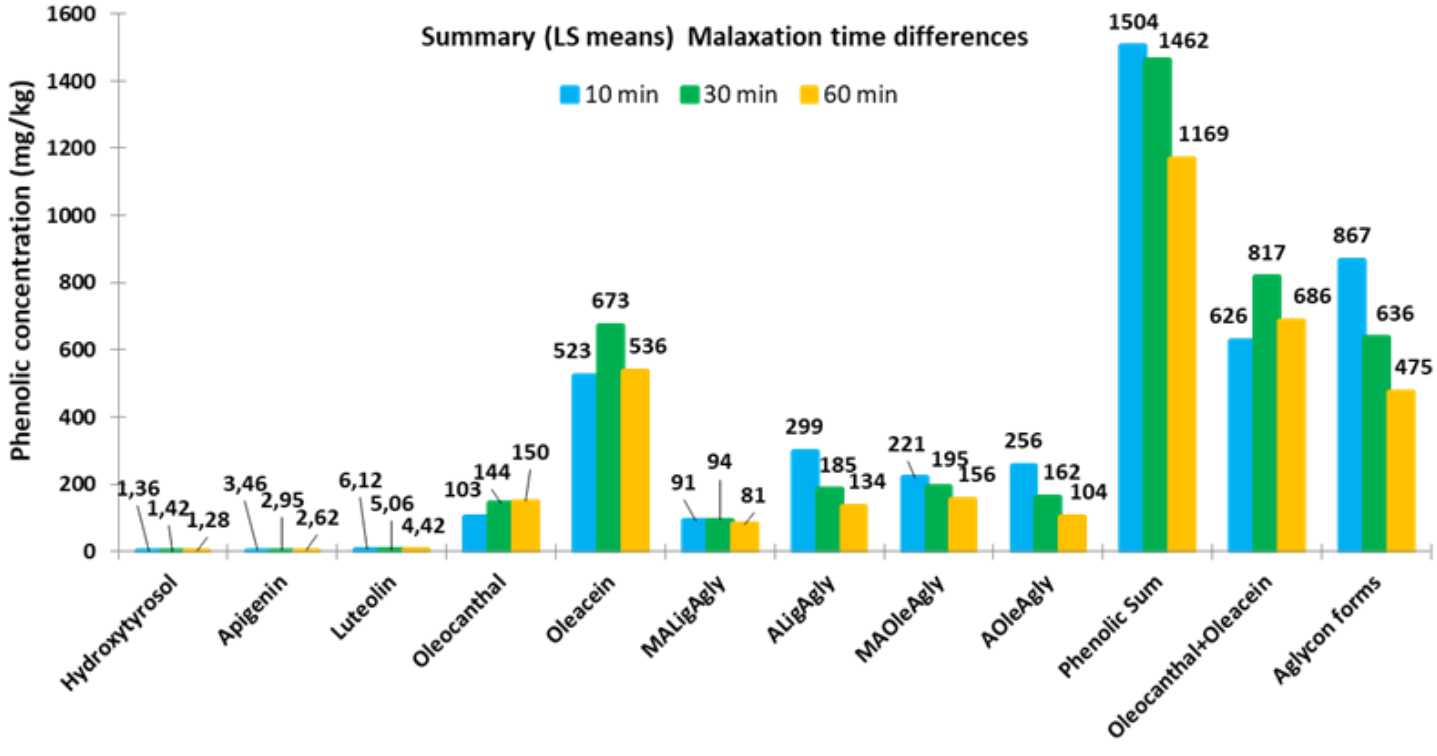
Supplementary table 9. RANCIMAT and fatty acids correlation matrix (Pearson).

Variables	RANCIMAT (h)	Palmitic C16:0	Stearic C18:0	Oleic C18:1	Linoleic C18:2	Linolenic C18:3
RANCIMAT (h)	**1**	**-0,6165**	**0,5389**	**0,8383**	**-0,8589**	-0,2421
Palmitic C16:0	**-0,6165**	**1**	**-0,8933**	**-0,8813**	**0,8023**	**0,3975**
Stearic C18:0	**0,5389**	**-0,8933**	**1**	**0,7013**	**-0,5830**	**-0,3752**
Oleic C18:1	**0,8383**	**-0,8813**	**0,7013**	**1**	**-0,9777**	-0,3336
Linoleic C18:2	**-0,8589**	**0,8023**	**-0,5830**	**-0,9777**	**1**	0,2741
Linolenic C18:3	-0,2421	**0,3975**	**-0,3752**	-0,3336	0,2741	**1**

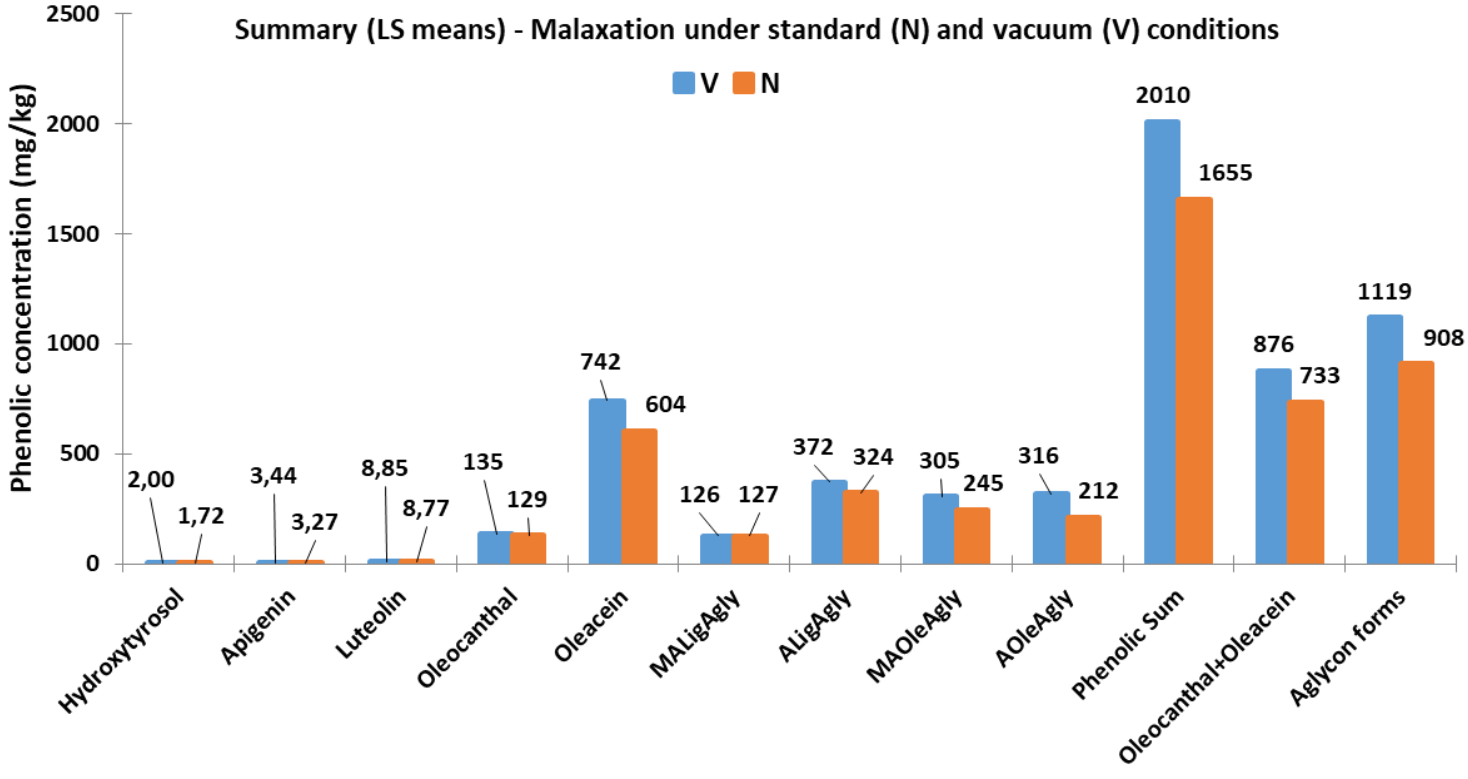
Values in bold are different from 0 with a significance level alpha=0,05

SUPPLEMENTARY FIGURES

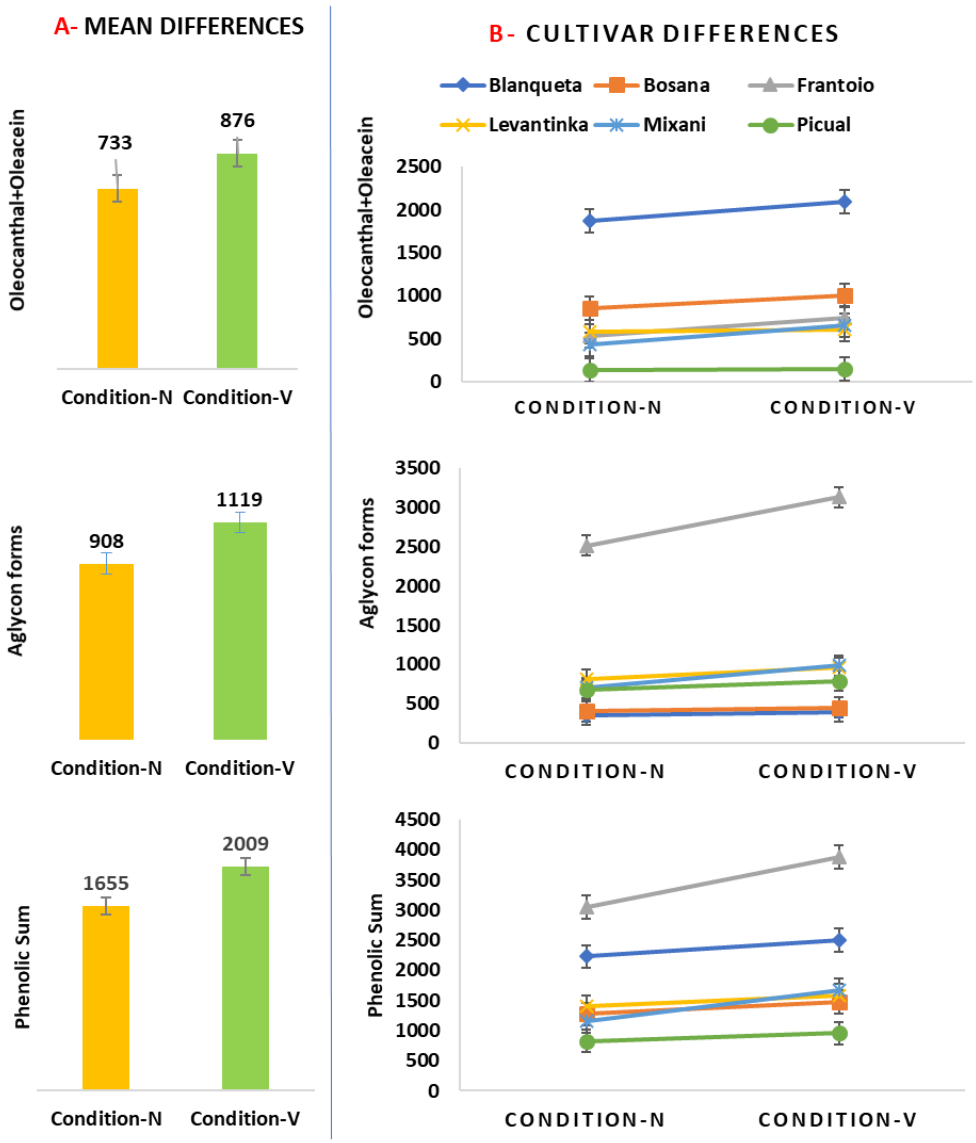
Supplementary figure 1. Experimental set-up installed in the Abencor system to test the effect of vacuum in the malaxation step (left) versus standard conditions (right).



Supplementary figure 2. Mean concentration of phenolic compounds in VOO from six cultivars at three malaxation times.



Supplementary figure 3. Mean concentration of phenolic compounds in VOO from six cultivars obtained by applying vacuum during malaxation or under atmospheric pressure.



Supplementary figure 4. A- Mean differences for the main groups of phenolic compounds found in olive oil from six cultivars processed at two different malaxation conditions: under vacuum or standard conditions. **B**- Phenolic concentrations for each cultivar processed at two different malaxation conditions. Phenolic concentrations are expressed in mg/kg. (N – standard or atmospheric condition; V – vacuum condition).

DISCUSIÓN GENERAL

GENERAL DISCUSSION

5. DISCUSIÓN GENERAL

Esta Tesis Doctoral ha contribuido considerablemente a un mejor conocimiento de los compuestos fenólicos del AOV, analizando y comprobando su diversidad y variabilidad inter-varietal durante tres años consecutivos. Por primera vez se ha realizado una clasificación exhaustiva de un numero amplio y representativo de variedades a nivel mundial en base a su composición fenólica. Finalmente, esta Tesis ha avanzado en el estudio del efecto de ciertos factores tecnológicos en la obtención de aceites de mayor calidad, y en la exploración del papel decisivo que tienen los fenoles en la vida útil del AOV.

Esta investigación se articula en los cuatro capítulos que componen esta Tesis Doctoral. Concretamente, en el Capítulo I, a modo de introducción, se ha realizado una revisión completa sobre la influencia de los factores precosecha en la calidad del aceite. En los Capítulos II y III se analiza el contenido fenólico del AOV y su variabilidad causada por el factor genético y ambiental. Por último, en el Capítulo IV se muestra como los factores tecnológicos tiempo de batido y extracción en vacío afectan al perfil fenólico del aceite y como el perfil contribuye a la estabilidad oxidativa del aceite.

Tal como se ha detallado en el Capítulo I, el cultivo del olivo (*Olea europaea* L.) se caracteriza por un número superior a 1200 variedades que representan un patrimonio inestimable de variabilidad genética seleccionado a lo largo de más de 5500 años de cultivo en los países mediterráneos (Muzzalupo, Vendramin, & Chiappetta, 2014; Rallo, Barranco, et al., 2018). Asimismo, el AOV es uno de los productos más importantes y destacados de la dieta mediterránea, ganando cada vez más popularidad en todo el mundo debido a sus excelentes propiedades organolépticas y nutracéuticas entre otras (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Servili et al., 2014). Según varios estudios, el consumidor está dispuesto a pagar hasta 6,02 euros más por cada litro de AOV si este tiene sus valores nutricionales específicamente etiquetados (Casini, Contini, Marinelli, Romano, & Scozzafava, 2014).

Las propiedades únicas del AOV se deben a su composición bioquímica donde, comparando con otros aceites vegetales, destaca su alto contenido en ácidos grasos monoinsaturados (ácido oleico) y la fracción "minoritaria" que es exclusiva del AOV y que engloba un conjunto de compuestos pertenecientes a diferentes familias químicas como

los compuestos fenólicos, volátiles, terpenos, tocoferoles, fitoesteroles, pigmentos, etc (Piroddi et al., 2017).

Los compuestos fenólicos aportan al AOV importantes características como riqueza sensorial, propiedades nutricionales y antioxidantes que contribuyen positivamente a la salud y también a una mejor conservación del propio aceite (Beauchamp et al., 2005; Bendini et al., 2007; Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012; Parkinson & Keast, 2014; Piroddi et al., 2017; Servili et al., 2016; Silva, Pinto, Carrola, & Paiva-Martins, 2010).

Sin embargo, a pesar de su importancia, la variabilidad fenólica de los aceites monovarietales así como los factores que determinan dicha variabilidad han sido escasamente estudiados. Investigaciones previas han analizado un número limitado de genotipos, ya sea de cultivares tradicionales con importancia regional en términos de producción de aceite o nuevos cultivares de programas de mejora genética. Por tanto, nuevas investigaciones que evalúen este aspecto de forma exhaustiva son más que necesarias (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Vinha et al., 2005).

Para el desarrollo del sector oleícola es fundamental conocer la riqueza fenólica del olivo a través del análisis de un número de variedades representativo de su diversidad genética. Este conocimiento facilitará el diseño de nuevas estrategias sostenibles de producción de aceites de alta calidad y, eventualmente, el diseño de programas de mejora para la obtención de variedades con perfiles fenólicos concretos (Rallo, Barranco, et al., 2018). Con este planteamiento, en el Capítulo II de esta Tesis Doctoral se analizaron los perfiles fenólicos de AOV de 80 variedades internacionales que representaban las principales zonas productoras del mundo. Todas las muestras analizadas se recogieron en el mismo campo (Banco Mundial de Germoplasma de Olivo de Córdoba- Colección UCO) garantizando la igualdad de las condiciones externas para todos los árboles. Asimismo, el proceso de la extracción de los aceites se realizó bajo un único protocolo para evitar cualquier interferencia en la evaluación de la influencia del factor genético en el perfil fenólico de los aceites (Peres, Martins, & Ferreira-Dias, 2014). Este protocolo fue el recomendado por el fabricante del sistema de extracción utilizado, concretamente, el Abencor. El muestreo de fruto se realizó para cada variedad en índice de madurez 2.0, que es el recomendado por el Consejo Oleícola Internacional como el momento más oportuno

para obtener el mejor rendimiento preservando la máxima calidad del aceite (International Olive Council, 2020). .

Los resultados obtenidos pusieron en evidencia la elevada diversidad en cuanto a concentraciones de los fenoles analizados en AOV de las variedades de olivo. Por ejemplo, en el caso del oleocantal, su concentración osciló entre 17 y 1600 mg/kg para las variedades 'Jabaluna' y 'Kalamon', respectivamente. Diferencias similares se observaron en el resto de los compuestos fenólicos donde destacaron por sus altas concentraciones los compuestos derivados de los secoiridoides como la oleuropeina y ligustrósido aglicona. Las tendencias obtenidas coincidieron con las señaladas por trabajos previos (García-Rodríguez, Belaj, Romero-Segura, Sanz, & Pérez, 2017; Karkoula, Skantzari, Melliou, & Magiatis, 2014). Sin embargo, nunca se habían observado concentraciones y diferencias tan elevadas, posiblemente debido al análisis de un número muy escaso de variedades. Esta variabilidad fenólica entre variedades estuvo principalmente vinculada al factor genético; concretamente, entre 83% y el 97% de la varianza observada estuvo explicada por el factor genético para los 9 compuestos fenólicos estudiados.

A través de diferentes análisis estadísticos (PCA y ANOVA) clasificamos las variedades estudiadas en cuatro grupos según sus perfiles fenólicos: a) G1 - variedades en las que predominan la oleuropeina y ligustrósido aglicona; b) G2 – variedades en las que predominan el oleocantal y la oleaceina; c) G3 – varieadades en las que predominan dos flavonoides como la luteolina y apigenina; y d) G4 – variedades equilibradas en las que no destaca ningún fenol concreto y con una riqueza fenólica relativamente baja.

Esta nueva clasificación de los fenoles en cuatro grupos, explica y sustenta las rutas metabólicas, anteriormente definidas por otros autores de la transformación fenólica en la aceituna (Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002; Servili et al., 2004). Concretamente, la formación de los compuestos agliconas esta inducida por las enzimas β–glucosidasas, mientras que, la formación de los compuestos oleocantal y oleaceina esta inducida por la combinación de las enzimas β–glucosidasas y metil-esterasas. En ambos casos, los precursores de dichos metabolitos son la oleuropeina y ligustrósido. Nuestros resultados del análisis PCA y las correlaciones entre los compuestos fenólicos indican y confirman la influencia del factor genético en dichas rutas metabólicas independientes.

El rol del factor genético como el factor determinante del perfil fenólico del aceite había sido destacado también por los estudios anteriores (Baiano, Terracone, Viggiani, & Nobile, 2013; De la Rosa, Arias-Calderón, Velasco, & León, 2016; El Riachy et al., 2011; Perez et al., 2014). Sin embargo, estos trabajos no se desarrollaron con un numero amplio de variedades y tampoco habían evaluado el papel de la 'variación interanual' en estos perfiles. De hecho, estos estudios se llevaron a cabo con muestras de una única campaña agronómica.

El Capítulo III de esta Tesis Doctoral profundizó en los resultados obtenidos en el Capítulo II evaluando la estabilidad de los perfiles fenólicos en 44 variedades de olivo durante tres campañas agronómicas consecutivas. La variabilidad fenólica entre variedades en las tres campañas de cosecha fue muy alta y significativa ($p < 0.05$). Por ejemplo, el promedio de la suma total de los fenoles para las variedades 'Cerezuela' y 'Royal de Cazorla' osciló entre 290 y 3208 mg/kg, respectivamente. Asimismo, de acuerdo con este ensayo y estudios de otros autores (Miho et al., 2018; Rodrigues et al., 2019) resultó que los compuestos fenólicos más abundantes en el AOV son los derivados secoiridoides (isómeros de oleuropeína y ligstrósido aglicona, oleocantal y oleaceina) .

Las variedades se clasificaron de acuerdo con sus concentraciones fenólicas mediante el test de Friedman. A continuación, se destacan las variedades más ricas en cada compuesto: en cuanto a la Suma de los Fenoles, las muestras de 'Cerezuela', 'Cornicabra' y 'Plementa Bjelica' dieron las concentraciones máximas de 3208, 2923 y 2841 mg/kg, respectivamente. Las variedades más ricas en oleocantal resultaron 'Kalamon', 'Plementa Bjelica' y 'Alfafara', con 1186, 936 y 852 mg/kg, respectivamente, mientras que 'Cerezuela', 'Mision Moojeski' y 'Kalamon' fueron las más ricas en oleaceína, con 1333, 904 y 875 mg/kg, respectivamente. Complementariamente, las variedades que destacaron en la concentración de isómeros de oleuropeína aglicona fueron 'Chetoui', 'Coratina' y 'Cornicabra', con 1170, 1065 y 933 mg/kg, respectivamente y, en cuanto a los isómeros de ligustrósido aglicona, estas fueron 'Cornicabra', 'Frantoio' y 'Manzanilla Prieta' con 925, 801 y 753 mg/kg, respectivamente.

En el Capítulo II donde se analizó la variabilidad fenólica durante una sola campaña resultó que el factor genético explicó entre el 83% y el 97% de la varianza. Sin embargo, durante tres años, involucrando también el factor 'variación interanual' (Capítulo III) el

peso del factor genético osciló entre el 31% y el 67%. Tal como estas cifras demuestran, el factor genético sigue siendo el más importante en la explicación de la varianza (explicando el 67% en el caso de la Suma de los Fenoles), pero, aun así, los valores absolutos de las concentraciones fenólicas oscilaron entre las campañas. Por lo tanto, es importante también considerar el comportamiento y determinar el peso del factor genético para cada variedad y cada fenol de forma individual.

Por otro lado, mediante el análisis de componentes principales (PCA) las variedades se agruparon en tres grupos, siendo la asignación de las variedades a cada uno de los grupos estable en el tiempo. Los grupos definidos fueron: a) G1 - variedades con alta concentración de oleuropeína y ligustrósido agliconas; b) G2 - variedades con predominancia de oleocantal y oleaceína; c) G3 – variedades equilibradas en las que no destacaba ningún fenol concreto y con una riqueza relativamente baja de estos compuestos. También un análisis estadístico discriminante confirmó que el 85% de las variedades mantiene su perfil fenólico en el tiempo; es decir, a pesar de que las concentraciones fenólicas absolutas de las variedades pueden variar en el tiempo, el perfil fenólico se mantiene estable. Estos resultados respaldan la posibilidad de diseñar futuros programas de mejora genética para la obtención de aceites de calidad y perfiles fenólicos diferenciados. Si se comparan los resultados derivados de ambos estudios (Capítulos II y III) se observa como en la investigación desarrollada durante tres campañas se pierde el grupo de variedades cuyo AOV destacaba en flavonoides. Observando los datos, este hecho potencialmente se explicaría por el pequeño número de variedades que destacaron significativamente en estos compuestos y debido a la relativa baja concentración encontrada en AOV.

El último Capítulo de esta Tesis (Capítulo IV), se centró en el efecto de factores tecnológicos en la composición fenólica de los aceites. Asimismo, en dicho Capítulo se estudió el rol de los perfiles fenólicos específicos sobre la estabilidad oxidativa de los aceites.

El primer factor estudiado fue el tiempo de batido debido a su efecto en la biotransformación y degradación de los compuestos fenólicos. En la literatura actual se confirmaba que debido a la actividad enzimática alta durante el batido, la concentración fenólica se reducía de manera genérica durante este proceso (Angerosa, Mostallino, Basti, & Vito, 2001; Jiménez, Sánchez-Ortiz, & Rivas, 2014; Kiritsakis & Shahidi, 2017; Taticchi et

al., 2013; Trapani et al., 2017). Sin embargo, otros estudios observaban que algunos compuestos fenólicos específicos aumentaban su concentración durante el proceso de batido (Germek et al., 2014; Gómez-Rico, Inarejos-García, Salvador, & Fregapane, 2009). Estas observaciones controvertidas que no se habían aclarado de forma explícita tienen origen en los procesos enzimáticos que ocurren durante la extracción de aceite. Específicamente, la activación inicial de las enzimas hidrolíticas como las β-glucosidasas y esterasas da lugar a la formación de los derivados secoiridoides en el aceite a partir de los glucósidos secoiridoides del fruto, mientras que, la activación de las oxidorreductasas (PPO y POX) da lugar a la degradación de los compuestos fenólicos (García-Rodríguez, Romero-Segura, Sanz, & Pérez, 2015; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012).

Para arrojar luz y aclarar dichas transformaciones fenólicas durante el proceso del batido, diseñamos un ensayo con seis variedades de diferentes perfiles fenólicos ('Arbosana', 'Blanqueta', 'Bosana', 'Coratina', 'Frantoio' y 'Mixani'), previamente caracterizados en el Capítulo II. Los aceites de estas variedades fueron extraídos en tres tiempos de batido diferentes (10, 30 y 60 minutos). Los resultados de este ensayo mostraron que no todos los compuestos fenólicos reducen su concentración con el aumento del tiempo de batido. Concretamente, dos compuestos abundantes y muy importantes de cara a sus efectos saludables (Beauchamp et al., 2005; Ghanbari et al., 2012; Parkinson & Keast, 2014), oleocantal y oleaceina, aumentaron su concentración hasta 3 veces desde el minuto 10 hasta el minuto 60 del batido. Sin embargo, este efecto no fue homogéneo para todas las variedades, probablemente debido al efecto del factor ´genotipo´. Precisamente, la concentración de oleocantal y oleaceina aumentó para todas las variedades del minuto 10 al minuto 30. Sin embargo, del minuto 30 al minuto 60 la mitad de los cultivares ('Coratina', 'Frantoio', 'Mixani') siguieron aumentando su concentración mientras que la otra mitad ('Arbosana', 'Blanqueta', 'Bosana') la redujeron. Probablemente, este comportamiento se explica por la diferente actividad enzimática entre las variedades; por ejemplo, en el caso en el que el contenido fenólico empiece a reducirse a partir del minuto 30 indica que las enzimas oxidorreductasas se vuelven predominantes sobre las hidrolasas (β-glucosidasas).

Este resultado evidenció por primera vez que el aumento del tiempo de batido puede influir positiva o negativamente para estos dos compuestos dependiendo de la variedad, por lo que, para definir el tiempo de batido óptimo para obtener el máximo contenido de oleocantal y oleaceína hacen faltas ensayos específicos para cada variedad. Sin embargo, el resultado deja claro que el tiempo de batido desde el inicio hasta el minuto 30 siempre favorece la formación de dichos compuestos.

Por otro lado, todos los demás compuestos fenólicos analizados (isómeros de oleuropeina y ligustrósido aglicona, luteonina y apigenina), incluyendo la suma total de los fenoles, redujeron su concentración con el aumento del tiempo de batido. Sin embargo, no se observaron diferencias significativas entre el minuto 10 y el minuto 30 para el conjunto de las variedades. Este resultado coincide con estudios anteriores de otros autores (Kiritsakis & Shahidi, 2017; Trapani et al., 2017).

Con respecto al peso de los factores ´genotipo´ y ´tiempo de batido´ en la variabilidad fenólica del AOV, concluimos que el factor genotipo sigue siento el más importante, explicando el 50.6% de la varianza para el hidroxitirosol y el 96.7% para la apigenina. El factor ´tiempo de batido´ llegó a explicar hasta el 20% de la varianza en el caso de la oleuropeina y ligustrósido agliconas. Por otro lado, hasta el 8% de la varianza fue explicada por la interacción de ambos factores, lo que como se comenta anteriormente, explica que la biotransformación de distintos fenoles es diferente entre las variedades.

En el segundo ensayo dentro del Capítulo IV de la Tesis, se estudió el efecto que tendría la implementación de las condiciones de vacío durante el proceso de batido en la composición fenólica de los aceites. Hasta la fecha no existía ninguna evidencia de dicho efecto, aunque el método de vacío es ampliamente utilizado para evitar la oxidación de los productos en la industria alimentaria (Castagnini, Betoret, Betoret, & Fito, 2015; Mushtaq, 2017). Estudios anteriores de otros autores coincidían en que la eliminación del aire durante el proceso de batido con otros gases inertes, como el nitrógeno o el dióxido de carbono, contribuye a preservar el contenido fenólico del AOV. Sin embargo, estas alternativas no se consideran rentables para la industria (Servili et al., 2008; Vierhuis et al., 2001).

Nuestro ensayo demostró que la implementación del sistema de vacío en el proceso de extracción puede ser una alternativa adecuada para preservar los compuestos fenólicos y obtener AOVs de mayor calidad. Concretamente, en el ensayo se compararon seis variedades cuyos aceites fueron extraídos paralelamente en condiciones estándar (atmosféricas) y condiciones de vacío. El sistema de vacío supuso un aumento significativo ($p < 0.005$) de 21.4% en la suma de los fenoles para el conjunto de las muestras analizadas. Asimismo, prácticamente todos los compuestos fenólicos individuales se vieron afectados positivamente.

En este ensayo se evaluó también el peso de los factores ´genotipo´ y ´condiciones de batido´ (aplicación del vacío) sobre la varianza observada en el conjunto de las muestras. De nuevo, el factor genético fue el predominante y explicó la mayor parte de la varianza desde 77,4% en el caso del hidroxitirosol al 98,2% para la luteolina; mientras que el factor ´condiciones de batido´ explicó hasta el 5,6% de la varianza total. Aunque el factor genético explica la mayor parte de la varianza debido a la gran variabilidad genética de las muestras elegidas, el factor ´condiciones de batido´ juega un papel importante en valor absoluto sobre las concentraciones fenólicas. Asimismo, hay que destacar que se observa una interacción entre ambos factores, lo que explica que no todas las variedades están afectadas por igual por las ´condiciones de batido´, aunque de forma general todas se ven afectas positivamente.

Por último, el tercer ensayo parte del Capítulo IV arrojó luz sobre el rol de los compuestos fenólicos individuales en la estabilidad oxidativa de los aceites. Se llegó a la conclusión de que la estabilidad oxidativa de una muestra de aceite se puede predecir mediante una ecuación matemática conociendo previamente su concentración de compuestos fenólicos y ácidos grasos específicos.

Los estudios previos dejaban claro que el perfil de los ácidos grasos y la riqueza fenólica de una muestra de aceite de oliva juegan un papel fundamental en su estabilidad oxidativa (Arcoleo et al., 1999; Bruscatto et al., 2017; Rallo, Díez, et al., 2018; Velasco, 2002). Asimismo, estos estudios indicaban que los aceites con una ratio ácido oleico/ácido linoleico alto tienden a ser más estables que los que presentan una ratio baja (Aparicio, Roda, Albi, & Gutiérrez, 1999; Spatari, De Luca, Ioele, & Ragno, 2017). Sin embargo, hasta la fecha no se habían definido posibles ratios de diferentes compuestos o perfiles fenólicos

que podrían estar correlacionados con la estabilidad oxidativa. Por otro lado, si bien la relación entre los fenoles totales y la estabilidad oxidativa estaba bien fundamentada, los efectos de los distintos compuestos fenólicos individuales a este contexto seguían siendo controvertidos (Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007; Kiritsakis & Shahidi, 2017). Con estas premisas, se diseñó un ensayo con 90 muestras de AOV de ocho variedades en las que se analizó la estabilidad oxidativa con el método Rancimat (horas), el perfil de ácidos grasos y el perfil fenólico.

Se encontraron compuestos fenólicos individuales correlacionados positivamente y otros negativamente con los valores Rancimat de estabilidad oxidativa. Este resultado señaló por primera vez que no simplemente la totalidad de los compuestos fenólicos es responsable de la estabilidad oxidativa, sino fenoles o perfiles fenólicos concretos. Precisamente, los fenoles totales mostraron una correlación positiva pero no significativa ($p > 0.05$) con la estabilidad oxidativa. Por lo tanto, de forma similar al ratio entre ácidos grasos anteriormente explicada, se diseñó un nuevo ratio fenólico (ratio "*f*") indicativo de la estabilidad de un aceite considerando su composición fenólica. Concretamente, el ratio "*f*" se definió como el cociente entre la suma de los compuestos agliconas y la suma de oleocantal y oleaceína. Cuando mayor es dicho ratio, mayor es la estabilidad oxidativa del aceite.

Por otro lado, los ácidos grasos que mostraron una correlación significativa y positiva con la estabilidad oxidativa resultaron ser el ácido oleico y esteárico, mientras que el resto de los ácidos grasos mostraron una correlación negativa. Este resultado coincide con estudios previos de otros autores (Aparicio et al., 1999; Baldioli, Servili, Perretti, & Montedoro, 1996; Romani et al., 2007; Velasco & Dobarganes, 2002).

Teniendo en cuenta dichas correlaciones, y trabajos anteriores de otros autores (Li & Wang, 2018), mediante un análisis de regresión lineal múltiple diseñamos y generamos un modelo matemático capaz de determinar la estabilidad oxidativa de una muestra de aceite conociendo solamente tres compuestos, hidroxitirosol, forma aldehídica de la oleuropeína aglicona y el ácido linoleico :

$$Y\ (horas) = 49.6+5.34(hidroxitirosol)+0.02(AOleAgly)-1.6(ácido\ linoleico)$$

Los resultados obtenidos en esta Tesis Doctoral subrayan la importancia de explorar los recursos genéticos del olivo y su papel crucial para la obtención de nuevas variedades y el desarrollo de productos finales de alta calidad y de propiedades nutraceúticas concretas. Asimismo, dichos resultados muestran la necesidad de seguir investigando sobre la composición fenólica del olivo, gran parte de la cual todavía es "materia oscura".

5. GENERAL DISCUSSION

This PhD Thesis has considerably contributed towards a better comprehension of the phenolic compounds in VOO, by analysing and verifying their diversity and cultivar variability during three consecutive harvest seasons. For the first time, an exhaustive classification on phenolic composition of a representative number of worldwide cultivars has been carried out. Finally, this Thesis has made progress in studying the effect of certain technological factors in obtaining high quality oils, and in exploring the decisive role of phenols in VOO shelf life.

This PhD Thesis is structured in the four Chapters. Specifically, in Chapter I as an introducing chapter, a complete review has been carried out about the influence of pre-harvest factors on the olive oil quality. Chapters II and III analyse the phenolic content of VOO and its variability caused by genetic and environmental factors. And lastly, Chapter IV demonstrates how technological factors such as malaxation time and extraction under vacuum conditions affect the phenolic profile of the olive oil and how the phenolic profile contribute into the oil oxidative stability.

As detailed in Chapter I, the olive tree (*Olea europaea* L.) comprises more than 1200 cultivars, comprehending a priceless heritage of genetic variability selected over more than 5500 years of cultivation in Mediterranean countries (Muzzalupo, Vendramin, & Chiappetta, 2014; Rallo, Barranco, et al., 2018). Likewise, VOO is one of the most important and outstanding products of the Mediterranean diet, becoming increasingly popular all over the world due to its excellent organoleptic and nutraceutical properties among others (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Servili et al., 2014). According to several studies, consumers are willing to pay up to 6.02 euros more for each litre of VOO if its nutritional values are clearly labelled (Casini, Contini, Marinelli, Romano, & Scozzafava, 2014).

The unique properties of VOO are attributed to thebiochemical composition. Compared to other vegetable oils, VOO´s biochemical composition outstands to its high content in monounsaturated fatty acids (oleic acid) and the "minor" fraction, which is exclusive to VOO. This fraction includes a set of compounds belonging to different chemical families such as phenolic compounds, volatile compounds, terpenes, tocopherols, phytosterols, pigments, etc. (Piroddi et al., 2017).

Phenolic compounds provide the VOO with important characteristics such as organoleptic richness, nutritional and antioxidants properties that contribute positively to human health and also to the better preservation of the oil itself (Beauchamp et al., 2005; Bendini et al., 2007; Ghanbari, Anwar, Alkharfy, Gilani, & Saari, 2012; Parkinson & Keast, 2014; Piroddi et al., 2017; Servili et al., 2016; Silva, Pinto, Carrola, & Paiva-Martins, 2010).

However, despite their importance, the variability of phenolic compounds in oils obtained from different olive cultivars, as well as the factors that determine such variability, remains poorly studied. Previous studies have analysed just a limited number of genotypes, either traditional cultivars of regional importance in terms of oil production or new cultivars from breeding programmes; so further studies are necessary (El Riachy, Priego-Capote, León, Rallo, & Luque de Castro, 2011; Vinha et al., 2005).

For the development of the olive sector, it is essential to know the phenolic richness of the olive oil through the analysis of a representative number of cultivars. This knowledge will facilitate the design of new sustainable strategies for the production of high quality olive oils and, eventually, the correct design of breeding programmes for obtaining cultivars with specific phenolic profiles. (Rallo, Barranco, et al., 2018). With this approach, in ChapterII of this PhD Thesis, the phenolic profiles of 80 international cultivars representing the main worldwide producing areas were analysed. All the samples analysed were collected in the same field (World Olive Germplasm Bank of Córdoba - UCO Collection) guaranteeing uniform field conditions for all the trees. Likewise, the oil extraction process was carried out under a unique protocol to avoid any interference in the evaluation of the influence of the genetic factor on the oil phenolic profile (Peres, Martins, & Ferreira-Dias, 2014). This protocol was recommended by the manufacturer of the extraction system used (Abencor system). Fruit sampling was carried out for each variety at maturity index 2.0, which is the maturity index recommended by the International Olive Oil Council as the most appropriate to obtain the best oil yield with the highest quality (International Olive Council, 2020).

The obtained results showed a high diversity in the concentrations of the phenols analysed in a large set of 80 monovarietal VOO. For example, in the case of oleocanthal, its concentration ranged from 17 to 1600 mg/kg for the 'Jabaluna' and 'Kalamon' cultivars, respectively. Similar differences were observed for the rest of the phenolic compounds,

where secoiridoids-derived compounds such as oleuropein and ligstroside aglycone stood out for their high concentrations. These trends were in line with previous studies (García-Rodríguez, Belaj, Romero-Segura, Sanz, & Pérez, 2017; Karkoula, Skantzari, Melliou, & Magiatis, 2014). However, such high concentrations and differences have never been previously observed, which was probably due to the examination of a very small number of cultivars. This phenolic variability was mainly associated to the genetic factor; precisely, between 83 and 97% of the observed variance was explained by the genetic factor for the 9 phenolic compounds studied.

Using different statistical analyses (PCA and ANOVA), we classified the studied cultivars into four groups according to their phenolic profiles: a) G1 - cultivars characterised by the predominance of oleuropein and ligstroside aglycone; b) G2 - cultivars characterised by the predominance of oleocanthal and oleacein; c) G3 - cultivars characterised by the predominance of flavonoids such as luteolin and apigenin; and d) G4 - balanced cultivars with a relative low phenolic content and not highlighted to any specific phenol.

This new classification of phenols in four groups explains and supports the metabolic pathways of olive fruit phenolic transformation previously defined by other authors (Ryan, Antolovich, Prenzler, Robards, & Lavee, 2002; Servili et al., 2004). Specifically, the formation of aglycone compounds is induced by β-glucosidase enzymes, while the formation of oleocanthal and oleacein compounds is induced by a combination of β-glucosidase and methyl esterase enzymes. In both cases, the precursors of these metabolites are oleuropein and ligstroside. Our results of PCA analysis and correlations within phenolic compounds indicate and confirm the influence of the genetic factor on these independent metabolic pathways.

The role of the genetic factor as the most relevant one in determining the oil's phenolic profile had also been highlighted by previous studies (Baiano, Terracone, Viggiani, & Nobile, 2013; De la Rosa, Arias-Calderón, Velasco, & León, 2016; El Riachy et al., 2011; Perez et al., 2014). However, these studies were not conducted on a large number of cultivars and the role of 'inter-annual variation' factor in the phenolic profiles was not evaluated. In fact, these studies were carried out with samples from a single cropping season.

Chapter III of this PhD Thesis further explored the results obtained in Chapter II, evaluating the phenolic content of 44 olive cultivars during three consecutive crop seasons. The phenolic variability between cultivars in the three crop seasons was very high and significant ($p < 0.05$). For example, the average of total phenols for the cultivars 'Cerezuela' and 'Royal de Cazorla' ranged between 290 and 3208 mg/kg, respectively. Likewise, according to this study and others conducted by other authors (Miho et al., 2018; Rodrigues et al., 2019), it was concluded that the most abundant phenolic compounds in VOO were the secoiridoids derivates (isomers of oleuropein and ligstroside aglycone, oleocanthal and oleacein).

The cultivars were classified according to their phenolic concentrations using the Friedman test. Next, the most highlighted cultivars in each phenolic compound are underlined; so, as regard to Total Phenolic content (or sum of all individual compounds), the cultivars 'Cerezuela', 'Cornicabra' and 'Plementa Bjelica' showed the highest concentrations of 3208, 2923 and 2841 mg/kg, respectively. The richest cultivars in oleocanthal were 'Kalamon', 'Plementa Bjelica' and 'Alfafara', with 1186, 936 and 852 mg/kg, respectively; while 'Cerezuela', 'Mision Moojeski' and 'Kalamon' were the richest in oleacein, with 1333, 904 and 875 mg/kg, respectively. In addition, the cultivars that stood out in the concentration of oleuropein aglycone isomers were 'Chetoui', 'Coratina' and 'Cornicabra', with 1170, 1065 and 933 mg/kg, respectively, and, in terms of ligstroside aglycone isomers, these were 'Cornicabra', 'Frantoio' and 'Manzanilla Prieta' with 925, 801 and 753 mg/kg, respectively.

In Chapter II, where phenolic variability was analysed during a single season, it turned out that the genetic factor explained between 83% and 97% of the variance. However, during three evaluation years, involving also the interannual variation factor (Chapter III), the weight of the genetic factor oscillated between 31% and 67%. As these figures show that the genetic factor remains the most important factor in explaining the variance (explaining 67% in the case of the Phenol Sum), but, even so, the absolute values of phenolic concentrations fluctuated among the seasons. Therefore, it is also important to consider the behaviour and determine the weight of the genetic factor for each variety and each phenol individually.

On the other hand, through principal component analysis (PCA) the cultivars were clustered into three groups, which resulted to be stable over time. The defined groups were a) G1 - cultivars with a high concentration of oleuropein and ligstroside aglycones; b) G2 - cultivars with a predominance of oleocanthal and oleacein; c) G3 - balanced and relatively low phenolic cultivars in which no specific phenol stood out. A statistical discriminant analysis confirmed that 85% of the cultivars remained stable in their phenolic profile over time. Understanding that, although the absolute phenolic concentrations of the cultivars may vary over time, they belong almost always in the same phenolic profile. These results confirm the possibility of designing future genetic breeding programmes for obtaining high-quality oils with specific phenolic profiles. If we compare the results derived from both studies (Chapters II and III), we can notice that in the second research carried out over three crop seasons, the group of cultivars rich in flavonoids disappeared. This fact could be potentially explained by the small number of rich in flavonoids cultivars and due to the relatively low concentration of these compounds in VOO.

The last chapter of this Thesis (Chapter IV) was focused on the effect of technological factors on the oils' phenolic composition. It also studied the role of specific phenolic profiles on the oils' oxidative stability.

The first technological factor studied was the malaxation time due to its effect on the biotransformation and degradation of phenolic compounds. The existing studies reported that the phenolic concentration generically decrease during the malaxation because of the high enzymatic activity (Angerosa, Mostallino, Basti, & Vito, 2001; Jiménez, Sánchez-Ortiz, & Rivas, 2014; Kiritsakis & Shahidi, 2017; Taticchi et al., 2013; Trapani et al., 2017). However, other studies found that specific phenolic compounds increased in their concentration during this process (Germek et al., 2014; Gómez-Rico, Inarejos-García, Salvador, & Fregapane, 2009). These controversial observations, which had not been explicitly clarified, are rooted in the enzymatic processes that occur during oil extraction. Specifically, the initial activation of hydrolytic enzymes such as β-glucosidases and esterases, that use as substrate the secoiridoid glycosides of the fruit, leads to the formation of secoiridoid derivatives in the oil, while the activation of oxidoreductases (PPO and POX) leads to the degradation all these phenolic compounds (García-Rodríguez,

Romero-Segura, Sanz, & Pérez, 2015; Romero-Segura, García-Rodríguez, Sánchez-Ortiz, Sanz, & Pérez, 2012).

To shed light on these phenolic transformations during the malaxation process, we designed an experiment with six cultivars of different phenolic profiles ('Arbosana', 'Blanqueta', 'Bosana', 'Coratina', 'Frantoio' and 'Mixani'), previously characterised in Chapter II. The oils of these cultivars were extracted at three different malaxation times (10, 30 and 60 minutes).

The results of this study showed that not all phenolic compounds reduce their concentration as malaxation time increases. In particular, two abundant compounds that are very important for their healthy properties, oleocanthal and oleacein, (Beauchamp et al., 2005; Ghanbari et al., 2012; Parkinson & Keast, 2014) increased in concentrations up to 3-fold from minute 10 to minute 60 of the malaxation. However, this effect was not homogeneous for all cultivars, probably due to the effect of the 'genotype' factor. Precisely, the oleocanthal and oleacein concentration increased for all cultivars from minute 10 to minute 30. But, from minute 30 to minute 60, half of the cultivars ('Coratina', 'Frantoio', 'Mixani') continued to increase their concentration while the remaining half ('Arbosana', 'Blanqueta', 'Bosana') reduced it. This behaviour is probably explained by the different enzymatic activity between the cultivars; for example, in the case in which the phenolic content starts to decrease from minute 30 onwards, it indicates that oxidoreductase enzymes become predominant over hydrolases (β-glucosidases).

This result showed for the first time that increasing the malaxation time can positively or negatively affect oleocanthal and oleacein depending on the variety. So, for defining the optimal malaxation time for these compounds, variety-specific trials are needed. However, it is evident that malaxation time up to 30 minutes always has a positive effect on the formation of these compounds.

On the other hand, all the rest of the phenolic compounds analysed (isomers of oleuropein and ligstroside aglycone, luteonin and apigenin), including the total sum of phenols, reduced their concentration by increasing the malaxation time, although no significant differences were observed between minute 10 and minute 30 for all the

cultivars. This result is in line with previous studies reported by other authors (Kiritsakis & Shahidi, 2017; Trapani et al., 2017).

Regarding to the weight of 'genotype' and malaxation time' factors in the olive oil phenolic variability, we concluded that the genotype factor is clearly the most important, explaining from 50.6% to 96.7% of the variance for the hydroxytyrosol and apigenin, respectively. The 'malaxation time' factor explained up to 20% of the variance in the case of oleuropein and ligstroside aglycones. On the other hand, up to 8% of the variance was explained by the interaction of both factors, which, as mentioned above, explains that the biotransformation of different phenols is different across cultivars.

In the second trial within Chapter IV of the Thesis, it was studied the effect that could have the implementation of vacuum conditions during the malaxation process on the VOO phenolic composition. To date, there was no evidence of such an effect, although the vacuum method is widely used to prevent oxidation of products in the food industry. (Castagnini, Betoret, Betoret, & Fito, 2015; Mushtaq, 2017). Previous studies concurred that replacement of atmospheric in the blender with other inert gases, such as nitrogen or carbon dioxide, during the malaxation process, contributes to preserve the phenolic content of the VOO. However, these alternatives are not considered cost-effective for the industry (Servili et al., 2008; Vierhuis et al., 2001).

Our trial demonstrated that the implementation of the vacuum system in the extraction process can be a suitable alternative to preserve phenolic compounds and obtain higher quality VOOs. Specifically, in the trial were compared six cultivars whose oils were simultaneously extracted under standard (atmospheric) and vacuum conditions. The vacuum system led to a significant increase ($p < 0.005$) of 21.4% in the sum of phenols for the group of samples analysed. Also, almost all individual phenolic compounds were positively affected.

In this trial, it was also evaluated the weight of variance for the 'genotype' and malaxation conditions' (vacuum conditions) factors. Once again, the genetic factor was predominant and explained most of the variance from 77.4% for hydroxytyrosol to 98.2% for luteolin; while the 'malaxation conditions' factor explained up to 5.6% of the total variance. Although the genetic factor explains most of the variance due to the high genetic

variability of the chosen samples, the vacuum conditions also play an important role in absolute values of phenolic concentrations. It should also be noted that an interaction between both factors is observed, which explains that not all cultivars are equally affected by the 'vacuum conditions', although in general they are all positively affected.

Finally, the third test part of Chapter IV shed light on the role of individual phenolic compounds in the oil's oxidative stability. It was concluded that the oxidative stability of an oil sample can be predicted through a mathematical equation based on specific phenolic compounds and fatty acids concentration.

Previous studies revealed that the fatty acid profile and phenolic richness of an olive oil sample are critical in determining its oxidative stability (Arcoleo et al., 1999; Bruscatto et al., 2017; Rallo, Díez, et al., 2018; Velasco, 2002). Furthermore, these studies suggest that oils with a high oleic acid/linoleic acid ratio tend to be more stable than those with a low ratio. (Aparicio, Roda, Albi, & Gutiérrez, 1999; Spatari, De Luca, Ioele, & Ragno, 2017). However, to date, no possible ratios of phenolic profiles that could be correlated with oxidative stability had been defined. On the other hand, while the correlation between total phenols and oxidative stability was well established, the effects of individual phenolic compounds in this context remained controversial (Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007; Kiritsakis & Shahidi, 2017).

With these premises, an assay was designed with 90 VOO samples of eight cultivars in which were analysed the oxidative stability with the Rancimat method (hours), the fatty acid and the phenolic content.

The results showed that different individual phenolic compounds correlated positively and others negatively with the oxidative stability Rancimat values. This result pointed out for the first time that not just the total phenolic content of phenolic compounds is responsible for oxidative stability, but rather individual phenols or phenolic profiles. Indeed, total phenols showed a positive but non-significant correlation ($p > 0.05$) with oxidative stability. Hence, similar to the fatty acid ratio explained above, a new phenolic ratio (*f*-ratio) indicative of the oil stability was designed considering oil phenolic composition. Specifically, the "*f*" ratio was defined as the ratio between the sum of the

aglycone compounds and the sum of oleocanthal and oleacein. As higher this ratio value, higher result to be the olive oil oxidative stability.

On the other hand, the fatty acids that showed a significant and positive correlation with oxidative stability were oleic and stearic acid, while the rest of the fatty acids showed a negative correlation. This result is in line with previous studies of other authors (Aparicio et al., 1999; Baldioli, Servili, Perretti, & Montedoro, 1996; Romani et al., 2007; Velasco & Dobarganes, 2002).

Taking into account these correlations, and previous studies by other authors (Li & Wang, 2018), we designed and generated a mathematical model able to determine the olive oil oxidative stability knowing only three compounds, hydroxytyrosol, aldehyde form of oleuropein aglycone and linoleic acid:

$$Y\ (hours) = 49.6+5.34(hydroxytyrosol)+0.02(AOleAgly)-1.6(linoleic\ acid)$$

The results obtained in this PhD Thesis underline the importance of exploring the olive genetic resources and their crucial role in breeding programs and in the production of high-quality end-products with specific nutraceutical properties. Furthermore, these results show the need for further research on the VOO phenolic composition, most of which is still "dark matter".

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CONCLUSIONES

CONCLUSIONS

6. CONCLUSIONES

Las conclusiones principales de esta Tesis Doctoral pueden resumirse como sigue:

1. Las diferencias en la riqueza fenólica entre las variedades de olivo son muy significativas; en cambio no lo fueron entre los dos árboles (réplicas) de una misma variedad analizados y cultivados en la misma parcela.

2. La variabilidad fenólica entre las variedades analizadas dentro de una misma campaña se explica casi en su totalidad por el factor genético.

3. La variabilidad fenólica entre las variedades analizadas durante más de una campaña de cultivo se explica principalmente por el factor genético (hasta un 67%). Sin embargo, una parte considerable de la varianza es debida a la variación interanual y a la interacción entre estos dos factores (hasta un 33%).

4. En función de los perfiles fenólicos, las variedades de olivo se distribuyen en tres grupos consistentes: G1 - rico en formas aldehídicas de oleuropeína y ligstrosido aglicona; G2 - rico en compuestos de oleocantal y oleaceína, y G3 - cultivares intermedios, equilibrados y no destacables por ningún compuesto fenólico específico. La composición de estos grupos resultó muy estable tras el análisis de tres campañas.

5. Nuestros resultados corroboran la teoría de las rutas de biotransformación fenólica basadas en la activación de reacciones enzimáticas de esterasas o β-glucosidasas; y que estas reacciones están genéticamente predefinidas para cada variedad.

6. El aumento del tiempo de batido generalmente reduce la concentración fenólica en el aceite de oliva. Por el contrario, el oleocanthal y la oleaceína tienden a comportarse de forma diferente y aumentan su concentración. No obstante, la magnitud de este último efecto también depende del factor genético ya que no todas las variedades se comportan de la misma manera.

7. La aplicación de las condiciones de vacío durante el proceso de batido aumenta significativamente el contenido fenólico total en al menos un 20% en comparación con las condiciones estándar.

8. La estabilidad oxidativa del aceite de oliva se define principalmente por su composición de ácidos grasos y fenoles. Los aceites ricos en las formas aldehídicas de la oleuropeína y el ligstrosido aglicona, y ricos en ácido oleico, tienden a ser mucho más estables que los aceites de otros perfiles. Asimismo, conociendo la composición fenólica y de ácidos grasos, es posible estimar la estabilidad oxidativa de los aceites aplicando un modelo matemático.

9. En conjunto, los resultados obtenidos en esta Tesis Doctoral confirman la viabilidad de diseñar programas novedosos de mejora genética para la obtención de aceites de oliva de alta calidad y con perfiles fenólicos específicos.

6. CONCLUSIONS

The most essential conclusions of this PhD Thesis can be summarized as follows:

1. The differences in phenolic richness among olive cultivars are very large and significant. Therefore, the possibilities to obtain olive oils of different nutritional, organoleptic and technological qualities are are quite extensive.

2. The phenolic variability among cultivars analysed within a single crop season is almost entirely explained by the genetic factor.

3. The phenolic variability among cultivars analysed during more than one crop season is mainly explained by the genetic factor (up to 67%). However, a considerable portion of the variation is caused by the interannual factor and the interaction between both factors (up to 33%).

4. Based on phenolic profiles, the olive cultivars are clustered in three consistent groups: G1 - rich in aldehydic forms of oleuropein and ligstroside aglycone; G2 - rich in oleocanthal and oleacein compounds, and G3 - intermediate, balanced and no remarkable cultivars to any specific phenolic compound. These groups were found to be very stable over three crop seasons.

5. Our results reinforce the phenolic biotransformation pathways theory based on the activation of esterase or β-glucosidase enzymatic reactions; and that these reactions are genetically predefined by the genetic factor.

6. The increase in the malaxation time generally reduces the phenolic concentration in the olive oil. But on the contrary, oleocanthal and oleacein increase their concentration. Nevertheless, the magnitude of this last effect also depends on the genetic factor and not all cultivars behave in the same way.

7. The implementation of the vacuum conditions during the malaxation process significantly increases the total phenolic content by at least 20% as compared to standard conditions.

8. The oxidative stability of olive oil is mainly defined by its fatty acid and phenolic composition. Oils richer in aldehydic forms of oleuropein and ligstroside aglycone,

and richer in oleic acid, tend to be much more stable than oils of other profiles. Likewise, knowing the phenolic and fatty acid composition, the oxidative stability of the VOO could be estimated throughout mathematical models.

9. Jointly, the findings obtained in this PhD Thesis confirm the feasibility of designing novels breeding programmes on obtaining high-quality olive oils with specific phenolic profiles.

ANEXOS / ANNEXES

Anexo I / Annex I

Publicación científica realizada en colaboración con otros miembros del Departamento de Química Analítica / Scientific publication carried out in collaboration with other members of the Analytical Chemistry Department.

Talanta 162 (2017) 24–31

Contents lists available at ScienceDirect

Talanta

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

Quantitative method for determination of oleocanthal and oleacein in virgin olive oils by liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Keywords:
Virgin olive oil
Oleocanthal
Oleacein
LC–MS/MS
Hemiacetal
Acetal

ABSTRACT

Oleocanthal and oleacein, two key secoiridoid derivatives present in virgin olive oil (VOO), are gaining clinical and nutritional interest thanks to their proved bioactivity; therefore, the determination of both phenols is a growing demanded application to increase the value of VOO. The main problem of previously reported liquid chromatography-based methods for oleocanthal and oleacein measurement is their interaction with water or other polar solvents such as methanol to promote the formation of hemiacetal or acetal derivatives. This interaction can occur during either sample extraction, basically liquid–liquid extraction, and/or chromatographic separation. The aim of this research was to evaluate the suitability of LC–MS/MS for absolute quantitation of oleocanthal and oleacein in VOO. For this purpose, both liquid–liquid extraction and chromatographic separation were studied as potential promoters of acetals and hemiacetals formation from oleocanthal and/or oleacein. The results showed that the use of methanol–water solutions for phenols extraction was not influential on the formation of these artifacts. Acetals and hemiacetals from oleocanthal and/or oleacein were only detected at very low concentrations when methanol gradients under acidic conditions were used for chromatographic separation. With this premise, a protocol based on extraction with acetonitrile and a reverse chromatographic gradient with methanol was established to quantify in absolute terms oleocanthal and oleacein in VOO samples. The resulting protocol was applied to three VOO samples characterized by high, medium, and low levels of these two phenols.

1. Introduction

Virgin olive oil (VOO) contains multiple minor components, such as sterols, volatile compounds, and phenols, among the most important families. Olive oil phenols comprise acids, phenolic alcohols, such as tyrosol (abbreviated as *p*-HPEA) and hydroxytyrosol (3,4-DHPEA), flavonoids, lignans, and secoiridoids (oleuropein, ligstroside and their derivatives). The bioactive capability of phenols present in VOO is a matter of great interest because of the proved or tentatively described healthy effects attributed to them. Additionally, olive oil phenols are major contributors to the long shelf-life and organoleptical characteristics of VOO [1,2]. Two secoiridoid derivatives should be mentioned in this regard, the dialdehydic forms of decarboxymethyl ligstroside and oleuropein aglycones, also known as oleocanthal (*p*-HPEA-EDA), and oleacein (3,4-DHPEA-EDA), respectively (Supplementary Fig. 1) [3]. These compounds are endowed with antimicrobial, anticancer, and hypoglycemic effects, and are considered key oxidation inhibitors among the main responsible for the antioxidant properties of VOO [4]. It is noteworthy to point out that oleacein has been declared a more potent antioxidant than hydroxytyrosol [4]; furthermore, the interest in these derivatives has been enhanced because of their reported anti-inflammatory properties. Thus, oleocanthal has shown intense anti-inflammatory effects comparable to ibuprofen thanks to its capability to inhibit cyclooxygenases COX-1 and COX-2 but not 15-lipooxygenase [5]. Indeed, some authors have pointed out that oleocanthal is one of the main components responsible for the therapeutic properties of VOO [6]. Recently, oleocanthal has also been proposed as a promising agent to induce selectively cancer cell death via lysosomal membrane permeabilization [7]. Concerning sensory properties, oleocanthal is responsible for the burning pungent sensation of VOO [8].

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http://dx.doi.org/10.1016/j.talanta.2016.09.056
Received 11 April 2016; Received in revised form 20 September 2016; Accepted 25 September 2016
Available online 24 September 2016

DOI: https://doi.org/10.1016/j.talanta.2016.09.056

Anexo II / Annex II

Comunicaciones a congresos y conferencias / Communications to congresses and conferences

V Congreso Científico de Investigadores en Formación
de la Universidad de Córdoba,
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La variabilidad de los compuestos fenólicos en aceites de oliva vírgenes extra

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DOI: https://doi.org/10.5281/zenodo.4415949

Resumen

El aceite de oliva virgen (AOV) es el zumo exprimido de las aceitunas frescas y sanas sin aditivos y sin aplicar procesos térmicos y químicos para su extracción. Los compuestos fenólicos que se encuentran en el AOV, determinan significativamente su calidad organoléptica y nutricional por lo que su estudio es objeto de gran interés. Sin embargo, la influencia de la variedad en los perfiles fenólicos de los AOV no ha sido extensivamente evaluada. Por este motivo, los objetivos fundamentales de este trabajo fueron la caracterización de los perfiles fenólicos de una amplia colección de variedades de olivo, la evaluación de la influencia de la variedad (genotipo) en estos perfiles y la agrupación de las variedades según la composición fenólica. Para abordar estos objetivos se analizaron 80 variedades de olivo del Banco de Germoplasma Mundial de Olivo localizadas en el Campus de Rabanales de la Universidad de Córdoba (UCO) y se analizaron 9 compuestos fenólicos individuales. Como resultado principal se obtuvo una gran gama de variación en los compuestos fenólicos analizados. Notablemente, el efecto de la variedad fue el más determinante en la composición del perfil fenólico del AOV, respaldando la posibilidad de obtener variedades con perfiles fenólicos determinados a través de la mejora y la posibilidad para producir aceites con características predeterminadas mediante los procesos de cupaje. A pesar del carácter preliminar de este estudio, cuyos resultados necesitan ser confirmados en próximas campañas, constituye un primer paso necesario para conocer la dimensión de la variabilidad fenólica en el AOV, así como para la selección de genitores idóneos para la obtención de nuevas variedades con perfiles fenólicos específicos.


VIII Congresso Ibérico
de Ciências Hortícolas
7>10 junho 2017
Centro de Congressos de Coimbra
Convento de São Francisco
Organização:
Associação Portuguesa de Horticultura
Sociedad Española de Ciencias Hortícolas
PROGRAMA
Dia
7
17.00>18.00h
Entrega de documentação
Colocação de painéis
18.30>19.30h
Cocktail de boas-vindas
http://www.aphorticultura.pt/iberico-2017.html

VIII Congreso Ibérico de Ciencias Hortícolas

Tema:

La variabilidad de los compuestos fenólicos en aceites de oliva vírgenes extra

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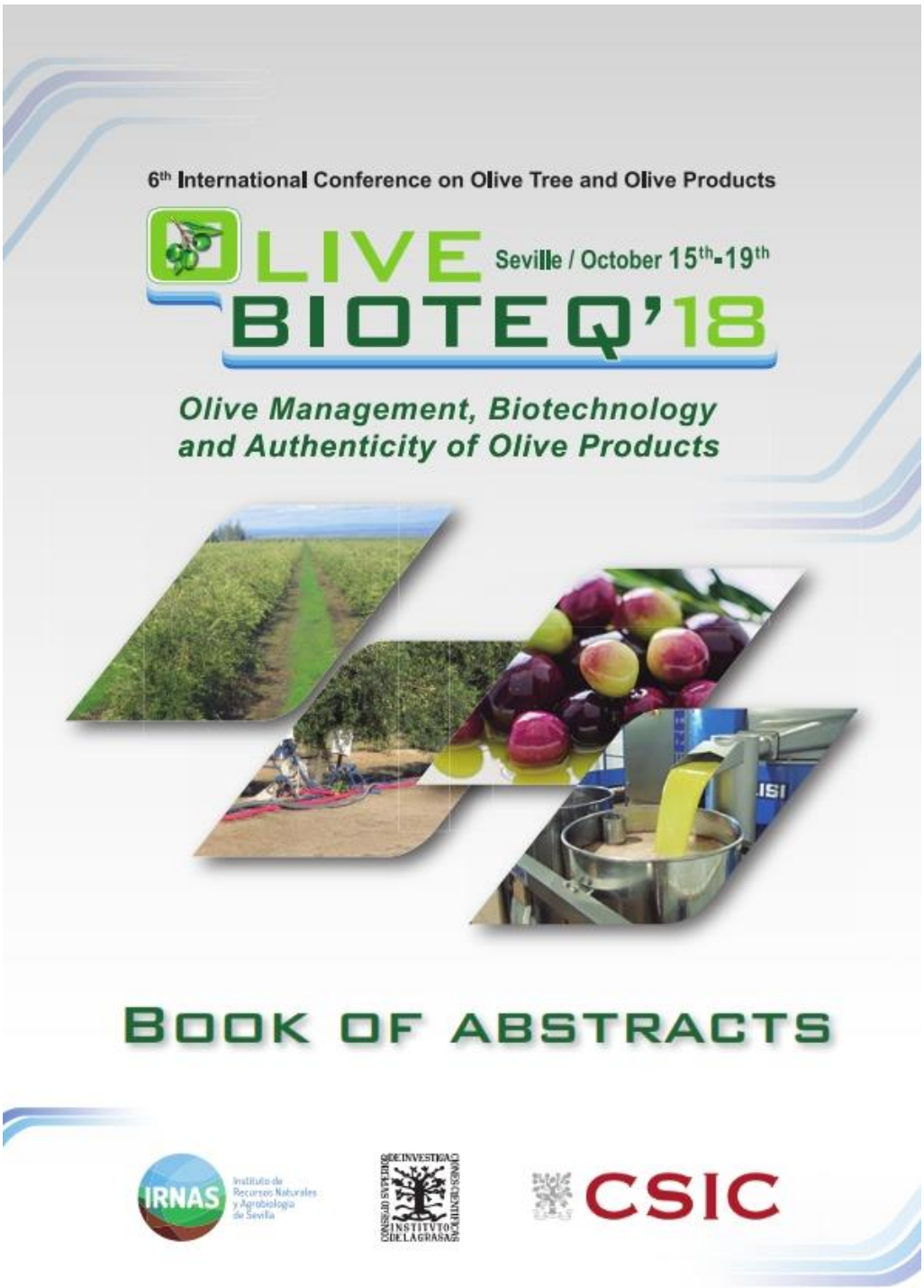
DOI: https://doi.org/10.5281/zenodo.4415983

Abstract

The extra virgin olive oil (VOO) is the juice of fresh and healthy olives fruits, with no additives and no thermal and/or chemical processes applied in the extraction process. The phenolic compounds found in the VOO significantly determine its organoleptic and nutritional quality and, therefore, their study is an object of big interest. However, the influence of the cultivar on the phenolic profile of the VOO has not been extensively evaluated. For this reason, the main objectives of this work were the characterization of the phenolic profile of a wide collection of olive cultivars, the evaluation of the genotype influence on these profiles and the grouping of cultivars according to the phenolic composition. In order to address these objectives, olive oil from 80 cultivars from the World Bank of Olive Germplasm located at the Rabanales Campus of the University of Córdoba (UCO) were analysed for quantitative determination of nine individual phenolic compounds. As a main result, we found a significant quantitative variation in the phenolic compounds analysed in the set of cultivars. Notably, the genetic effect was determinant in the composition of the phenolic profile of the olive oil, supporting the possibility of obtaining cultivars with specific phenolic profiles through the genetic improvement and the coupage processes. On the other hand, through the statistical analysis, the cultivars grouped into four main clusters according to their phenolic profile, where the group G1 was rich in aglycon isomers of oleuropein and ligstroside, G2 was rich in oleocanthal and oleacien, G3 rich in the most reprsentative flavonoids, apigenin and luteolin, and G4 presented a balanced composition. For a set of 36 cultivars, these analyses were repeated for two consecutive seasons that highlighted the consistency of the results.



Source: https://olivebioteqsevilla2018.com/wp-content/uploads/2018/10/ABSTRACTS-OLIVEBIOTEQ.pdf

CULTIVAR INFLUENCE ON VARIABILITY IN OLIVE OIL PHENOLIC PROFILES DETERMINED THROUGH AN EXTENSIVE GERMPLASM SURVEY

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DOI: https://doi.org/10.5281/zenodo.4049812

Abstract

Despite the evident influence of the cultivar on olive oil composition, few studies have been devoted to exploring the variability of phenols in a representative number of monovarietal olive oils. In this study, oil samples from 80 cultivars selected for their impact on worldwide oil production were analyzed to compare their phenolic composition by using a method based on LC–MS/MS. Secoiridoid derivatives were the most concentrated phenols in virgin olive oil, showing high variability that was significantly due to the cultivar. Multivariate analysis allowed discrimination between four groups of cultivars through their phenolic profiles: (i) richer in aglycon isomers of oleuropein and ligstroside; (ii) richer in oleocanthal and oleacein; (iii) richer in flavonoids; and (iv) oils with balanced but reduced phenolic concentrations. Additionally, correlation analysis showed no linkage among aglycon isomers and oleocanthal/oleacein, which can be explained by the enzymatic pathways involved in the metabolism of both oleuropein and ligstroside.

Anexo III / Annex III

Editoriales en revistas de divulgación / Editorials in dissemination magazines

Source: http://www.doopaper.com/visor_html5/edimarket/especialdiaolivo#page/34

Las variedades de olivo y la diversidad de los AOVEs

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El reconocimiento universal del aceite de oliva virgen extra (AOVE) se debe a sus efectos beneficiosos sobre la salud y a su diversidad de sabores. Ambos se fundamentan en las variedades, el medio de cultivo y la elaboración del aceite. La selección empírica local de las variedades por los olivareros durante más de 6.000 años constituye un legado de diversidad varietal que se conserva y estudia en los Bancos de Germoplasma.

En la actualidad, una Red de Bancos coordinada por el Consejo Oleícola Internacional (COI) incluye colecciones en 23 países que albergan más de 1.800 denominaciones varietales. Su autentificación es un paso imprescindible para su evaluación agronómica y oleotécnica. Un proyecto del COI, coordinado por la Universidad de Córdoba, autentificará las 191 denominaciones varietales comerciales que sustentan la producción oleícola de las Denominaciones de Origen Protegidas (DOP) en los países miembros.

También se precisan nuevas variedades. El cambio de paradigma que ha experimentado la olivicultura desde el final de la II Guerra Mundial requiere variedades para los nuevos olivares. Así, un programa conjunto UCO-IFAPA ha originado hasta la fecha tres nuevas variedades (sikitita, sikitita2 y sikitita3) y formado una generación de mejoradores. Nuevos programas cooperativos entre instituciones públicas y privadas tratan de seleccionar y obtener variedades adaptadas al medio, resistentes a plagas y enfermedades devastadoras, productivas y con AOVEs diversos que ampliarán la oferta varietal en la próxima década.

La diversidad y calidad de los AOVEs

El AOVE se define como el jugo natural de la aceituna extraído únicamente por procesos mecánicos de máxima calidad reglamentada máxima. Se trata del producto estrella de la Dieta Mediterránea por sus efectos saludables y sus características organolépticas singulares.

El AOVE se diferencia químicamente de otras grasa vegetales por su alto contenido en grasas monoinsaturadas (ácido oleico: 60-80%); y por su gran riqueza en compuestos minoritarios, que desempeñan el papel fundamental en las propiedades saludables y organolépticas. Los principales del AOVE son los fenoles, terpenos, esteroles, pigmentos y los compuestos volátiles. Diferentes estudios han demostrado que estos compuestos aportan beneficios para la salud por sus efectos antiinflamatorios, antioxidantes, anticancerígenos, cardioprotectores y reguladores del colesterol y del sistema endocrino del organismo.

Asimismo, dichos compuestos aportan su sabor único al AOVE mediante sensaciones de picor, amargor, astringencia y diversos aromas frutados. Las variedades proporcionan una gran variabilidad de base genética en la composición química y en las percepciones sensoriales de sus aceites. Por ejemplo, en estudios recientes del Grupo UCOLIVO se han observado diferencias varietales desde 290 hasta 3.208 mg./kg. en los compuestos fenólicos totales entre 80 variedades en análisis repetidos durante tres años. Numerosos estudios de composición, clínicos y sensoriales en todo el mundo evidencian la diversidad de los AOVEs.

En suma, la caracterización de los aceites varietales y de sus coupages proporcionan una diversidad ilimitada de los aceites de oliva vírgenes extra para el consumidor y una gran oportunidad de negocio para el sector oleícola.

Abreviaturas / Abbreviations

Abreviaturas / Abbreviations:

3,4-DHPEA	Hydroxytyrosol; 4-(2-Hydroxyethyl) benzene-1,2-diol; 2-(3,4-Dihydroxyphenyl) ethanol; 3,4-Dihydroxyphenylethano.
3,4-DHPEA-EA	Oleuropein-aglycone mono-aldehyde; (Oleuropein-aglycone major form); 3,4-DHPEA-Elenolic acid mono-Aldehyde.
3,4-DHPEA-EDA	Oleacein; 3,4-DHPEA-Elenolic acid Di-Aldehyde Oleuropein-aglycone di-aldehyde; (Decarboxymethyl oleuropein-aglycone major form).
ALigAgly	Aldehydic open forms of Ligstroside Aglycone.
ANOVA	Analysis of Variance.
AOleAgly	Aldehydic open forms of Oleuropein Aglycone.
AOV	Aceite de Oliva Virgen.
AOVE	Aceite de Oliva Virgen Extra.
DA	Discriminant Analysis.
DAFB	Days After Full Bloom.
EEC	European Economic Community.
EFSA	European Food Safety Authority.
ESI	Electrospray Ionization.
EVOO	Extra Virgin Olive Oil.
FA	Free Acidity.
FAEE	Fatty Acid Ethyl Esters.
FATH	Fruit Abscission Threshold.
FR	Fruit Ripening.
IOC	International Olive Council.
LC - MS/MS	Liquid Chromatography - tandem Mass Spectrometry.
LC	Liquid Chromatography.
LDL	Low-Density Lipoprotein.
Ligstroside	p-HPEA-Elenolic acid glucoside.

MALigAgly	Monoaldehydic closed form of Ligstroside Aglycone.
MAOleAgly	Monoaldehydic closed form of Oleuropein Aglycone.
MeOH	Methanol water.
MI	Maturity Index.
MRM	Multiple Reaction Monitoring.
MS	Mass Spectrometry.
MT	Malaxation Time.
MUFA	Monounsaturated Fatty Acid.
Oleuropein	3,4-DHPEA-Elenolic acid glucoside.
PCA	Principal Component Analysis.
p-HPEA	Tyrosol; 4-(2-Hydroxyethyl) phenol; 2-(4-Hydroxyphenyl) ethanol; 2,4-Hydroxyphenyl-ethyl-alcohol; 4-Hydroxyphenylethanol.
p-HPEA-EA	Ligstroside-aglycone mono-aldehyde; (Ligstroside-aglycone major form); p-HPEA-Elenolic acid mono-Aldehyde.
p-HPEA-EDA	Oleocanthal; p-HPEA-Elenolic acid Di-Aldehyde; Ligstroside-aglycone di-aldehyde; (Decarboxymethyl ligstroside-aglycone major form).
PL	Phospholipids.
POX	Peroxidase.
PPO	Polyphenol Oxidase.
PUFA	Polyunsaturated Fatty Acid.
QqQ	Triple Quadrupole.
RDI	Regulated Deficit Irrigation.
VOO	Virgin Olive Oil.

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