UNIVERSITAT DE BARCELONA

Determinación por cromatografía líquida acoplada a espectrometría de masas de polifenoles y triterpenos pentacíclicos en aceituna de mesa y aceite de oliva. Estudio de su ingesta en ratas: concentración de dichos compuestos en plasma y contenido intestinal y efecto de la aceituna de mesa sobre lesiones preneoplásicas en el colon

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DETERMINACIÓN POR CROMATOGRAFÍA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE MASAS DE POLIFENOLES Y TRITERPENOS PENTACÍCLICOS EN ACEITUNA DE MESA Y ACEITE DE OLIVA. ESTUDIO DE SU INGESTA EN RATAS: CONCENTRACIÓN DE DICHOS COMPUESTOS EN PLASMA Y CONTENIDO INTESTINAL Y EFECTO DE LA ACEITUNA DE MESA SOBRE LESIONES PRENEOPLÁSICAS EN EL COLON

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Barcelona, 2020

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Departament de Bioquímica i Fisiologia
Secció de Fisiologia

Programa de doctorado:
ALIMENTACIÓ I NUTRICIÓ

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Memoria presentada por **Rocío Moreno González** para optar al Título de Doctor por la Universitat de Barcelona.

Esta tesis ha sido realizada en el grupo de *Fisiologia i Nutrició Experimental* bajo la supervisión de la Dra. **Joana Maria Planas Rosselló** y la Dra. **Maria Emília Juan Olivé**.

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Barcelona, 2020

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INFORMAN

Que la memoria titulada ***Determinación por cromatografía líquida acoplada a espectrometría de masas de polifenoles y triterpenos pentacíclicos en aceituna de mesa y aceite de oliva. Estudio de su ingesta en ratas: concentración de dichos compuestos en plasma y contenido intestinal y efecto de la aceituna de mesa sobre lesiones preneoplásicas en el colon*** presentada por **Rocío Moreno González** para optar al Título de Doctor por la Universitat de Barcelona, ha estado realizada bajo nuestra dirección en la Secció de Fisiologia del Departament de Bioquímica i Fisiologia y, considerándola concluida, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

Y porque así conste, firmamos el presente en Barcelona, 28 de Febrero del 2020

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a mis hermanos Irene, Gerardo y Mari Carmen

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ABREVIATURAS

AC	Acrónimo del inglés *aberrant crypt,* criptas aberrantes
ACF	Acrónimo del inglés *aberrant crypt foci*, foco de criptas aberrantes
AOM	Azoximetano
AOV	Aceite de oliva virgen, en inglés *virgin olive oil (VOO)*
AOVE	Aceite de oliva virgen extra, en inglés *extra virgin olive oil (EVOO)*
APC	Acrónimo del inglés de *adenomatous polyposis coli*, poliposis adenomatosa coli
APCI	Acrónimo del inglés *atmospheric pressure chemical ionization*, ionización química a presión atmosférica
API	Acrónimo del inglés *atmospheric pressure ionization*, ionización a presión atmosférica
BA	Acrónimo del inglés *betulinic acid*, ácido betulínico
CE	Acrónimo del inglés *collision energy*, energía de colisión
CK1	Acrónimo del inglés *casein kinase 1*, caseína quinasa 1
Cmax	Concentración máxima en plasma
cox-2	Ciclooxigenasa-2
CRC	Cáncer colorectal, en inglés *colorectal cancer*
CXP	Acrónimo del inglés *collision cell exit potential*, potencial de salida de la celda de colisión
CYP	Citocromo P450
DAD	Acrónimo del inglés *diode array detector*, detector de diodos
3,4-DHPA	3,4-Dihidroxifenilacetato
3,4-DHPEA	Hidroxitirosol

3,4-DHPEA-EA	Oleuropeína aglicona o forma aldehídica de ácido elenólico unido a hidroxitirosol
3,4-DHPEA-EDA	Oleaceína
DMAPP	Dimetilalil difosfato
DMH	1,2-Dimetilhidrazina
DMSO	Dimetilsulfóxido
ESI	Acrónimo del inglés *electrospray ionization,* ionización por electrospray
EDTA	Ácido etilendiaminotetraacético
E4P	Eritrosa 4-fosfato
ERY	Acrónimo del inglés *erythrodiol*, eritrodiol
FAP	Acrónimo del inglés *familial adenomatous polyposis*, poliposis adenomatosa familiar
FCE	Acrónimo del inglés *food conversion efficiency*, eficiencia de conversión de alimentos
GC-FID	Acrónimo del inglés *gas chromatography - flame ionization detector*, cromatografía de gases con detector de ionización de llama
GLUT2	Acrónimo del inglés *glucose transporter 2*, transportador de glucosa 2
GPP	Geranil difosfato
GSK3β	Acrónimo del inglés *glycogen synthase kinase 3β*, glucógeno sintasa quinasa 3β
HID-AB	Diamina con alto contenido en hierro / azul alcián / rojo neutro
HMG-CoA	3-Hidroxi-3-metilglutaril-CoA

4-HPA	4-Hidroxifenilacetaldehído
***p*-HPAA**	Ácido *p*-hidroxifenilacético
***p*-HPEA**	Tirosol
***p*-HPEA-EA**	Ligstrósido aglicona o forma aldehídica de ácido elenólico unido a tirosol
***p*-HPEA-EDA**	Oleocantal
HPLC	Acrónimo del inglés *high performance liquid chromatography*, cromatografía de líquidos de alta eficacia
***p*-HPPA**	Ácido *p*-hidroxifenilpirúvivo
IPP	Isopentenil difosfato
IS	Acrónimo del inglés *internal standard*, patrón interno
LC	Acrónimo del inglés *liquid chromatography*, cromatografía de líquidos
LC-APCI-MS	Acrónimo del inglés *liquid chromatography - atmospheric pressure chemical ionization - mass spectrometry*, cromatografía de líquidos acoplada a espectrometría de masas con fuente de ionización química a presión atmosfética
LC-APCI-QTRAP-MS	Acrónimo del inglés *liquid chromatography - atmospheric pressure chemical ionization - quadropule ion trap - mass spectrometry*, cromatografía de líquidos acoplada a un espectrómetro de masas de masas con analizador triple cuadrupolo de trampa de iones lineal y fuente de ionización química a presión atmosférica
LC-ESI-MS/MS	Acrónimo del inglés *liquid chromatography - electrospray ionization - tandem mass spectrometric*, cromatografía de líquidos acoplada a espectrometría de masas en tándem con fuente de ionización por electrospray

LC-ESI -QTRAP-MS/MS	Acrónimo del inglés *liquid chromatography – electrospray ionization - quadropule ion trap - tandem mass spectrometric*, cromatografía de líquidos acoplada a espectrometría de masas en tándem con analizador triple cuadrupolo de trampa de iones lineal y fuente de ionización por electrospray
LC-LTQ-Orbitrap	Acrónimo del inglés *liquid chromatograply - linear ion trap quadrupole - Orbitrap*, cromatografía de líquidos acoplada a un espectrómetro de masas híbrido compuesto de un analizador cuadrupolo de trampa de iones lineal y un Orbitrap
LC-MS	Acrónimo del inglés *liquid chromatograply – mass spectrometry,* cromatografía de líquidos acoplada a espectrometría de masas
LC-QqQ-MS	Acrónimo del inglés *liquid chromatography – triple quadropule - mass spectrometry*, cromatografía de líquidos acoplada a un espectrómetro de masas con analizador triple cuadupolo
LC-QTRAP-MS	Acrónimo del inglés *liquid chromatography - quadropule ion trap - mass spectrometry*, cromatografía de líquidos acoplada a espectrometría de masas con analizador triple cuadrupolo de trampa de iones lineal
L-DOPA	L-3,4-Dihidroxifenilalanina
iNOS	Acrónimo del inglés, *inducible nitric oxide synthase*, sintasa de óxido nítrico inducible
LOD	Acrónimo del inglés *limit of detection*, límite de detección
LOQ	Acrónimo del inglés *limit of quantification*, límite de cuantificación
LLOQ	Acrónimo del inglés *lower limit of quantification*, límite inferior de cuantificación
MA	Acrónimo del inglés *maslinic acid*, ácido maslínico
MAM	Metilazoximetanol

MAPK	Acrónimo del inglés *mitogen-activated protein kinase*, proteína quinasa activada por mitógeno
MDAC	Acrónimo del inglés *mucin-depleted aberrant crypts,* criptas aberrantes con depleción de mucina
MDF	Acrónimo del inglés, *mucin depleted foci*, foco con depleción de mucina
MEK	Acrónimo del inglés *mitogen-activated protein kinase kinase*, proteina quinasa quinasa activada por mitógeno
MEP	2-C-metil-d-eritrodiol 4-fosfato
MRM	Acrónimo del inglés *multiple reactions monitoring*, monitoreo de reacciones múltiples
MS	Acrónimo del inglés *mass spectrometry*, espectrometría de masas
MVA	Mevalonato o ácido mevalónico
MVAPP	Mevalonato difosfato
NaCl	Cloruro sódico
m/z	Relación masa/carga
NF-κB	Acrónimo del inglés nuclear factor *κ-light-chain-enhacer of activated B cells*, factor de transcripción nuclear κB
OA	Acrónimo del inglés *oleanolic acid*, ácido oleanólico
p53	Factor de transcripción nuclear p53
PBS	Acrónimo del inglés *phosphate-buffered saline*, tampón fosfato salino
PEP	Fosfoenolpiruvato
PHA	Acrónimo del inglés, *polycyclic aromatic hydrocarbon*, hidrocarburo policíclico aromático

PI3K	Acrónimo del inglés, *phosphoinositide 3-kinase*, fosfoinositida 3-quinasa
PT	Acrónimo del inglés *pentacyclic triterpenes*, triterpenos pentacíclicos
PTFE	Politetrafluoroetileno
6466 QqQ MS/MS	Acrónimo del inglés *6466 triple quadrupole tandem mass spectrometry*, espectrometría de masas en tándem con un triple cuadrupolo 6466
RSD (%)	Desviación estándar relativa (%) o coeficiente de variación (%)
RT	Acrónimo del inglés *retention time*, tiempo de retención
SEM	Acrónimo del inglés, *standard error of the mean*
SGLT1	Acrónimo del inglés *sodium/glucose cotransporter 1*, cotransportador de sodio/glucosa 1
SH-CoA	Coenzima A
SIM	Acrónimo del inglés *selected ion monitoring*, monitoreo de un ión seleccionado
STZ-induced	Acrónimo del inglés *streptozotocin-induced,* inducción con estreptozotocina
TCF	Acrónimo del inglés *T-cell factor*, factor de transcripción de células T
TGF-β	Acrónimo del inglés *transforming growth factor beta*, factor de crecimiento transformante beta
TIC	Acrónimo del inglés *total ion chromatogram*, cromatograma de iones totales
tmax	Tiempo al que se consigue la concentración máxima en plasma
UA	Acrónimo del inglés *ursolic acid*, ácido ursólico

UPLC	Acrónimo del inglés *ultra pressure liquid chromatography*, cromatografía líquida de alta presión
UHPLC	Acrónimo del inglés *ultra high pressure liquid chromatography*, cromatografía líquida de ultra alta presión
UV	Ultravioleta
UVA	Uvaol
WNT	Acrónimo del inglés *wingless and int,* sin traducción en castellano
XDB-C18	Acrónimo del inglés, *extra dense bonding-C18*, unión extra densa-*C18*
XIC	Acrónimo del inglés *extracted ion chromatogram,* cromatograma de iones extraídos

RESUMEN DE LA TESIS

Los triterpenos pentacíclicos y polifenoles son compuestos bioactivos con propiedades saludables que se encuentran en plantas medicinales y comestibles, incluyendo *Olea europaea* L. En la tesis presentada se ha llevado a cabo un desarrollo de nuevos métodos para el análisis de estos compuestos en aceitunas de mesa, aceite de oliva virgen extra (AOVE), así como en el plasma y el contenido de colon después de la ingesta de estos alimentos. Además, también se ha investigado los efectos antitumorales *in vivo* del consumo de aceitunas de mesa sobre las lesiones preneoplásicas de colon. En primer lugar, se estableció un método para determinar los polifenoles en las aceitunas de mesa mediante extracción líquida y posterior análisis por LC-ESI-MS/MS. La validación mostró unos resultados satisfactorios de linealidad, sensibilidad, exactitud, precisión y recuperación. En segundo lugar, la extracción se validó para la determinación simultánea de triterpenos pentacíclicos y polifenoles a partir de la misma muestra de aceitunas previamente al análisis por LC-APCI-MS para triterpenos pentacíclicos y LC-ESI-MS/MS para polifenoles. El procedimiento sirvió para analizar tres variedades, encontrándose valores de triterpenos pentacíclicos más elevados en las aceitunas de mesa Marfil (3,62 ± 0,29 g/kg), seguido de las Arbequina (3,28 ± 0,07 g/kg) y Empeltre (2,77 ± 0,20 g/kg). El triterpeno pentacíclico más abundante fue el ácido maslínico (62-77%) y posteriormente el ácido oleanólico (23-38%) y eritrodiol (< 0,3%). En cuanto a los polifenoles, se detectaron dieciséis con concentraciones ligeramente más altas en Arbequina (1,04 ± 0,03 g/kg) con respecto a Marfil (0,87 ± 0,08 g/kg) y Empeltre (0,83 ± 0,05g/kg). El hidroxitirosol fue el mayoritario (~45%), seguido por verbascósido (28%) y luteolina (9%) tanto en Arbequina como en Empeltre. Aunque Marfil tiene porcentajes similares de hidroxitirosol y luteolina, se encontró un perfil diferente para otros polifenoles como verbascósido, que presentó un 0,10% y, tirosol un 23%. En tercer lugar, se investigaron las concentraciones plasmáticas de compuestos bioactivos después de la administración de AOVE de Arbequina a ratas Sprague-Dawley. El AOVE contiene 4 triterpenos pentacíclicos (149 ± 30 mg/kg) y 13 polifenoles (16,8 ± 0,3 mg/kg). Las concentraciones plasmáticas obtenidas después de la extracción líquida y el análisis LC-QTRAP-MS, revelaron la presencia de 1 triterpeno pentacíclico y 7 polifenoles. Todos los analitos alcanzaron su máxima concentración (Cmax) a los 15 minutos (tmax), disminuyeron de forma constante a lo largo del tiempo y aún se detectaron a los 120 min. Finalmente, se investigó el efecto de las aceitunas de mesa (3,85 g/kg) en las primeras etapas de la carcinogénesis de colon inducido por 1,2-dimetilhidrazina en ratas Sprague-Dawley. La administración de aceitunas Arbequina durante 49-días redujo los focos de criptas aberrantes y los focos con depleción de mucina en el colon total por un 54,1% y 35,7%, respectivamente. Concluyendo, las aceitunas de mesa contienen compuestos bioactivos en cantidades altas que, junto con las bajas concentraciones medidas en el plasma y la disminución de las lesiones preneoplásicas en el colon, sugeriría que su consumo regular, en el contexto de un estilo de vida saludable, podría contribuir a la prevención de enfermedades crónicas.

Pentacyclic triterpenes and polyphenols are bioactive compounds with health-protecting activities found in different medicinal and edible plants, among which stands out *Olea europaea* L. This thesis develops new methods for their analysis in table olives, extra virgin olive oil (EVOO) as well as in plasma and colon content after the intake of these foods. Moreover, the *in vivo* antitumoral effects of table olives was investigated on colonic pre-neoplastic lesions. Firstly, a new method to determine polyphenols in table olives by liquid extraction and LC-ESI-MS/MS was established. Validation showed satisfactory linearity, sensibility, accuracy, precision and recoveries. Secondly, the extraction was also validated for the determination of pentacyclic triterpenes concurrently with polyphenols from the same olives prior to LC-APCI-MS for pentacyclic triterpenes and LC-ESI-MS/MS for polyphenols. The analysis of three varieties yielded the greatest values of pentacyclic triterpenes in Marfil (3.62 ± 0.29 g/kg), followed by Arbequina (3.28 ± 0.07 g/kg) and Empeltre (2.77 ± 0.20 g/kg) table olives. The most abundant pentacyclic triterpene was maslinic acid (62-77%), ensued by oleanolic acid (23-38%) and erythrodiol (< 0.3%). Sixteen polyphenols were detected with concentrations slightly higher in Arbequina (1.04 ± 0.03 g/kg) with respect to Marfil (0.87 ± 0.08 g/kg) and Empeltre (0.83 ± 0.05 g/kg). Hydroxytyrosol was the predominant (~45%), succeeded by verbascoside (28%) and luteolin (9%) in Arbequina and Empeltre. Although Marfil hold similar percentages of hydroxytyrosol and luteolin, a different profile was found for other polyphenols with verbascoside showing a 0.10% and tyrosol reaching a 23%. Thirdly, the plasmatic concentrations of bioactive compounds after the administration of Arbequina EVOO to Sprague-Dawley rats was investigated. EVOO contained 4 pentacyclic triterpenes (149 ± 30 mg/kg) and 13 polyphenols (16.8 ± 0.3 mg/kg). Plasmatic concentrations obtained after liquid extraction and LC-QTRAP-MS analysis revealed the presence of 1 pentacyclic triterpene and 7 polyphenols. All analytes showed maximum concentration (Cmax) at 15 minutes (tmax) followed by a steady decrease and still were detected at 120 min. Finally, the effect of table olives (3.85 g/kg) in early stages of colon carcinogenesis induced by 1,2-dimethylhydrazine in Sprague-Dawley rats was investigated. The administration of Arbequina olives for 49-days reduced aberrant crypt foci and mucin depleted foci in total colon in a 54.1% and 35.7%, respectively. In conclusion, table olives contain bioactive compounds in high amounts that together with their low plasmatic concentrations and the decrease of preneoplastic lesions suggests that a regular consumption of this food in the context of a healthy lifestyle, could contribute to the prevention of chronic diseases.

I. INTRODUCCIÓN

1.1. El olivo

El olivo (*Olea europaea* subsp. *europaea* var. *Europaea* L.) pertenece a la familia de las oleáceas, a la subespecie *europaea* y a la variedad *europaea* L. que designa el olivo cultivado para distinguirlo de la variedad *sylvestris* (Mill.) Lehr, que es el olivo silvestre que crece de forma espontánea, y que recibe el nombre de acebuche (Green, 2002). El olivo es un árbol perennifolio de crecimiento lento, cuya longevidad puede llegar a ser de más de mil años. Su elevada adaptación al clima mediterráneo junto con su robustez, le permite resistir la sequía estival y soportar las heladas invernales moderadas.

Los orígenes del olivo se remontan al Neolítico (Breton *et al*., 2012). Sin embargo, no es hasta los años comprendidos entre 5000 y 1400 a. C. en que se tiene evidencia de su cultivo. Este se inició en Israel, Siria y Creta, extendiéndose a las zonas del sur de Turquía, Chipre, Egipto y Grecia. Con la expansión de las colonias griegas, el cultivo del olivo se propagó al sur de Italia y al norte de África. El posterior dominio romano facilitó su cultivo en toda la cuenca Mediterránea (Kaniewski *et al*., 2012). Con el descubrimiento de América, se expandió este patrimonio hasta dicho continente, siendo introducido primero en Colombia y Perú y, con posterioridad, en la costa oeste de los Estados Unidos. El cultivo del olivo ha continuado creciendo hasta lugares tan distantes de sus orígenes como Sudáfrica, Australia y, más tarde, China, Japón, Argentina y Chile (Breton *et al*., 2012). Sin embargo, el 98% del cultivo del olivo se halla todavía en los países de la cuenca Mediterránea, donde tiene una gran importancia no solo desde el punto de vista económico sino también cultural (Lucena *et al*., 2017).

En 2017, la Unión Europea disponía de una superficie de cultivo dedicada al olivar de 4,6 millones de hectáreas (Eurostat 2017). Los principales países productores son España (55%) e Italia (23%), que representan más de las tres cuartas partes del área total, seguidos de Grecia (15%) y Portugal (7%). Francia, Croacia, Chipre y Eslovenia tienen una superficie de cultivo de olivos de aproximadamente un 1% (Figura 1.1).

En España, se cultiva el olivo en todas las regiones autónomas excepto en la de Asturias y Cantabria. La región de España con mayor producción de aceituna es Andalucía, seguida de Extremadura, Castilla-La Mancha, Cataluña y Comunidad Valenciana. El 91% de la producción de aceitunas se utiliza para la obtención de aceite y solo el 9% se destina a aderezo (MAGRAMA, anuario de estadística 2017).

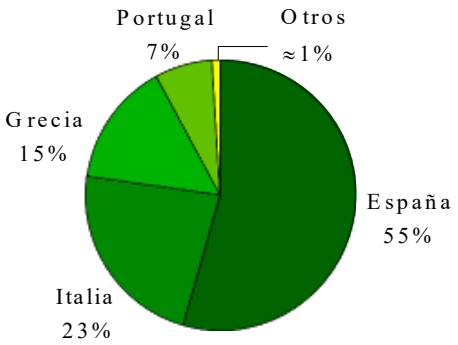

Figura 1.1. Superficie de cultivo de olivo en 2017 en la Unión Europea (Eurostat, 2017).

En Cataluña hay un 15% de la superficie agrícola dedicada al cultivo del olivo, con un total de 113069 hectáreas utilizadas para este fin (Generalitat de Catalunya, 2017). Las comarcas con mayor superficie de olivos son las Garrigues y Baix Ebre, seguidas por el Montsià (Ninot *et al*., 2019). En Cataluña se cultivan diferentes variedades de olivos, entre las que destacan Arbequina, Empeltre y Marfil, las cuales serán objeto de estudio de la tesis que se presenta (Figura 1.2)

La variedad Arbequina es la más extendida en el mundo debido a sus características de gran resistencia al frio y adaptabilidad a diferentes condiciones de temperatura y suelo. Aún cuando es originaria del municipio de Arbeca (Lérida), se ha extendido a diferentes comarcas de Cataluña, como Ribera d'Ebre (Tarragona). Actualmente es muy cultivada en diversas regiones del mundo como es el caso de California (Ninot *et al*., 2019). Su fruto se utiliza mayoritariamente para hacer aceite de oliva y en menor proporción como aceituna de mesa. La variedad Empeltre se caracteriza por adaptarse fácilmente a terrenos de baja calidad y sometidos a sequedad. Se cultiva en Cataluña, principalmente en la comarca denominada Terra Alta (Tarragona) y su fruto es muy valorado tanto para la producción de aceite como de aceitunas de mesa. La variedad Marfil es una variedad que se halla principalmente en el Montsià. Es una variedad poco productiva y de maduración lenta que debe su nombre a que el fruto maduro presenta una coloración parecida al marfil. Mayoritariamente se utiliza para obtener aceite de oliva que está muy valorado por su alto contenido de polifenoles (Ninot *et al*., 2019)

Figura 1.2. Localización de las comarcas de Cataluña de donde proceden las aceitunas estudiadas. Arbequina (verde), Empeltre (gris) y Marfil (amarillo)

1.2. La aceituna

El fruto del olivo, la aceituna, es una drupa de forma oval que tiene un tamaño medio comprendido entre 1 y 4 cm de longitud y de 0,6 a 2 cm de diámetro, dependiendo de la variedad. Su peso puede variar de 2 a 12 g e incluso llegar a 20 g en algunas variedades. En la aceituna se pueden distinguir tres partes: el epicarpio o exocarpio (piel), el mesocarpio (pulpa) y el endocarpio (hueso) (Figura 1.3). El epicarpio, es la capa exterior del fruto que es rica en clorofila, carotenoides, antocianinas, así como en ceras (Bianchi, 2003; Ghanbari *et al*., 2012). Durante la maduración, la concentración de estos componentes varía dando lugar a un cambio de color de la piel, pasando de verde claro a

una tonalidad morada-marrón o incluso negra (Giuliani *et al*., 2011; Ghanbari *et al*., 2012). El mesocarpio es el tejido carnoso que concentra la mayor parte de componentes de la aceituna, incluyendo el agua y el aceite. Está formada por células mesenquimáticas poco diferenciadas, pero con alta capacidad de crecimiento (Conde *et al*., 2008; Rapoport y Moreno-Alías, 2017). El endocarpio, constituye la parte leñosa de la aceituna que encierra la semilla. A diferencia del mesocarpio que crece hasta la maduración, el crecimiento del endocarpio se detiene a los dos meses de la fecundación (Rapoport y Moreno-Alías, 2017). El crecimiento y la maduración de la aceituna puede demorar aproximadamente unos 5 meses, aunque en condiciones climáticas frías, el crecimiento es más lento (Ghanbari *et al*., 2012). La composición media del fruto es de un 50% de agua que dependerá del contenido de grasa según la variedad (siendo de un 14-15% en aceitunas verdes de mesa y un 30% en aceitunas negras maduras), un 1,6% de proteínas, un 22% de lípidos, un 19,1% de carbohidratos, un 5,8% de celulosa, un 1,5% de minerales (cenizas) y entre un 1 y 3% de compuestos fenólicos y triterpenos pentacíclicos. Otros componentes son pectinas, ácidos orgánicos y pigmentos (Ghanbari *et al*., 2012).

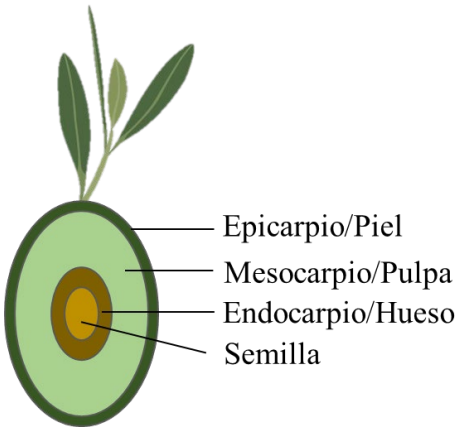

Figura 1.3. Estructura de una aceituna

1.3. La aceituna de mesa como fuente de polifenoles y triterpenos pentacíclicos

La aceituna constituye un fruto de alto valor nutritivo con un contenido importante de diferentes compuestos con efectos beneficiosos para la salud (Guo *et al*., 2018). Entre estos componentes, cabe destacar los polifenoles y triterpenos pentacíclicos, que son metabolitos secundarios de la planta, capaces de protegerla frente a diferentes agresiones bióticas y abióticas (Boskou, 2017). Además, estos compuestos dan una serie de características al fruto cosechado de la *Olea europaea* L. como son el aroma, sabor y textura dependiendo de la variedad y de la concentración de estos compuestos en el fruto. La composición de la aceituna depende de diversos factores tales como la localización geográfica, técnicas de cultivo, riego y maduración del fruto, entre otros (Boskou, 2017). Por otro lado, la aceituna debe ser procesada para poder ser consumida y el método de procesamiento de la aceituna afectará a su composición. Entre los métodos de elaboración, hay que destacar los tres tipos más tradicionales que son el estilo Español, el Californiano y el Griego. Este último proceso es al que se han sometido las aceitunas de mesa que han sido estudiadas durante el desarrollo de esta tesis doctoral. Las aceitunas, una vez recolectadas del árbol y seleccionadas, se han sumergido en una solución de NaCl (4-15%; p/v) para que se produzca una fermentación

espontánea (Kailis y Kiritsakis, 2017). Durante este proceso, es notable la reducción del amargor principalmente debido a la hidrólisis del compuesto fenólico denominado oleuropeína, produciendo así cambios en la composición (Guo *et al*., 2018). Transcurridos entre 2 y 3 meses, las aceitunas se extraen y se colocan en tarros con NaCl a una concentración de 3,5-4% para su consumo (Kailis y Kiritsakis, 2017). Este alimento fermentado contiene altas cantidades de polifenoles y triterpenos pentacíclicos a los que se les ha atribuido actividades biológicas a través de los estudios realizados *in vitro* e *in vivo* en animales e intervenciones nutricionales, entre las que cabe destacar efectos antioxidantes, antiinflamatorios, actividades antimicrobianas, cardioprotectoras y antitumorales (Sánchez-Quesada *et al*., 2013; Pedret *et al*., 2018). Así pues, la ingesta regular de aceitunas de mesa podría aportar cantidades suficientes de estos fitoquímicos dando lugar a efectos beneficiosos sobre la salud.

1.4. Polifenoles

El término *polifenol* ha sido adoptado por diferentes autores para hacer referencia a una amplia clase de compuestos que albergan en su estructura al menos un anillo fenólico (Frank *et al.*, 2019). Bajo este término se incluye tanto a *compuestos fenólicos* que contienen un único anillo aromático, como a *polifenoles* que albergan en su estructura múltiples anillos fenólicos (Frank *et al.*, 2019). Aunque los polifenoles naturales se pueden agrupar siguiendo diferentes clasificaciones, una de las más empleadas es la establecida por Harborne (Evans & Harbone, 1990) en base a su estructura química que recientemente Tsimogiannis y Oreopoulou (2019) simplificaron en tres categorías. De este modo, los polifenoles pueden estar formados por un simple anillo aromático con un grupo hidroxilo (C6), por un anillo aromático unido a una cadena de carbono (C6-Cn) o por dos anillos aromáticos unidos por una cadena de carbono (C6-Cn-C6). Asimismo, cada una de estas clases se subdivide en categorías, como por ejemplo la clase C6-Cn-C6 que incluye cuatro subgrupos, de los que destaca el C6-C3-C6 que contiene solo a los flavonoides. En las plantas, muchos de estos compuestos se hallan como glucósidos, y otros derivados. Además, estos polifenoles pueden dar lugar a estructuras de mayor complejidad que se agrupan en dímeros, oligómeros y polímeros (Tsimogiannis y Oreopoulou, 2019). A partir de la clasificación en base a su estructura química y para facilitar su comprensión, los polifenoles se pueden dividir en dos grandes grupos, los no flavonoides y los flavonoides (Durazzo *et al*., 2019).

Los polifenoles no flavonoides se pueden subdividir en las siguientes subclases: alcoholes fenólicos, ácidos fenólicos, secoiridoides, lignanos, estilbenos, xantonas y taninos (Durazzo *et al*., 2019). Sus estructuras pueden ser sencillas, como las que corresponden a un anillo aromático con un grupo hidroxilo (clase C6) o con una cadena de carbono (C6-Cn) y, de mayor complejidad, como los esqueletos formados por dos anillos aromáticos enlazados por una cadena de carbono (clase C6-

Cn-C6), excluyendo C6-C3-C6 que sería específica para los flavonoides (Tsimogiannis y Oreopoulou, 2019).

Los polifenoles flavonoides constituyen un amplio grupo de polifenoles que se dividen en seis subclases: antocianos, flavanoles, flavanonas, flavonoles, flavononas e isoflavonas (Durazzo *et al*., 2019). Se han identificado más de 4000 flavonoides en alimentos que son consumidos habitualmente en una dieta rica en frutas y verdura. La estructura general consta de un esqueleto básico C6-C3-C6 en donde las dos unidades C6 son anillos fenólicos (anillo A y B) unidos por un anillo heterocíclico, que a menudo es un piranósido (anillo C) (Tsao, 2010) (Figura 1.4). Estos compuestos se pueden encontrar como agliconas, unidos a azúcares o a ácidos orgánicos, lo que da lugar a los diferentes tipos de flavonoides con sus correspondientes propiedades químicas, biológicas y de biodisponibilidad (Durazzo *et al*., 2019; Teng y Chen, 2019).

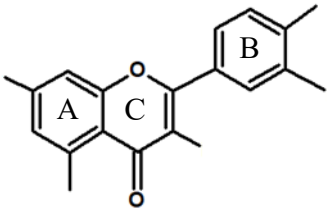

Figura 1.4. Esqueleto de los flavonoides.

Los polifenoles naturales se sintetizan como metabolitos secundarios con funciones involucradas en la protección de la planta frente a la radiación ultravioleta, así como a agentes patógenos (Manach *et al*. 2004). Los estudios de localización en las células y en los tejidos de los diferentes polifenoles han proporcionado cierta información de su papel en las plantas. Así, la presencia de polifenoles que dan color a órganos y tejidos reproductivos como flores, frutos y semillas tiene la finalidad de atraer a polinizadores y agentes que favorezcan la dispersión de las semillas, mientras que los compuestos no pigmentados confieren resistencia a las infecciones fúngicas y bacterianas. Para ejercer estas funciones, los polifenoles, se acumulan en la periferia y en la superficie de los órganos de las plantas (Piccolella *et al.*, 2019).

Se han identificado más de 8000 compuestos fenólicos en plantas comestibles y los alimentos más ricos en ellos son las frutas, las verduras y los frutos secos, entre otros (Tsao, 2010). Es este sentido, diferentes estudios tanto epidemiológicos como de intervención nutricional han indicado que dietas ricas en fruta y verdura, que contienen concentraciones elevadas de polifenoles, se han asociado con un menor riesgo de aparición de enfermedades crónicas y degenerativas, tales como el cáncer y enfermedades cardiovasculares y neurodegenerativas (Manach *et al*., 2004).

1.4.1. Polifenoles en *Olea europaea* L.

Las aceitunas de mesa y el aceite de oliva son fuentes de una gran variedad de compuestos fenólicos con importantes actividades beneficiosas para la salud (Romani *et al*., 2019). En el caso de las plantas pertenecientes a la familia de las Oleáceas, como la *Olea europaea* L., se sintetizan un conjunto de polifenoles específicos, tales como los hidroxitirosol, tirosol y ácido elenólico que dan lugar a estructuras más complejas que se agrupan bajo el término de secoiridoides y tienen en la

oleuropeína y el ligstrósido a sus compuestos más representativos. La presencia de hidroxitirosol y sus derivados confiere a los alimentos procedentes del olivo unas características especiales, tal como ha quedado indicado en la alegación de salud aprobada por la *European Food and Segurity Safety* (EFSA, 2011) que indica que el consumo diario de 20 g de aceite de oliva virgen extra que con un contenido de 5 mg de hidroxitirosol y derivados (por ejemplo, oleuropeína o tirosol) contribuyen a la protección de los lípidos de la sangre frente al daño oxidativo (Reg. EU nº 432/2021). Esta declaración confiere a este alimento un papel importante en la disminución del riesgo de padecer enfermedades cardiovasculares.

Las aceitunas contienen un conjunto importante de polifenoles en un porcentaje que oscila entre el 1 y el 3% (Charoenprasert y Mitchell, 2012). La composición fenólica de las hojas, las aceitunas de mesa y el aceite de oliva, puede diferir de acuerdo con factores como la variedad de cultivo, las condiciones ambientales, la región, la etapa de maduración y el modo de procesamiento para obtener las aceitunas de mesa y el aceite de oliva (Laguerre *et al*., 2009; Volpe *et al*., 2014; D'Antuono *et al*., 2018). Así, por ejemplo, en lo que se refiere a la madurez del fruto, en la primera fase del desarrollo de la drupa, se produce la acumulación de uno de los principales polifenoles del olivo, la oleuropeína. Durante la maduración tiene lugar una reducción de la oleuropeína que va hidrolizándose, así como de la clorofila. En estadios más avanzados aparecen las antocianinas, lo que permite que el fruto adquiera un color cada vez más oscuro (Soler-Rivas *et al*., 2000). El aceite de oliva es el derivado de la *Olea europaea* L. que más se ha estudiado y en él se han determinado hasta 36 polifenoles diferentes (Charoemprasert y Mitchell, 2012; Olmo-García *et al*., 2019a). Atendiendo a las estructuras químicas de estos compuestos fenólicos, se han descrito 4 subclases de no flavonoides (alcoholes fenólicos, ácidos fenólicos, secoiridoides, lignanos) y 2 subclases de flavonoides (flavonas y flavonoles).

1.4.1.1. Síntesis de polifenoles

Tal como se ha comentado, los polifenoles constituyen uno de los grupos más grandes de metabolitos secundarios de las plantas que varían desde los que presentan en su estructura los anillos aromáticos más simples, a los más complejos como los secoiridoides. Por este motivo, en la síntesis de polifenoles en *Olea europaea* L. están implicadas diferentes vías que actúan como una red interconectada. Entre ellas se encuentran implicadas las vías del 2-C-metil-d-eritrodiol 4-fosfato (MEP), mevalonato (MVA), shikimato y fenilpropanoide (Figura 1.5 y Figura 1.6).

a) Síntesis de los alcoholes fenólicos, ácidos fenólicos, flavonoides y lignanos

La vía del shikimato y fenilpropanoide es la responsable de la síntesis de los alcoholes fenólicos (incluido hidroxitirosol y tirosol), ácidos fenólicos, flavonoides y lignanos (Guodong *et al*., 2019) (Figura 1.5). Estos grupos de polifenoles tienen su origen en la tirosina y la fenilalanina, cuya

síntesis se encuentra precedida por la ruta del ácido shikímico. Dicha vía utiliza como sustratos el fosfoenolpiruvato (PEP) originario de la glucólisis y la eritrosa 4-fosfato (E4P) procedente de la ruta de las pentosas fosfato y, a través de siete intermediarios metabólicos, genera el producto final corismato. Este último es un metabolito central en plantas que permite generar aminoácidos aromáticos y otros compuestos como el tetrahidrofolato (vitamina B9) (Tzin y Galili, 2010). Así, el corismato se transforma en prefenato y posteriormente en arogenato, el cual da lugar a tirosina y fenilalanina. La tirosina es clave para generar los alcoholes fenólicos tirosol e hidroxitirosol que participan en la síntesis de secoiridoides. De este modo, la síntesis de tirosol desde este aminoácido puede ocurrir por dos ramas: a través de la descarboxilación de la tirosina para dar tiramina y posteriormente 4-hidroxifenilacetaldehído (4-HPA) o bien a través de los ácidos *p*-hidroxifenilpirúvico (*p*-HPPA) y *p*-hidroxifenilacético (*p*-HPAA), donde la descarboxilación ocurre en el paso final que da lugar al tirosol (Guodong *et al*., 2019). Asimismo, el alcohol salidrosida podría proceder del tirosol generado por la rama tiramina/4-HPA (Saimaru y Orihara, 2010). La síntesis de hidroxitirosol desde la tirosina ocurre a través de 3,4-dihidroxifenialalanina (L-DOPA), que se descarboxila para formar dopamina y, posteriormente, 3,4-dihidroxifenilacetato (3,4-DHPA) (Guodong *et al*., 2019) (Figura 1.5).

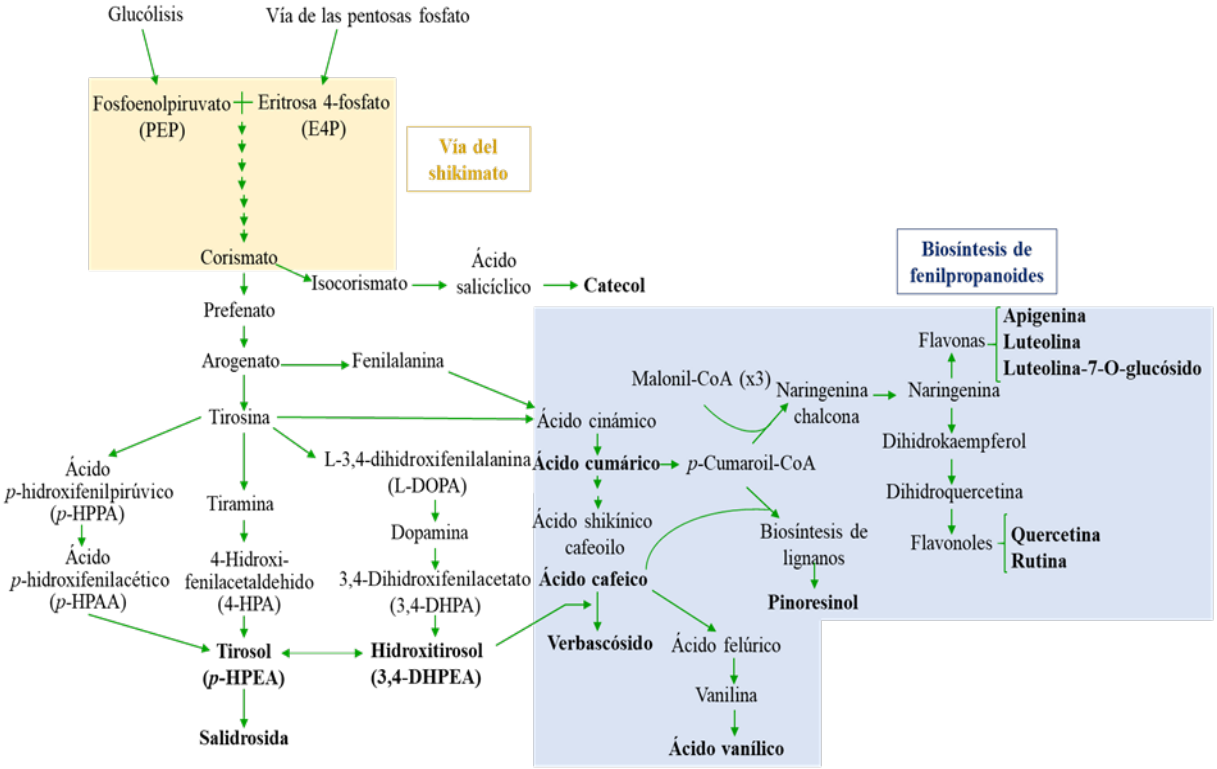

Figura 1.5. Representación esquemática de las rutas biosintéticas de los alcoholes fenólicos, ácidos fenólicos, flavonoides y lignanos en *Olea europaea* L.

La tirosina junto con la fenilalanina, ingresan en la vía de los fenilpropanoides para sintetizar el ácido cinámico, el precursor central que da lugar a los ácidos fenólicos, los flavonoides y los lignanos. El ácido cinámico se transforma en ácido cumárico y puede seguir dos vías. Por un lado, puede generar el intermediario ácido shikínico cafeoilo, que se transforma en ácido cafeico (Guodong *et al*., 2019), el cual junto al hidroxitirosol genera verbascósido (Ryan *et al*., 2003; Saimaru y Orihara, 2010; Alagna *et al*., 2012). Este es el motivo por el cual el fruto puede presentar concentraciones tan elevadas de este compuesto (Medjkouh *et al*., 2018). El ácido cafeico también se puede transformar en ácido ferúlico (Guodong *et al*., 2019), que según la ruta que presentan las plantas, podría dar lugar a ácido vanílico (Anantharaju *et al*., 2016).

La otra vía que puede seguir el ácido cumárico, es su transformación en *p*-cumaroil-CoA, el cual participa en la síntesis de flavonoides y lignanos. Para la síntesis de los flavonoides, una unidad de *p*-cumaroil-CoA y tres de malonil-CoA forman la naringenina chalcona que genera naringenina. Ésta última se bifurca en dos ramas para dar lugar por un lado a las flavonas apigenina, luteolina y luteolina-7-O-glucósido y, por otro, a los flavonoles quercetina y rutina (Guodong *et al*., 2019). Asimismo, el *p*-cumaroil CoA junto con el ácido cafeico desembocarían en la síntesis del lignano pinoresinol, aunque los intermediarios metabólicos de esta vía en *Olea europaea* L. no han sido estudiados (Alagna *et al*., 2012).

En cuanto al catecol, su metabolismo no ha sido dilucidado en *Olea europaea* L., aunque en otras plantas se forma a partir del ácido salicílico derivado del corismato (Akhtar y Pichersky, 2013).

b) Síntesis de los secoiridoides

La biosíntesis de los secoiridoides, al igual que sucede para los esteroles y los terpenoides, se inicia a partir de isopentenil difosfato (IPP), el cual se tranforma en geranil difosfato (GPP), un intermediario común de ambos grupos de compuestos (Figura 1.6). El IPP puede proceder de la vía del MVA o de la vía del MEP. La primera se encuentra en organismos complejos como animales, hongos, arqueas y algunas bacterias, mientras que la segunda es característica de organismos procariotas y parásitos apicomplexos (Frank y Groll, 2017). En las plantas, se han descrito ambas vías, pero en compartimentos independientes. Las enzimas para la ruta del MVA se encuentran en el citosol, mientras que las de MEP se encuentran en los plástidos (Tetali, 2019). Se ha sugerido que la biosíntesis de secoiridoides podría iniciarse a partir del IPP procedente de la vía MEP (Alagna *et al*., 2012; Ali *et al*., 2019). De este modo, el IPP seguiría la rama de iridoides cuya secuencia es: ácido deoxilogánico, ácido 7-epilogánico, ácido 7-cetologánico y 7-cetologanina (Gutierrez-Rosales *et al*., 2012). Éste último iridoide carbocíclico da lugar al oleósido-11 metil éster que se transforma en 7-β-1-glucopiranosil-11 metil oleósido, el cual bajo una reacción de esterificación con tirosol que aporta la fracción fenólica, genera ligstrósido. Seguidamente, el ligstrósido se hidroxila dando lugar a la oleuropeína (Gutierrez-Rosales *et al*., 2012). El papel desempeñado por

los compuestos loganina y secologanina en la biosíntesis de secoiridoides oleósidos en *Olea europaea* L. sigue siendo una controversia, ya que podrían ser intermedios de la oleuropeína (Guoodong *et al*., 2019) (Figura 1.6).

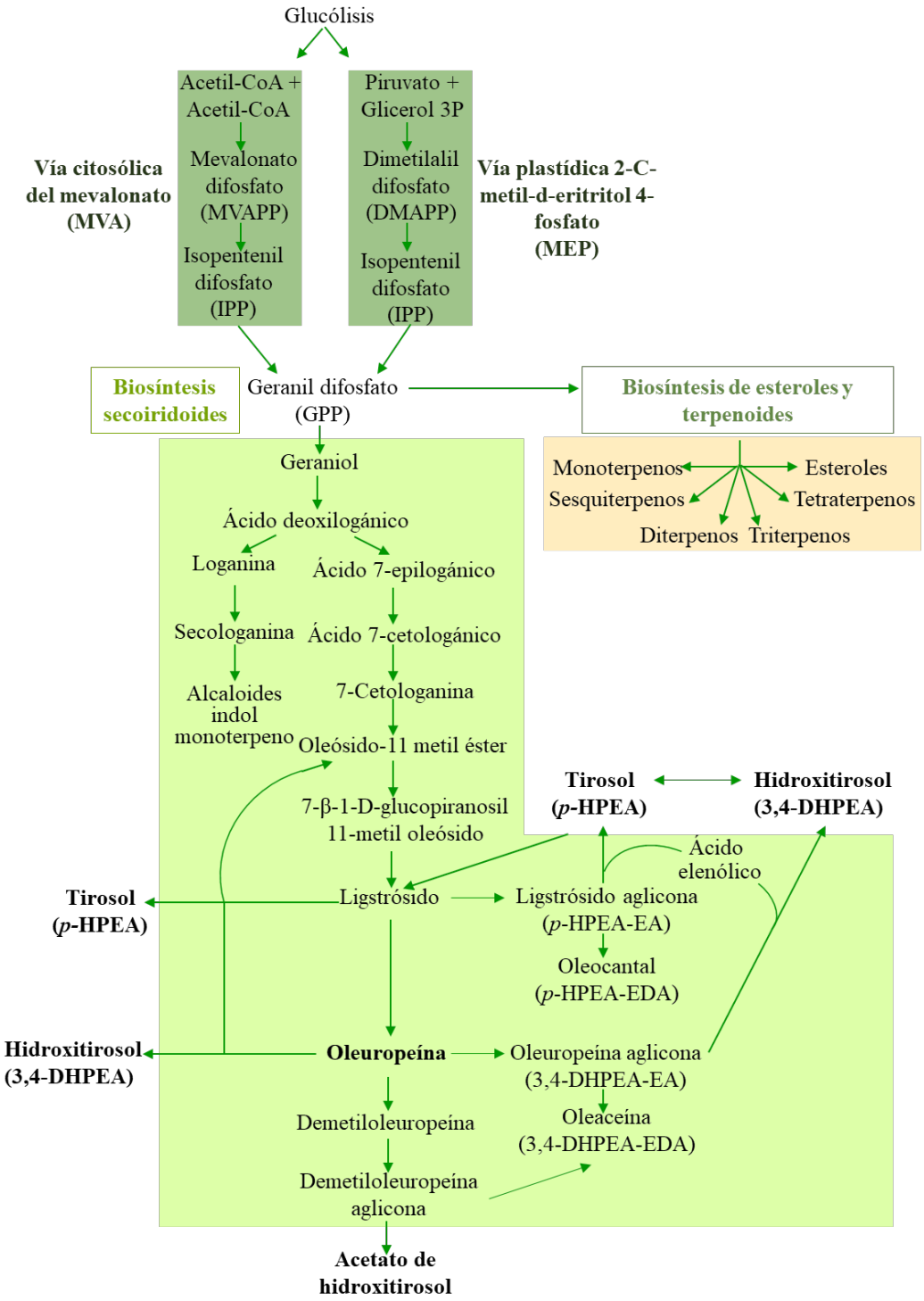

Figura 1.6. Representación esquemática de la ruta biosintética de los secoiridoides en *Olea europaea* L.

El ligstrósido y la oleuropeína se acumulan durante la maduración. La hidrólisis de estos compuestos por la enzima β-glucosidasa da lugar a las formas aglicona de ligstrósido y oleuropeína. De este modo, la aglicona de ligstrósido sería la forma aldehídica de ácido elenólico unido a tirosol (*p*-HPEA-EA), mientras que la aglicona de oleuropeína sería la forma aldehídica de ácido elenólico unido a hidroxitirosol (3,4-DHPEA-EA). Las agliconas de ligstrósido y oleuropeína bajo una posterior hidrólisis por esterasas darían lugar a ácido elenólico y a tirosol e hidroxitirosol, respectivamente. Alternativamente, estas agliconas de ligstrósido y oleuropeína pueden sufrir una descarboxilación dando lugar a la correspondiente forma dialdehídica de ácido elenólico unido a tirosol, también denominada oleocantal (*p*-HPEA-EDA) y a la forma dialdehídica de ácido elenólico unido a hidroxitirosol, también designada como oleaceína (3,4-DHPEA-EDA) (Gutierrez-Rosales *et al*., 2012; Johnson *et al*., 2018). Otros estudios sugieren que la síntesis de oleaceína tendría lugar a partir de la demetiloleuropeína, que es un derivado de la oleuropeína (Obied *et al*., 2008). Asimismo, parece que la demetiloleuropeína también podría dar lugar al alcohol fenólico acetato de hidroxitirosol (Sivakumar *et al*., 2007) (Figura 1.6).

El ligstrósido y la oleuropeína también pueden sufrir una hidrólisis directa del enlace éster dando lugar a oleósido 11-metil éster y a tirosol e hidroxitirosol (Johnson *et al*., 2018) (Figura 1.6).

1.4.1.2. No flavonoides

Los polifenoles no flavonoides de las aceitunas pertenecen a las subclases: alcoholes fenólicos, ácidos fenólicos, secoiridoides y lignanos. Entre los alcoholes fenólicos se encuentran el hidroxitirosol, tirosol, acetato de hidroxitirosol, salidrosida y catecol. Los ácidos fenólicos mayoritarios son el ácido vanílico, ácido *p*-cumárico, ácido *o*-cumárico, ácido cafeico y verbascósido. El serocoiridoide más abundante es la oleuropeína y, entre los lignanos, destaca el (+)-pinoresinol.

a) Alcoholes fenólicos

Los alcoholes fenólicos poseen un anillo aromático unido a un hidroxilo tal como se muestra en la Figura 1.7.

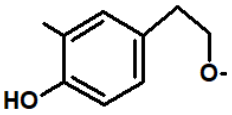

Figura 1.7. Esqueleto de los alcoholes fenólicos.

El hidroxitirosol (2-(3,4-dihidroxifenil)etanol) se encuentra en el fruto y las hojas de *Olea europaea* L. (Fernández-Bolaños *et al*., 2012). Su fuente dietética mayoritaria son las aceitunas de mesa (Boskou, 2010) y el aceite de oliva (Waterman y Lockwood, 2007), aunque también se han detectado en el vino tinto y blanco pudiendo producirse a partir del tirosol durante la fermentación alcohólica (Fernández-Mar *et al*. 2012). Este polifenol ha sido ampliamente estudiado, demostrándose que posee poder antioxidante, cardioprotector, anticancerígeno, neuroprotector, antidiabético, regulador de lípidos, antimicrobiano, antiviral, antiinflamatorio, modulador de la

alergia y protector de los huesos, así como frente a la radiación UV-B (Karković Marković *et al*., 2019).

El tirosol (2-(4-hidroxifenil)etanol) es un alcohol primario y al igual que el hidroxitirosol, está presente concretamente en el fruto y las hojas de *Olea europaea* L. (Ghanbari *et al*., 2012). Su fuente dietética primordial son las aceitunas de mesa (Boskou *et al*., 2006) y el aceite de oliva (Waterman y Lockwood, 2007), aunque también se han encontrado en los vinos blanco y tinto, en el vermut y en la cerveza, posiblemente generado mediante la degradación de aminoácidos (Almeida *et al*., 2006; Fernández-Mar *et al*. 2012; Chen *et al*., 2014). Se ha sugerido que posee propiedades antioxidantes, aunque menos intensas que el hidroxitirosol (Waterman y Lockwood, 2007). También goza de capacidad, antiinflamatoria, cardioprotectora, neuroprotectora, antidiabética, regulador de lípidos, antibacteriana, antiparasitaria, protector de los huesos, así como de la radiación UV-B (Karković Marković *et al*., 2019).

El acetato de hidroxitirosol (2-(3,4-dihidroxifenil)etil acetato) es un derivado de hidroxitirosol presente en el fruto y las hojas de *Olea europaea* L. (Morelló *et al*., 2004; Goulas *et al*., 2009). Su ingesta dietética tiene lugar a través de las aceitunas de mesa (Romero *et al* 2004) y aceite de oliva (Brenes *et al*., 1999), pudiendo ser su concentración igual o incluso más alta que la del hidroxitirosol. Los efectos beneficiosos más conocidos incluyen la capacidad antioxidante, neuroprotectora y antiinflamatoria (Rosillo *et al*., 2015).

La salidrosida (2-(4-hidroxifenil)etil β−D-glucopiranósido) se encuentra en plantas del género Rhodiola, principalmente en la raíz de *Rhodiola rosea* L. (Perfumi *et al*., 2007), aunque también se ha detectado en otras plantas como *Ligustrum lucidum* (Shi *et al*., 1998) y en las semillas del fruto de *Olea europaea* L. (Obied *et al*., 2008). Su ingesta alimentaria está restringida principalmente a las aceitunas de mesa. Se han descrito diversos efectos beneficiosos tales como anticancerígeno, antifatiga, antioxidante, antiinflamatorio, antiviral, cardioprotector, neuroprotector, hepatoprotector, así como protector renal (Xie *et al*., 2020).

El catecol (1,2-bencenodiol) es un fenol alcohólico presente en frutas (p. ej. manzana, albaricoque, aguacate, uvas, mango, melocotón, pera y ciruela), vegetables (p. ej. patata) y setas (Corzo-Martínez *et al*., 2012). También se ha detectado en los alimentos derivados de *Olea europaea* L., tales como las aceitunas de mesa (Romero *et al*., 2004) y el aceite de oliva (García *et al*., 2006). El catecol se utiliza como colorante, como reactivo para fotografía, en la producción de caucho y plásticos, en la síntesis de insecticidas, perfumes y algunos fármacos (Balderas-Hernández *et al*., 2014). Entre las propiedades biológicas, destaca su capacidad antioxidante y antiinflamatoria (Huber *et al*., 2018).

b) Ácidos fenólicos

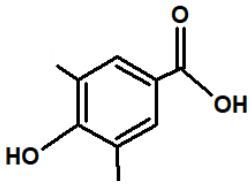

Figura 1.8. Esqueleto de los ácidos hidroxibenzoicos.

Los ácidos fenólicos contienen en su esqueleto un anillo fenólico unido a un ácido orgánico. Estos compuestos pueden derivar del ácido benzoico, cuya estructura química básica es de C6-C1 (Figura 1.8), del ácido cinámico (Figura 1.9), con una estructura química C6-C3 o de otros ácidos fenólicos y derivados. Los más predominantes en el fruto incluyen el ácido cafeico, los ácidos clorogénicos (ferúlico, vanílico, cumárico y siríngico), y el verbascósido, que es un complejo formado por hidroxitirosol y ácido cafeico esterificado mediante un azúcar y, puede llegar a concentraciones de 3 g/kg (peso seco) en el fruto sin procesar.

El ácido vanílico (ácido 4-hidroxi-3-metoxibenzoico) es un derivado del ácido cafeico, que se encuentra principalmente en extractos de *Vanilla planifolia*, una planta nativa de México (Salazar-Rojas *et al*., 2012), aunque también puede estar presente en otras plantas y frutos (Calixto-Campos *et al*., 2015). Debido a su aroma característico, se utiliza frecuentemente en fragancias y como aditivo alimentario. Además, es uno de los principales metabolitos que se encuentran en sangre en personas consumidoras de té verde (Gitzinger *et al*., 2012). Se ha detectado en el fruto y las hojas de *Olea europaea* L. (Romani *et al*., 1999; Mohamed *et al*., 2018), así como en las aceitunas de mesa (D'Antuono *et al*., 2018) y el aceite de oliva (Ricciutelli *et al*., 2017). Entre sus propiedades biológicas destacan su capacidad quimiopreventiva, antioxidante, antimicrobiana y antiviral (Stanely Maizen Prince *et al*., 2011).

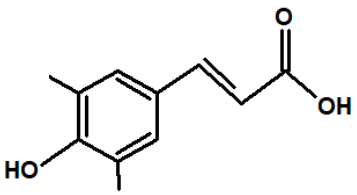

Figura 1.9. Esqueleto de los ácidos hidroxicinámicos.

El ácido *p*-cumárico (ácido *trans*-4-hidroxicinámico) es un metabolito intermediario de la síntesis de una gran variedad de fenoles y se encuentra presente de forma esterificada o libre en hongos y plantas que incluyen las gramíneas (p. ej. maíz, avena y trigo), frutas (p. ej. manzana, peras, uvas, bayas, naranjas y tomates) y vegetales (p. ej. cebollas, frijoles y papas) (Pei *et al*., 2016). También se ha detectado en las hojas y el fruto de *Olea europaea* L. (Bianco y Ucella, 2000; Brahmi *et al*., 2013) así como en sus productos derivados, las aceitunas de mesa (Cabrera-Bañegil *et al*., 2017) y el aceite de oliva (Olmo-García *et al*., 2019a). Entre las actividades biológicas que ejerce, se encuentran su poder antioxidante, antimicrobiano, antiviral, anticancerígeno, antidiabético, antipirético, analgésico, hipopigmentante, protector de la úlcera gástrica, inhibidor de la agregación plaquetaria y sedante (Pei *et al*., 2016).

El ácido *o*-cumárico (ácido *trans*-2-hidroxicinámico) es un isómero del ácido *p*-cumárico encontrado en plantas como la alfalfa, la ortiga, el "guaco" (*Mikania laevigata* Schultz Bip. Ex. Baker (Asteraceae) y *Mikania glomerata* Spreng) y en otras especies herbáceas como *Caucalis*

platycarpos L. (Sen *et al*., 2015). Está presente en alimentos como el café, el té y las nueces (Sen *et al*., 2015), aunque también se ha detectado en el fruto de *Olea europaea* L., principalmente en las aceitunas de mesa (Aktas *et al*., 2014), no habiendo evidencia de su presencia en el aceite de oliva. Posee actividades antioxidantes, antilipidémicas y anticancerígenas (Sen *et al*., 2015).

El ácido cafeico (ácido 3,4-dihidroxicinámico) es un fenol ampliamente distribuido en tejidos vegetales, estando presente en una variedad de frutas (p. ej. arándanos, fresas, manzanas, naranja, albaricoque y manzana), vegetales (p. ej. coles de Bruselas, brócoli, repollo y zanahorias), bebidas (p. ej. vino, café y sidra) e hierbas medicinales asiáticas (Magnani *et al*., 2014; Lucci *et al*., 2015). Su presencia en las hojas y en el fruto de *Olea europaea* L. ha sido demostrada (Ryan *et al*., 2003; Mohamed *et al*., 2018), así como en las aceitunas de mesa (Boskou *et al*., 2006) y en el aceite de oliva (Monasterio *et al*., 2017). Entre sus propiedades biológicas, destaca su poder antioxidante, antimicrobiano, antibacteriano, antiviral, antiinflamatorio, citotóxico, anticancerígeno y atenuante del desarrollo de la enfermedad de Alzheimer (Magnini *et al*., 2014)

El verbascósido (acteósido), es un éster heterosídico del ácido cafeico e hidroxitirosol. Se encuentra ampliamente extendido en la naturaleza, siendo predominante en las especies del género *Verbascum* (Alipieva *et al*., 2014). Sin embargo, su fuente más conocida procede de los productos derivados de *Olea europaea* L. (Caturla *et al*., 2010) y las hojas de *Lippia citriodora* (hierba luisa) (Etemad *et al*., 2016). Las propiedades biológicas de este compuesto incluyen la actividad antioxidante, antiinflamatoria, anticancerígena, neuroprotectora, antibacteriana y antiandrogénica (Alipieva *et al*., 2014).

c) Secoiridoides

La oleuropeína ((2S,3E,4S)-3-etilideno-2-(β-D-glucopiranosiloxi)-3,4-dihidro-5-(metoxicarbonil)-2H-piran-4-ácido acético 2-(3,4-dihidroxifenil)etil éster) constituye el secoiridoide más abundante que se encuentra en *Olea europaea* L., siendo altamente concentrado en las fases tempranas de crecimiento del fruto y las hojas (Soler-Rivas *et al*., 2000). También está presente en otros géneros de la familia Oleaceae como *Fraxinus excelsior*, *F. angustifolia*, *F. chinensis* y *F. mandshurica* var. *japónica*, *Syringa josikaea* y *S. vulgaris*, *Phillyrea latifolia*, *Ligustrum ovalifolium* y *L. vulgare*, *Jasminum polyanthum* y *Osmanthus asiaticus* (Soler-Rivas *et al*., 2000). La oleuropeína tiene una gran variedad de actividades biológicas, entre las que destacan su capacidad anticancerígena, antimicrobiana, hipoglucemiante, antioxidante, antiviral, antihipertensiva, hepatoprotectora, neuroprotectora, vasodilatadora, antiinflamatoria, antireumática, antiaterogénica y antipirética (Haloui *et al*., 2011; Przychodzen *et al*., 2019).

d) Lignanos

Los lignanos se caracterizan por tener una estructura no bien definida y se forman por la condensación de aldehídos aromáticos. Los lignanos principales en aceitunas son el (+)-pinoresinol y el (+)-1-acetopinoresinol.

El (+)-pinoresinol (4,4′-((1S,3aR,4S,6aR)-hexahidrofuro[3,4-c]furan-1,4-diil)bis(2-metoxifenol)), se encuentra también en los cereales (cebada, trigo, mijo, avena y centeno), frutas (uvas, kiwi, limón y naranjas), frutos secos y semillas (nueces, anacardos, almendras, castañas, pistachos, semillas de lino y semillas de sésamo) y vegetales (espárragos, pepino, berenjena, tomate y rábano). En *Olea europaea* L. está presente en el fruto (Bodoira *et al*., 2016) y las hojas (Olmo-García *et al*., 2018a), así como en las aceitunas de mesa (Perpetuini *et al*., 2018) y el aceite de oliva (Olmo-García *et al*., 2019a). Se ha sugerido que el pinoresinol posee actividades beneficiosas como anticancerígeno, antioxidante, antiinflamatorio, antifúngico, hipoglucemiante y neuroprotector (López-Biedma *et al*., 2016; Yu *et al*., 2019).

1.4.1.3. Flavonoides

Los flavonoides predominantes en las aceitunas pertenecen a dos subclases, las flavononas (flavonas) y los flavonoles. Entre las flavonas se encuentran principalmente la apigenina, la luteolina y la luteolina-7-glucósido, mientras que entre los flavonoles están la rutina, la quercetina y la quercetina 3-ramnósido.

a) Flavononas

La apigenina (4′,5,7-trihidroxiflavona) es una flavona natural presente en muchos alimentos que incluyen frutas (p. ej. pomelo y naranjas), verduras (p. ej. cebolla, pimiento y apio), cereales (p. ej. maíz, arroz y germinados de trigo), plantas medicinales y culinarias (p. ej. perejil y manzanilla) y bebidas alcohólicas (p. ej. vino y cerveza). También se han encontrado en otras plantas de uso medicinal o culinario (Ali *et al*., 2017). Se han identificado tanto la forma aglicona como la glicosilada en las hojas y el fruto de *Olea europaea* L. (Romani *et al*., 1999; Yorulmaz *et al*., 2012; Mohamed *et al*., 2018; Guex *et al*., 2019), así como en las aceitunas de mesa (Aktas *et al*., 2014; Selli *et al*., 2018) y el aceite de oliva (Inarejos-García *et al*., 2010; Olmo-García *et al*., 2019a). Posee diversas actividades biológicas, entre las que destacan su poder antidiabético, anticancerígeno, analgésico frente a dolores óseos, quimiopreventivo frente a enfermedades neurológicas como la enfermedad de Alzheimer y/o de Parkinson y reductor de los niveles de ansiedad, depresión e insomnio (Shalei *et al*., 2019).

La luteolina (3′,4′,5,7-tetrahidroxiflavona) en una flavona ampliamente extendida en la naturaleza, cuyas fuentes dietéticas proceden de vegetales (p. ej. lechuga, brócoli, cebolla, pepino, nabo, zanahorias, pimientos, coles, apio y alcachofas), frutas (p. ej. granada y manzana), cereales (p. ej.

brotes de trigo sarraceno), chocolate, té de rooibos, plantas medicinales y culinarias (p. ej. orégano, perejil, tomillo, menta, romero y albahaca), así como de otras plantas asiáticas con diversos usos (p. ej. *Chrysanthemum indicum* var. albescens, *Codariocalyx motorius* (Houtt.) H. Ohashi, y *Artemisia asiática* Nakai) (Aziz *et al*., 2018). También se encuentra presente en las hojas y el fruto de *Olea europaea* L. (Romani *et al*., 1999; Mohamed *et al*., 2018), así como en las aceitunas de mesa (D'Antuono *et al*., 2018) y el aceite de oliva (Olmo-García *et al*., 2019a). Entre sus actividades biológicas, destacan su capacidad antioxidante, antiinflamatoria, antimicrobiana, quimiopreventiva, cardioprotectora, antidiabética, neuroprotectora y antialérgica (López-Lázaro, 2009).

La luteolina-7-O-glucósido (3',4′,5,7-tetrahidroxiflavona 7-glucósido) es una forma β-D-glicosilada de la luteolina que se encuentra en diversos alimentos como las aceitunas, salvia, perejil, tomillo, comino, alcachofas, apio, cacao y alcaparras. También se ha detectado en las hojas y el fruto de *Olea europaea* L. (Yorulmaz *et al*., 2012; Mohamed *et al*., 2018), así como en las aceitunas de mesa (Aktas *et al*., 2014) y el aceite de oliva (Olmo-García *et al*., 2019a). Entre sus actividades biológicas, destaca su capacidad antitumoral, antioxidante, antiinflamatoria y neuroprotectora (Chen *et al*., 2018).

b) Flavonoles

Entre los flavonoles, destaca la quercetina (2-(3,4-dihidroxifenil)-3,5,7-trihidroxi-4H-1-benzopiran-4-ona), que es la aglicona de la rutina. Este compuesto se encuentra en vegetales (p. ej., cebolla, espárragos y pimientos), frutas (p. ej., arándanos, manzanas, uvas y cerezas) y bebidas como el vino y el té negro o verde. No obstante, en muchas plantas se encuentra glicosilada, como es el caso de las cebollas, la principal fuente de quercetina de la dieta, las cuales contienen mayoritariamente quercetina-4-glucósido y quercetina-3,4-diglucósido, mientras que las manzanas pueden contener quercetina-3-O-rutinósido (rutina), quercetina-3-O-glucósido, quercetina-3-O-galactósido y quercetina-3-O-ramósido (Erlund, 2004; Huang *et al*., 2020). El fruto y las hojas de *Olea europaea* L., así como las aceitunas de mesa y el aceite de oliva, contienen quercetina en su forma aglicona (Dell´Agli *et al*., 2008; Bali *et al*., 2014; Maalej *et al*., 2017; D'Antuono *et al*., 2018). Se ha sugerido que la biodisponibilidad de la quercetina tiene lugar fundamentalmente en su forma aglicona, para lo cual, las enzimas intestinales y las bacterias se encargan de dicha hidrólisis (Wein *et al*., 2018). Entre las actividades biológicas que caracterizan a este polifenol, se encuentra su capacidad antiinflamatoria, antioxidante, cardioprotectora, neuroprotectora, anticancerígena, gastroprotectora, antibacterana, antiviral y antialergénica (Anand David *et al*., 2016).

La rutina (quercetina 3-rutinósido) se compone de quercetina con un grupo hidroxi sustituido por el disacárido rutinosa (glucosa y ramnosa). Generalmente se suele encontrar en los mismos alimentos que la quercetina, tales como las cebollas, manzanas, bayas, vino y té (Chua, 2013). Se ha detectado su presencia en las hojas y el fruto de *Olea europaea* L. (Romero *et al*., 2017;

Mohamed *et al*., 2018) así como en las aceitunas de mesa (Melliou *et al*., 2015), estando ausente en el aceite de oliva (Artajo *et al*., 2007; Olmo-García *et al*., 2019a). Entre sus propiedades biológicas destacan su capacidad antioxidante, antimicrobiana, antiinflamatoria, anticancerígena, antidiabética, antialergénica, neuroprotectora y cardioprotectora (Gullón *et al*., 2017).

1.5. Triterpenos

Los triterpenos son una de las principales clases de productos naturales que contienen seis unidades de isopreno con la fórmula molecular básica de $C_{30}H_{48}$. Los triterpenos se clasifican en triterpenos acíclicos, monocíclicos, bicíclicos, tricíclicos, tetracíclicos y pentacíclicos (Hill y Connolly, 2018). Pueden hallarse en su en forma libre, formando glucósidos u otros compuestos habiéndose identificado más de 20000 compuestos diferentes en la naturaleza (Thimmappa *et al.*, 2014). La mayoría de estos productos naturales se sintetizan y acumulan en las plantas como metabolitos secundarios que contribuyen a su protección (Szakiel *et al*, 2012). Desde la antigüedad, las plantas que contienen estos metabolitos secundarios se han utilizado en la medicina tradicional para tratar diferentes enfermedades humanas (Ghosh, 2016). En las últimas décadas, se han llevado a cabo numerosos estudios para dilucidar los efectos beneficiosos sobre la salud, habiéndose descrito numerosas actividades biológicas, tales como antitumorales, antiinflamatorias, antidiabéticas, antivirales y hepatoprotectoras, entre otras (Sheng y Sun, 2011; Lozano-Mena *et al*., 2014; Jesus *et al*., 2015; Lin *et al*., 2016; Sharma *et al*., 2018; Ghante y Jamkhande, 2019; Juan *et al*., 2019).

1.5.1. Síntesis de triterpenos pentacíclicos

En las plantas superiores, los triterpenos se forman predominantemente a través de la vía del MVA, llamada así por formar como intermedio al ácido mevalónico (MVA) (Figura 1.10). El acetil-CoA se transforma en 3-hidroxi-3-metilglutaril-CoA (HMG-CoA) y la enzima HMG-CoA reductasa da lugar al MVA, que es el precursor de la vía (Buhaescu y Izzedine, 2007). La participación de dos quinasas conduce a la generación de IPP que se isomeriza a dimetilalil difosfato (DMAPP) por la acción de la enzima isopentenil difosfato isomerasa. IPP y DMAPP son dos compuestos clave en la síntesis de terpenos (Tetali, 2019).

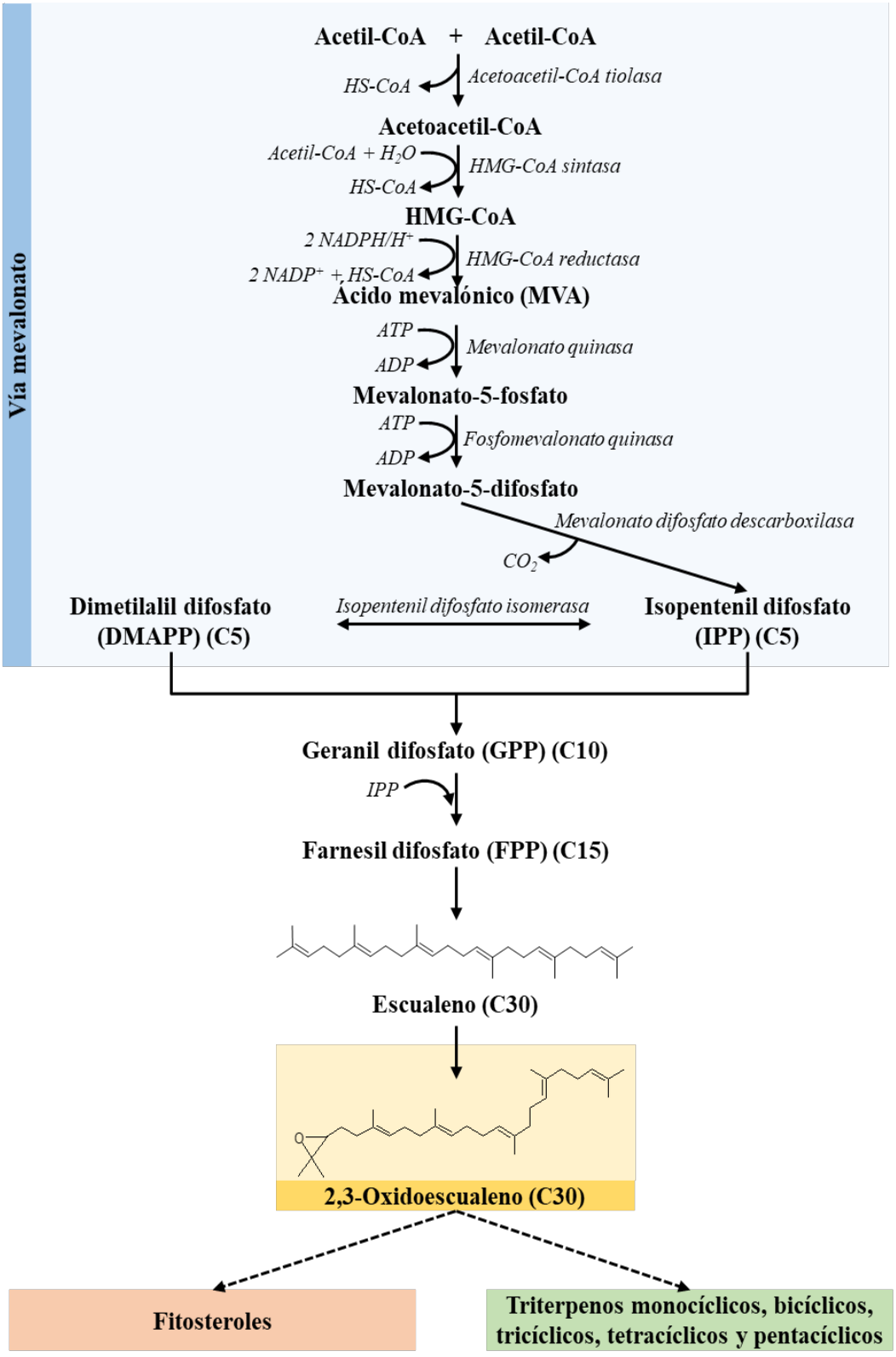

Figura 1.10. Biosíntesis de triterpenos a través de la ruta del mevalonato en el citosol de plantas superiores. Las flechas simples representan la conversión de un paso, las flechas discontinuas representan múltiples pasos (Juan *et al*., 2020).

Durante muchos años, se pensó que IPP y DMAPP se formaban exclusivamente a través de la vía del MVA en las plantas superiores. Sin embargo, actualmente se conoce la existencia de la vía MEP que se origina a partir de los sustratos ácido pirúvico y gliceraldehído-3-fosfato (Frank y Groll, 2017). Esta vía participaría principalmente en la formación de monoterpenos (C10), diterpenos (C20), sesterpenos (C25), carotenoides (C40) y fitol de cadena larga (Ghosh, 2016). En cambio, la vía citosólica de MVA sería utilizada en la formación de sesquiterpenos (C15), triterpenos (C30) y politerpenos (>45) (Ghosh, 2016).

La síntesis de triterpenos en las plantas tiene lugar mediante una reacción de condensación de 2 unidades de IPP y 1 unidad de DMAPP para producir farnesil difosfato (FPP) (C15) en un proceso de dos pasos que incluye la formación del intermediario GPP (Figura 1.10). Luego, dos unidades de FPP se fusionan para formar escualeno (C30) que sirve como precursor de triterpenos acíclicos en las plantas (Ghosh, 2016)

El escualeno se somete a una reacción de epoxidación, sintetizando el 2,3-oxidosqualeno (C30), que es el punto en el que se inicia tanto la vía para la biosíntesis de metabolitos primarios como secundarios (Figura 1.10). La actividad de las oxidoescualeno ciclasas, también conocidas como triterpeno sintasas, cataliza las reacciones de formación de anillos para formar los diversos triterpenos monocíclicos, bicíclicos, tricíclicos, tetracíclicos y pentacíclicos (Ghosh, 2016). De lo contrario, la ciclación de 2,3-oxidosqualeno a cicloartenol a través de cicloartenol sintasa sirve para la formación de fitoesteroles de membrana y hormonas esteroides (Ghosh, 2016).

Dentro del grupo de triterpenos, se hallan los pentacíclicos que tienen importantes actividades biológicas como antitumoral, antiinflamatorio, antidiabético, antiviral y hepatoprotector, entre otras (Sheng y Sun, 2011; Lozano-Mena *et al*., 2014; Jesus *et al*., 2015; Lin *et al*., 2016; Sharma *et al*., 2018; Ghante y Jamkhande, 2019; Juan *et al*., 2019). Estos compuestos se agrupan en tres grupos principales: oleanano, ursano y lupano. Los más abundantes en las plantas superiores son los compuestos oleanano, siendo el ácido oleanólico uno de los triterpenos más ampliamente distribuidos en la naturaleza, seguido del ácido maslínico (Hill y Connolly, 2018). Estos compuestos se originan a partir de la ß-amirina, que forma eritrodiol que es el precursor de los ácidos oleanólico y maslínico.

1.5.2. Triterpenos pentacíclicos en *Olea europaea* L.

Los derivados de la *Olea europaea* L., la aceituna de aderezo y el aceite de oliva, constituyen unos componentes fundamentales de la dieta, principalmente en los países que se hallan a lo largo de la costa Mediterránea (Guo *et al*., 2018). En consecuencia, *Olea europaea* L. ha proporcionado tradicionalmente importantes beneficios económicos y dietéticos para la población de la región. Además, el aceite de oliva y las hojas del olivo se han utilizado ampliamente en medicina

tradicional, destacando su uso como hipoglucemiante, antihipertensivo, antimicrobiano y antiaterosclerótico, entre otros (EI SN y Karakaya, 2009).

La síntesis de los triterpenos pentacíclicos en *Olea europaea* L. fue estudiada por Stiti *et al*. (2007) y postularon una vía biosintética a partir del 2,3-oxidoscualeno (Figura 1.11). En el olivo, la producción de triterpenos pentacíclicos requiere la generación de diferentes intermedios carbocatiónicos. De este modo, la ciclación del 2,3-oxidoescualeno conduce a la formación del catión dammarenilo tetracíclico que se transforma en el catión baccharenilo y lupenilo antes de la formación de los cationes oleanilo y ursanilo (Stiti *et al*., 2007) originando los tres grupos de triterpenos pentacíclicos: lupano, oleanano y ursano. En *Olea europaea* L., los principales triterpenos pentacíclicos surgen de la estabilización del catión oleanilo a ß-amirina (olean-12-en-3ß-ol). Este compuesto se oxida secuencialmente en la posición C-28 por una enzima P450 del citocromo (CYP) para producir, en primer lugar, el alcohol eritrodiol y, en segundo lugar, el ácido oleanólico (Ghosh, 2016). Finalmente, una enzima diferente CYP P450 cataliza la adición de un grupo hidroxilo formando ácido maslínico (Dai *et al*., 2019). Los triterpenos pentacíclicos del grupo oleanano se producen en mayores cantidades que los de las clases ursano o lupano, tal como describieron Stiti *et al.* (2007). Aunque estos autores identificaron 19 triterpenos pentacíclicos diferentes, demostraron que los triterpenoides del grupo oleanano representaban el 99.2% de los triterpenos de las aceitunas (Stiti *et al*., 2007).

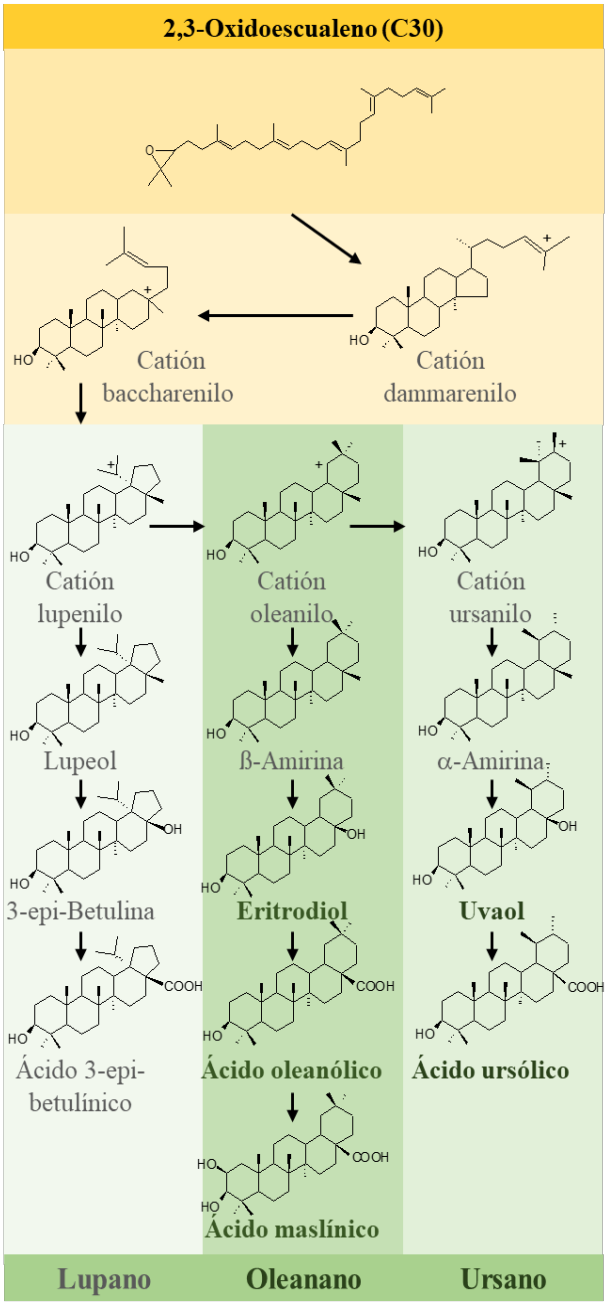

Figura 1.11. Síntesis de triterpenos pentacíclicos en la *Olea europaea* L.

1.5.2.1. Alcoholes triterpénicos pentacíclicos

Se ha encontrado eritrodiol o 3ß-olean-12-en-3,28-diol (Figura 1.12) en muy pocas especies, aparte de *Olea europaea* L., donde se ha detectado en la fruta y el aceite en bajas cantidades (Szakiel *et al*, 2012). A pesar de la escasa información sobre este compuesto, se ha sugerido que el eritrodiol ejerce actividades antitumorales, antiinflamatorias y cardioprotectoras (Sánchez-Quesada, *et al.*, 2013).

ERY

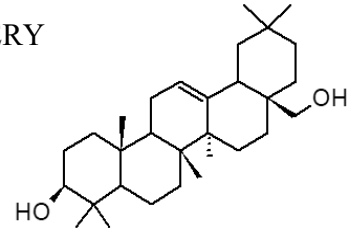

Figura 1.12. Estructura química del eritrodiol (ERY).

1.5.2.2. Ácidos triterpénicos pentacíclicos

El ácido oleanólico o el ácido 3ß-hidroxi-olean-12-en-28-oico (Figura 1.13) se forma después de la oxidación del alcohol en la posición C28 del eritrodiol. Este compuesto ha sido aislado de más de 1600 especies, incluidos alimentos comestibles y plantas medicinales (Hill y Connolly, 2018). Este triterpeno pentacíclico recibe el nombre de oleanólico debido a su presencia en *Olea europaea* L. (Simonsen y Ross, 1957). El ácido oleanólico posee actividades farmacológicas prominentes, siendo las actividades hepatoprotectoras, antiinflamatorias, antioxidantes, antidiabéticas y antitumorales las más destacadas (Pollier y Goossens, 2012).

OA

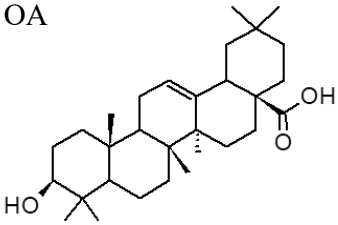

Figura 1.13. Estructura química del ácido oleanólico (OA).

El ácido maslínico o el ácido 2α, 3ß-hidroxi-olean-12-en-28-oico (Figura 1.14) se sintetiza a partir del ácido oleanólico y difiere solo en un grupo hidroxilo adicional en la posición del carbono 2. El ácido maslínico se aisló en 1927 de las hojas de *Crataegus oxycantha* L. y se denominó ácido cratególico (Bächler, 1927). Este compuesto se ha detectado en 30 especies diferentes siendo uno de los principales triterpenos pentacíclicos encontrados en *Olea europaea* L. (Giménez *et al*., 2015). El ácido maslínico ha recibido menos atención en comparación con los numerosos estudios dedicados a su precursor, el ácido oleanólico, a pesar de poseer propiedades prometedoras para la salud, tales como antitumoral, antidiabético, antioxidante, cardioprotector, neuroprotector, antiparasitario y estimulante del crecimiento (Lozano-Mena *et al*. 2014).

MA

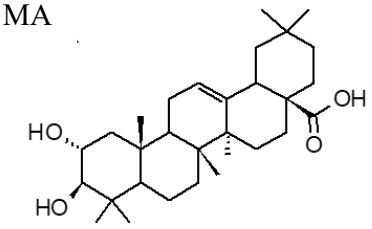

Figura 1.14. Estructura química del ácido maslínico (MA).

II. PLANTEAMIENTO Y OBJECTIVOS

La aceituna y el aceite de oliva son alimentos típicos de la Dieta Mediterránea cuya composición incluye una fuente valiosa de compuestos bioactivos con múltiples propiedades de interés terapéutico (Ghanbari *et al*., 2012). En los años precedentes, la comunidad científica ha centrado su investigación en demostrar los efectos beneficiosos que posee la ingesta de aceite de oliva en la salud. Sin embargo, han sido pocos los estudios realizados sobre el consumo del fruto del cual se extrae el aceite, la aceituna. Dicho fruto no se consume al ser recogido, sino que es procesado mediante métodos específicos que dan lugar a los diferentes tipos de aceitunas de mesa con una determinada composición y características organolépticas específicas (Johnson *et al*., 2018).

Los beneficios de las aceitunas de mesa están asociados, además de a los ácidos grasos poliinsaturados, a componentes minoritarios tales como los triterpenos pentacíclicos y los polifenoles (Boskou *et al*., 2006; Alexandraki *et al*., 2014). Estos compuestos son microconstituyentes no nutritivos de la dieta, cuya actividad beneficiosa ha sido demostrada en ensayos *in vitro* e *in vivo*, utilizando ya sean los compuestos aislados o bien el aceite obtenido de la aceituna (Bazoti *et al*., 2010; Ruiz-Canela y Martínez-González, 2011; López de las Hazas *et al*., 2017; Juan *et al*., 2019).

El objetivo global de la tesis que se presenta se centra en el estudio de los triterpenos pentacíclicos y polifenoles contenidos en la aceituna de mesa y en el aceite de oliva virgen, así como las concentraciones que se alcanzan tanto en plasma como en el contenido intestinal al ser administrados por vía oral a ratas. A continuación, se describen los siguientes objetivos planteados:

1. Puesta a punto de un método de extracción líquida seguido de análisis por cromatografía líquida acoplada a espectrometría de masas para determinar los polifenoles representativos en aceituna de mesa.

Para ello, se desarrolla y valida un método analítico que permita extraer de forma conjunta y con un mínimo tratamiento de la muestra, los polifenoles de diferentes familias tales como los alcoholes fenólicos, ácidos fenólicos, flavonoides, secoiridoides y lignanos, presentes en la aceituna. Tras la extracción, se identifica y cuantifica cada compuesto por LC-ESI-MS/MS. El método descrito se aplica a la aceituna de mesa Marfil, una variedad nativa de la región de Montsià (Tarragona). La caracterización de polifenoles en aceitunas de mesa es clave para evaluar si la ingesta diaria de una cierta variedad, así como de un número determinado de aceitunas puede contribuir a la protección de los lípidos de la sangre frente al estrés oxidativo (EFSA, 2011).

2. Estudio del perfil de triterpenos pentacíclicos y polifenoles en las aceitunas de mesa por cromatografía líquida acoplada a espectrometría de masas.

En este objetivo, se valida un método que realiza la extracción conjunta de triterpenos pentacíclicos y polifenoles de una misma muestra de aceitunas seguida de la identificación y cuantificación de dichos compuestos por LC-APCI-MS y LC-ESI-MS/MS, respectivamente. Los triterpenos pentacíclicos se determinan por una técnica puesta a punto en el grupo de investigación (Giménez

et al., 2015), mientras que los polifenoles serán analizados por el método descrito en el primer objetivo de esta tesis (Moreno-González *et al*., 2020a). Estas técnicas se aplican al análisis de diferentes aceitunas de mesa que incluyen las variedades Arbequina y Empeltre procedentes de Cataluña, donde se emplean tanto como aceitunas de mesa como para producir aceite de alta calidad. Además, para completar el análisis de las aceitunas Marfil de la región del Montsià, se determinan los triterpenos pentacíclicos.

3. Determinación por cromatografía líquida acoplada a espectrometría de masas de los triterpenos pentacíclicos y polifenoles contenidos en aceite de oliva virgen extra de la variedad Arbequina y en plasma tras su administración oral en rata.

La elevada concentración de triterpenos pentacíclicos y polifenoles hallada en las aceitunas de mesa hizo plantear una nueva cuestión sobre la contribución de la ingestión de un número determinado de aceitunas y el posible efecto cardioprotector descrito para el aceite de oliva virgen. Así se realiza un estudio para conocer las concentraciones plasmáticas de triterpenos pentacíclicos y polifenoles al ser administrados por vía oral a rata.

4. Estudio quimiopreventivo de la ingesta de aceitunas de mesa de la variedad Arbequina sobre lesiones preneoplásicas en colon de rata inducidas con 1,2-dimetilhidrazina (DMH).

Estudios previos demostraron la actividad pro-apoptótica y antiproliferativa de los triterpenos pentacíclicos en cultivos celulares de cáncer de colon (Juan *et al*., 2008a; 2008b), así como un efecto quimiopreventivo en modelos animales con lesiones preneoplásicas colónicas inducidas (Juan *et al*., 2019). Para establecer si la ingesta de aceitunas de mesa tiene efectos *in vivo*, se inducen lesiones preneoplásicas en ratas por la administración intraperitoneal del carcinógeno 1,2-dimetilhidrazina y se evalúa el efecto que tiene la administración oral diaria de aceituna de la variedad Arbequina. Asimismo, se analiza por cromatografía líquida acoplada a espectrometría de masas, las concentraciones de triterpenos pentacíclicos y polifenoles en el contenido de colon y en plasma. Este análisis persigue determinar la cantidad de compuestos bioactivos que se absorben, y correlacionar una baja biodisponibilidad con una alta concentración en el intestino grueso, lo que favorece su actividad antitumoral en el colon de rata.

III. RESULTADOS

El apartado de resultados de este trabajo se encuentra subdividido en cuatro capítulos, cada uno de los cuales corresponden a los objetivos planteados inicialmente. La introducción, el material y métodos, los resultados, la discusión y las conclusiones de los capítulos 1 y 2 se recogen en dos artículos publicados, y en los capítulos 3 y 4 en dos manuscritos en formato de artículo. Además, el capítulo 2 tiene un segundo apartado redactado en castellano.

III. Resultados ~ Capítulo 1

3.1. CAPÍTULO 1. PUESTA A PUNTO DE UN MÉTODO DE EXTRACCIÓN LÍQUIDA SEGUIDO DE ANÁLISIS POR CROMATOGRAFÍA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE MASAS PARA DETERMINAR POLIFENOLES EN ACEITUNAS DE MESA

Los resultados presentados en este capítulo se encuentran recogidos en el artículo 1:

Table olive polyphenols: A simultaneous determination by liquid chromatography-mass spectrometry

Moreno-González, R., Juan, M.E., Planas, J.M.

Journal of Chromatography A (2020), 1609: 460434

(doi: 10.1016/j.chroma.2019.460434)

Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- *Combined determination of polyphenols and pentacyclic triterpenes from Olea europaea* L. *in rat plasma by HPLC-MS*

  Gómez-Contreras, A., Moreno-González, R., Lenard, J., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  XVII Reunión de la Sociedad Española de Nutrición (SEÑ)
  Santiago de Compostela, España, 2 - 5 de noviembre de 2016

- *Determination of biophenols in table olive of the Marfil variety by HPLC-ESI-MS/MS*

  Moreno-González, R., Kundisová, I., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  XL Congreso de la Sociedad Española de Bioquímica y Biología Molecular (SEBBM) + 1st Joint Meeting of the French-Porguese-Spanish B&MB Societies (FEBS3+)
  Barcelona, España, 23 - 26 de octubre de 2017

Polifenoles de las aceitunas de mesa y su determinación por cromatografía líquida-espectrometría de masas

Moreno-González, R., Juan, M.E., Planas, J.M.
Journal of Chromatography A (2020), 1609: 460434
(doi: 10.1016/j.chroma.2019.460434)

3.1.1. Resumen del artículo 1

Objetivos: La aceituna de mesa es un producto tradicional de la dieta Mediterránea que alberga una gama de compuestos fenólicos asociados con numerosos efectos beneficiosos para la salud, entre los que destacan las actividades antioxidantes, cardioprotectoras y anticancerígenas. Dichos componentes fenólicos varían desde monofenoles simples hasta estructuras más complejas con múltiples anillos fenólicos, que pueden presentar azúcares y ácidos orgánicos en sus moléculas. Teniendo en cuenta la complejidad de la matriz de la aceituna, así como de los propios polifenoles, el objetivo del siguiente estudio fue la puesta a punto y validación de un método de extracción, seguido del análisis por cromatografía líquida acoplada a espectrometría de masas en tándem utilizando una fuente de ionización por electrospray (LC-ESI-MS/MS). Esta técnica permite identificar y cuantificar simultáneamente los compuestos fenólicos biológicamente activos presentes en las aceitunas de mesa. El método se aplicó al análisis de las aceitunas de mesa de la variedad Marfil.

Material y métodos: Los polifenoles fueron extraídos de las aceitunas Marfil pertenecientes a la cosecha 2014/2015 y procesadas al estilo-Griego (El Molí de la Creu, La Galera, Tarragona). Para ello, se trituraron 10 g de pulpa en un Polytron homogeneizador con 18 mL de agua Milli-Q. Se tomó 1 g de la suspensión resultante y se sometió a tres extracciones líquidas con etanol:metanol (1:1; v/v) que contenía el patrón interno 2-(3-hidroxifenil)-etanol. Los tres sobrenadantes se juntaron, se filtraron y se realizaron dos diluciones en metanol 80%. La dilución 1/50 sirvió para determinar hidroxitirosol y la dilución 1/4 se utilizó para analizar el resto de los polifenoles.

Seguidamente, tuvo lugar el análisis por LC-ESI-MS/MS. La separación cromatográfica se llevó a cabo utilizando una columna Zorbax Eclipse-XDB-C18 (150 mm × 4,6 mm, 5 μm) y una fase móvil compuesta por agua Milli-Q con 0,025% de ácido acético (fase A) y acetonitrilo con 5% de acetona (fase B), la cual eluyó en gradiente a un flujo de 0,8 mL/min. El análisis por espectrometría de masas (MS) se realizó en un analizador triple cuadrupolo con una fuente de ionización por electrospray (ESI) a 350°C en modo negativo y monitoreo de reacciones múltiples (MRM), considerando tanto las transiciones cuantificadora como cualificadora para cada compuesto.

La validación del método siguió las recomendaciones de la *European Medicines Agency* (EMA, 2011) e incluyó la evaluación de los parámetros de linealidad, precisión, exactitud, recuperación, sensibilidad, selectividad, efecto matriz y arrastre (carry-over). Para ello, se prepararon estándares

de calibración en el rango de concentraciones de 0,025 a 5 µM, añadiendo directamente soluciones de trabajo que contenían una mezcla de 17 polifenoles (apigenina, ácido cafeico, catecol, ácido *o*-cumárico, ácido *p*-cumárico, hidroxitirosol, acetato de hidroxitirosol, luteolina, luteolina-7-O-glucósido, oleuropeína, (+)-pinoresinol, quercetina, rutina, salidrosida, tirosol, ácido vanílico y verbascósido) a los extractos de aceitunas filtrados a la misma concentración que en las muestras (1/50 o 1/4).

Resultados: Para realizar el análisis de los polifenoles por LC-ESI-MS/MS, se llevó a cabo previamente el establecimiento de las condiciones óptimas para conseguir una separación y resolución cromatográfica adecuadas que permitiesen obtener una elevada sensibilidad e intensidad de la señal. En primer lugar, se realizaron pruebas con diversas columnas cromatográficas a múltiples temperaturas utilizando diferentes fases móviles, y se determinó la fuente de ionización a utilizar. Asimismo, se precisaron los solventes de lavado de la aguja inyectora de muestras en el cromatógrafo, para evitar el efecto de arrastre. Posteriormente, se ajustó las condiciones del espectrómetro de masas mediante el ensayo de diferentes voltajes del spray de ionización, así como de las temperaturas de la fuente ESI. Por último, para determinar la fragmentación y las transiciones MRM de cada compuesto, se inyectaron estándares individuales de los polifenoles y el patrón interno directamente en la fuente y se ajustaron los siguientes parámetros de espectrometría de masas en tándem: potencial de desagregación (*declustering potential*), potencial de entrada (*entrance potential*) y las transiciones MRM cualificadoras y cuantificadoras (*qualifier* y *quantifier transitions*), así como sus respectivas energías de colisión.

La linealidad del método se determinó mediante el análisis de tres curvas de calibrado preparadas en tres días diferentes en el rango de concentraciones de 0,05 a 5 µM. Los resultados mostraron linealidad desde 0,05 a 5 µM para el ácido ácido *o*-cumárico, ácido *p*-cumárico, hidroxitirsol, acetato de hidroxitirosol, luteolina y tirosol, mientras que el resto de los polifenoles tuvieron una respuesta lineal hasta 2,5 µM. El coeficiente de correlación (R^2) para todos los polifenoles fue superior a 0,9967. La precisión y exactitud se evaluaron en los niveles de concentración 0,05, 0,5 y 1,5 µM, manifestando resultados inferiores al 15% recomendado. Concretamente, la precisión intradía e interdía, determinada como la desviación estándar relativa (%RSD; coeficiente de variación), presentó valores por debajo del 12,9% y 9,58% respectivamente, mientras que la exactitud (% de error relativo de la concentración medida respecto a la nominal) fue menor del 12,7%. La recuperación, ensayada a las concentraciones de 25, 75 y 180 µM para el hidroxitirosol y a los niveles de 2, 6 y 12 µM para el resto de los polifenoles, fue superior al 95%. El método mostró una alta sensibilidad, ya que el límite inferior de cuantificación (LLOQ) establecido como el punto más bajo de la recta de calibración con una relación señal-ruido 10:1, se encontró entre 0,8 y 7,1 nM para 12 de los 17 polifenoles, siendo ligeramente superior para tirosol, ácido vanílico, salidrosida y pinoresinol (de 0,0281 a 0,0622 µM). Catecol fue el polifenol que presentó el LLOQ más alto, con un valor de 0,1167 µM. El LLOQ también se validó mediante el análisis de seis

muestras de aceitunas con concentraciones teóricas añadidas después de la extracción. Los resultados mostraron unos valores de precisión y exactitud inferiores al 20% recomendado. El ensayo tuvo una alta selectividad para los polifenoles, evitando la interferencia con otros picos. El efecto matriz, medido a partir de la comparación de las pendientes de las curvas preparadas con extracto de aceitunas fortificado con diferentes polifenoles a distintas concentraciones (de 0,025 a 5 μM), con aquellas obtenidas con estándares de calibración preparados a las mismas concentraciones en metanol 80%, presentó valores entre 0,92 (hidroxitirosol y luteolina) y 1,13 (quercetina), indicando la ausencia de interferencias matriciales (Matuszewski, 2006). Además, las soluciones blanco interpuestas a lo largo del proceso no presentaron picos al mismo tiempo de retención de los polifenoles, evidenciando así la carencia arrastre.

Una vez que las condiciones analíticas se establecieron y el método se validó, éste se utilizó para identificar y cuantificar el perfil fenólico en aceitunas de mesa de la variedad Marfil. Dicha variedad presentó 15 polifenoles, siendo los mayoritarios el hidroxitirosol (384 ± 81,2 mg/kg), el tirosol (201± 23,2 mg/kg), la luteolina (88,0 ± 3,8 mg/kg) y la salidrosida (85,9 ± 3,2 mg/kg). Los resultados obtenidos indican que el consumo diario de 7 aceitunas, que corresponde a 8 g de porción comestible, proporciona la cantidad de 5,41 mg de hidroxitirosol y derivados (por ejemplo, el tirosol y la oleuropeína) necesarios para ejercer un efecto protector de lípidos frente al estrés oxidativo según la declaración de propiedades beneficiosas para la salud (Reg. EU nº 432/2012).

Conclusiones: El procedimiento desarrollado permite la determinación de polifenoles de diferentes clases en aceitunas de mesa mediante un tratamiento mínimo de las muestras y en un tiempo cromatográfico inferior a 9 min. Uno de los problemas principales en el análisis de polifenoles en una matriz tan compleja como la aceituna, es la preparación de la muestra. En este caso, la extracción de los polifenoles de las aceitunas de mesa se realizó mediante un procedimiento directo que no requirió pasos intermedios como la evaporación o el baño de ultrasonidos. Además, se obtuvo una buena resolución cromatográfica con la selección de la columna Zorbax Eclipse-XDB-C18 y de la fase móvil. Asimismo, el uso de la espectrometría de masas en tándem permitió una precisa identificación y cuantificación.

La validación indica que el método es lineal, preciso, exacto, sensible, robusto, presentando una buena recuperación, ausencia de efecto matriz, así como de arrastre.

El método fue aplicado al estudio de la composición de polifenoles de aceitunas de mesa de la variedad Marfil, en las que se destaca la presencia de 15 polifenoles biológicamente activos. Teniendo en cuenta nuestros resultados, esta variedad contiene un alto contenido de polifenoles que representan un total de 0.87 ± 0.08 g/kg. Por tanto, en vista de los resultados, se podría afirmar que las aceitunas de mesa son una excelente fuente de polifenoles, emergiendo como un prometedor alimento funcional.

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Table olive polyphenols: A simultaneous determination by liquid chromatography–mass spectrometry

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ABSTRACT

Table olives contain a wide range of polyphenols responsible for protective effects on health that have been associated with a lower prevalence of chronic diseases. A new method to identify and quantify these compounds in table olives, by means of methanol:ethanol (1:1; v/v) extraction followed by LC-ESI-MS/MS, has been developed and validated. The chromatographic column Eclipse-XDB-C18, never used before in this kind of application, provided the best results using Milli-Q water with 0.025% acetic acid and acetonitrile with 5% acetone as eluents. This method allows the quantification of 17 polyphenols, namely, hydroxytyrosol, tyrosol, salidroside, hydroxytyrosol acetate, catechol, vanillic acid, caffeic acid, *o*-coumaric acid, *p*-coumaric acid, verbascoside; oleuropein; pinoresinol, apigenin, luteolin, luteolin-7-O-glucoside, quercetin and rutin. The new method has been validated and shows linear correlations (R^2>0.996), recoveries superior to 95%, high sensitivity, adequate precision and accuracy (RSD < 15%) as well as a short chromatographic analysis of 9 min. Its application to the analysis of Marfil table olives enabled the quantification of 15 polyphenols, among which hydroxytyrosol (384.1 ± 81.2 mg/kg), tyrosol (201.2 ± 3.8 mg/kg), luteolin (88.0 ± 3.8 mg/kg) and salidroside (85.9 ± 3.2 mg/kg) stand out. Furthermore, this method allows to assess whether the intake of a certain number of olives can meet the health claim associated to olive oil polyphenols (Reg. EU n.432/2012). Our results indicate that the daily intake of only 7 olives, which corresponds to 8 g of edible portion, provide an amount of hydroxytyrosol and derivatives (e.g. oleuropein complex and tyrosol) of 5 mg, according to the health claim of the EU. In view of the results, it could be stated that table olives are an excellent source of bioactive compounds, thus emerging as a promising functional food.

1. Introduction

Table olives are a significant product of the Mediterranean diet, which is considered one of the healthiest nutritional patterns in the prevention of chronic ailments including cancer, cardiovascular and neurodegenerative diseases [1,2]. The fruits of *Olea europaea* L. are not only an important source of nutrients, but also of components with nutraceutical value, due to their composition rich in unsaturated fatty acids, vitamin E, carotenoids, minerals, pentacyclic triterpenes and polyphenols [3]. The latter represents a complex group that is synthesized through the shikimate pathway as secondary metabolites that act as phytoalexins with a crucial role in the plant response to environmental stress, mainly as a defence against pathogens, insects and ultraviolet radiation [4,5]. The regular intake of table olives could be meaningful to human health due to the multiple biological activities exerted by these compounds with antioxidant, anti-inflammatory, antimicrobial and antitumoral [6–8]. The cardioprotective effect was substantiated with the acknowledgement of the health claim that the daily consumption of 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil contributes to the protection of blood lipids from oxidative stress [9]. However, the concentrations in some olive oils may be too low to achieve the intake of the recommended amounts in the context of a balanced diet [7]. Hence, the fruits of *Olea europaea* L. which hold a content of these bioactive compounds higher than the oil, arise as a putative functional food. Then, the first step for their consideration is to gain a thorough knowledge on the content of polyphenols.

Despite the fact that at least 36 polyphenols from five different classes have been identified in the leaves and fruits of *Olea*

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europaea L., most of the studies have focussed on olive oil, and the ones existing for table olives determine only some of these compounds [3–5,10]. In addition, it must be borne in mind that the composition of polyphenols in table olives differs from the one described for olive oil or unprocessed olives [10]. In this sense, the secoiridoids, oleuropein and ligstroside attain very high concentrations in the raw fruits, and due to their contribution to the bitter taste, they must be removed to render olives suitable for consumption. Therefore, their amounts in the commercial edible fruits are relatively low, in contrast to the large values achieved by their hydrolysis products, namely hydroxytyrosol and tyrosol [5,10]. Moreover, table olives contain phenolic acids like vanillic and caffeic acids, flavonoids as luteolin, apigenin and quercetin and their glucosides as well as the lignan pinoresinol which is found in minor quantities [3–5]. Therefore, the diversity of polyphenols in table olives with complex characteristics that includes diverse molecular weights and polarities hinders their analysis. Those facts could explain, in part, the lack of a procedure that could provide a fast and high-resolution method for the simultaneous assessment of a large number of polyphenols in table olives [11].

Up to now, the determination of polyphenols in *Olea europaea* L., that is, olive leaves, fruits and oil was accomplished by various analytical strategies [12], being the techniques based on liquid chromatography (LC) the most widely used [13–21]. Among them, LC coupled to either UV or DAD are extensively applied for the identification and quantification of polyphenols in olive oils or table olives. However, these methods hold the drawbacks of long run times (from 30 to 100 min) and high limits of quantification that do not allow to determine the distinct classes of polyphenols in *Olea europaea* L., especially those found at low concentrations [13,17,19,20]. Furthermore, there is not a standard method to measure these compounds, although the International Olive Council (IOC) adopted a procedure with LC-DAD analysis for the determination of biophenols in olive oil [21]. The method presents some limitations, such as a long run time of 60 min. Moreover, it holds the disadvantage of quantifying all the polyphenols using a unique calibration curve with tyrosol as the standard and assuming that all compounds have the same response factor with the consequent deficient accuracy in the results [21]. These shortcomings could be surpassed when LC separation is combined with MS detection, due to its significant increment in the sensitivity, along with the selectivity in the detection of several compounds in complex samples, thus becoming a powerful tool for the analysis of polyphenols in table olives. Consequently, the purpose of the present study was the development of a liquid extraction procedure followed by LC-ESI-MS/MS analysis to identify and quantify key biologically active polyphenols in table olives. The method was validated by spiking samples with standards of polyphenols and following the European Medicines Agency (EMA) Guideline [22] and was applied to the analysis of table olives of the Marfil variety. The developed technique allows the characterization of polyphenols in the fruit of *Olea europaea* L. through a rapid, highly sensitive accurate and precise method that constitutes a useful tool for the routine analysis of these bioactive compounds.

2. Materials and methods

2.1. Chemicals

Apigenin, luteolin, luteolin 7-O-glucoside and tyrosol were provided by Extrasynthèse (Genay, France). Caffeic acid, catechol, *o*-coumaric acid, *p*-coumaric acid, 2-(3-hydroxyphenyl)-ethanol (internal standard, I.S.), oleuropein, (+)-pinoresinol, quercetin, rutin, salidroside, vanillic acid and verbascoside were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Hydroxytyrosol and hydroxytyrosol acetate were from Seprox BIOTECH (Madrid, Spain). Acetone, acetonitrile, methanol, 2-propanol and tetrahydrofuran were obtained from Panreac Química SLU (Castellar del Vallés, Spain). Acetic acid was from Merck (Darmstadt, Germany) and ethanol from J.T. Baker (Devender, Holland). All solvents were LC–MS grade. Water with a conductivity lower than 0.05 mS cm^{-1} was obtained using a Milli-Q water purification system from Millipore (St. Louis, Missouri, USA).

2.2. Stock solutions and working standards

Separate stock solutions for each polyphenol were prepared in methanol 80% to a final concentration of 250 μM. They were used to make working standards containing a mixture of 17 polyphenols at 1 and 10 μM. Further dilution of the working standards yielded to the calibration standards at final concentrations that ranged from 0.025 to 5 μM. The internal standard (I.S.) 2-(3-hydroxyphenyl)-ethanol was prepared as a stock solution of 250 μM and diluted to produce the working standard at 50 μM employed in the preparation of the calibration standards points. Stock solutions were stored at −20 °C, whereas working and calibration standards were freshly prepared before use. Methanol 80% was always employed as solvent in the preparation of stock solutions and working standards.

2.3. Extraction of phenolic compounds from table olives

Fruits from *Olea europaea* L. of the Marfil variety were from the 2014/2015 season (El Molí de la Creu, La Galera, Tarragona, Spain) and were processed as natural olives following the Greek-style. Briefly, olives were harvested, cleaned and classified before being placed in brine for natural fermentation that turns the sugars into lactic acid, lowering the pH and guaranteeing a correct conservation process.

For the extraction of polyphenols, table olives were dried with filter paper and weighed prior and after being destoned. 10 g of olive pulp were placed in 50 mL tubes and 18 mL of Milli-Q water was added. Samples were carefully grinded with 6 short pulses of 30 s with a Polytron homogenizer (PTA 20 TS rotor, setting 5; Kinematica AG, Lucerne, Switzerland) that yielded a fine and homogeneous olive suspension. Analyses of polyphenols were performed in an aliquot of the homogenate. Next the finely grinded olive pulp was stirred in a vortex and 1 g of the uniform suspension (checked by weight) was taken with a Pasteur pipette and deposited in a 15 mL conical centrifuge tube. The aliquot was diluted with 6 mL of ethanol/methanol (1:1; v/v) containing the I.S. at the final concentration of 4 μM. The mixture was vortexed for 5 min and centrifuged at 3345 × *g* for 30 min at 4 °C (Megafuge 1.0R, Heraeus Instruments GmbH, Hanau, Germany). The supernatant was transferred to another tube, and two additional extractions of the pellet were performed by adding 3 mL of ethanol/methanol (1:1; v/v), followed by vigorous shaking in a vortex for 5 min and centrifugation at 3345 × *g* for 30 min at 4 °C (Megafuge 1.0R). The three supernatants were pooled and centrifugated at 27190 × *g* for 30 min at 2 °C (Centrifuge 5417 R, Eppendorf AG, Hamburg, Germany). The clean supernatant was filtered with a 0.45 μM PTFE syringe filters and two dilutions were prepared, 1/50 in methanol 80% to determine hydroxytyrosol and 1/4 to measure the other polyphenols. The samples were placed in amber vials for immediate LC–MS analysis.

2.4. Liquid chromatography-mass spectrometry analysis

2.4.1. Instrument

The analyses were performed on an Acquity UPLC system equipped with a binary solvent manager, sample manager, and column heater (Waters, Milford, Michigan, USA) coupled to an API 3000 triple quadrupole (AB Sciex, Toronto, Canada). The Analyst

Table 1
MRM parameters corresponding to each polyphenol and the internal standard set or obtained by LC-ESI-MS/MS. The quantification and qualification transitions were monitored for the different compounds, while only one MRM transition was used for the internal standard.

Compound	Retention time (min)	Parent ion (*m/z*)	MRM transitions		Declustering potential (V)	Entrance potential (V)	Collision energy	
			Quantifier (*m/z*)	Qualifier (*m/z*)			Quantifier (V)	Qualifier (V)
Apigenin	8.93	269.1	117.1	151.1	65	10	48	34
Caffeic acid	6.92	179.1	135.2	107.1	40	5	25	35
Catechol	7.21	108.8	91.0	65.0	53	10	29	36
o-Coumaric acid	8.12	163.1	119.2	93.0	40	4	20	40
p-Coumaric acid	7.49	163.1	119.2	93.0	40	4	20	40
Hydroxytyrosol	6.23	153.0	123.0	95.0	40	5	25	25
Hydroxytyrosol acetate	8.00	195.0	59.1	135.1	40	4	25	15
Luteolin	8.42	285.0	133.0	151.0	75	10	50	35
Luteolin 7-O-glucoside	7.05	447.2	285.0	327.0	80	10	40	35
Oleuropein	7.53	539.5	275.0	307.0	50	11	33	33
(+)-Pinoresinol	8.61	357.1	151.1	135.9	70	6	45	25
Quercetin	8.50	301.0	151.0	179.1	55	4	30	25
Rutin	6.84	609.0	301.0	271.0	55	10	50	80
Salidroside	6.10	299.1	119.0	89.0	50	4	20	20
Tyrosol	6.79	137.1	106.1	118.8	45	4	25	25
Vanillic acid	7.03	167.1	108.0	152.0	50	6	25	20
Verbascoside	6.91	623.5	161.1	461.2	85	10	50	40
2-(3-Hydroxyphenyl)-ethanol (I.S.)	7.02	137.0	107.0	–	40	5	20	–

software 1.4.2 (AB Sciex) operated the instrument and executed the data analyses. The equipment was available at the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB).

2.4.2. Liquid chromatography conditions

Samples were kept at 10 °C prior to injection in the full-loop mode set at 10 μL. Chromatographic separation of polyphenols was effectuated on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm, 5 μm), preceded by a guard column of the same material (12.5 mm × 4.6 mm, 5 μm), both provided by Agilent Technologies (Santa Clara, CA, USA). The temperature of the column was 30 °C. The mobile phase consisted of Milli-Q water with 0.025% acetic acid (phase A) and acetonitrile with 5% acetone (phase B) delivered at 0.8 mL/min. Separation of polyphenols was performed using the following gradient: 0 min, 97.5% A and 2.5% B; 2 min, 97.5% A and 2.5% B; 3 min, 90% A and 10% B; 8 min, 35% A and 65% B; 8.5 min, 0% A and 100% B. Carry-over was prevented by programming three washing cycles consisting of 2 min of 100% organic phase interleaved with re-equilibration to the initial conditions. Finally, the system was re-equilibrated for 6 min at the initial conditions before the next injection. To avoid further carry-over the needle strong-wash (5000 μL) consisted of isopropanol:tetrahydrofuran:milli-Q water (1:1:1; v/v) whereas acetonitrile:water (10:90; v/v) was used as weak-wash solvent (5000 μL).

2.4.3. Mass spectrometry conditions

The MS analysis was carried out using an electrospray ionization (ESI) source, in negative mode with temperature set at 350 °C and multiple reactions monitoring (MRM). The effluent from the chromatographic column was split approximately at a ratio before entering the mass spectrometer. The ESI source was used with the following settings: nebulizer gas (N_2) at 10 (arbitrary units), curtain gas (N_2) at 12 (arbitrary units) and collision gas (N_2) at 4 (arbitrary units). The ionization spray voltage was −4200 V. To determine the fragmentation and MRM transitions of each compound, individual polyphenols and I.S. at 50 μM were directly infused into the ESI source by a Model 11 syringe (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 10 μL/min. Subsequently, the tandem-mass spectrometry parameters were optimized for efficient isolation of the precursor ions and their selective fragments; the declustering potential, the entrance potential, and quantifier and qualifier transitions with their corresponding collision energy are shown in Table 1. The dwell time for the quantifier transition was 60 ms, and for the qualifier transition it was 10 ms.

2.4.4. Identification and quantification of polyphenols in table olives

Identification of the different polyphenols in the samples was carried out comparing the retention times of each analyte with those of a standard and considering the quantifier and qualifier transitions obtained with the MRM mode (Table 1). Quantification was performed using the standard addition method. Therefore, calibration standards were prepared by directly adding working solutions to the filtered supernatants at the same dilution as the samples (1/50 or 1/4). The calibration curves were constructed using the peak area ratio of the individual polyphenols to the I.S. (2-(3-hydroxyphenyl)-ethanol) versus the concentration of the analyte. Within each analytical run, a full set of calibration standards, which included reagent blank, were injected.

2.5. Method validation

The developed method was validated in accordance with the EMA Guideline for matrix effect, linearity, recovery, sensitivity, precision, selectivity and carry-over [22]. Calibration standards at concentrations ranging from 0.025 to 5 μM were prepared by adding working standards to the post-extracted olive samples at a 1/50 dilution for hydroxytyrosol or 1/4 for the determination of the other polyphenols.

2.5.1. Matrix effect

Relative matrix effect was measured in terms of standard line slope as described by Matuszewski [23]. Hence, samples of table olives were spiked after extraction with the polyphenols at different concentrations ranging from 0.025 to 5 μM and the slopes of the calibrations curves were compared to those obtained when the standards were prepared at the same concentrations in methanol 80%. Three independent calibration curves were analysed in different LC-ESI-MS/MS runs. No matrix effect was considered if the slope ratio were within 0.8 and 1.2. Values lower than 0.8 indicated signal suppression, whereas ratios higher than 1.2 meant an increase on ionization. EMA Guideline considers that the variability calculated as the coefficient of variation (%) should be lower than 15% [22].

2.5.2. Linearity

The linearity of the method was studied by injecting calibration standards in triplicate at 0, 0.05, 0.10, 0.25, 0.50, 0.75, 1, 2.5 and 5 μM prepared in three different days.

2.5.3. Recovery

Recovery of the extraction process was evaluated by adding to 1 g of grinded de-stoned olive, calibration standards of hydroxytyrosol at the concentrations of 25, 75 and 180 μM of hydroxytyrosol as well as 2, 6 and 12 μM of the other polyphenols. Six independent samples for the three levels of concentrations evaluated were prepared and extracted as described in Section 2.3. The recovery, evaluated as a percentage, was determined by comparing the values obtained after fortifying the samples at the beginning of the process with the ones found from post-extraction spiked at the end of the process at the expected concentrations.

2.5.4. Sensitivity

The sensitivity allowed by the method was assessed by calculating the limit of detection (LOD) that was defined as the analyte concentration that provided a signal-to-noise ratio of 3:1. The lowest limit of quantification (LLOQ) was established as the lowest point in the calibration curve and fulfilled the criteria of a signal 10 times higher than the noise level, a precision (%RSD; coefficient of variation) not exceeding 20% and an accuracy between 80% and 120%. The LLOQ was validated by performing the analysis of six olives samples spiked after extraction with the theoretical concentrations.

2.5.5. Precision and accuracy

The precision, defined as relative standard deviation (%RSD; coefficient of variation) was evaluated by analysing the calibration standards prepared at 0.05, 0.5 and 1.5 μM, which correspond to the low, medium and high range of concentrations of polyphenols analysed by LC-ESI-MS/MS. Three independent calibration standards were prepared in three different days to establish the intra- and inter-day precision, respectively. Accuracy of the analytical method was calculated as the percentage of relative error of the measured concentration with respect to the nominal one at the three levels of 0.05, 0.5 and 1.5 μM. A limit of 15% was considered acceptable for both precision and accuracy [22].

2.5.6. Carry-over

The carry-over of one sample to another on the LC-ESI-MS/MS instrument was evaluated within the validation of the method by injecting a blank reagent after the injection of the highest calibration standard. Moreover, carry-over was routinely assessed 6 times in each analytical run by injecting a vial with methanol 80% always at regular intervals based on the total number of samples included in the batch.

2.6. Statistical analysis

Data were given as means ± standard error of the means (SEM). Polyphenols concentrations were expressed in mg/kg of flesh. Chauvenet's criterion was used to identify outliers. Data evaluation, statistical analysis and elaboration of graph were performed with a commercially available package (Prism version 6; GraphPad Software Inc., San Diego, CA). D'Agostino-Pearson omnibus test was used for the assessment of normality. Data were analysed by one-way ANOVA followed by Tukey's Multiple Comparison tests. A $p < 0.05$ level was taken as significant.

3. Results

3.1. Set-up of the liquid chromatography-mass spectrometry conditions for the determination of polyphenols in table olives

Separation of bioactive compounds was attempted in first place with the LiChrospher® 100RP-18 column (250 mm × 4 mm, 5 μm, Merck KGaA, Darmstadt, Germany) that gave a short retention time for hydroxytyrosol that eluted at 6.98 min. However, this silica based reverse-phase support was discarded due to poor chromatographic separation, and polyphenols such as tyrosol and oleuropein were scarcely retained, yielding low peak intensities. To improve the chromatographic resolution, the performance of other reverse phase C18 columns, such as Luna® (50 × 2 mm; 5 μm, Phenomenex, Torrance, CA, USA) and Synergi™ Fusion-RP (150 mm × 4.6 mm; 4 μm, Phenomenex) were assessed with the concomitant evaluation of different mobile phases as well as temperatures of the column and the ionization source. The use of the Luna® column improved the peak intensity of oleuropein, but decreased the sensitivity for hydroxytyrosol, and tyrosol failed to be detected. The shift for the Synergi™ column that is recommended for the separation of mixtures containing both polar and non-polar compounds although enhanced detection still gave a weak signal for tyrosol and also produced poor peak shapes. In contrast, enhanced separation of the different polyphenols was attained when the Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) was used. Thus, the column was selected to develop the chromatographic method.

For the selection of the suitable aqueous phase either milli-Q water alone or in combination with acetic acid (0.025, 0.05, 0.1, 1 and 3%), formic acid (0.1%) or ammonium acetate (5 mM) were evaluated. Milli-Q water alone induced peak splitting for hydroxytyrosol and oleuropein that was prevented with acidification. Formic acid inhibited the ionization of tyrosol and ammonium acetate rendered low peak intensities, since the areas were in average a 90% lower than the ones obtained with the final selected conditions. Acetic acid was the modifier that provided the best peak resolution at the lowest concentration of 0.025%. Concerning the organic phase, different solvents were investigated to better promote ionization of polyphenols. Hence, methanol and acetonitrile were evaluated either alone or in combination with dioxane (5%) and acetone (5 or 10%). Tyrosol was not detected when dioxane was used as a modifier in both solvents. Further, the use of methanol involved a carry-over effect. After the injection of a standard at the concentration of 5 μM, hydroxytyrosol, tyrosol and oleuropein were found in the reagent blank in a 2.1%, 0.7% and 0.4%. When acetonitrile with 5% acetone was used, the retention decreased to a 0.8%, 0.3% and 0.20% for hydroxytyrosol, tyrosol and oleuropein, respectively, thus being selected as organic solvent. The column temperature was also evaluated at 25, 30 or 40 °C and a reliable separation, along with improved sensitivity was achieved when 30 °C was used.

Once the mobile phase was defined as milli-Q water with 0.025% acetic acid and acetonitrile with 5% acetone, the solvents that formed the needle wash were evaluated since a small carry-over effect still persisted in the system. The needle strong-wash was initially composed by acetonitrile:water (1:1; v/v) and a weak-wash of acetonitrile:milli-Q water (1:9; v/v) both delivered at 300 μL. Therefore, the composition of the strong-wash as well as the volume were assessed to prevent carry-over. Firstly, the percentage of acetonitrile was increased to 90%, and different volumes were also tested (500, 1000 or 5000 μL). Although the use of a higher volume decreased the hold of polyphenols, it was not sufficient to eradicate it. Therefore, isopropanol:milli-Q water (1:1; v/v), tetrahydrofuran:milli-Q water (1:1; v/v) or isopropanol:tetrahydrofuran:milli-Q water (1:1:1; v/v) at the volumes of 500, 1000 or 5000 μL were assayed. Carry-over was

Table 2
Validation parameters of the analytical method: linearity, matrix effect and sensitivity.

Compound	Matrix effect		Linearity			Sensitivity	
	Slope ratio	RSD (%)	Range (μM)	Equations	R^2	LOD (μM)	LLOQ (μM)
Apigenin	0.93	7.51	0.010-2.5	$y = (1.414 \pm 0.204)x + (-3.27e^{-5} \pm 0.58e^{-5})$	0.9983	0.0003	0.0010
Caffeic acid	1.08	0.99	0.010-2.5	$y = (1.108 \pm 0.534)x + (-5.10e^{-6} \pm 0.10e^{-6})$	0.9967	0.0002	0.0008
Catechol	1.03	4.14	0.120-2.5	$y = (0.056 \pm 0.002)x + (1.59e^{-7} \pm 0.22e^{-7})$	0.9969	0.0350	0.1167
o-Coumaric acid	1.01	4.70	0.015-5.0	$y = (1.6489 \pm 0.439)x + (-5.56e^{-3} \pm 0.81e^{-3})$	0.9982	0.0005	0.0015
p-Coumaric acid	0.94	4.89	0.025-5.0	$y = (0.956 \pm 0.111)x + (-4.59e^{-6} \pm 2.49e^{-6})$	0.9974	0.0008	0.0027
Hydroxytyrosol	0.92	5.09	0.030-5.0	y = (6.674 ± 0.333)x + (5.37e-5 ± 2.05e-6)	0.9971	0.0009	0.0028
Hydroxytyrosol acetate	1.05	13.1	0.010-5.0	$y = (0.456 \pm 0.109)x + (-1.60e^{-5} \pm 0.38e^{-5})$	0.9976	0.0021	0.0071
Luteolin	0.92	3.67	0.005-5.0	$y = (1.065 \pm 0.175)x + (-4.45e^{-5} \pm 1.89e^{-5})$	0.9976	0.0003	0.0009
Luteolin 7-O-glucoside	0.95	12.5	0.010-2.5	$y = (2.610 \pm 1.507)x + (-7.74e^{-7} \pm 0.21e^{-7})$	0.9976	0.0003	0.0010
Oleuropein	1.06	6.98	0.025-2.5	$y = (0.236 \pm 0.079)x + (3.28e^{-7} \pm 0.15e^{-7})$	0.9967	0.0008	0.0027
(+)-Pinoresinol	0.94	4.72	0.075-2.5	$y = (0.030 \pm 0.004)x + (2.34e^{-7} \pm 0.92e^{-7})$	0.9967	0.0186	0.0622
Quercetin	1.13	7.24	0.005-2.5	$y = (0.869 \pm 0.079)x + (-1.78e^{-6} \pm 0.45e^{-6})$	0.9969	0.0014	0.0046
Rutin	0.99	9.26	0.005-2.5	$y = (0.290 \pm 0.110)x + (-3.15e^{-7} \pm 0.26e^{-7})$	0.9972	0.0005	0.0017
Salidroside	0.96	5.15	0.050-2.5	$y = (0.044 \pm 0.008)x + (-3.20e^{-7} \pm 1.05e^{-7})$	0.9970	0.0164	0.0547
Tyrosol	1.08	3.36	0.025-5.0	$y = (0.015 \pm 0.002)x + (4.36e^{-5} \pm 1.72e^{-5})$	0.9969	0.0084	0.0281
Vanillic acid	0.96	1.77	0.050-2.5	$y = (0.127 \pm 0.043)x + (4.94e^{-6} \pm 0.91e^{-6})$	0.9971	0.0111	0.0370
Verbascoside	0.97	1.49	0.025-2.5	$y = (0.307 \pm 0.019)x + (6.24e^{-7} \pm 1.14e^{-7})$	0.9971	0.0007	0.0023

y, Peak area ratio (analyte/internal standard); x, concentration of polyphenols (μM); RSD, relative standard deviation or coefficient of variation; LLOD, lowest limit of detection (signal to noise ratio of 3:1); LOQ, limit of quantification (signal to noise ratio of 10:1).

completely avoided when isopropanol:tetrahydrofuran:milli-Q water as strong-wash and acetonitrile:milli-Q water (1:9; v/v) as weak-wash were employed, both at the volume of 5000 μL.

The performance of the mass spectrometer was adjusted, once the chromatographic conditions were settled by evaluating different ionization spray voltages (−3000 to −4500 V) being −4200 V the one that provided the best intensity of the peaks. Finally, the sensitivity of the detector was increased by evaluating the influence of the temperature (250, 300, 350, 400 and 450 °C) on the ion intensity. The temperature of the ESI source that yielded the best signal intensity was 350 °C.

3.2. Method validation

3.2.1. Matrix effect

The liquid-liquid extraction procedure was able to eliminate the matrix effect since the slope ratio ranged from 0.92 (hydroxytyrosol and luteolin) to 1.13 (quercetin) (Table 2). The variability observed in the matrix effect, was calculated as RSD (%) and was below 15% for all the polyphenols (Table 2).

3.2.2. Linearity

The parameters obtained after the LC-ESI-MS/MS analysis of 3 independent calibration curves are displayed on Table 2. The response of the instrument over a specific concentration range was different depending on the analyte. *o*-Coumaric acid, *p*-coumaric acid, hydroxytyrosol, hydroxytyrosol acetate, luteolin, tyrosol and verbascoside were linear from 0.05 to 5 μM, whereas the other polyphenols hold a linear response up to 2.5 μM. The calibration coefficients (R^2) were higher than 0.9967 for all the compounds.

3.2.3. Recovery

Recovery was evaluated by comparing the values obtained in olives spiked prior to the extraction to the ones fortified after the preparation of the samples, taking into account that hydroxytyrosol was analysed by LC-ESI-MS/MS at the 1/50 dilution whereas the other polyphenols were measured at the 1/4 dilution. The recoveries obtained for all the compounds at the three levels of concentrations tested were ≥ 95%.

3.2.4. Sensitivity

The developed chromatographic conditions yielded very low values for 11 out of the 17 polyphenols analysed, since their LOD's ranged from 0.0002 μM to 0.0021 μM, consequently, their LLOQ were between 0.0010 and 0.0071 μM (Table 2). Slightly higher amounts were observed for tyrosol and vanillic acid with LLOQ of 0.0281 and 0.0370 μM, whereas the concentrations of salidroside and pinoresinol at the LLOQ were 0.0547 and 0.0622 μM, being of 0.1167 μM for catechol.

3.2.5. Precision and accuracy

Table 3 summarizes the performance data of the method measured at three concentration levels. For all the polyphenols, precision, evaluated as relative standard deviation (%RSD; coefficient of variation) were inferior to 12.9% and 9.58% for the intra-day and inter-day assays, thus fulfilling the acceptable limits established by the guidelines. The developed LC-ESI-MS/MS conditions were accurate at the concentrations of 0.05, 0.05 and 1.5 μM, since the deviance between the theoretical and calculated concentrations did not surpass the 15% permitted by the guidelines [22].

3.2.6. Carry-over

The analysis of blank samples injected immediately after a calibration standard at high concentrations revealed no peak at the retention times of the analytes or the I.S., thus ensuring the reliability of the method at low concentrations.

3.3. Application to the analysis of polyphenols in table olives of the Marfil variety

Once the analytical conditions were settled and subsequently validated, the method was used to identify and quantify the phenolic profile in table olives of the Marfil variety. Peak identification was carried out by comparing the retention time of polyphenols from the samples compared to the ones obtained after the injection of commercial standards. Fig. 1 shows a representative MRM-extracted chromatogram that demonstrates that the developed method is able to simultaneously determine different classes of polyphenols in Marfil table olives within a short chromatographic separation time of 9 min. Salidroside is the polyphenol that elutes in first place at 6.10 min being the last apigenin at 8.93 min. The similar transitions hold by tyrosol (137.1→106.1) and the internal standard, 2-(3-hydroxyphenyl)-ethanol (137.0→107.0), could be appreciated in the extracted ion chromatogram of tyrosol, where it elutes at 6.79 min and the internal standard appears as a small peak at 7.02 min, being both of them well resolved. The isomers

Table 3
Validation parameters of the analytical method: precision (RSD, %) and accuracy.

Compound	Precision (RSD, %)												Accuracy (%)		
	Low (0.05 μM)				Medium (0.5 μM)				High (1.5 μM)				Low 0.05 μM	Medium 0.5 μM	High 1.5 μM
	Day 1	Day 2	Day 3	Inter-day	Day 1	Day 2	Day 3	Inter-day	Day 1	Day 2	Day 3	Inter-day			
Apigenin	4.36	0.14	7.27	4.74	3.89	0.44	8.52	4.70	2.69	0.53	1.54	1.63	−1.73	−1.32	2.04
Caffeic acid	1.48	2.66	4.49	3.15	5.02	10.21	5.80	6.02	3.11	3.76	5.05	3.43	0.80	−3.76	2.95
Catechol	0.12	8.45	2.11	7.95	8.27	7.07	9.03	7.24	9.07	2.33	3.29	4.29	−2.01	1.04	0.14
o-Coumaric acid	3.04	3.00	8.85	6.40	6.15	7.49	0.55	5.80	6.32	0.81	1.30	2.91	1.09	−12.70	1.68
p-Coumaric acid	0.85	2.43	0.60	1.50	1.02	1.46	1.40	2.50	5.00	1.98	4.02	3.11	−0.90	−1.03	1.57
Hydroxytyrosol	0.91	4.10	5.31	3.64	3.42	1.90	8.86	5.11	3.23	1.44	7.97	5.80	0.28	0.83	−1.01
Hydroxytyrosol acetate	0.95	4.78	9.19	5.16	4.44	6.99	3.34	7.00	3.71	0.15	1.11	1.86	−3.47	2.87	1.19
Luteolin	0.28	2.20	5.46	3.49	4.38	0.77	2.21	2.38	1.17	0.16	4.76	2.24	−1.58	0.72	0.35
Luteolin 7-O-glucoside	2.76	1.48	4.92	3.90	3.02	3.98	4.92	4.15	0.10	1.87	3.15	3.14	3.78	−0.15	2.35
Oleuropein	2.59	4.60	10.29	6.38	0.38	3.43	7.43	4.78	3.10	3.17	5.61	5.20	1.98	−1.81	4.98
(+)-Pinoresinol	0.75	2.22	1.59	6.72	8.23	3.48	1.62	9.58	3.79	9.10	4.73	6.18	−3.33	3.53	2.51
Quercetin	4.18	4.10	4.22	4.47	5.14	2.63	2.26	2.98	1.16	1.48	2.73	1.98	−2.34	−1.07	1.02
Rutin	6.95	2.05	6.92	8.83	0.43	6.11	7.92	7.16	0.77	1.97	2.07	2.30	1.80	−4.92	2.71
Salidroside	2.29	1.39	0.59	1.48	6.00	2.29	0.77	4.61	0.95	1.90	11.50	6.76	−0.09	−1.71	0.38
Tyrosol	0.17	0.83	12.93	7.49	5.04	1.63	4.26	3.68	6.92	2.72	2.84	4.30	−0.62	−0.68	1.57
Vanillic acid	1.57	0.46	4.16	2.69	5.19	0.52	2.05	2.57	8.25	1.41	2.47	4.36	−1.41	−0.55	4.61
Verbascoside	2.04	1.90	1.49	4.09	4.17	0.88	5.37	5.31	4.04	1.57	2.40	2.65	4.50	−1.83	1.68

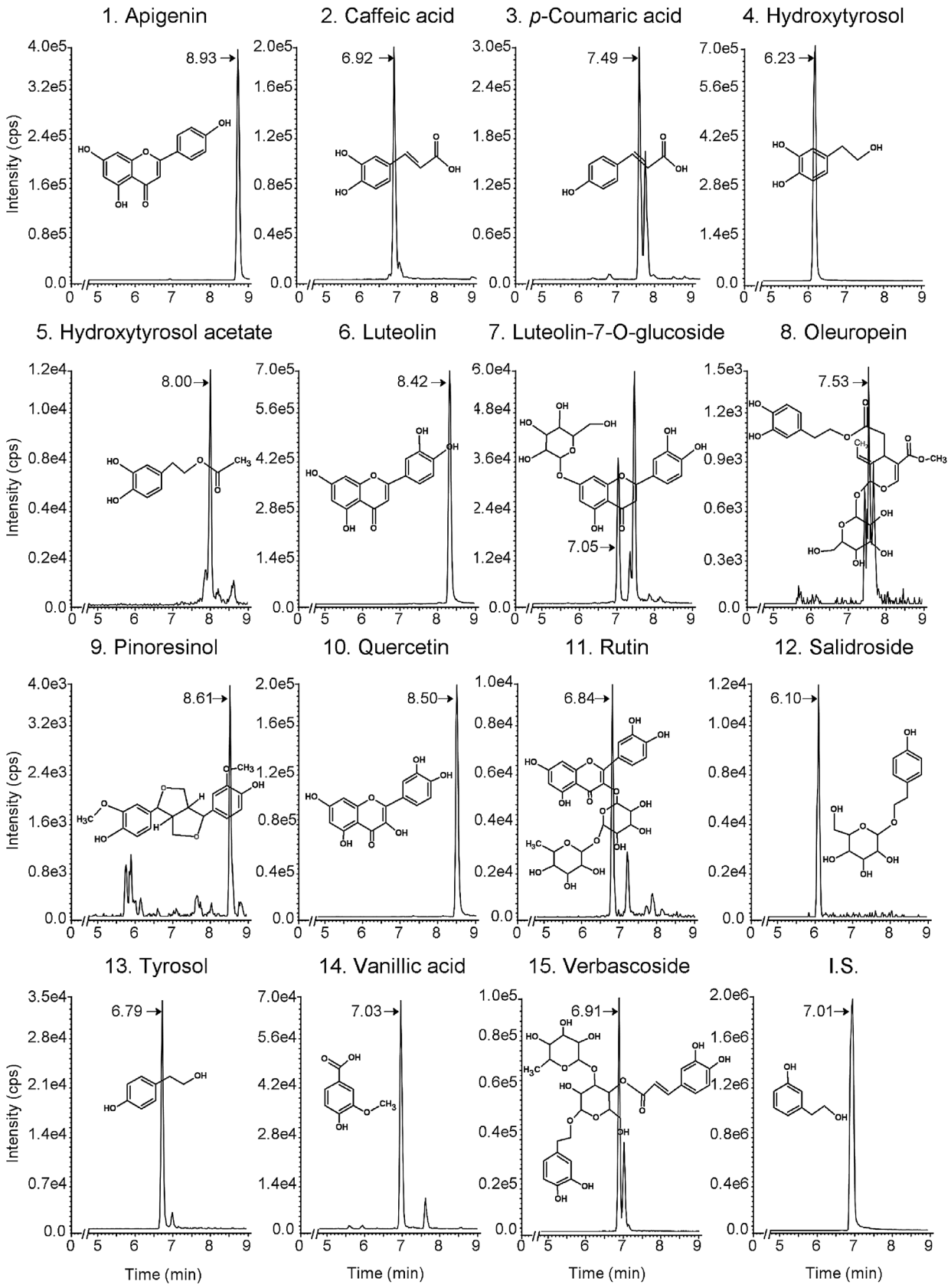

Fig. 1. Representative LC-ESI-MS/MS extracted ion chromatograms of the polyphenols from the drupe of table olives from the Marfil variety. 1) Apigenin, *m/z* 269.1/117.1; 2) Caffeic acid, *m/z* 179.1/135.2; 3) *p*-Coumaric acid, *m/z* 163.1/119.2; 4) Hydroxytyrosol, *m/z* 153.0/123.0; 5) Hydroxytyrosol acetate, *m/z* 195.0 /59.1; 6) Luteolin, *m/z* 285.0/133.0; 7) Luteolin 7-O-glucoside, *m/z* 447.2/285.0; 8) Oleuropein, *m/z* 539.5/275.0; 9) Pinoresinol, *m/z* 357.1/151.1; 10) Quercetin, *m/z* 301.0/151.0; 11) Rutin, *m/z* 609.0/301.0; 12) Salidroside, *m/z* 299.1/119.0; 13) Tyrosol, *m/z* 137.1/106.1; 14) Vanillic acid, *m/z* 167.1/108.0; 15) Verbascoside, *m/z* 623.5/161.1; IS) 2-(3-hyroxyphenyl)-ethanol, *m/z* 137.0/107.0.

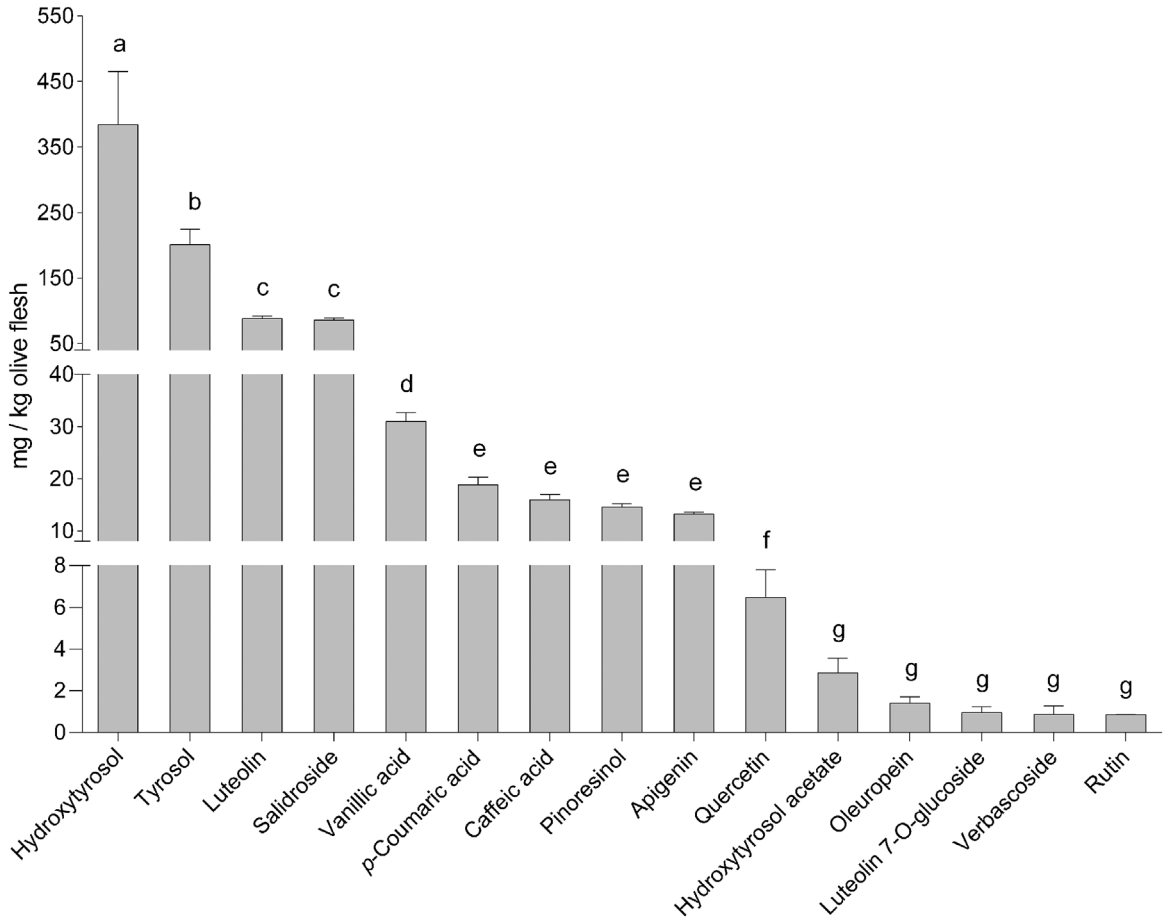

Fig. 2. Concentrations of polyphenols in the drupe of table olives of the Marfil variety collected in December during the 2014/2015 season and determined by LC-ESI-MS/MS. Results are expressed as means + SEM of seven independent samples extracted in duplicate. Different letters on the bars indicate significant differences according to one-way ANOVA analysis followed by Tukey's Multiple Comparison tests ($p < 0.05$).

o-coumaric acid and *p*-coumaric acid that have the same MRM transitions of 163.1→119.2 and 163.1→93.0 could be unambiguously identified due to their different retention in the chromatographic column. Analysis of standards revealed that *p*-coumaric acid elutes at 7.49 min, whereas its isomer *o*-coumaric acid is retained a little longer and appears at 8.12 min. However, in the samples of table olives of the Marfil variety, no traces of *o*-coumaric acid or catechol were observed.

Fig. 2 shows the concentrations of polyphenols identified in table olives of the Marfil variety. Hydroxytyrosol and tyrosol were the main compounds with values of 384.1 ± 81.2 and 201.2 ± 23.2 mg/kg of flesh, respectively. They were followed by luteolin with 88.0 ± 3.8 mg/kg of flesh and salidroside, which is a tyrosol glucoside that reached the amount of 85.9 ± 3.2 mg/kg of flesh. Vanillic acid gave concentrations with values that were half of the ones observed for luteolin and salidroside. *p*-Coumaric acid and caffeic acid were both found at similar quantities of 18.9 ± 1.4 and 16.0 ± 1.0 mg/kg of flesh, respectively. The lignan, pinoresinol and the flavonoid apigenin showed similar results. Concentrations dropped to 6.45 ± 1.34, 2.85 ± 0.71 and 1.40 ± 0.31 mg/kg of flesh for quercetin, hydroxytyrosol acetate and oleuropein, respectively. Finally, luteolin-7-O-glucoside, verbascoside and rutin had a content lower than 1 mg/kg of flesh in table olives of the Marfil variety.

4. Discussion

The diversity of polyphenols in *Olea europaea* L. encompassing molecules with different chemical structures and polarities have hindered their analysis and, consequently, a thorough knowledge of their content in table olives [11]. Then, the aim of the present study was the development of a fast and high-resolution analytical method for routine screening and quality assurance of these bioactive compounds in table olives. One of the bottlenecks in the analysis of polyphenols in a complex matrix such as the fruit of *Olea europaea* L. is the sample preparation step. Here, the extraction of polyphenols from table olives was performed through a straightforward procedure that was established previously for the analysis of pentacyclic triterpenes in table olives [24]. The method consists of a first step in which the de-pitted olives were homogenised to disrupt the cells, thus facilitating the isolation of the bioactive compounds in the second step that includes three consecutive extractions with methanol:ethanol (1:1; v/v) prior to LC-ESI-MS/MS analysis. Previous methods described in the literature were also based on extraction with solvents, employing the majority of them methanol at 100% [17,18,25] or with different percentages of water, such as 80% [16,26], 70% [15], 60% [14,27] or 50% [28,29] and in one case, in combination with acetone at equal volumes [30]. Other solvents were ethanol 80% [19,31–33], DMSO [13,34] and in some procedures, water was used [6,35,36]. Although numerous methods have been described, only a few evaluated the extraction yield of the different solvents [17,25,34]. The highest efficiency in the extraction of polyphenols was achieved when methanol 100% or DMSO 100% were employed [17,25,34]. Given that the extraction of polyphenols was enhanced with the lower amount of water within the extraction solvent, in the present study we evaluated the use of methanol:ethanol (1:1; v/v) to perform the

concurrent extraction of different classes of polyphenols in table olives, rendering good recoveries as substantiated in the validation of the extraction process. Moreover, the analytical methods already published frequently incorporate to the liquid extraction different strategies to improve the efficiency. The most used is the evaporation of the solvent to concentrate the sample and enhance sensitivity [15,29,30–33]. Ultrasound is also employed to facilitate the release of polyphenols from the cells [16,17,25]. Sometimes solid-phase extraction is used [19,34] and, finally, Kanakis *et al.* [18] uses a combination of all of these methods. In contrast, our method holds minimal sample treatment, with only grinding the olives to release polyphenols prior to extraction with a reduced solvent use, yielding a robust, affordable and environmentally friendly process.

In addition to the extraction process, the method of analysis constitutes a cornerstone for the accurate identification and quantification of polyphenols in table olives belonging to different classes in a short run time. Consequently, in the establishment of the LC-ESI-MS/MS conditions, specific care was taken to achieve good chromatographic resolution, which usually is a key element in the reduction of the analysis time given the differences in polarity and chemical structure of the compounds to be detected. Thus, the selection of the column was carefully considered. Most studies that determine polyphenols in table olives use the Spherisorb ODS-2 C18 reverse-phase column. Although this stationary phase renders a satisfactory separation of compounds, the times of analyses are long, ranging from 60 to 80 min in LC [6,13,25,32,35,37] and up to 50 min in LC–MS [15]. Consequently, different stationary phases were evaluated concomitantly to diverse mobile phases to achieve a compromise between retention times, resolution performance and enhanced ionization at the ESI source. Then, solvents commonly used for the LC–MS analysis of polyphenols in table olives [14–16,18,20,27,30,33,38], such as milli-Q water and methanol or acetonitrile in combination with formic or acetic acid were evaluated. The best signal intensities, enhanced separation and improved peak resolution were achieved when the stationary phase was the Zorbax Eclipse XDB C-18 column, and the mobile phase was formed with milli-Q water acidified with acetic acid 0.025% and acetonitrile with acetone at 5%. The developed method allows a comprehensive analysis of their composition in table olives, in a shorter time than the existing LC–MS methods which have analyses time ranging from 33 to 75 min [14,15,18,20,30,33,38]. Melliou *et al.* [16] described a procedure with a similar run time than ours; however, unlike our work, it presented the drawback of the lack of sensitivity to detect tyrosol, which is a predominant phenol in table olives, and it only detected 12 compounds. Recently, the same group has published a method with an analysis time of 5 min, but it was restricted to the detection of 9 polyphenols focused on oleuropein and ligstroside and their related hydrolysis products [27]. Hence, the optimized chromatographic and mass spectrometry conditions enabled the selective detection of 17 compounds from different classes of polyphenols in less than 9 min. Moreover, the use of standards and determination by LC-ESI-MS/MS in MRM mode allows a precise identification of polyphenols as well as proper quantifications, ensuring their analysis in table olives.

A systematic validation of the extraction process and the LC-ESI-MS/MS analysis was performed to assess the suitability of the developed methodology. It is worth mentioning that despite the number of published methods, only a few have been validated [16–18,27,34,36]. All the polyphenols were assessed during the recovery of the extraction procedure, obtaining values above 95% at the three levels of concentrations evaluated. Hence, the extraction process was considered adequate for the analysis of polyphenols. Solely a few studies provide values of recoveries [16,18,36], being only superior to 95% in the method described by Kanakis *et al.* [18]. Zoidou *et al.* [36] evaluated this parameter at three concentrations and reported that they oscillated from 82 to 89% for hydroxytyrosol and between 70 and 80% for oleuropein. On the other hand, Meillou *et al.* [16] reported that at low concentrations, their recoveries were between 50–60%, increasing to 85–95% at high concentrations of added standards. Matrix effect was evaluated, since table olives contain many compounds that could interfere, and results could be adversely affected due to ion suppression or enhancement at the ESI source. The present method avoids the presence of interfering molecules given that the obtained slope ratios ranged from 0.92 to 1.13, with RSD (%) below 15%, thus demonstrating that the effect is consistent and does not compromise the quantification of the different analytes. Moreover, the sensitivity achieved under the developed chromatographic conditions is superior to the ones described in the literature for the analysis of polyphenols in table olives by LC [17,36] or LC–MS [16,18,27]. The LOQ obtained by LC-LTQ-Orbitrap analysis for hydroxytyrosol, oleuropein and tyrosol was 78, 15 and 94 nM [18]. Similar sensitivity was achieved when UHPLC coupled to a 6466 QqQ MS/MS was used since the LOQ reported for hydroxytyrosol, luteolin 7-O-glucoside, *o*-coumaric acid, oleuropein, rutin and verbascoside were 65, 112, 305, 46, 82 and 320 nM [16]. Johnson *et al.* [27], with the same equipment, improved the LOQ with values for hydroxytyrosol of 3.8 nM that is similar to the 2.8 nM that we have found. However, their LOQ for oleuropein and tyrosol were 9.3 nM and 80 nM, which are higher to the 2.7 nM and 28 nM that we obtained. Consequently, the validation indicates that the method is accurate, precise, with adequate linearity and selective. Noteworthy, the enhanced sensitivity in relation to the existing techniques and shorter analysis time. Considering the results obtained, the LC–MS method could be applied not only to the determination of polyphenols in table olives, or other food matrices, but also to the analysis of these bioactive molecules in bioavailability studies, were the detection of polyphenols in plasma or urine require sensitive techniques.

The validated method was applied to the study of the phenolic composition of table olives from the Marfil variety, which is native to the Montsià region (Tarragona, Spain). Its name refers to its elongated shape and white colour that the olive takes when it ripens. Marfil table olives possess a fruit mass of 1.57 ± 0.07 g and a 27.1 ± 0.7% of stone [24]. This variety have been previously studied from the point of view of pentacyclic triterpenes composition [24], but their content of polyphenols had not been published until now. The application of the method allowed the identification of 15 polyphenols with a total content that represents approximately a 1% of the fresh pulp weight. The main polyphenols were the phenyl alcohols hydroxytyrosol and tyrosol with amounts representing a 44.3% and 23.2%, respectively. The concentrations obtained for hydroxytyrosol were in agreement with the values reported for commercial table olives treated with a natural debittering process in different varieties from Spain [17,35], Italy [20,29] and Greece [32,36,39]. Conversely, the concentration of tyrosol measured in the Marfil variety nearly double the ones reported previously in different varieties [17,20,32,35,36,39]. Higher values of tyrosol were only reported in the Italian variety, *Cellina di Nardò* [29]. Luteolin was found in Marfil olives in concentrations representing a 10.2% of all the polyphenols quantified. This flavone that had been barely determined in table olives, generally reported to display lower values [17,20,32], except for the ones indicated in black dry salted olives [15] and in *Cellina di Nardò* olives [29]. Salidroside was quantified in concentrations of 85.9 ± 3.20 mg/kg, a value that represents a 9.9% of polyphenols. This tyrosol glucoside has been previously described in naturally processed Kalamata and Thassos varieties [40] and in Manzanilla and Hojiblanca varieties where salidroside was described to remain in the olives after oxidation treatment [41]. Moreover, this compound was also detected in non-processed olives from the Picual and Arbequina varieties [13].

Marfil table olives presents a distinctive composition in polyphenols, out of which their content in phenolic acids stand out,

since the concentrations of vanillic acid, *p*-coumaric acid, caffeic acid were higher to the ones previously described [15,17,29,39]. Noteworthy, pinoresinol that achieved values of 14.6 ± 0.7 mg/kg and only had been described at concentration of around 1 mg/kg in one study in which Italian olives underwent different natural fermentation processes [42]. With regards to the flavonoids, apigenin and quercetin were found at concentrations of around 1%, which are in accordance with the values described previously in other varieties [15,20,29]. Finally, hydroxytyrosol acetate, oleuropein, luteolin-7-O-glucoside, verbascoside and rutin represented less than 1% of the polyphenols quantified in the Marfil variety, being these values lower than the ones previously found in other varieties [15–17,20,29]. Considering our results, this variety contains a high range of polyphenols accounting for a total of 866.2 mg/kg. It is noteworthy that the amount of hydroxytyrosol tyrosol and oleuropein corresponds to 675.4 mg/kg, which means that one olive could yield 0.774 mg of them. Thus, the daily consumption of only 7 olives (8 g of edible portion) from the Marfil variety provides 5.41 mg, which is higher than the quantity of hydroxytyrosol, tyrosol and oleuropein that according to EFSA, exerts a protection of blood lipids from oxidation [43]. Moreover, Marfil table olives also contain other polyphenols as well as pentacyclic triterpenes such as maslinic acid, oleanolic acid and erythrodiol [24], which can enlarge the beneficial effects when olives are ingested. Even though olives are preserved in brine, the amount of sodium provided by 7 olives or 8 g of pulp of the Marfil variety would be of 107 mg. The Food and Drug Administration (FDA) considers 2300 mg as the recommended daily intake of sodium. Hence, the consumption of 7 olives of the Marfil variety yields less than the 140 mg of sodium per serving that the FDA considers that could be displayed in the Nutritional Fact label as "low sodium" [44]. In view of the results, it could be stated that table olives, and in particular, the Marfil variety emerge as an excellent source of bioactive compounds, and that the daily ingestion of a few number of them may provide protection against cardiovascular disease, as indicated in the EFSA claim [43]. The impact on health achieved by the regular consumption of these bioactive compounds cannot be underestimated since table olives are ingested habitually as part of the diet, either as an appetizer, in a salad or as an ingredient in different recipes. Consequently, the knowledge of the composition of table olives may contribute to a better understanding of their influence in the quality and the biological properties of this food, typical in the Mediterranean Diet.

5. Conclusions

A new liquid extraction procedure followed by liquid chromatography-mass spectrometry strategy has been developed for fast analysis and improved characterization of polyphenols in table olives in order to assess if the intake of a certain number of olives can parallel the health claim attained for olive oil. The method allows the determination of polyphenols in table olives with minimal sample treatment. The developed chromatographic conditions allow a reduced analysis time of less than 9 min, without compromising the chromatographic quality, with high resolution and reproducibility. The results obtained in the validation showed satisfactory precisions, detection limits, as well as good recoveries. The robustness of the method and its good selectivity allowed its successful application to the analysis of polyphenols in table olives of the Marfil variety, thus demonstrating that the daily consumption of only 7 olives, which corresponds to 8 g of edible fruit, supplies the 5 mg of hydroxytyrosol, tyrosol and oleuropein indicated to exert a protection of blood lipids from oxidative stress. Noteworthy, the enhanced sensitivity of the developed methodology in relation to the existing ones allows to be applied not only to the analysis of polyphenols in table olives, but also in biological fluids after the ingestion of this food in animals or humans for pharmacokinetic studies and the evaluation of their impact on health.

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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III. Resultados ~ Capítulo 2

3.2. CAPÍTULO 2. ESTUDIO DEL PERFIL DE TRITERPENOS PENTACÍCLICOS Y POLIFENOLES EN ACEITUNAS DE MESA POR CROMATOGRAFÍA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE MASAS

Los resultados presentados en este capítulo se encuentran recogidos en el artículo 2 y en un apartado ampliado de este estudio:

a) Profiling of pentacyclic triterpenes and polyphenols by LC-MS in Arbequina and Empeltre table olives

Moreno-González, R., Juan, M.E., Planas, J.M.

LWT – Food Science and Technology (2020), 126: 109310

(doi: 10.1016/j.lwt.2020.109310)

b) Estudio de los triterpenos pentacíclicos por LC-MS en aceitunas de mesa Marfil

Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- *Simultaneous determination of triterpenic acids and alcohols in Arbequina table olives by HPLC-MS*

  Moreno-González, R., Camps, C., Bergna, M., Kundisová, I., Lozano-Mena, G., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  XXXVIII Congreso de la SEBBM

  Valencia, España, 7 - 10 de septiembre de 2015

- *Combined determination of polyphenols and pentacyclic triterpenes from table olives by HPLC-MS*

  Moreno-González, R., Kundisová, I., Gómez-Contreras, A., Ekşi, H., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  II Workshop Anual del Institut de Recerca en Nutrició i Seguretat Alimentària – Universitat de Barcelona (INSA-UB)

  Santa Coloma de Gramanet, España, 9 de noviembre de 2016

- *Identificación y cuantificación de los polifenoles en aceitunas de mesa de las variedades Arbequina y Empeltre mediante cromatografía líquida acoplada a espectrometría de masas*

  Moreno-González, R., Juan M.E., Planas J.M.

  Comunicación presentada como póster que fue premiado en el congreso:

  VI Jornada Nacional del grupo de Olivicultura de la Sociedad Española de Ciencias Hortícolas (SECH)

  Madrid, España, 26 - 27 de junio de 2019

- *Bioactive compounds in table olives from the Conservolea variety analyzed by LC-MS*
  Juan M.E., Moreno-González, R., Kundisová, I., Moschochoritis, K., Vallis, N., Planas, J.M.

  Comunicación presentada como oral y seleccionada en el congreso:

  2nd International Yale Symposium on Olive Oil and Health
  Delfos, Grecia, 1 - 4 de diciembre de 2019

a) Perfil de triterpenos pentacíclicos y polifenoles en las aceitunas de mesa Arbequina y Empeltre por LC-MS

Moreno-González, R., Juan, M.E., Planas, J.M.
LWT- Food Science and Technology, (2020), 126: 109310
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3.2.1. Resumen del artículo 2

Objetivos: España es el principal productor de aceitunas de mesa, un producto con una importante contribución económica y un poderoso valor nutritivo. Su composición, además de incluir un alto contenido en ácido oleico, alberga compuestos minoritarios con propiedades beneficiosas, como los triterpenos pentacíclicos y los polifenoles (Sánchez-Quesada *et al*., 2013; Pedret *et al*., 2018). Sin embargo, el perfil de estos componentes puede variar principalmente por factores como la variedad de cultivo, el grado de maduración del fruto y el procesamiento llevado a cabo para que se pueda consumir (Boskou, 2017). Con el objetivo de identificar y cuantificar los triterpenos pentacíclicos y los polifenoles en aceitunas de mesa, se ha adaptado un método analítico que permite la extracción conjunta de ambos tipos de compuestos de una misma muestra, seguido del análisis por LC-MS. El procedimiento ha sido ensayado en dos de las variedades principales de aceitunas de mesa en Cataluña; Arbequina y Empeltre.

Material y métodos: Se utilizaron aceitunas de mesa Arbequina (Cooperativa del Camp Foment Maialenc SCCL, Maials, Lleida) y Empeltre (Agrícola Sant Isidre SCCL, La Fatarella, Tarragona), procesadas como aceitunas naturales en salmuera al estilo-Griego y pertenecientes a las cosechas 2014/2015 y 2015/2016. Los compuestos bioactivos fueron extraídos siguiendo el procedimiento previamente descrito (Moreno-González *et al*., 2020a) y utilizando los patrones internos ácido betulínico (triterpenos pentacíclicos) y 2-(3-hidroxifenil)-etanol (polifenoles). El sobrenadante resultante se filtró y se prepararon alícuotas, diluidas en metanol 80% (1/50 y 1/4) y sin diluir, para el análisis de los diferentes compuestos.

La determinación de triterpenos pentacíclicos se realizó por cromatografía líquida acoplada a espectrometría de masas con una fuente de ionización a presión atmosférica (LC-APCI-MS) siguiendo el procedimiento de Giménez *et al*. (2015) con algunas modificaciones que optimizaron el proceso debido a una bajada de señal. Así, la separación cromatográfica se efectuó con la columna ajustada a 40°C y la elución en gradiente, empleando agua Milli-Q con ácido acético 0,05% (fase A) y metanol con ácido acético 0,05% (fase B) como fase móvil. El análisis en el MS se llevó a cabo con la fuente de ionización programada a 500°C en modo negativo para los ácidos y a 450°C en modo positivo para los alcoholes. La determinación de los polifenoles se llevó a cabo por LC-ESI-MS/MS siguiendo la metodología de Moreno-González *et al*. (2020a).

Finalmente, el cálculo de las concentraciones se realizó a partir de curvas de calibrado preparadas mediante la adición de soluciones de trabajo de triterpenos pentacíclicos y polifenoles a los sobrenadantes filtrados de aceitunas Arbequina o Empeltre a la misma dilución que las muestras (1/50 o 1/4), en el rango de concentraciones de 0,025 a 10 µM.

La validación del método siguió los criterios de la *Eurachem Guide* (Eurachem, 2014), evaluándose los parámetros de sensibilidad, linealidad, precisión, exactitud, efecto matriz y arrastre.

Resultados: Los resultados de la validación mostraron que el método es sensible, con un límite de cuantificación (LOQ) para el ácido maslínico de 8 nM, para los ácidos oleanólico y ursólico de 12 nM y para los alcoholes eritrodiol y uvaol, de 40 y 70 nM, respectivamente. En cuanto a los 17 polifenoles analizados, 12 de ellos mostraron un LOQ inferior a 5 nM, 4 analitos (ácido vanílico, salidrosida, tirosol y pinoresinol) presentaron resultados entre 11 a 39 nM y 1 compuesto (catecol) obtuvo el mayor LOQ, con un valor de 120 nM. La precisión y exactitud del LOQ fue evaluada mediante el análisis de seis muestras de aceitunas enriquecidas después de la extracción con soluciones de trabajo de triterpenos pentacíclicos y polifenoles. Los resultados mostraron una precisión y exactidud inferiores al 20% recomendado. La linealidad se determinó mediante el análisis de tres curvas de calibrado elaboradas en tres días independientes con un rango de concentración de 0,025 a 10 µM. Los triterpenos pentacíclicos mostraron linealidad desde el LOQ hasta 10 µM. La mayoría de los polifenoles fueron lineales desde el LOQ hasta 2,50 µM, excepto el hidroxitirosol, acetato de hidroxitirosol, tirosol, ácido *o*-cumárico, ácido *p*-cumárico y luteolina, que lo fueron hasta 5 µM, mientras que la apigenina hasta 2 µM. Los R^2 de las curvas de calibrado fueron superiores a 0,995 para todos los analitos. La precisión intradía e interdía y la exactitud en todos los niveles de concentración, estuvieron por debajo del 15% recomendado. No se observó efecto matriz, ya que la relación de las pendientes de las rectas preparadas con extracto de aceitunas con soluciones de triterpenos pentacíclicos y polifenoles añadidas frente a aquellas elaboradas con metanol 80% a la misma concentración, se encontraron entre los valores recomendados de 0,80 a 1,20 (Matuszewski, 2006) para todos los compuestos, estando su variabilidad (%RSD) dentro del ± 15% recomendado. El método tampoco mostró arrastre, ya que tanto las soluciones blanco como el metanol 80% interpuestos a lo largo de la secuencia, no mostraron picos al mismo tiempo de retención que los analitos problema.

El análisis de estas aceitunas de mesa en ambas cosechas determinó 3 triterpenos pentacíclicos y 16 polifenoles. Los primeros, alcanzaron concentraciones totales de 3,28 ± 0,07 g/kg en Arbequina y de 2,77 ± 0,2 g/kg en Empeltre, mientras que los segundos alcanzaron una concentración total de 1,04 ± 0,03 g/kg y 0,83 ± 0,05 g/kg, respectivamente. Dentro de la misma variedad de aceituna, la cantidad de compuestos bioactivos de ambas cosechas no mostraron diferencias estadísticas significativas ($p > 0,05$). Sin embargo, al comparar las dos variedades se encontraron diferencias. El ácido maslínico fue el analito mayoritario en ambos tipos de aceitunas (2,51 ± 0,07 g/kg en

Arbequina y 1,86 ± 0,18 g/kg en Empeltre), seguido del ácido oleanólico y, en menor concentración, el eritrodiol. Los polifenoles presentaron un perfil variable tanto dentro de la misma variedad, como entre ellas. La concentración total para Arbequina fue de 1,04 ± 0,03 g/kg, mientras que la de Empeltre fue de 0,83 ± 0,05 g/kg. El hidroxitirosol fue el analito más abundante, con 0,51 ± 0,05 g/kg en Arbequina y 0,39 ± 0,03 g/kg en Empeltre.

Conclusiones: La validación del método aplicado ha presentado unos resultados que permiten la fiabilidad de los análisis de triterpenos pentacíclicos y polifenoles en aceitunas de mesa. Su determinación en las variedades Arbequina y Empeltre, mostró que los compuestos mayoritarios son el ácido maslínico, ácido oleanólico e hidroxitirosol. Asimismo, la variedad Arbequina presentó concentraciones totales de triperpenos pentacíclicos y polifenoles más elevadas que las de Empeltre. No obstante, ambas variedades son ricas en triterpénicos pentacíclicos que, aunque han sido menos estudiados que los polifenoles, poseen múltiples efectos beneficiosos para la salud (Sánchez-Quesada *et al*., 2013).

Por tanto, nuestros resultados indican que las aceitunas de mesa son una fuente de compuestos bioactivos, ya que el consumo diario de 8 aceitunas Arbequina o 5 Empeltre, aportarían la cantidad necesaria de polifenoles que establece la declaración de propiedades saludables de la Unión Europea (Reg. EU nº 432/2012). De igual forma, esta cantidad de aceitunas aportaría la porción necesaria de triterpenos pentacíclicos que se ha sugerido que podría ejercer un efecto beneficioso (Sánchez-Quesada *et al*., 2

b) Estudio de los triterpenos pentacíclicos por LC-MS en las aceitunas de mesa Marfil

3.2.2. Resumen de resultados del estudio

Objetivos: En el objectivo 1 se analizaron los polifenoles de las aceitunas de mesa Marfil. Para completar el estudio de compuestos bioactivos de esta variedad, se han determinado los triterpenos pentacíclicos.

Material y métodos: Se utilizaron aceitunas Marfil (El Molí de la Creu, La Galera, Tarragona) procesadas como aceitunas naturales al estilo-Griego y pertenecientes a las cosechas 2013/2014 y 2014/2015. La preparación de la muestra y el análisis, se ha llevado a cabo de la misma forma que para las variedades de aceituna Arbequina y Empeltre. La validación del método se llevó a cabo siguiendo los parámetros de la guía Eurachem (2014).

Resultados: Los resultados de validación del método para el análisis de los triterpenos pentacíclicos de las aceitunas Marfil mostraron que este es sensible, lineal, preciso, exacto, con ausencia de efecto matriz y arrastre. Los resultados de concentración total de triterpenos pentacíclicos fueron de 3,77 ± 0,15 g/kg, destacando el ácido maslínico (2,53 ± 0,09 g/kg), seguido del ácido oleanólico y en menor concentración el eritrodiol.

Conclusiones: La cantidad total de triterpenos pentacíclicos en las aceitunas de mesa Marfil, fue mayor que la cuantificada en las variedades Arbequina y Empeltre. La diferencia más notable se encontró en el ácido oleanólico, que supera casi el doble a la concentración en Arbequina y en menor proporción a Empeltre.
Por tanto, se puede confirmar que las aceitunas de mesa Marfil son una fuente de triterpenos pentacíclicos y, como se ha confirmado en el capítulo 1, de polifenoles.

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Profiling of pentacyclic triterpenes and polyphenols by LC-MS in Arbequina and Empeltre table olives

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ABSTRACT

Pentacyclic triterpenes (PT) and polyphenols from two varieties of table olives, Arbequina and Empeltre, were determined using a unique extraction from the same olive samples prior to their analysis by LC-APCI-MS and LC-ESI-MS/MS, respectively. Validation provided linear correlation and high sensitivity, as well as adequate precision and accuracy (RSD < 15.0%). The analysis of these table olives allowed the identification and quantification of 3 PT and 16 polyphenols. The most abundant were PT, with a total content of 3.28 g/kg in Arbequina and 2.77 g/kg in Empeltre, ahead of 1.04 g/kg and 0.83 g/kg of polyphenols, respectively. The main PT was maslinic acid, with 2.51 g/kg in Arbequina and 1.86 g/kg in Empeltre, followed by oleanolic acid and finally, erythrodiol. The main polyphenol was hydroxytyrosol, with 0.51 g/kg in Arbequina and 0.39 g/kg in Empeltre, succeeded by luteolin and verbascoside. Bioactive compounds were found in concentrations high enough to exert beneficial effects on health, thus suggesting that a regular consumption of table olives, in the context of a healthy lifestyle, could contribute to the prevention of chronic diseases.

1. Introduction

Table olives are an important product of the countries bordering the Mediterranean Sea not only for their significant contribution to their cultural heritage and economies, but also to nutrition and health (Guo et al., 2018). The fruit of *Olea europaea* L. constitutes a relevant food as a dietary source of pentacyclic triterpenes (PT) and polyphenols, which are plant secondary metabolites with protective effects. The skin is rich in PT that act as insect antifeedants and antimicrobial agents (Diarte et al., 2019; Szakiel, Pączkowski, Pensec, & Bertsch, 2012). Polyphenols are mainly located in the pulp and perform a protective role against pathogens, insects and ultraviolet radiations (Boskou, 2017; Guo et al., 2018). The composition of table olives is influenced by geographical localization, agronomical and technological practices, fruit ripeness and crop season (Boskou, 2017). Moreover, the processing method is an important factor that affect their composition. The harvested fruit of *Olea europaea* L. has to be treated to be edible to modify the aroma, flavor and texture of olives, converting them into a safe, tasty and nutritious food. The three principal types of processing methods are Spanish-style, Californian-style and Greek-style. The latter consists on a traditional elaboration in which fruits are placed in NaCl (4–15%, w/v) and a spontaneous fermentation occurs (Kailis & Kiritsakis, 2017). During this process, it is noteworthy the reduction of bitterness mostly due to the hydrolysis of oleuropein (Guo et al., 2018). Finally, olives are placed in fresh brine before consumption (Kailis & Kiritsakis, 2017).

This fermented food contains high amounts of PT and polyphenols and different *in vitro* mechanistic studies as well as *in vivo* experiments in animals and human interventions have demonstrated that both groups of compounds perform antioxidant, anti-inflammatory, antimicrobial, cardioprotective and antitumoral activities (Pedret et al., 2018; Sánchez-Quesada et al., 2013). Notwithstanding the beneficial effect on health described for PT and polyphenols, within the European Union only polyphenols from olive oil have been granted an authorized health claim related to the reduction of cardiovascular risk (Reg. EU nº 432/2012). The daily intake of 20.0 g of olive oil containing 5.00 mg of hydroxytyrosol and derivatives such as tyrosol and oleuropein protects blood lipids from oxidative stress. Hence, the importance of knowing the content of hydroxytyrosol and its derivatives that would allow the fruit of *Olea europaea* L. to be considered as a functional food. Additionally, table olives contain PT and other polyphenols, thus conferring on this food a distinctive and relevant phytochemical profile that would benefit health conditions (Giménez et al., 2015; Romero et al., 2010). Consequently, the aim of the present study was to assess the profile of PT and polyphenols in Arbequina and Empeltre table

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olives processed following the Greek-style. These varieties are used both as table olives and to produce high-quality oil (Romero, Medina, Mateo, & Brenes, 2017). To achieve this objective, a method that allows the simultaneous extraction of PT and polyphenols was applied to the same olive samples, prior to analysis by LC-MS (Giménez et al., 2015; Moreno-González, Juan, & Planas, 2020). The procedures were validated following the recommendations of the Eurachem Guide (2014), prior to being applied for the identification and quantification of the main PT and polyphenols in Arbequina and Empeltre table olives, from two different seasons.

2. Material and methods

2.1. Chemicals

Apigenin, betulinic acid (internal standard (IS) of PT), luteolin, luteolin-7-*O*-glucoside, oleanolic acid, tyrosol, ursolic acid and uvaol were supplied by Extrasynthèse (Genay, Francia). Caffeic acid, catechol, *o*-coumaric acid, *p*-coumaric acid, 2-(3-hydroxyphenyl)-ethanol (IS of polyphenols), erythrodiol, maslinic acid, oleuropein, (+)-pinoresinol, quercetin, rutin, salidroside, vanillic acid and verbascoside were purchased from Sigma-Aldrich (Tres Cantos, Spain). Hydroxytyrosol and hydroxytyrosol acetate came from Seprox BIOTECH (Madrid, España). Acetone, acetonitrile and methanol were obtained from Panreac Química (Castellar del Vallés, Spain). Acetic acid was procured from Merck (Darmstadt, Germany) and ethanol from J.T. Baker (Deventer, Netherlands). All solvents were LC-MS grade. Ultrapure water was used in all the experiments (Millipore, Madrid, Spain).

2.2. Table olives of Arbequina and Empeltre varieties

Analyses were performed on two different varieties of olives, Arbequina (Cooperativa del Camp, Maials, Lleida) and Empeltre (Agrícola Sant Isidre SCCL, La Fatarella, Tarragona) harvested during the 2014/2015 and 2015/2016 seasons and processed following the Greek-style. The *Olea europaea* L. cultivars of the Arbequina variety were situated in Ribera d'Ebre (Tarragona) and the ones of Empeltre, in Terra Alta (Tarragona), in orchards with drip irrigation. The harvest of olives, in perfect sanitary conditions, was performed for the Arbequina variety in the green-yellow stage of maturation and ripe for the Empeltre variety. Samples were transported to the factory where they were separated from leaves and selected those of extra quality according to their size, medium for Arbequina and extra-large for Empeltre. Then, they were placed in fermentation vessels with 8% (w/v) of NaCl for a period superior to 2 months. After this treatment, olives were washed and placed in the final brine composed of 3.5% (w/v) of NaCl.

Arbequina are small-sized olives with an average weight of 1.55 ± 0.03 g/fruit (n = 83). The weight of destoned olive per fruit is 1.10 ± 0.02 g that corresponds to a 70.6 ± 0.32% of the total. The Empeltre variety are black, medium-sized olives weighting 2.76 ± 0.06 g/fruit (n = 39) and 2.13 ± 0.05 g of destoned olive/fruit which represents a 77.2 ± 0.40%.

2.3. Simultaneous extraction of pentacyclic triterpenes and polyphenols from Arbequina and Empeltre table olives

Polyphenols and PT were concurrently extracted from the same olive sample (Moreno-González et al., 2020). Briefly, 10.0 g of destoned olives were mixed with 18.0 mL of Milli-Q water and ground with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). An aliquot of 1.00 g of the homogeneous olive suspension was mixed with 6.00 mL of ethanol:methanol (1:1, v/v) that contained betulinic acid (IS of PT) and 2-(3-hydroxyphenyl)-ethanol (IS of polyphenols), to a final concentration of 100 μM and 4.00 μM, respectively. Betulinic acid and 2-(3-hydroxyphenyl)-ethanol were used as IS due to their similar chemical and physical properties as the studied analytes and were not detected in olives samples. The mixture was vigorously shaken in a vortex for 5 min, centrifuged at 3345 × *g* for 30 min at 4 °C (Megafuge 1.0R, Heraeus Instruments GmbH, Hanau, Germany) and the pellet was subjected to two additional extractions with 3 mL of ethanol:methanol (1:1, v/v). The pooled supernatants were further centrifuged and filtered with a 0.45 μm PTFE syringe filter. Before LC-MS analysis, two dilutions of the filtrate were performed, using methanol 80% as a solvent. Maslinic acid, oleanolic acid, hydroxytyrosol, verbascoside and luteolin were determined in a 1/50 dilution. The other polyphenols, present in minor concentrations, were measured in a 1/4 dilution. Finally, a non-diluted aliquot was used to evaluate erythrodiol.

2.4. Instrumentation

The LC-MS analyses were carried out in an Acquity UPLC (Waters, Milford, USA) coupled to an API 3000 triple quadrupole (AB Sciex, Toronto, Canada). The UPLC was equipped with a binary pump delivering a flow rate of 0.80 mL/min, a refrigerated auto-sampler kept at 10 °C, an automatic injector set at 10.0 μL, a continuous degassing system and a thermostatted column compartment. The equipment was controlled by the Analyst software 1.4.2 (AB Sciex) that also performed data acquisition and processing. The instrumentation was accessible at the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB).

2.5. LC-APCI-MS conditions for the analysis of pentacyclic triterpenes

PT were analyzed with the LC-APCI-MS conditions previously established by Giménez et al. (2015) that were slightly modified. Chromatographic separation was carried out on a Zorbax Eclipse PAH column (150 mm × 4.60 mm, 3.50 μm) kept at 40 °C, coupled to a guard column of the same material (12.5 mm × 4.60 mm, 5.00 μm) (Agilent Technologies, Santa Clara, USA). The mobile phase consisted of Milli-Q water with acetic acid at 0.05% (phase A) and methanol with acetic acid at 0.05% (phase B) that eluted as follows: 0 min, 17% A and 83% B; 22.5 min, 17% A and 83% B; 23.0 min, 0% A and 100% B. The column was washed for 4 min before setting the gradient back to initial conditions. There was a delay of 6 min prior to the injection of the next sample to ensure equilibration of the column.

Ionization of acids was achieved in an APCI source set at 500 °C in negative mode from 0 min to 17 min, whereas alcohols were ionized at 450 °C in positive mode from 17 min to 23 min. The previously established MS conditions demonstrated the poor fragmentation of PT that made the selected ion monitoring (SIM) mode the most suitable to attain the highest sensitivity (Giménez et al., 2015). Then, maslinic acid was determined as $[M\text{-}H]^-$ ions at *m/z* 471.3 whereas oleanolic acid, ursolic acid and betulinic acid (IS) were measured as $[M\text{-}H]^-$ ions at *m/z* 455.3. The dwell time of 1000 ms was employed for all them. On the other hand, erythrodiol and uvaol were monitored at $[M+H–H_2O]^+$ at *m/z* of 425.3 and a dwell time of 4000 ms.

2.6. LC-ESI-MS/MS conditions for the analysis of polyphenols

Polyphenols were analyzed using the LC-ESI-MS/MS conditions previously established (Moreno-González et al., 2020). In short, the stationary phase was a Zorbax Eclipse XDB-C18 column (150 mm × 4.60 mm, 5.00 μm) kept at 30 °C protected with a guard column of the same material (Agilent Technologies). The mobile phase included solvent A, Milli-Q water with 0.025% acetic acid and solvent B, acetonitrile with 5% acetone. Separation of analytes was achieved using an 8.50 min gradient prior to ionization in an electrospray ionization (ESI) source set in negative mode and at a temperature of 350 °C. Table 1 displays the optimized mass spectrometry parameters of individual analytes. MS was operated in multiple reaction monitoring

Table 1
Optimized MRM conditions for the analysis of polyphenols in table olives by LC-ESI-MS/MS.

Class	Compound	Parent ion (*m/z*)	MRM transitions		Declustering potential (V)	Entrance potential (V)	Collision energy	
			Quantifier (*m/z*)	Qualifier (*m/z*)			Quantifier (V)	Qualifier (V)
Phenolic alcohols	Hydroxytyrosol	153.0	123.0	95.0	40.0	5.00	25.0	25.0
	Tyrosol	137.1	106.1	118.8	45.0	4.50	25.0	25.0
	Hydroxytyrosol acetate	195.0	59.1	135.1	40.0	4.50	25.0	15.0
	Salidroside	299.1	119.0	89.0	50.0	4.50	20.0	20.0
	Catechol	108.8	91.0	65.0	53.0	10.0	29.0	36.0
Phenolic acids	Vanillic acid	167.1	108.0	152.0	50.0	6.00	25.0	20.0
	o-Coumaric acid	163.1	119.2	93.0	40.0	4.50	20.0	40.0
	p-Coumaric acid	163.1	119.2	93.0	40.0	4.50	20.0	40.0
	Caffeic acid	179.1	135.2	107.1	40.0	5.00	25.0	35.0
	Verbascoside	623.5	161.1	461.2	85.0	10.0	50.0	40.0
Flavonoids	Luteolin	285.0	133.0	151.0	75.0	10.5	50.0	35.0
	Luteolin-7-O-glucoside	447.2	285.0	327.0	80.0	10.5	40.0	35.0
	Apigenin	269.1	117.1	151.1	65.0	10.0	48.0	34.0
	Quercetin	301.0	151.0	179.1	55.0	4.50	30.0	25.0
	Rutin	609.0	301.0	271.0	55.0	10.5	50.0	80.0
Secoiridoids	Oleuropein	539.5	275.0	307.0	50.0	11.0	33.0	33.0
Lignans	(+)-Pinoresinol	357.1	151.1	135.9	70.0	6.50	45.0	25.0
	IS: 2-(3-Hydroxyphenyl)-ethanol	137.0	107.0	–	40.0	5.00	20.0	–

(MRM) mode with 60.0 ms as dwell time for the quantifier transition and 10.0 ms for the qualifier transition.

2.7. Identification and quantification of pentacyclic triterpenes and polyphenols in table olives

Analytes were identified by comparing the retention times of each compound with those of the standards obtained at their corresponding *m/z*. Quantification was accomplished with the standard addition method. Firstly, individual stock solutions for each polyphenol and PT were prepared in methanol 80% at 250 μM and stored at −20 °C. Stock solutions were used to make working solutions of polyphenols, containing 17 analytes, and PT, with 5 compounds, at the concentrations of 1.00, 10.0 and 50.0 μM. Then, calibration standards were constructed by adding working solutions to filtered supernatants of either Arbequina or Empeltre olives at the same dilution (1/50 or 1/4) as the samples. The final concentrations of analytes in the calibration standards, spiked post-extraction into Arbequina or Empeltre table olives, were 0.025, 0.05, 0.25, 0.50, 1.00, 2.50, 5.00 and 10.0 μM. Working and calibration standards were freshly prepared before use. Within each analytical run a full set of calibration standards, which included reagent blank, were injected.

2.8. Statistical analysis

Results are presented as mean ± standard error of the means (SEM). Analyte concentrations in table olives are expressed in mg/kg of destoned olives. Chauvenet's criterion was used to reject outliers. Data evaluation, statistical analysis and elaboration of graphs were performed with a commercially available package (Prism version 6; GraphPad Software Inc., San Diego, USA). Normality was assessed with D'Agostino-Pearson omnibus and Shapiro-Wilk tests. Differences in the concentrations of PT between varieties and seasons were analyzed by two-way ANOVA followed by Tukey's multiple comparison test, whereas the concentrations of polyphenols were assessed by the non-parametric Kruskal-Wallis test, followed by Dunn's multiple comparisons test. A $P < 0.05$ level was taken as significant.

3. Results

3.1. Validation of the method for the analysis of pentacyclic triterpenes and polyphenols in table olives

3.1.1. Matrix effect

Matrix effect was assessed as described by Matuszewski (2006) in terms of relative standard line slope. Hence, the slope of calibration curves prepared in post-extracted Arbequina and Empeltre table olives were compared to those obtained by preparing calibration standards at the same concentrations in methanol 80%. The liquid extraction process avoided a matrix effect since the slope ratios were above 0.80 and below 1.20 for PT (Table 2) and polyphenols (Table 3). Moreover, the variability, expressed as relative standard deviation (%RSD), was within the ± 15.0% recommended by Eurachem Guide (2014).

3.1.2. Sensitivity

The limit of detection (LOD) was defined as the concentration with a signal 3 times above the noise level, whereas the limit of quantification (LOQ) was the signal 10 times greater than the baseline noise. LOQ was validated by the analysis of six samples of olives spiked after extraction with the theoretical concentrations, meeting the criteria of accuracy and precision with a %RSD within the ± 20.0%. LOQ for PT (Table 2) and polyphenols (Table 3) were inferior to 15 nM, except for erythrodiol (40 nM), pinoresinol (40 nM), uvaol (70 nM) and catechol (120 nM).

3.1.3. Linearity

Three independent calibration curves were constructed and analyzed on different days at the concentration range from 0.025 to 10.0 μM. PT hold the wider concentration range, since linearity was kept in all the interval (Table 2). On the other hand, hydroxytyrosol, hydroxytyrosol acetate, tyrosol, *o*-coumaric acid, *p*-coumaric acid and luteolin were linear up to 5.00 μM, whereas the other polyphenols held a linear response up to 2.50 μM, except for apigenin, which was linear only up to 2.00 μM (Table 3). The calibration coefficients (R^2) were higher than 0.995 for all the compounds (Tables 2 and 3).

Table 2
Validation parameters of the analytical method: matrix effect, linearity, sensitivity and precision, and accuracy.

Compound	Table olive	Matrix effect		Linearity		Sensitivity		Precision (RSD; %)		Accuracy (%)
		Slope ratio	RSD (%)	Calibration curves	R^2	LOD (nM)	LOQ (nM)	Intra-day	Inter-day	
Maslinic acid	A	1.00	12.1	$y = (0.342 \pm 0.004)x + (2.263e^{-5} \pm 1.342e^{-5})$	0.9975	2.56	7.68	2.28	2.92	0.64
	E	1.04	8.10	$y = (0.340 \pm 0.009)x + (-4.71e^{-5} \pm 3.61e^{-5})$	0.9977	2.63	7.91	1.40	4.00	0.40
Oleanolic acid	A	0.95	5.98	$y = (0.487 \pm 0.020)x + (2.288e^{-5} \pm 2.028e^{-5})$	0.9983	3.75	11.3	1.15	3.14	1.73
	E	0.97	5.78	$y = (0.488 \pm 0.016)x + (-4.56e^{-5} \pm 1.97e^{-5})$	0.9983	3.68	11.1	1.59	2.42	0.21
Ursolic acid	A	0.96	14.8	$y = (0.390 \pm 0.022)x + (-9.22e^{-3} \pm 5.02e^{-3})$	0.9979	3.54	11.8	3.20	5.59	−1.91
	E	0.96	14.9	$y = (0.373 \pm 0.032)x + (-3.90e^{-3} \pm 3.29e^{-3})$	0.9975	3.93	12.0	4.28	4.74	−3.51
Erythrodiol	A	1.00	11.2	$y = (0.337 \pm 0.015)x \pm (-1.75e^{-2} \pm 7.68e\text{-}3)$	0.9977	13.3	40.0	3.21	7.44	1.66
	E	1.02	12.3	$y = (0.318 \pm 0.019)x + (-1.27e^{-2} \pm 6.68e\text{-}3)$	0.9976	12.6	37.7	2.54	3.11	0.59
Uvaol	A	1.06	11.4	$y = (0.142 \pm 0.028)x + (-1.17e^{-2} \pm 8.06e^{-3})$	0.9974	23.3	69.9	5.78	5.95	7.14
	E	0.99	12.2	$y = (0.183 \pm 0.038)x + (-1.39e^{-2} \pm 5.62e^{-3})$	0.9963	22.3	66.9	2.63	5.39	−0.50

Variety of table olive, A, Arbequina, E, Empeltre; *y*, Peak area ratio (analyte/internal standard); *x*, concentration of polyphenols (μM); RSD, relative standard deviation or coefficient of variation; LOD, limit of detection (signal to noise ratio of 3:1); LOQ, limit of quantification (signal to noise ratio of 10:1).

3.1.4. Precision and accuracy

Repeatability (%RSD), was assessed by the analysis of 6 replicates within the same day. Reproducibility was attained by the evaluation of 6 replicates on 3 separate days. Accuracy was established as the percentage of relative error of the measured concentration compared to the nominal ones. Results showed satisfactory repeatability, reproducibility and accuracy since RSD were below the 15.0% limit allowed by Eurachem Guide (2014) for both PT (Table 2) and polyphenols (Table 3).

3.1.5. Carryover

Carryover from one sample to another was checked by injecting the highest calibration standard followed by a blank reagent and was routinely controlled 6 times per run by programming an injection with a vial of methanol 80% at regular intervals based on the total number of samples. In none of the blank reagents were detected any peaks with the same retention time of analytes.

3.2. Pentacyclic triterpenes in table olives of the Arbequina and Empeltre varieties

The chromatograms at Fig. 1 showed that only maslinic and oleanolic acids could be detected in both Arbequina and Empeltre varieties at the dilution 1/50, with no traces of ursolic acid, erythrodiol and uvaol. Therefore, the pooled supernatants obtained after the extraction of table olives were directly injected to the LC-APCI-MS in an attempt to improve the sensitivity. In non-diluted samples, erythrodiol was found in both Arbequina and Empeltre table olives.

Maslinic acid was found to be the most abundant bioactive compound (Table 4). The Arbequina variety supplied the highest amount ($P < 0.05$) of this pentacyclic triterpene with values of approximately 2.5 g/kg for both seasons. The results obtained for Empeltre olives were of 1.86 ± 0.18 and 1.47 ± 0.06 g/kg for the harvest of 2014/2015 and 2015/2016, respectively. On the other hand, no significant differences were observed for oleanolic acid between varieties and crops, yielding concentrations that ranged from 0.76 to 0.90 g/kg. Finally, quantification of erythrodiol gave values of about 10 mg/kg for both varieties and seasons ($P > 0.05$).

3.3. Polyphenols in table olives of the Arbequina and Empeltre varieties

Fig. 2 show the representative chromatograms obtained by LC-ESI-MS/MS for the 16 polyphenols identified in both Arbequina and Empeltre table olives. *o*-Coumaric was not detected in any variety, whereas catechol was found in Arbequina and Empeltre olives, but only in the 2015/2016 season.

Hydroxytyrosol is the most abundant polyphenol with values around 500 mg/kg in the Arbequina table olives in both seasons (Table 4). In contrast, in the Empeltre variety, significant differences were found between harvests, since 200 ± 36.6 mg/kg was found in the yield of 2014/2015, increasing to 389 ± 28.3 mg/kg ($P < 0.05$) in the olives obtained in the ensuing period.

The following polyphenols in attaining important concentrations were verbascoside, luteolin, tyrosol, luteolin-7-*O*-glucoside, salidroside and rutin (Table 4). Verbascoside was found at 303 ± 20.0 mg/kg in Arbequina from 2014/2015 and 229 ± 19.2 mg/kg in Empeltre table olives from 2015/2016 ($P < 0.05$). Conversely, in Arbequina olives harvested in 2015/2016 and Empeltre from 2014/2015, the concentrations of verbascoside dropped to 16.8 ± 2.02 mg/kg and 4.75 ± 0.76 mg/kg, respectively. On the other hand, luteolin remained constant in the different varieties and seasons with values from 71.3 ± 0.10 mg/kg to 84.0 ± 7.20 mg/kg ($P > 0.05$). The next compound in abundance was tyrosol that achieved the highest amount in the crop of 2015/2016, being 38.5 ± 4.15 mg/kg and 31.6 ± 3.02 mg/kg for Arbequina and Empeltre with lower amounts in the season 2014/2015 ($P < 0.05$). Although luteolin-7-*O*-glucoside was found at 130 ± 0.26 mg/kg in the Empeltre olives from 2014/2015, results dropped from 33.8 ± 6.88 mg/kg to 5.90 ± 1.97 mg/kg (Table 4). Salidroside yielded relevant amounts of 40.0 ± 0.70 mg/kg in Arbequina from 2014/2015 and 27.7 ± 0.83 mg/kg in Empeltre for 2015/2016 ($P < 0.05$). Rutin was found at 40.1 ± 3.82 mg/kg in the Empeltre variety from the 2014/2015 season decreasing to 10 mg/kg for Arbequina (2014/2015) and Empeltre (2015/2016) and dropping to nearly 1 mg/kg in Arbequina harvested in 2015/2016. Finally, the phenolic alcohols (hydroxytyrosol acetate and catechol), phenolic acids (vanillic acid, *p*-coumaric acid and caffeic acid), flavonoids (apigenin and quercetin) and the secoiridoid, oleuropein and the lignan, pinoresinol were found at concentrations lower than 20 mg/kg (Table 4).

4. Discussion

The table olives content of PT and polyphenols has been extensively studied (Alexandraki et al., 2014; Blekas, Vassilakis, Harizanis, Tsimidou, & Boskou, 2002; Cabrera-Bañegil et al., 2017; D'Antuono et al., 2018; García, Romero, & Brenes, 2018; Durante et al., 2018; Guinda, Rada, Delgado, Gutiérrez-Adánez, & Castellano, 2010; Medina, García-Garcia, Romero, de Castro, & Brenes, 2019; Romero et al., 2010; Romero et al., 2017). However, research has generally been focused on the determination of only one of these groups of bioactive compounds. Considering the important beneficial effects on health of these components (Pedret et al., 2018; Sánchez-Quesada et al., 2013), the knowledge of the composition in the same olive sample of PT and polyphenols will contribute to a better understanding of the convenience of eating table olives. It is noteworthy that their content may

Table 3
Validation parameters of the analytical method: matrix effect, linearity, sensitivity and precision, and accuracy.

Class	Compound	Table olive	Matrix effect		Linearity		Sensitivity		Precision (RSD; %)		Accuracy (%)
			Slope ratio	RSD (%)	Calibration curves	R^2	LOD (nM)	LOQ (nM)	Intra-day	Inter-day	
Phenolic alcohols	Hydroxytyrosol	A	0.92	5.09	$y = (6.674 \pm 0.333)x + (5.37e^{-5} \pm 2.05e^{-6})$	0.9971	0.68	2.28	2.77	2.74	0.33
		E	0.94	3.00	$y = (6.131 \pm 0.891)x + (7.31e^{-5} \pm 2.47e^{-5})$	0.9971	1.18	3.95	3.51	3.38	−0.35
	Tyrosol	A	0.80	6.94	$y = (0.012 \pm 0.002)x + (1.01e^{-4} \pm 0.99e^{-4})$	0.9975	4.23	14.4	1.51	2.10	0.46
		E	1.00	13.8	$y = (0.012 \pm 0.004)x + (-1.65e^{-7} \pm 6.55e^{-8})$	0.9965	4.53	15.1	5.19	5.67	0.81
	Hydroxytyrosol acetate	A	0.93	13.0	$y = (0.721 \pm 0.090)x + (1.33e^{-5} \pm 0.33e^{-5})$	0.9973	1.50	4.99	1.78	5.19	−0.21
		E	1.04	13.4	$y = (0.355 \pm 0.058)x + (-8.91e^{-7} \pm 4.04e^{-6})$	0.9964	1.63	4.90	5.08	6.56	0.56
	Salidroside	A	0.85	14.8	$y = (0.075 \pm 0.009)x + (2.01e^{-6} \pm 0.88e^{-6})$	0.9974	3.96	13.2	5.11	5.83	−0.26
		E	1.07	11.5	$y = (0.082 \pm 0.002)x + (-1.52e^{-6} \pm 2.72e^{-6})$	0.9982	5.06	15.0	3.71	5.17	−1.39
	Catechol	A	0.89	11.5	$y = (0.063 \pm 0.002)x + (2.50e^{-6} \pm 0.50e^{-6})$	0.9962	28.6	95.2	4.21	3.33	1.58
		E	1.09	14.9	$y = (0.059 \pm 0.008)x + (-3.61e^{-7} \pm 4.65e^{-7})$	0.9963	40.0	120	2.73	2.50	1.49
Phenolic acids	Vanillic acid	A	1.16	12.7	$y = (0.110 \pm 0.037)x + (-1.638e^{-8} \pm 1.56^{-6})$	0.9972	3.45	11.5	2.62	4.19	1.03
		E	0.85	1.31	$y = (0{,}091 \pm 0.017)x + (-1.43e^{-7} \pm 2.58e^{-6})$	0.9964	4.99	15.0	4.47	5.69	−0.95
	o-Coumaric acid	A	0.99	9.12	$y = (1.651 \pm 0.446)x + (2.98\ e^{-2} \pm 0.62\ e^{-2})$	0.9970	1.05	3.50	2.24	4.59	−0.05
		E	1.03	6.93	$y = (1.446 \pm 0.430)x + (2.52e^{-6} \pm 2.18e^{-6})$	0.9962	1.34	4.48	5.67	5.89	1.60
	p-Coumaric acid	A	0.92	8.85	$y = (0.995 \pm 0.208)x + (3.88e^{-6} \pm 2.14e^{-6})$	0.9981	1.30	4.32	3.00	5.50	0.75
		E	0.91	4.28	$y = (0.990 \pm 0.248)x + (1.98e^{-5} \pm 2.81e^{-6})$	0.9949	1.52	4.58	3.72	4.97	−2.33
	Caffeic acid	A	0.92	10.2	$y = (1.017 \pm 0.340)x + (1.79e^{-6} \pm 0.19e^{-6})$	0.9977	1.11	3.71	3.49	5.12	−0.05
		E	0.86	6.00	$y = (0.482 \pm 0.041)x + (7.35e^{-4} \pm 7.20e^{-4})$	0.9971	1.20	4.00	3.37	3.52	1.30
	Verbascoside	A	0.96	12.6	$y = (0.294 \pm 0.039)x + (5.62e^{-6} \pm 1.56e^{-6})$	0.9974	0.91	3.03	2.93	4.10	−0.90
		E	0.98	1.70	$y = (0.450 \pm 0.003)x + (3.61e^{-6} \pm 2.24e^{-6})$	0.9952	1.52	4.55	2.59	4.07	−0.18
Flavonoids	Luteolin	A	0.92	9.94	$y = (1.287 \pm 0.419)x + (1.52e^{-4} \pm 0.61e^{-4})$	0.9972	0.09	0.31	2.46	2.90	0.32
		E	0.99	14.6	$y = (1.349 \pm 0.119)x + (7.68e^{-5} \pm 3.34e^{-5})$	0.9952	0.82	2.47	3.49	3.85	0.40
	Luteolin-7-*O*-glucoside	A	0.95	9.13	$y = (2.153 \pm 0.886)x + (4.05e^{-7} \pm 0.67e^{-7})$	0.9979	0.28	0.93	2.32	5.75	0.12
		E	1.04	2.53	$y = (1.528 \pm 0.411)x + (3.74e^{-6} \pm 1.27e^{-6})$	0.9981	0.94	3.12	4.96	6.67	0.20
	Apigenin	A	0.99	8.66	$y = (1.411 \pm 0.491)x + (4.31e^{-5} \pm 1.36e^{-5})$	0.9977	0.22	0.72	2.52	4.44	1.64
		E	1.10	3.20	$y = (1.783 \pm 0.283)x + (-1.57e^{-6} \pm 1.33e^{-6})$	0.9982	0.53	1.77	2.70	4.19	0.67
	Quercetin	A	1.00	7.97	$y = (0.831 \pm 0.130)x + (4.58e^{-6} \pm 7.71e^{-6})$	0.9981	0.34	1.15	2.40	4.54	−0.92
		E	1.01	11.1	$y = (0.883 \pm 0.047)x + (2.81\ e^{-6} \pm 9.33e^{-6})$	0.9962	1.33	4.43	3.45	3.93	−0.81
	Rutin	A	0.93	13.3	$y = (0.308 \pm 0.069)x + (2.88e^{-7} \pm 1.42e^{-7})$	0.9981	0.28	0.93	3.46	5.25	−2.17
		E	1.03	7.70	$y = (0.393 \pm 0.094)x + (-7.35e^{-7} \pm 4.00e^{-7})$	0.9974	0.88	2.93	5.40	5.99	0.85
Secoiridoids	Oleuropein	A	1.03	10.9	$y = (0.230 \pm 0.050)x + (3.75e^{-7} \pm 0.99e^{-7})$	0.9974	0.19	0.64	3.38	5.89	1.25
		E	1.12	6.04	$y = (0.165 \pm 0.038)x + (-5.09e^{-8} \pm 2.01e^{-7})$	0.9974	1.49	4.48	2.26	3.09	−0.14
Lignans	(+)-Pinoresinol	A	0.81	12.0	$y = (0.035 \pm 0.002)x + (1.38e^{-5} \pm 0.19e^{-5})$	0.9964	10.6	31.7	4.74	6.51	1.37
		E	0.81	14.9	$y = (0.030 \pm 0.004)x + (5.27e^{-8} \pm 2.50e^{-8})$	0.9962	11.7	39.1	5.34	7.92	−1.90

Variety of table olive, A, Arbequina, E, Empeltre; y, Peak area ratio (analyte/internal standard); x, concentration of polyphenols (μM); RSD, relative standard deviation or coefficient of variation; LOD, limit of detection (signal to noise ratio of 3:1); LOQ, limit of quantification (signal to noise ratio of 10:1).

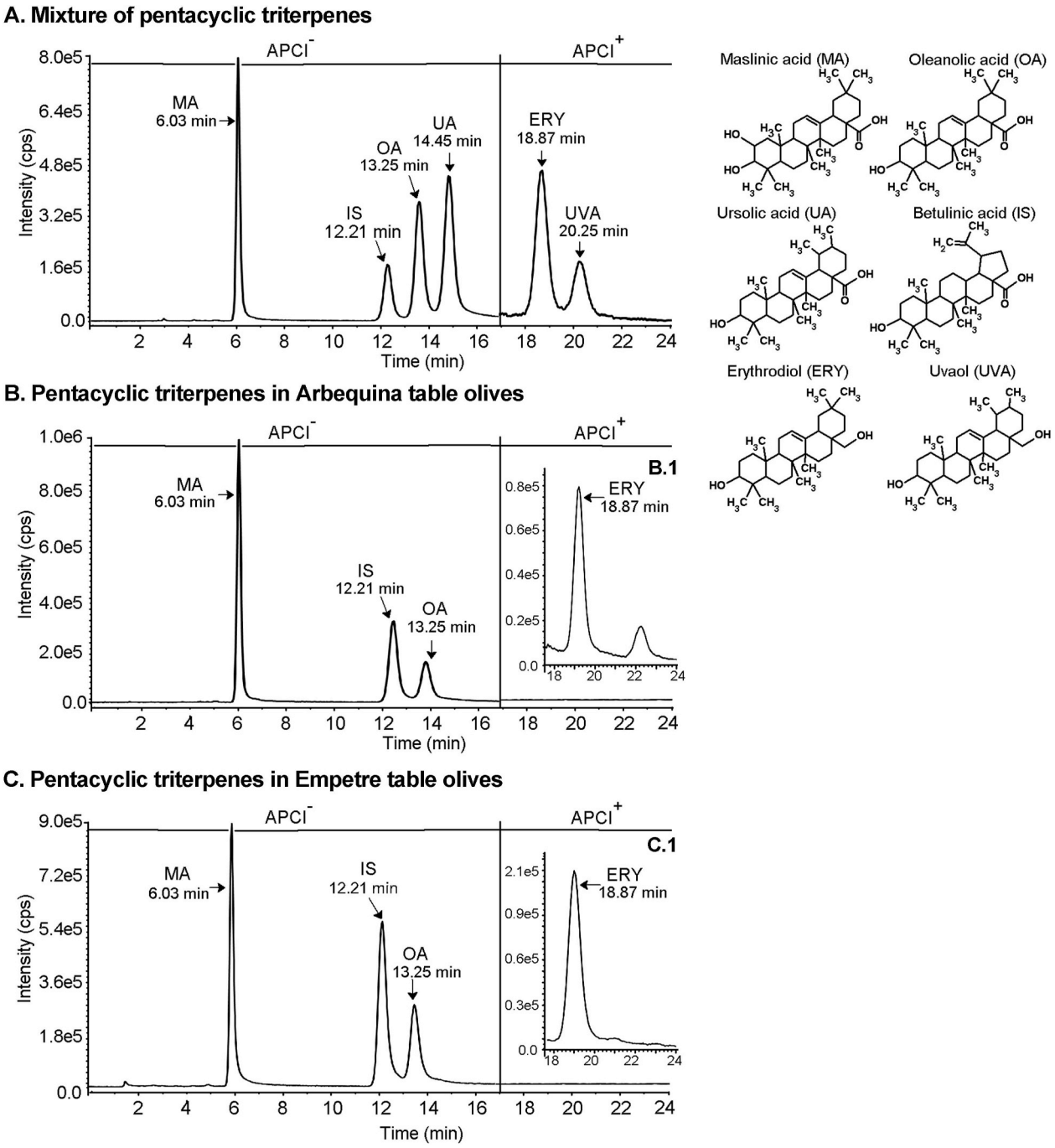

Fig. 1. Total ion chromatograms (TIC) obtained by LC-APCI-MS. (A) A mixture of standards of maslinic acid, oleanolic acid, ursolic acid, erythrodiol and uvaol at 5.00 μM and betulinic acid (IS) at 2.00 μM, in methanol 80%. Pentacyclic triterpenes detected in Arbequina (B) and Empeltre (C) table olives at a 1/50 dilution. The inserts (B.1) and (C.1) depict the extracted ion chromatogram (XIC) of the non-diluted extract.

vary based on different factors (Boskou, 2017) being of great interest to discern the profile of PT and polyphenols in the different varieties of olives from different regions and crop season. The methodologies reported in the literature for the determination of both groups of compounds in the same sample are limited and not easily accessible (Olmo-García et al., 2019). Here, we describe a rapid method that enables the concurrent extraction of PT and polyphenols in the same olive sample before LC-MS analysis. Bearing in mind the existing techniques, our method holds several advantages, such an extraction deploying a unique process which avoids the use of solvent, generating less waste, thus rendering an eco-friendly and cost-effective procedure. Once extracted, PT were determined by LC-APCI-MS, fine-tuned with conditions that not only permit the simultaneous assessment of acids and alcohols but also separate isomers (Giménez et al., 2015). Hence, oleanolic and ursolic acids, as well as erythrodiol and uvaol, which only differ by the position of one methyl group, could be chromatographically separated and detected by MS. In addition, the determination of 17 polyphenols from different classes as diverse as phenyl alcohols, phenolic acids, secoiridoids, flavonoids and lignans in table olives, carried out by LC-ESI-MS/MS, completed the analysis (Moreno-González et al., 2020). Hence, we propose an analytical strategy readily available and affordable for both quality control analysis and nutritional studies, with instruments that are common in many laboratories.

The validation of the methods showed no matrix effect, good

Table 4
Content of pentacyclic triterpenes and polyphenols (mg/kg of destoned olives) in table olives of the Arbequina and Empeltre varieties, harvested during the 2014/2015 and 2015/2016 seasons.

Class	Compound	Arbequina		Empeltre	
		2014/2015	2015/2016	2014/2015	2015/2016
Pentacyclic triterpenes					
Triterpenic acids	Maslinic acid	2417 ± 79.6^{a} (n = 6)	2508 ± 68.8^{a} (n = 6)	1862 ± 183^{b} (n = 6)	1468 ± 55.0^{b} (n = 6)
	Oleanolic acid	781 ± 44.6^{a} (n = 6)	758 ± 17.6^{a} (n = 6)	898.6 ± 60.9^{a} (n = 6)	811 ± 23.5^{a} (n = 6)
	Ursolic acid	< LOD	< LOD	< LOD	< LOD
Triterpenic alcohols	Erythrodiol	12.2 ± 1.02^{a} (n = 6)	9.48 ± 0.34^{a} (n = 6)	10.8 ± 1.51^{a} (n = 6)	11.3 ± 0.68^{a} (n = 6)
	Uvaol	< LOD	< LOD	< LOD	< LOD
	Total	**3210 ± 89.7^{a}**	**3276 ± 66.9^{a}**	**2771 ± 197^{ab}**	**2290 ± 73.3^{b}**
Polyphenols					
Phenolic alcohols	Hydroxytyrosol	507 ± 53.3^{a} (n = 3)	484 ± 32.2^{a} (n = 7)	200 ± 36.6^{b} (n = 3)	389 ± 28.3^{a} (n = 7)
	Tyrosol	15.8 ± 1.15^{b} (n = 3)	38.5 ± 4.15^{a} (n = 7)	3.48 ± 0.95^{b} (n = 3)	31.6 ± 3.02^{a} (n = 7)
	Hydroxytyrosol acetate	8.05 ± 0.61^{a} (n = 3)	9.32 ± 0.51^{a} (n = 7)	0.90 ± 0.05^{b} (n = 3)	8.59 ± 0.88^{a} (n = 7)
	Salidroside	40.0 ± 0.70^{a} (n = 3)	8.26 ± 0.16^{c} (n = 7)	6.90 ± 1.18^{c} (n = 3)	27.7 ± 0.83^{b} (n = 7)
	Catechol	Φ	5.11 ± 0.19^{a} (n = 7)	Φ	2.81 ± 0.36^{b} (n = 7)
Phenolic acids	Vanillic acid	6.08 ± 0.38^{b} (n = 3)	12.4 ± 0.36^{a} (n = 7)	5.52 ± 0.12^{b} (n = 3)	2.98 ± 0.31^{c} (n = 7)
	o-Coumaric acid	< LOD	< LOD	< LOD	< LOD
	p-Coumaric acid	4.87 ± 0.06^{a} (n = 3)	0.26 ± 0.01^{b} (n = 7)	1.09 ± 0.27^{b} (n = 3)	3.68 ± 0.46^{a} (n = 7)
	Caffeic acid	2.58 ± 0.26^{b} (n = 3)	0.46 ± 0.05^{c} (n = 7)	2.91 ± 0.98^{b} (n = 3)	9.50 ± 1.06^{a} (n = 7)
	Verbascoside	303 ± 20.0^{a} (n = 3)	16.8 ± 2.02^{c} (n = 7)	4.75 ± 0.76^{c} (n = 3)	229 ± 19.2^{b} (n = 7)
Flavonoids	Luteolin	84.0 ± 7.20^{a} (n = 3)	74.4 ± 1.35^{a} (n = 7)	71.3 ± 0.10^{a} (n = 3)	75.1 ± 5.91^{a} (n = 7)
	Luteolin-7-O-glucoside	33.8 ± 6.88^{b} (n = 3)	5.90 ± 1.97^{b} (n = 7)	130 ± 0.3^{a} (n = 3)	12.5 ± 8.74^{b} (n = 7)
	Apigenin	3.78 ± 0.31^{b} (n = 3)	5.44 ± 0.09^{a} (n = 7)	1.53 ± 0.06^{d} (n = 3)	2.33 ± 0.17^{c} (n = 7)
	Quercetin	1.19 ± 0.27^{c} (n = 3)	5.19 ± 0.09^{b} (n = 7)	1.16 ± 0.18^{c} (n = 3)	19.4 ± 1.24^{a} (n = 7)
	Rutin	10.5 ± 0.26^{b} (n = 3)	1.31 ± 0.03^{c} (n = 7)	40.1 ± 3.82^{a} (n = 3)	7.18 ± 2.03^{b} (n = 7)
Secoiridoids	Oleuropein	15.8 ± 0.52^{a} (n = 3)	1.41 ± 0.07^{c} (n = 7)	3.90 ± 0.33^{b} (n = 3)	3.14 ± 0.67^{b} (n = 7)
Lignans	(+)-Pinoresinol	7.17 ± 0.36^{a} (n = 3)	1.96 ± 0.28^{b} (n = 7)	2.07 ± 0.22^{b} (n = 3)	3.33 ± 0.02^{b} (n = 7)
	Total	**1044 ± 29.5^{a}**	**671 ± 30.7^{b}**	**476 ± 45.6^{c}**	**828 ± 50.8^{d}**

Results are expressed as means ± SEM. Pentacyclic triterpenes were compared by two-way ANOVA analysis followed by Tukey's multiple comparison tests, whereas polyphenols were assessed by non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison ($P < 0.05$). Within the same compound, means without a common letter differ. Φ, non-measured. LOD, limit of detection.

linearity, adequate sensitivity and optimal precision and accuracy, thus sustaining their adequacy for the determination of PT and polyphenols in table olives. Concerning PT, no data have been previously reported about processed Arbequina olives. Our results showing an amount of 3.30 g/kg for both crops reveled that this variety harbors a high content of maslinic acid followed by oleanolic acid. Noteworthy the fact that olives were naturally fermented in brine, a process that have been described in Conservolea and Kalamata olives to preserve maslinic and oleanolic acids, in contrast to the decrease observed when debittering is performed using the Spanish-style (Alexandraki et al., 2014). Consequently, our values in debittered Arbequina olives are consistent with existing research in the raw fruit that reports values ranging from 2.40 to 3.10 g/kg of triterpenic acids (Guinda et al., 2010; Romero et al., 2010, 2017). Referring to the Empeltre variety, our results of 2.77 and 2.29 mg/kg in the harvest of 14/15 and 15/16, respectively, agree with those recently published for the same variety that showed concentrations of triterpenic acids of between 2.30 and 2.50 mg/kg (Medina et al., 2019). Our results are also consistent with those described for other varieties, such as Manzanilla (2.10 mg/kg), Leccino (2.18 mg/kg), Cellina di Nardò (2.48 mg/kg), Hojiblanca (3.00 mg/kg), Conservolea (3.02 mg/kg), Marfil (3.12 mg/kg) and Kalamata (3.28 mg/kg) (Durante et al., 2018; García et al., 2018; Giménez et al., 2015). Finally, it is noteworthy that the extraction method and LC-APCI-MS conditions that we used enabled the detection of erythrodiol in amounts that ranged from 9.48 to 12.2 mg/kg for both crops. The fact that other authors did not detect erythrodiol could be attributed to the use of LC-DAD (Durante et al., 2018; Medina et al., 2019; Romero et al., 2010, 2017) or GC-FID analysis (Guinda et al., 2010), which are less sensitive than the MS detector employed in our study, along with the fact that the methods used were not validated for this alcohol.

Table olives also contain polyphenols which are quantitatively less abundant than PT, but qualitatively more numerous, since 16 members of this class were identified and quantified. The total concentration of polyphenols was approximately 1.04 and 0.67 g/kg in Arbequina, whereas 0.48 and 0.83 g/kg were detected in Empeltre, for the seasons 2014/2015 and 2015/2016, respectively. Raw olives must be treated to remove the bitterness associated with the secoiridoids, a process that affects the profile of polyphenols in the edible fruit. Although Greek-style processing retains more polyphenols in the fruit than the Spanish or Californian styles (Boskou, 2017; D'Antuono et al., 2018), a hydrolysis of glycosides takes place, thus decreasing the content of oleuropein, ligstroside and verbascoside as well as hydroxytyrosol and tyrosol glucosides, which leads to an increase in the content of the aglycons (Boskou, 2017). Moreover, polyphenols are water-soluble compounds that easily diffuse in brine, so the content of these molecules is not only influenced by the type of treatment but also by the different washings applied to the fruit (Boskou, 2017; Romero et al., 2004). These factors could explain why raw Arbequina olives harvested at different degrees of maturation during the 2012/2013 season have been reported to contain approximately 12.0–14.0 g/kg of total phenols. This value is an order of magnitude higher than those we found for the same variety processed as natural olives in brine (Romero et al., 2017). In contrast, Cabrera-Bañegil et al. (2017) found 0.19 g/kg in Arbequina olives (crop of 2014/2015) debittered following the Spanish style. The results obtained for polyphenols in Empeltre olives are consistent with those reported for the same variety also processed following the Greek style, of between 0.43 and 1.40 g/kg (Medina et al., 2019). Our results for polyphenols are in accordance with those

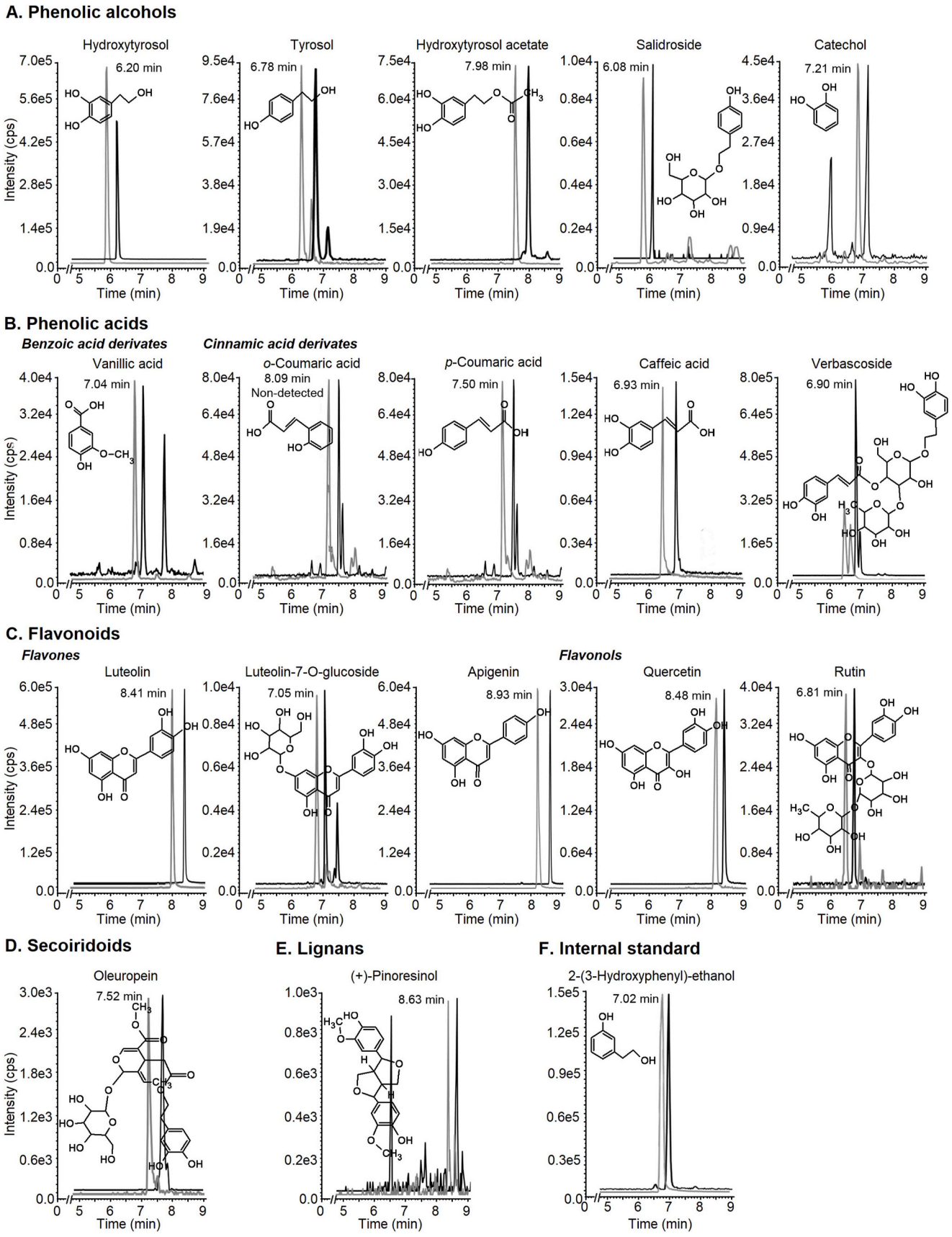

Fig. 2. Representative LC-ESI-MS/MS extracted ion chromatograms (XIC) for polyphenols from Arbequina (gray trace) and Empeltre (black trace) table olives.

indicated for other varieties treated with the Greek style, as in Cellina de Nardo (2.2 g/kg), Bella di Cerignola (0.61 g/kg), Termite di Bitetto (0.35 g/kg) (D'Antuono et al., 2018) or Manzanilla (1.20 g/kg) (Romero et al., 2004).

Finally, the present findings validate table olives as a source of polyphenols that could meet the health claim from the European Union (Reg. EU nº 432/2012) more reliably than extra virgin olive oil as previously suggested (D'Antuono et al., 2018). The claim states that 5.00 mg of hydroxytyrosol and derivatives protects blood lipids against oxidation (Reg. EU nº 432/2012). Taking into account the weight of the olives studied as well as the total amount of hydroxytyrosol and derivatives found, we can conclude that the consumption of 8 Arbequina or 5 Empeltre olives would confer this benefit by providing the requisite quantity of polyphenols. In addition, the consumption of 8 small-sized Arbequina olives would contribute with 25.0 mg of maslinic acid and 7.20 mg of oleanolic acid, whereas 5 medium-sized Empeltre would supply 19.0 mg and 9.00 mg, respectively. These PT, although investigated less than polyphenols have also been acknowledged as beneficial to health, having multiple effects (Sánchez-Quesada et al., 2013). Considering the health relevance of these bioactive compounds and their variability, the knowledge of the content of PT and polyphenols in table olives may well be of great benefit to consumers and health professionals that could recommend, with a scientific basis, an amount of intake of this food in reinforcing healthiness.

5. Conclusions

In this study, a novel approach to the analysis of PT and polyphenols from table olives have been described and validated. The method enables their concurrent extraction from the same olive sample avoiding expensive purification steps while being time-efficient. In addition to the advantages of a convenient sample preparation and short LC-MS runs, the results obtained in the validation revealed its high precision, accuracy and sensitivity. The application of the validated methods assessed the qualitative and quantitative profiling of key phytochemicals in two of the most widely consumed varieties of table olives, Overall, our results allow to indicate that the consumption of 8 Arbequina or 5 Empeltre olive supplies the amount of bioactive compounds relevant to human health, thus providing the basis for considering table olives as a functional food.

CRediT authorship contribution statement

Rocío Moreno-González: Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **M. Emília Juan:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision. **Joana M. Planas:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

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b) Estudio de los triterpenos pentacíclicos por LC-MS en las aceitunas de mesa Marfil

3.2.2. Resultados del estudio

3.2.2.1 Objetivos

En el capítulo 1 de esta tesis, se analizaron los polifenoles de las aceitunas de mesa Marfil pertenecientes a la cosecha 2014/2015 y procesadas según el estilo griego. En este apartado del capítulo 2 se presentan los resultados del análisis de los triterpenos pentacíclicos de esta misma variedad de aceitunas (El Molí de la Creu, La Galera, Tarragona) procedentes de las cosechas 2013/2014 y 2014/2015.

3.2.2.2. Material y métodos

La preparación de la suspensión de aceitunas, la extracción y la identificación y cuantificación de los triterpenos pentacíclicos por LC-APCI-MS, se llevó a cabo de la misma forma que se ha explicado anteriormente (Moreno-González *et al*., 2020b). De la cosecha 2013/2014 se han analizado cuatro muestras de aceitunas independientes y de la cosecha 2014/2015 tres muestras, todas ellas extraídas por duplicado. El método se validó siguiendo las recomendaciones de la guía Eurachem (2014), donde se tuvieron en cuenta los parámetros de sensibilidad, linealidad, precisión, exactitud, efecto matriz y arrastre.

3.2.2.3. Resultados

Los resultados de validación del método para el análisis de los triterpenos pentacíclicos de las aceitunas Marfil mostraron una sensibilidad óptima, ya que el LOQ del ácido maslínico fue de 9 nM, para los ácidos oleanólico y ursólico de 11 nM y para el eritrodiol y uvaol de 38 nM y 72 nM, respectivamente. El LOQ se validó mediante el análisis de seis muestras de aceitunas enriquecidas después de la extracción con concentraciones teóricas de triterpenos pentacíclicos, dando resultados de precisión (%RSD: coeficiente de variación) y exactitud (% de error relativo de la concentración medida respecto a la nominal) inferior al ±20.0%. La linealidad medida en tres días independientes estuvo en el rango de LOQ a 10 μM para todos los triterpenos pentacíclicos. La precisión intradía e interdía fue inferior a 3,49% y 5,0% respectivamente, mientras que la exactitud en todos los puntos de la recta fue inferior a 7,27%. No se observó efecto matriz (Matuszewski, 2006), ya que la relación de pendientes se encontró entre 0,80 y 0,92 para todos los analitos, con un %RSD dentro del ± 15%. Tampoco hubo efecto de contaminación por arrastre de una muestra a otra, ya que las soluciones blanco y de metanol 80% colocadas a lo largo de la secuencia, no mostraron picos al mismo tiempo de retención que los analitos problema.

La figura 1 muestra un cromatograma representativo de una muestra de aceitunas Marfil, donde se puede observar que en la dilución 1/50 se pudo cuantificar el ácido maslínico y oleanólico, mientras que el ácido ursólico, el eritrodiol y el uvaol no se detectaron. Cuando se analizó el extracto de aceitunas sin diluir, se pudo encontrar eritrodiol. Los resultados de concentración total de

triterpenos pentacíclicos fueron de 3,77 ± 0,15 g/kg en la cosecha 2013/2014 y de 3,62 ± 0,29 g/kg en la 2014/2015. La Tabla 1, muestra que en ambas cosechas el ácido maslínico fue el compuesto mayoritario, seguido del ácido oleanólico, y en menor concentración el eritrodiol. No se detectaron ni el ácido ursólico ni el uvaol.

Tabla 1. Contenido en triterpenos pentacíclicos en aceitunas de la variedad Marfil analizadas por LC-APCI-MS.

Compuesto	Marfil	
	2013/2014	2015/2016
Ácido maslínico	2526 ± 86.4[a] (n = 4)	2217 ± 240[a] (n = 3)
Ácido oleanólico	1222 ± 81.9[a] (n = 4)	1394 ± 62.9[a] (n = 3)
Ácido ursólico	< LOD	< LOD
Eritrodiol	20.9 ± 0.94[a] (n = 4)	11.6 ± 0.76[a] (n = 3)
Uvaol	< LOD	< LOD

Los resultados se expresan como medias ± SEM. Los triterpenos pentacíclicos fueron comparados por la prueba no paramétrica de Mann-Whitney ($p < 0.05$). Dentro de cada compuesto, las medias con una letra común indican que no hay diferencias. LOD (límite de detección).

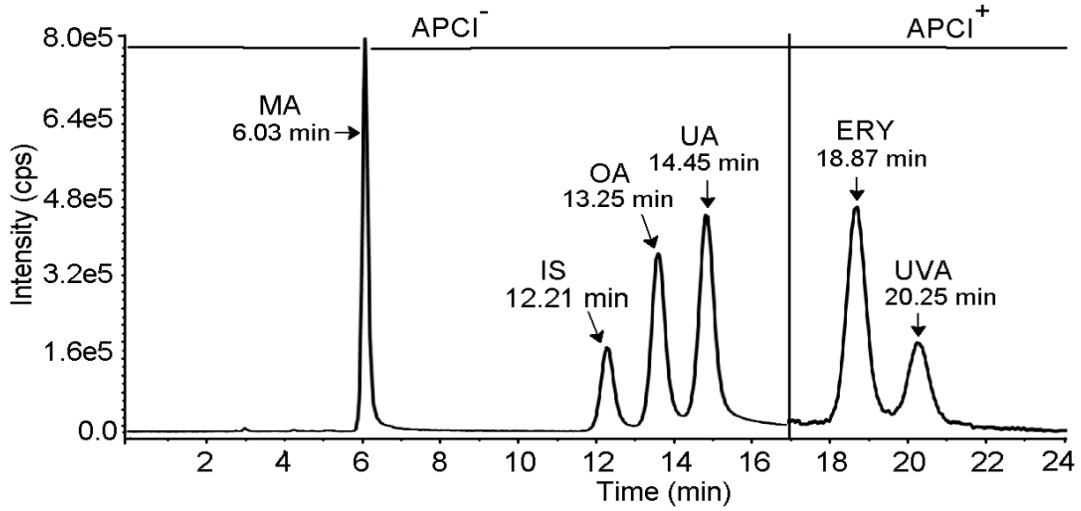
3.2.2.4. Discusión

Las aceitunas de mesa Marfil son una variedad local poco conocida fuera de Cataluña, pero no por ello menos importantes en cuanto a componentes bioactivos se refiere que otras variedades más extendidas. La cantidad total de triterpenos pentacíclicos en estas aceitunas fue mayor que la cuantificada en las variedades Arbequina y Empeltre. El ácido maslínico fue superior al determinado en Empeltre (1,86 ± 0,18 g/kg) y similar a Arbequina (2,51 ± 0,07 g/kg). Sin embargo, la diferencia más notable se encontró en el ácido oleanólico, el cual supera casi el doble a la concentración en Arbequina (0,78 ± 0,02 g/kg) y en menor grado a Empeltre (0,90 ± 0,06 g/kg). El eritrodiol en las aceitunas Marfil fue bastante cambiante entre cosechas, a diferencia de la constancia que conservan Arbequina y Empeltre. En la cosecha 2013/2014 la concentración de este analito duplicó a las variedades Arbequina (0,01 ± 0,001 g/kg) y Empeltre (0,01± 0,0007 g/kg), aunque en la recolecta 2014/2015 los valores de eritrodiol de las 3 variedades de aceitunas fueron muy similares.

3.2.2.5. Conclusiones

Se puede confirmar que las aceitunas de mesa Marfil son una fuente de componentes bioactivos. Su riqueza en triterpenos pentacíclicos y, como se ha estudiado en el capítulo 1, en polifenoles, hacen que sean una buena elección dietética para obtener la cantidad de componentes bioactivos necesarios para ejercer efectos beneficiosos sobre la salud (Reg. EU nº 432/2012; Sánchez-Quesada *et al*., 2013).

(A) Mezcla de triterpenos pentacíclicos



(B) Triterpenos pentacíclicos en aceitunas de mesa Marfil

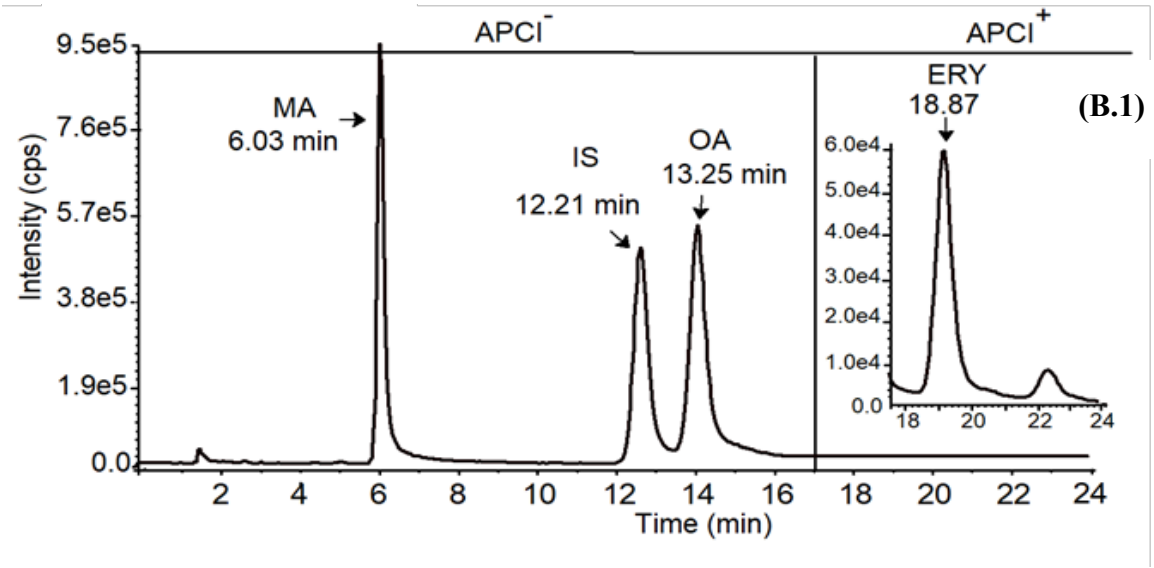


Figura 1. Cromatogramas de iones totales (TIC) obtenidos por LC-APCI-MS de (**A**) un patrón conteniendo una mezcla de triterpenos pentacíclicos (ácido maslínico, *m/z* 471,6 (MA); ácido oleanólico, *m/z* 455.5 (OA); ácido ursólico, *m/z* 455.5 (UA); eritrodiol, *m/z* 425.7 (ERY) y uvaol *m/z* 425.7 (UVA)) a 5 µM y el patrón interno ácido betulínico, *m/z* 455.5 (IS) a 2 µM disueltos en metanol 80% y (**B**) una muestra de aceitunas Marfil a la dilución 1/50. El inserto (**B.1**) muestra un cromatograma de iones extraídos (XIC) del extracto de aceitunas no diluido donde se puede apreciar el eritrodiol.

III. Resultados ~ Capítulo 3

3.3. CAPÍTULO 3. DETERMINACIÓN POR CROMATOGRAFÍA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE MASAS DE LOS TRITERPENOS PENTACÍCLICOS Y POLIFENOLES CONTENIDOS EN ACEITE DE OLIVA VIRGEN EXTRA DE LA VARIEDAD ARBEQUINA Y EN PLASMA TRAS SU ADMINISTRACIÓN ORAL A RATA

Los resultados presentados en este capítulo se encuentran recogidos en el artículo 3:

Determination by LC-MS of pentacyclic triterpenes and polyphenols in extra virgin olive oil and its bioavailability after oral administration in rats

Moreno-González, R., Juan, M.E., Planas, J.M.

Manuscrito en preparación

Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- *Analysis of bioactive triterpenic compounds in Arbequina extra virgin olive oil and in plasma of rats by HPLC-MS*

  Moreno-González, R., Planas, J.M., Juan, M.E.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  III Workshop Oli d'Oliva - INSA-UB

  Santa Coloma de Gramanet, España, 19 de septiembre de 2017

- *The fruit of Olea europaea* L. *as a dietary source of pentacyclic triterpenes*

  Moreno-González, R., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  III Workshop Oli d'Oliva - INSA-UB

  Santa Coloma de Gramanet, España, 19 de septiembre de 2017

Determinación por LC-MS de triterpenos pentacíclicos y polifenoles en aceite de oliva virgen extra y biodisponibilidad tras la ingesta en ratas

Moreno-González, R., Juan, M.E., Planas, J.M.

Manuscrito en preparación

3.3.1. Resumen del artículo 3

Objetivos: El aceite de oliva es el principal aceite vegetal producido y consumido en la cuenca mediterránea, cuyo procesamiento mecánico sin refinar (virgen y virgen extra) permite conservar tanto el perfil de ácidos grasos monoinsaturados, como otros componentes bioactivos minoritarios tales como los esteroles, las vitaminas, el escualeno, los polifenoles y los triterpenoides. Cada vez más estudios confirman los efectos beneficiosos que aportan los triterpenos pentacíciclicos y los polifenoles del fruto de *Olea Europaea* L. En consecuencia, el objetivo del presente estudio fue evaluar las concentraciones plasmáticas de triterpenos pentacíclicos y polifenoles por LC-MS tras la administración oral de aceite de oliva virgen extra (AOVE) Arbequina a ratas Sprague-Dawley. Para ello, en primer lugar, se llevó a cabo el análisis de AOVE Arbequina con un método novedoso que permite la extracción simultánea de ambos grupos de compuestos para su posterior análisis por LC-MS.

Material y métodos: Los triterpenos pentacíclicos y los polifenoles se extrajeron de una alícuota de 0,5 g de AOVE de Arbequina (Cooperativa del Camp Foment Maialenc SCCL, Maials, Lleida) a través de una triple extracción líquido-líquido, utilizando un volumen total de 1,5 mL de solvente etanol:metanol (1:1; v/v) que contenía el ácido betulínico (triterpenos pentacíclicos) y 2-(3-hidroxifenil)-etanol (polifenoles) como patrones internos. Los tres extractos se juntaron y se evaporaron a sequedad (45°C), previa incorporación de ácido ascórbico 10%. El residuo resultante se reconstituyó en 250 µL de metanol 80% y tras centrifugarse, el sobrenadante se filtró y se prepararon muestras diluidas en metanol 80% (1/50 y 1/4) y sin diluir, para determinar los diferentes compuestos por LC-MS. Los triterpenos pentacíclicos se evaluaron de acuerdo al procedimiento descrito en el capítulo 2 (Moreno-González *et al*., 2020b), mientras que los polifenoles se estudiaron siguiendo el método establecido por Moreno-González *et al*. (2020a).

Posteriormente, el AOVE de Arbequina se administró a ratas a una dosis de 6,16 mL/kg y se sacó sangre a los 0, 15, 30, 60, 90, y 120 min post-administración para evaluar las concentraciones plasmáticas de triterpenos pentacíclicos y polifenoles. Dichos compuestos fueron extraídos de una alícuota de 200 µL de plasma mediante una doble extracción líquido-líquido, empleando 4 mL de acetato de etilo acidificado con ácido acético 1% que incluía los patrones internos ácido betulínico y 2-(3-hidroxifenil)-etanol, así como ácido ascórbico 10%. Los sobrenadantes se mezclaron y se evaporaron a sequedad (45°C). El sedimento se reconstituyó en 100 µL de metanol 80%, se centrifugó y el sobrenadante se colocó directamente en viales para el análisis por LC-MS siguiendo

el mismo procedimiento que para el aceite, pero con un programa de elución en gradiente ligeramente diferente para ambos grupos de compuestos bioactivos. Además, también se llevó a cabo el estudio de los metabolitos derivados de estos compuestos.

Resultados: En el AOVE de Arbequina se cuantificaron un total de 17 compuestos, de los cuales, 4 pertenecían a triterpenos pentacíclicos y 13 a polifenoles. La concentración total de triterpenos pentacíclicos fue de 149 ± 30 mg/kg, destacando el ácido maslínico (116 ± 9,83 mg/kg) seguido del ácido oleanólico (29,3 ± 0,83 mg/kg) y en menor concentración el eritrodiol (3,73 ± 0,35 mg/kg) y uvaol (0,22 ± 0,02 mg/kg). Los polifenoles sumaron un total de 16,8 ± 0,3 mg/kg, predominando el acetato de hidroxitirosol (4,60 ± 0,19 mg/kg), el hidroxitirosol (3,68 ± 0,08 mg/kg), la luteolina (3,63 ± 0,11 mg/kg), el tirosol (2,19 ± 0,07 mg/kg) y la apigenina (1,61 ± 0,06 mg/kg). El catecol, ácido vanílico, pinoresinol y ácido *p*-cumárico mostraron valores entre 0,43 y 0,14 mg/kg, mientras la quercetina, ácido cafeico, verbascósido y salidrosida presentaron los valores más bajos.

El análisis del plasma de rata a diferentes tiempos post-administración de AOVE Arbequina a una dosis de 6,16 mL/kg, mostró 8 compuestos, de los cuales, 1 correspondía al triterpeno pentacíclico ácido maslínico y 7 a los polifenoles ácido *p*-cumárico, ácido vanílico, apigenina, pinoresinol, hidroxitirosol, luteolina y tirosol, detectándose metabolitos derivados de los tres últimos. Los compuestos tuvieron la máxima concentración (Cmax) a los 15 min (tmax), disminuyeron progresivamente con el tiempo y, excepto pinoresinol, todos se midieron aún a los 120 min. El ácido maslínico, presentó un valor de 21,2 ± 5,13 nM a los 15 min y de 5,62 ± 0,82 nM a los 120 min. El ácido vanílico, mostró una concentración de 59,8 ± 7,34 nM a los 15 min y de 8,25 ± 3,03 nM a los 120 min, mientras que el tirosol, que contó con 40,8 ± 4,02 nM a los 15 min y con 4,68 ± 1,50 nM a los 120 min, presentó un derivado sulfato a mayor concentración que el compuesto padre (259,6 ± 26,7 nM a los 15 min y 38,1 ± 0,06 nM a los 120 min). El hidroxitirosol, alcanzó una concentración de 28,8 ± 1,64 nM a los 15 min y de 5,12 ± 0,45 nM a los 120 min, mientras que su metabolito sulfato, mostró un valor de 17,4 ± 3,34 nM a los 15 min y de 1,07 ± 0,005 nM a los 120 min. El ácido *p*-cumárico, la luteolina y la apigenina, fueron encontrados a concentraciones menores. Concretamente, el ácido *p*-cumárico obtuvo un valor de 16,1 ± 6,57 nM a los 15 min y de 0,35 ± 0,15 nM a los 120 min, mientras que la luteolina se encontró a 7,10 ± 1,11 nM a los 15 min y a 1,19 ± 0,69 nM a los 120 min, detectándose un derivado glucurónido a muy baja concentración (0,57 ± 0,10 nM a los 15 min y 0,14 ± 0,005 nM a los 120 min). Por último, la apigenina fue de 3,21 ± 0,75 nM a los 15 min y de 0,72 ± 0,13 nM a los 120 min. Finalmente, (+)-pinoresinol se detectó solo a los 15 y 30 min con valores de 4,75 ± 1,29 nM y 1,01 ± 0,26 nM, respectivamente.

Conclusiones: El análisis de los triterpenos pentacíclicos y polifenoles en AOVE de Arbequina, permite afirmar que es una fuente de compuestos bioactivos, a pesar de que sus concentraciones sean inferiores a las determinadas en aceitunas de mesa de esta misma variedad (Moreno-González *et al*., 2020b). La evaluación del plasma tras la administración de AOVE de Arbequina a ratas,

detectó 1 triterpeno pentacíclico (ácido maslínico) y 7 polifenoles (ácido *p*-cumárico, ácido vanílico, apigenina, pinoresinol, hidroxitirosol, luteolina y tirosol). Además, también se detectaron metabolitos derivados del ácido maslínico, del hidroxitirosol, del tirosol y de la luteolina.

Por tanto, esta determinación permite ampliar el conocimiento del destino de estos componentes bioactivos y proporciona un apoyo convincente de que el AOVE es una fuente dietética no solo de polifenoles, sino lo más importante, de triterpenos pentacíclicos.

Determination by LC-MS of pentacyclic triterpenes and polyphenols in extra virgin olive oil and its bioavailability after oral administration in rats

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Abstract: Extra virgin olive oil (EVOO), a recognized healthy food, contains pentacyclic triterpenes and polyphenols, that are minor compounds with neuroprotective, cardioprotective and antitumoral activities, among others. Despite their beneficial effects on health, evidences from *in vivo* research are still needed for a better understanding their role in disease prevention. To this aim, the present study evaluates the plasmatic concentrations of pentacyclic triterpens and polyphenols by LC-MS after the oral administration of Arbequina EVOO to Sprague-Dawley rats. Firstly, EVOO was analyzed with a novel method that enables the simultaneous determination of both groups of compounds by means of liquid-liquid extraction with ethanol-methanol (1:1; v/v) prior to LC-MS analysis. Pentacyclic triterpenes were the most abundant compounds with 149 mg/kg, while polyphenols were found at 16.8 mg/kg. The main pentacyclic triterpene was maslinic acid (116 ± 9.83 mg/kg), followed by oleanolic acid (29.3 ± 0.83 mg/kg), erythrodiol (3.73 ± 0.35 mg/kg) and minor amounts of uvaol (0.22 ± 0.02 mg/kg). Concerning polyphenols, 13 compounds were identified, of which the higher concentrations were reached by hydroxytyrosol acetate (4.60 ± 0.19 mg/kg), hydroxytyrosol (3.68 ± 0.08 mg/kg), luteolin (3.63 ± 0.10 mg/kg), tyrosol (2.19 ± 0.06 mg/kg) and apigenin (1.61 ± 0.05 mg/kg). Catechol, vanillic acid, pinoresinol and *p*-coumaric acid showed values between 0.43 and 0.14 mg/kg. Minor amounts were detected for quercetin, caffeic acid, verbascoside and salidroside. Sprague-Dawley rats were orally administered a dose of 6.16 mL oil/kg (equivalent to the consumption of 60 mL in a 60 kg-human), and blood was extracted at 0, 15, 30, 60, 90 and 120-min post-administration. Pentacyclic triterpenes and polyphenols were concurrently extracted from plasma by liquid-liquid extractions with ethyl acetate prior LC-QTRAP-MS analysis. Maslinic acid was the only pentacyclic triterpene in rat plasma, whereas vanillic acid, tyrosol, hydroxytyrosol, *p*-coumaric acid, luteolin, apigenin and pinoresinol were the polyphenols detected. The analytes found in plasma followed a similar pattern, the maximum concentration (Cmax) was observed at 15 minutes (tmax) followed by a steady decrease, yet still was detected at 120 min. The Cmax ranged from 59.8 nM (vanillic acid) to 3.21 nM (apigenin). In conclusion, the present results improve the knowledge on the content of pentacyclic triterpenes and polyphenols in EVOO of the Arbequina variety, as well as their fate after the ingestion of this food.

Keywords Extra virgin olive oil (EVOO); *Olea europaea* L.; Arbequina; maslinic acid; hydroxytyrosol; vanillic acid; tyrosol; bioavailability; rats

1. Introduction

Mediterranean diet is characterized by using olive oil as the main source of fat, a high consumption of vegetables, legumes, cereals, fruits, pasta, bread, nuts and seeds, moderate amounts of fish, white meat, dairy products and eggs and small ingestion of red meat and wine (Boskou, 2012). Olive oil has an important value due to its unique composition, rich in monounsaturated fatty acids, mostly oleic acid and minor bioactive compounds such as tocopherols, squalene, polyphenols and triterpenoids (Olmo-García *et al*., 2019a). However, the profile of olive oil depends on several different factors such as geographical origin, the ripening stage, the soil features, the olive cultivar, the technological production process and storage conditions (López-Yerena *et al*., 2019).

Hydrophilic phenols are the most abundant natural antioxidants of extra virgin olive oil (EVOO) and they can be grouped in different classes that include phenolic alcohols (hydroxytyrosol, hydroxytyrosol acetate, tyrosol), phenolic acids (vanillic, *p*-coumaric, *o*-coumaric, ferulic, syringic, protocatechuic, synaptic, *p*-hydroxybenzoic, gallic and caffeic acids), flavonoids (luteolin, luteolin-7-O-glucoside and apigenin), secoiridoids (oleuropein and ligstroside) and lignans (pinoresinol, acetoxypinoresinol) (Olmo-García *et al*., 2019b). Phenolic compounds, in addition to providing quality and stability to the oil, also contribute to the organoleptic properties such as bitterness, astringency and pungency (Vitaglione *et al*., 2015). On the other hand, the data concerning the concentration of pentacyclic triterpenes in olive oil is scarce, although the presence of maslinic and oleanolic acids is known of, as well as minor amounts of erythrodiol and uvaol (Pérez-Camino & Cert, 1999; Allouche *et al*., 2009; Allouche *et al*., 2010; Olmo-García *et al*., 2018). Noteworthy that triterpenic dialcohols (erythrodiol and uvaol) are found in minor concentrations, but they are determined as an authenticity parameter to detect fraudulent mixtures with pomace olive oil (Bajoub *et al*., 2016).

Nowadays, a growing number of studies point out that both pentacyclic triterpenes and polyphenols from *Olea europaea* L. could have an important role in the prevention of chronic diseases (Reboredo-Rodríguez *et al*., 2018; Visioli *et al*., 2018). Despite the knowledge of the beneficial effects on health of pentacyclic triterpenes and polyphenols present in EVOO, the information regarding both groups of compounds in the food is scarce, as well as the data on their oral bioavailability after the consumption of EVOO. This lack of information could be due to the difficulties encountered in developing sensitive and accurate methods that measure simultaneously measured pentacyclic triterpene and polyphenols in the food as well as in biological fluids. Consequently, the first aim of the present study was to develop an analytical strategy that allows the concurrent determination of pentacyclic triterpenes and polyphenols present in EVOO by LC-MS. The determinations were performed in EVOO of the Arbequina variety, one of the main Spanish olive oils, which is well known in the international market for its excellent taste and flavor. In addition, the second objective consisted on the development of a method that allows the simultaneous measurement of pentacyclic triterpenes and polyphenols in plasma by LC-QTRAP-

MS. The analytical approach was applied to the determination of the plasmatic concentrations of both groups of compounds after the oral administration of this EVOO to Sprague-Dawley.

2. Materials and Methods

2.1. Chemicals

Apigenin, betulinic acid (internal standard (IS) of pentacyclic triterpenes), luteolin, luteolin-7-O-glucoside, oleanolic acid, tyrosol, ursolic acid and uvaol were supplied by Extrasynthèse (Genay, France). L-ascorbic acid, caffeic acid, catechol, *o*-coumaric acid, *p*-coumaric acid, 2-(3-hydroxyphenyl)-ethanol (IS of polyphenols), erythrodiol, maslinic acid, oleuropein, (+)-pinoresinol, quercetin, rutin, salidroside, vanillic acid and verbascoside were purchased from Sigma-Aldrich (St. Louis, USA). Hydroxytyrosol and hydroxytyrosol acetate came from Seprox BIOTECH (Madrid, Spain). Acetone, acetonitrile, methanol, 2-propanol and tetrahydrofuran were obtained from Panreac Química SLU (Castellar del Vallés, Spain). Acetic acid was purchased from Merck (Darmstadt, Germany) while ethyl acetate and ethanol came from J. T. Baker (Deventer, Holland). All solvents were LC-MS grade. Water with a conductivity lower than 0.05 $mS \cdot cm^{-1}$ was obtained using a Milli-Q water purification system from Millipore (St. Louis, Missouri, USA).

2.2. Animals and Diets

Male adult Sprague-Dawley rats weighting 235-275 g were from the Animal House Facility of the Facultat de Farmàcia i Ciències de l'Alimentació (Universitat de Barcelona, UB). Animals were randomly distributed in cages (n = 2/cage) with free access to a commercial diet (2014 Teklad Global 14%, Harlan, Barcelona, Spain) and water. The animal room was kept under controlled conditions of temperature (22 ± 2˚C), relative humidity (40%-70%) and lighting (12 h light/dark cycle). Experiments were performed in the morning to minimize the influence of circadian rhythms. EVOO of Arbequina variety was administered as a single p.o. dose to rats deprived from food for 12 h before the experiments, but with free access to water. All animal manipulations fulfilled the Guidelines established by the European Community for the care and management of laboratory animals. The study was approved by the Ethic Committee of Animal Experimentation of the Generalitat de Catalunya with reference number 9468.

2.3. Extra virgin olive oil of the Arbequina variety

Experiments were performed with EVOO of the Arbequina variety (Cooperativa del Camp Foment Maialenc SCCL, Maials, Lleida, Spain) from olives harvested during the 2015/2016 season. Olive trees were cultivated in Ribera d'Ebre (Tarragona, Spain) in orchards with drip irrigation. The oil was kept refrigerated and protected from light until analysis. The EVOO fulfilled the quality parameters of acidity lower than 0.2º, peroxide value of 15 meq O_2/Kg of oil and specific extinction coefficients at 232 nm (K_{270}) and 270 nm (K_{232}) of 0.10 and 2.50, respectively. All quality parameters

were evaluated following the European Union standards methods (European Community Regulation EEC/2568/91).

2.4. Dose administration and sample collection

Animals received by gavage a single dose of 6.16 mL EVOO/kg of body weight. The dose administered was equivalent to the consumption of 60 mL for a person weighting 60 kg according to the dose translation between animal to human based on body surface areas, as indicated by Reagan-Shaw *et al*. (2017). After oral administration, blood was withdrawn from the lateral saphenous vein in both hindlegs through a puncture performed with a 21G needle (Hem *et al*., 1998). Blood was collected at 15, 30, 60 and 90 min and was directly placed into EDTA-K_2 tubes (Microvette CB 300 K2E, Sarstedt, Nümbrecht, Germany) to avoid coagulation. Plasma was separated by centrifugation at 27190 x *g* for 15 min at 4°C (Centrifuge 5417 R, Eppendorf AG, Hamburg, Germany) and it was stored at −20°C until its analysis. At 120 min post-administration of EVOO, blood was extracted by cardiac puncture (20G needle) with animals previously anesthetized i.p. with 90 mg/kg of ketamine (Imalgene 100 mg/mL, Merial Laboratorios S.A., Barcelona, Spain) and 10 mg/kg of xylacine (Rompun 20 mg/mL, Bayer Hispania S.L., Barcelona, Spain). Blood collected in the syringes was immediately placed into 4 mL EDTA-K_3 tubes (Sarstedt, Nümbrecht, Germany), centrifuged at 3345 x *g* for 15 min at 4°C (Megafuge 1.0R, Heraeus Instruments GmbH, Hanau, Germany) and the supernatant was stored at −20°C until analysis.

2.5. Analysis of EVOO of the Arbequina variety

2.5.1. Simultaneous extraction of pentacyclic triterpenes and polyphenols from EVOO

Pentacyclic triterpenes and polyphenols were simultaneously extracted from the same Arbequina EVOO sample prior to the administration to rats. For that, 0.50 g of oil (checked by weight) was taken with a Pasteur pipette, placed in a 2 mL conical tube and 25 µL of freshly prepared ascorbic acid 10% was added. EVOO was submitted to a first extraction with 0.50 mL of ethanol:methanol (1:1; v/v) containing betulinic acid (IS of pentacyclic triterpenes) and 2-(3-hydroxyphenyl)-ethanol (IS of polyphenols) to a final concentration of 1.2 and 2.0 µM, respectively. The content was vigorously vortexed for 5 min and introduced in an ultrasonic bath for 10 min. The mixture was centrifuged at 27190 × *g* for 10 min at 2˚C (Centrifuge 5417 R) and the supernatant was reserved in a new tube. Two additional extractions of the pellet were performed by adding 0.50 mL of ethanol:methanol (1:1; v/v). The three extracts were pooled and 25 µL of ascorbic acid 10 % was added before the evaporation by dryness in a Concentrator 5301 (Eppendorf AG, Hamburg, Germany) at 45˚C. Then, the residue was reconstituted in 250 µL of methanol 80%, agitated in a vortex for 5 min, sonicated for 2 min and centrifuged at 27190 x *g* for 30 min at 2˚C (Centrifuge 5417 R). The clean supernatant was filtered with a 0.45 µm PTFE syringe filter and two dilutions were performed before analysis using methanol 80% as a solvent. Maslinic acid,

oleanolic acid and erythrodiol were determined in a 1/50 dilution, while most polyphenols were measured in a 1/10 dilution. Finally, a non-diluted sample was used to evaluate the compounds present at a very low concentration (uvaol, catechol, caffeic acid, salidroside, quercetin and verbascoside). All samples were transferred into sealed amber vials prior to LC-MS analysis of pentacyclic triterpenes and polyphenols.

2.5.2. Instrumentation

Analyses were performed on an Acquity UPLC system composed with a binary solvent manager, sample manager, and a column heater (Waters, Milford, Michigan, USA) coupled to an API 3000 triple quadrupole (AB Sciex, Toronto, Canada). The Analyst software 1.4.2 (AB Sciex) operated the instrument and performed the data acquisition and processing. The equipment was available at the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB).

2.5.3. Determination of pentacyclic triterpenes by LC-APCI-MS

LC-MS analyses were performed as previously indicated (Moreno-González *et al*., 2020b). Briefly, chromatographic separation of pentacyclic triterpenes was performed on a Zorbax Eclipse PAH column (150 mm x 4.6 mm, 3.5 µm) kept at 40˚C and preceded by a guard column of the same material (12.5 mm × 4.6 mm, 5.0 µm) (Agilent Technologies, Santa Clara, USA). The injection volume was 10 µL. The mobile phase consisted of Milli-Q water with acetic acid at 0.05% (phase A) and methanol with acetic acid at 0.05% (phase B). Mobile phase eluted as follows (0.8 mL/min): 0 min, 17% A and 83% B; 22.5 min, 17% A and 83% B; 23 min, 0% A and 100% B. The column was washed for 4 min with 100% B and the system was re-equilibrated during 6 min prior to the injection of the next sample. To avoid carry-over effect, the needle was washed after each injection with a strong solution composed of acetonitrile:Milli-Q water (90:10; v/v) followed by a weak-wash solution of methanol:Milli-Q water (80:20; v/v), both delivered at 300 µL.

The ionization of the triterpenic compounds was carried out using an APCI source programmed in two modes: from 0 to 16 min, the source operated in negative mode with a temperature set at 500˚C allowing the detection of pentacyclic triterpenic acids (maslinic acid, oleanolic acid, ursolic acid and betulinic acid (IS)) and from 16 to 22 min, the source was kept at 450˚C in positive mode for the detection of alcohols (erythrodiol and uvaol). MS was programmed in the selected ion monitoring (SIM) mode. The mass-to-charge ratio (*m/z*) corresponding to $[M-H]^-$ was 471.6 for maslinic acid and 455.5 for oleanolic, ursolic and betulinic acids (IS) whereas erythrodiol and uvaol were detected at $[M+H-H_2O]^+$ with a *m/z* 425.7. The dwell time for acids was 1000 ms whereas the dwell time for alcohols was of 4000 ms.

2.5.4. Determination of polyphenols by LC-ESI-MS/MS

Determination of polypohenols were carried out following the LC-MS conditions previously established by Moreno-González *et al*. (2020a). Briefly, chromatographic separation was performed on a Zorbax Eclipse XDB-C18 column (150 mm x 4.60 mm x 5.0 µm) kept at 30˚C coupled to a

guard column of the same material and injection volume of 10 µL. The mobile phase was composed of Milli-Q water with 0.025% acetic acid (phase A) and acetonitrile with 5% acetone (phase B) and was delivered in a gradient elution program of 9.5 min. Polyphenols were ionized using an electrospray ionization (ESI) source in negative mode and temperature set at 350˚C. MS was operated in multiple reaction monitoring (MRM) mode, with a dwell time for the quantifier and qualifier transitions of 60 ms and 10 ms, respectively.

2.5.5. Quantification of pentacyclic triterpenes and polyphenols in olive oil

Quantification was performed with calibration curves built using the peak area ratios of analyte to internal standard versus the concentration of analyte. Therefore, individual stock solutions for each analyte were prepared in methanol 80% to a final concentration of 250 µM and stored at −20˚C. They were used to prepare working standards at 1 and 10 µM, for both the mixture containing 5 pentacyclic triterpenes and the 17 polyphenols. Further dilution of the working standards resulted in calibration standards at final concentrations of 0; 0.05; 0.10; 0.50; 1.00; 2.00; 2.50, and 3.00 µM. Internal Standard (IS) of triterpenes (betulinic acid) and polyphenols (2-(3-hydroxyphenyl)-ethanol) were prepared as a stock solution of 250 µM and diluted to produce final concentrations of 1.2 and 2 µM. All working and calibration standards were freshly prepared before use. The concentration of pentacyclic triterpenes and polyphenols in EVOO were calculated from the peak area ratio of the individual compound to the IS interpolated on the calibration curve. Within each analytical run, a full set of calibration standards, which included reagent blank, were injected.

2.6. Analysis of pentacyclic triterpenes and polyphenols in rat feed

The commercial diet (2014 Teklad Global 14%) supplied to the animals was analyzed to establish their content in presence of pentacyclic triterpenes and polyphenols. Thus, the food was crushed with a mortar and subsequently 5 short pulses of 30 s with a homogenizer polytron (PTA 20 TS rotor, setting 3; Kinematica AG, Lucerne, Switzerland) were performed until an ultrafine powder was obtained. An aliquot of 1 g of grinded sample was placed in 15 mL conical tubes and diluted with 6 mL of ethanol:methanol (1:1; v/v) containing betulinic acid (IS triterpens) and 2-(3-hydroxyphenyl)-ethanol (IS polyphenols) at final concentration of 1 µM. The mixture was vortexed for 5 min and centrifuged at 3345 × *g* for 30 min at 4˚C (Megafuge 1.0R). The supernatant was reserved in a new tube and two additional extractions of the pellet with 3 mL ethanol:methanol (1:1; v/v) were performed. The extracts were pooled and further centrifuged at 27190 × *g* for 30 min at 2˚C (Centrifuge 5417 R) to remove solid particles. The clean supernatant was filtered with a 0.45 µm PTFE syringe filter and directly placed in amber vials for the LC-MS analysis, carried out as indicated in the subheadings 2.5.3 for pentacyclic triterpenes and 2.5.4 for polyphenols.

2.7. Analysis of plasma samples

2.7.1. Simultaneous extraction of pentacyclic triterpenes and polyphenols from rat plasma samples

Blank plasma obtained from rats that have never received EVOO and plasma samples collected after the administration of EVOO were processed as follows: a 200 µL aliquot of plasma was added 10 µL of ascorbic acid 10%, 10 µL of of betulinic acid (2 µM) and 2-(3-hydroxyphenyl)-ethanol (10 µM), 10 µL acetic acid 1% and 2 mL of ethyl acetate in 15 mL tubes. The mixture was vigorously shaken for 5 min, sonicated for 10 min and centrifuged at 3345 x *g* for 10 min at 2˚C (Megafuge 1.0R). The supernatant was reserved in a new tube and the pellet was subjected to a second extraction with 2 mL of ethyl acetate. Both supernatants were combined and evaporated to dryness at 45˚C. The residue was reconstituted in 100 µL of methanol 80% and vortexed for 5 min. Samples were introduced in an ultrasonic bath for 2 min and centrifuged at 27190 x *g* for 30 min at 2˚C (Centrifuge 5471 R). Then the supernatant was transferred into sealed amber vials for the subsequent by LC-MS.

2.7.2. Instrumentation

The analyses were performed in an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, California, USA) equipped with a binary solvent manager, sample manager and column heater coupled to a 4000 QTRAP system (AB Sciex, Toronto, Canada). The Analyst 1.6.2 software (AB Sciex) controlled the instrument and performed data acquisition and processing. The equipment was available at the Scientific and Technological Centers of the Universitat de Barcelona (CCiTUB).

2.7.3. Determination of pentacyclic triterpenes by LC-APCI-QTRAP-MS

The measurement of plasma samples was performed with the most sensible instrumentation that allowed lowest limits of quantification. Consequently, the method was based on the conditions indicated in the subheading 2.4.3, with some modifications, regarding the gradient elution that was enlarged to allow the determination of metabolites and the MS instrument. The mobile phase included Milli-Q water with acetic acid 0.05% (phase A) and methanol with 0.05% acetic acid (phase B), which eluted under gradient conditions at a flow rate of 0.8 mL/min as follows: 0 min, 95% A and 5% B; 2 min, 95% A and 5% B; 4 min, 40% A and 60% B; 19 min, 17% A and 83% B; 40 min, 17% A and 83% B; 40.5 min, 0% A and 100% B. Lastly, the column was washed for 4 min with 100% B and the system was re-equilibrated during 6 min before the next injection. The needle was washed with acetonitrile:Milli-Q water (90:10; v/v). The ionization of the compounds was carried out using an APCI source programmed in two periods: from 0 to 34.5 min operated at 500˚C in negative mode allowing the detection of pentacyclic triterpenic acids (maslinic, oleanolic, ursolic and betulinic acids) and from 34.5 to 40.5 min, the source was kept at 450˚C and positive mode for alcohols (erythrodiol and uvaol). Nitrogen with a nebulizing flow set at −5 and 5 arbitrary units for negative and positive ion mode, respectively, served as curtain gas (25 arbitrary units) and gas 1 and gas 2 (50 and 0 arbitrary units, respectively). The optimal MS conditions in the negative mode for the detection of pentacyclic acids had a declustering potential, with −175 V for maslinic acid and −150 V for betulinic acid, oleanolic acid and ursolic acid and the entrance potential, with −10

V for all of them. The triterpenic alcohols (positive ion mode) erythrodiol and uvaol shared the same values of declustering potential and entrance potential that were 70 V and 10 V, respectively.

Identification was performed using the selected ion monitoring (SIM) mode at *m/z* 471.6 or maslinic acid and 455.5 for oleanolic acid, ursolic acid and betulinic acid (IS) with a dwell time of 50 ms for all of them. Triterpenic alcohols erythrodiol and uvaol were analyzed at *m/z* 425.7 with a dwell time of 2000 ms. The *m/z* corresponding to the metabolites from phase I and phase II from maslinic and oleanolic acids were added, with a dwell time set at 50 ms.

2.7.4. Determination of polyphenols by LC-ESI-QTRAP-MS/MS

The method was based on the conditions indicated in the subheading 2.5.4, with some modifications, regarding the volume of injection that was set at 2 µl, the gradient elution that was enlarged to allow the determination of metabolites and the MS instrument.

Table 1. MRM parameters corresponding to each polyphenol and the internal standard set or obtained by LC-ESI-QTRAP-MS/MS.

Compound	Parent ion (*m/z*)	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)	Declustering potential (V)	CXP (V)	CE Quantifier (V)	CE Qualifier (V)
Apigenin	269.0	117.1	151.1	−120.0	−10.0	−55.0	−35
Caffeic acid	179.1	135.1	107.1	−75.0	−10.0	−23.0	−30
Catechol	109.0	91.2	65.0	−80.0	−10.0	−28.0	−40
o-Coumaric acid	163.2	119.2	93.2	−80.0	−15.0	−22.0	−45
p-Coumaric acid	163.2	119.2	93.2	−80.0	−15.0	−22.0	−45
Hydroxytyrosol	153.2	122.8	94.8	−78.0	−10.0	−20.0	−30
Hydroxytyrosol acetate	195.0	59.0	134.9	−85.0	−10.0	−17.0	−20
Luteolin	285.2	133.2	150.9	−100.0	−10.0	−50.0	−75
Luteolin-7-O-glucoside	447.3	285.2	327.1	−130.0	−15.0	−40.0	−36
Oleuropein	539.5	275.0	307.3	−110.0	−10.0	−30.0	−30
(+)-Pinoresinol	357.3	151.1	136.1	−97.0	−10.0	−27.0	−25
Quercetin	301.2	151.1	178.1	−110.0	−10.0	−30.0	−35
Rutin	609.5	300.1	271.0	−300.0	−10.0	−50.0	−75
Salidroside	299.2	119.2	89.3	−74.0	−15.0	−22.0	−20
Tyrosol	137.1	106.2	118.8	−70.0	−15.0	−20.0	−20
Vanillic acid	167.0	152.0	107.9	−70.0	−10.0	−20.0	−20
Verbascoside	623.5	161.3	461.3	−140.0	−10.0	−50.0	−48
IS	137.0	107.0		−70.0	−15.0	−18.0	--

The gradient elution was as follows: 0 min, 95% A and 5% B; 1 min, 90% A and 10% B; 10 min 35% A and 65% B and 10.50 min 0% A and 100% B. The column was washed during 5 min with 100% organic phase and the system was re-equilibrated during 6 min to the initial conditions before the next injection. A solution of isopropanol:tetrahydrofuran:Milli-Q water (1:1:1; v/v) was utilized to wash the needle. Polyphenols were ionized in negative mode using an electrospray (ESI)

source set at 600°C. Nitrogen was used as curtain gas (25 arbitrary units), ionization gas 1 and gas 2 (50 arbitrary units for both) and collision gas (−2 arbitrary units). The ionization spray voltage was established at −3500 V. The analyzer was operated in multiple reaction monitoring (MRM) mode with a dwell time of 30 ms for all the compounds. Table 1 shows the optimal mass spectrometry parameters of analytes studied. The entrance potential was −10.0 V for all analytes. Within each analytical run a full set of calibration standards, which included a blank plasma sample and a reagent blank, were analyzed.

2.7.5. Quantification of pentacyclic triterpenes and polyphenols in rat plasma

Quantification of pentacyclic triterpenes and polyphenols was performed by interpolation of the peak area ratio of analytes versus IS on a calibration curve built with calibration standards prepared at different concentration using plasma samples from rats that had never received EVOO. Hence, working solutions of triterpenes and polyphenols prepared as described in the subheading 2.5.5, were further diluted to obtain 0.2; 0.5; 1; 2 and 3 µM and were used to prepare the calibration standards. Then, different pools of blank rat plasma were spiked with 10 µL of working solutions and subsequently extracted according to the procedure described in 2.7.1.

Calibration curves were prepared at the concentrations of 0; 10; 25; 50; 100 and 150 nM. Stock solutions of IS at 250 µM were diluted to obtain the working standards at 2 µM for pentacyclic triterpenes and 10 µM for polyphenols and added to calibration standards at 10 µl. Metabolites were quantified with calibration curves corresponding to their precursors to due to the lack of standards for them.

2.8. Statistical analysis

The results are expressed as the mean ± standard error of the means (SEM). Analyte concentrations of Arbequina EVOO are presented in mg/kg of EVOO, whereas plasma results were given in nM. Chauvenet's criterion was used to reject outliers. Data evaluation, statistical analysis and elaboration of graphs were performed with a commercially available package (Prism version 6; GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Determination of pentacyclic triterpenes and polyphenols in EVOO of the Arbequina variety

3.1.1. Method validation

The method employed to determine pentacyclic triterpenes and polyphenols in EVOO was validated following the Eurachem Guide (2014) for sensitivity, linearity, accuracy, precision and carry-over. Table 2 displays the results obtained for the analysis of pentacyclic triterpenes by LC-APCI-MS as well as the determination of polyphenols that were measured with different analytical conditions (LC-ESI-MS/MS). Sensitivity was evaluated through the determination of the limit of detection (LOD) and limit of quantification (LOQ) that were established as the concentrations that

meet the criteria of a signal-to-noise ratio of 3:1 and 10:1, respectively (Table 2). Concerning pentacyclic triterpenes, maslinic acid hold the lower LOQ of 10.8 nM, while double values were found for oleanolic and ursolic acids. The alcohols had the highest LOQ with concentrations of 46.6 and 71.8 nM for erythrodiol and uvaol, respectively. Concerning polyphenols, the sensitivity was higher since most of the compounds showed a LOQ inferior to 10 nM. Only catechol, salidroside, tyrosol and vanillic acid yield a LOQ close to 30 nM.

Table 2. Validation parameters of the analytical methods used for the analysis of pentacyclic triterpenes and polyphenols in EVOO: sensitivity, linearity, accuracy and precision.

Compound	Sensitivity		Linearity			Accuracy (%)	Precision (%)
	LOD (nM)	LOQ (nM)	Range (µM)	Equations	R^2		
Pentacyclic triterpenes analyzed by LC-APCI-MS							
Maslinic acid	3.23	10.8	0-3.0	y = 0.369x - 0.034	0.9980	2.19	1.99
Oleanolic acid	5.86	19.5	0-3.0	y = 0.947x + 0.043	0.9984	1.24	3.04
Ursolic acid	7.38	24.6	0-3.0	y = 0.948x − 0.036	0.9988	2.65	4.53
Erythrodiol	14.0	46.6	0-3.0	y = 0.624x − 0.023	0.9963	1.51	3.12
Uvaol	21.5	71.8	0-3.0	y = 0.198x − 0.012	0.9946	1.22	2.80
Polyphenols analyzed by LC-ESI-MS/MS							
Apigenin	0.35	1.17	0-3.0	y = 0.134x − 0.002	0.9970	3.41	5.27
Caffeic acid	1.09	3.63	0-2.5	y = 0.352x + 0.005	0.9975	4.49	3.52
Catechol	8.59	28.6	0-2.5	y = 0.0045x + 0.002	0.9984	9.28	1.91
o-Coumaric acid	0.37	1.23	0-3.0	y = 0.439x + 0.002	0.9988	1.78	3.36
p-Coumaric acid	1.07	3.58	0-3.0	y = 0.159x + 0.008	0.9955	8.73	7.86
Hydroxytyrosol	1.01	3.37	0-3.0	y = 0.301x − 0.009	0.9987	4.19	6.35
Hydroxytyrosol acetate	3.06	10.2	0-3.0	y = 0.288x + 0.005	0.9979	8.50	5.90
Luteolin	1.33	4.43	0-2.5	y = 0.318x − 0.006	0.9954	1.01	4.12
Luteolin-7-O-glucoside	0.56	1.87	0-3.0	y = 0.620x + 0.005	0.9962	6.72	7.32
Oleuropein	0.75	2.51	0-2.5	y = 0.139x + 0.007	0.9952	2.63	6.78
(+)-Pinoresinol	3.57	11.9	0-2.5	y = 0.016x − 0.006	0.9957	4.57	4.09
Quercetin	0.61	2.05	0-3.0	y = 0.265x − 0.001	0.9974	4.59	5.21
Rutin	0.14	0.45	0-2.5	y = 0.138x + 0.004	0.9962	9.09	5.51
Salidroside	8.36	27.9	0-2.5	y = 0.090x + 0.003	0.9960	7.05	9.28
Tyrosol	7.56	25.2	0-3.0	y = 0.0085x + 0.002	0.9966	4.93	3.27
Vanillic acid	7.56	25.2	0-3.0	y = 0.0374x + 0.003	0.9960	4.18	6.47
Verbascoside	1.59	5.30	0-2.5	y = 0.139x + 0.001	0.9975	2.38	5.93

Moreover, the calibration curves for pentacyclic triterpenes were linear up to 3.0 µM while for some polyphenols they were linear up to 2.5 µM as it is shown in Table 2. For all compounds, the correlation coefficient (R^2) was higher than 0.9952.

The precision (%RSD: coefficient of variation) and accuracy (percentage of relative error of the measured concentration with respect to the nominal one) was calculated for all the calibrations

standards at the concentrations of 0.05; 0.1; 0.5; 1.0; 2.0; 2.5 and 3.0 µM. Table 2 reports the means obtained for the 7 levels of concentrations and the values were lower to 10% for both precision and accuracy, thus fulfilling the requirements of being inferior to 15% that are indicated in the Eurachem Guide (2014).

Finally, the carryover of one sample to another on the LC-MS instrument was evaluated within the validation of the method by injecting the highest calibration standard followed by a blank reagent. Carry-over was assessed 6 times by injecting a vial of methanol 80% at regular intervals based on the total number of samples of the batch. Blank reagents and methanol 80% did not show peaks at the same retention time than pentacyclic triterpenes, polyphenols and IS.

3.1.2. Identification and quantification of pentacyclic triterpenes by LC-APCI-MS

Pentacyclic triterpenic acids were determined with negative ionization and single ion monitoring (SIM) mode in which only the precursor ion was observed $[M-H]^-$. Of these, maslinic acid was the pentacyclic triterpene that eluted in first place, with a retention time of 5.85 min and the highest peak intensity (Figure 1). This compound was clearly identified in the EVOO samples of the Arbequina variety at the dilution 1/50 at the *m/z* of 471.6.

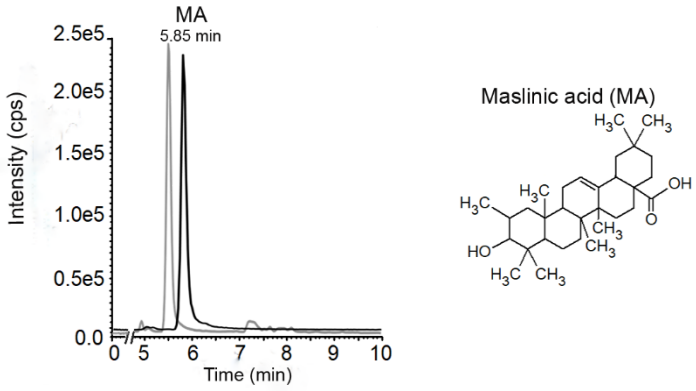

Figure 1. LC-MS extracted ion chromatogram (XIC) of maslinic acid ($[M-H]^-$; *m/z* of 471.6) at 1 µM in a standard solution in methanol 80% (gry trace) and in EVOO of Arbequina variety analyzed at a 1/50 dilution (black trace).

The following pentacyclic triterpenic acid to be detected was oleanolic acid, with a retention time of 12.88 min and separated 1.14 min from its positional isomer, ursolic acid that elutes at 14.02 min (Figure 2). These compounds, as well as the internal standard, betulinic acid, were measured as the precursor ions ($[M-H]^-$) at *m/z* of 455.5. Oleanolic acid was unambiguously detected in the EVOO sample of the Arbequina variety at the dilution 1/50, while ursolic acid was not found either in the diluted sample nor in the sample obtained after the reconstitution of the residue and directly injected to the LC-MS.

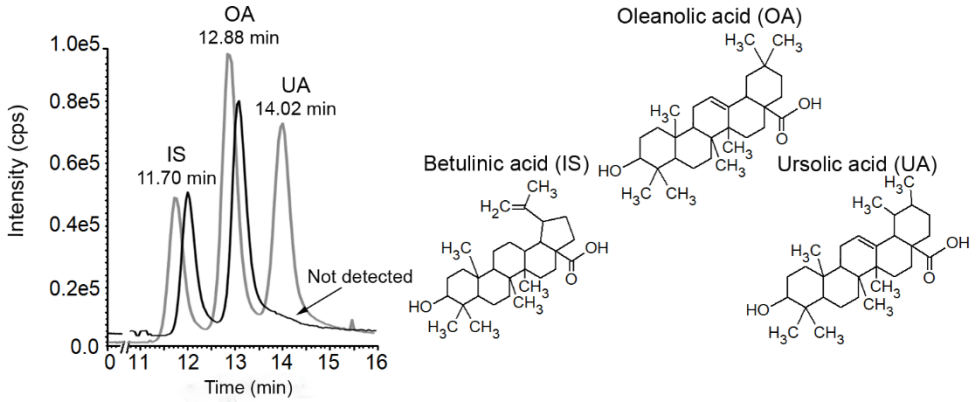

Figure 2. LC-MS extracted ion chromatogram (XIC) of oleanolic and ursolic acids ($[M-H]^-$; *m/z* of 455.5) at 1 µM in a standard solution in methanol 80% (gray trace) and in EVOO of Arbequina variety analyzed at a 1/50 dilution (black trace). The internal standard, betulinic acid ($[M-H]^-$; *m/z* of 455.5) was added at 1.2 µM in both, the standard solution and the EVOO samples.

On the other hand, the pentacyclic triterpenic alcohols, erythrodiol and uvaol were detected with positive ionization in SIM mode, and the principal mass corresponded to the one of the parent compound that had lost a water molecule $[M+H-H_2O]^+$ at the *m/z* of 425.7 (Figure 3). Erythrodiol eluted at 17.71 min while uvaol had a retention time of 19.22 min. Both alcohols were identified in EVOO samples but in different amounts, while erythrodiol was found at the 1/50 dilution, uvaol was only detected in the non-diluted samples.

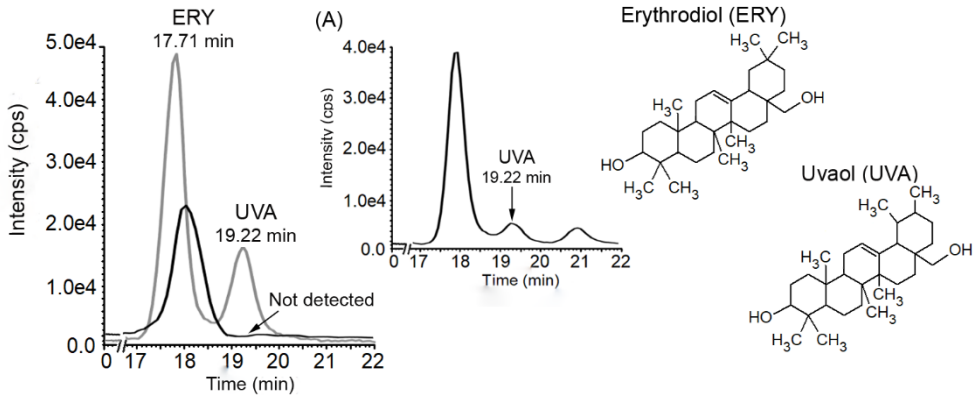

Figure 3. LC-MS extracted ion chromatogram (XIC) of erythrodiol and uvaol ($[M+H-H_2O]^+$; m/z of 425.7) at 1 µM in a standard solution in methanol 80% (gray trace) and in EVOO of Arbequina variety analyzed at a 1/50 dilution (black trace). The insert (A) depicts the XIC of the non-diluted EVOO sample.

The concentration obtained for the pentacyclic triterpenes found in EVOO of the Arbequina variety are depicted in Figure 4. Maslinic acid was the main compound with values of 116.2 ± 9.83 mg/kg of oil, followed by oleanolic acid with 29.3 ± 0.83 mg/kg. Erythrodiol and uvaol gave the lowest concentrations being 3.73 ± 0.35 mg/kg and 0.22 ± 0.02 mg/kg, respectively. Then, the total amount of pentacyclic triterpenes was 149 mg/kg.

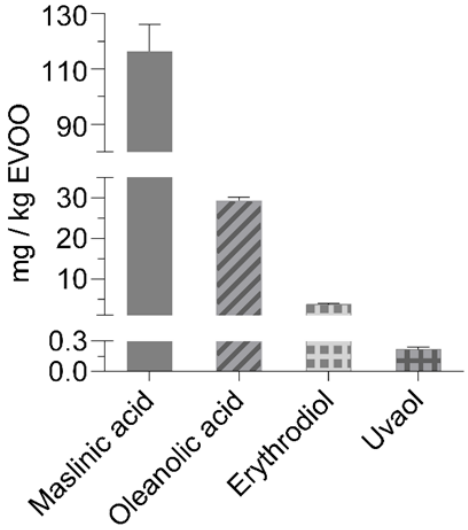

Figure 4. Concentrations of pentacyclic triterpenes in EVOO of the Arbequina variety. Results are expressed as means ± SEM of three independent samples extracted in duplicate.

3.1.3. Identification and quantification of polyphenols by LC-ESI-MS/MS

The analysis of polyphenols in EVOO was attempted with a LC-ESI-MS/MS method that enabled the determination of 17 polyphenols from different classes, as shown in Figure 5 that depicts the chromatographic profile of standards in methanol 80% at 1 µM. Contrary to pentacyclic triterpenes that were determined in the SIM mode, polyphenols were analyzed by multiple reaction monitoring (MRM) mode, that possesses the advantage of unambiguously identifying compounds even though they hold overlapping chromatographic retention times, as well as enabling a short chromatographic run of 9 minutes. Although two pairs of compounds had similar transitions, they were adequately resolved chromatographically. The first one involved the isomers *o*-coumaric and *p*-coumaric acid, both of them with *m/z* of 163.1 and quantifier and qualifier transitions of 119.2 and 93.0. In this case, *p*-coumaric acid elutes in the first place with a retention time of 7.53 min, while *o*-coumaric acid comes up 0.58 min later, at a retention time of 8.11 min. The second one took place with tyrosol (137.1→106.1) and the internal standard (137.0→107.0), but they could be adequately separated by the gradient elution, since tyrosol turned up at 6.79 min and the IS at 7.03 min.

The analysis of EVOO of the Arbequina variety enabled the identification of 13 phenolic compounds (Figure 5). Polyphenols were found at inferior concentrations than pentacyclic triterpenes; therefore, a different dilution was used. Apigenin, *p*-coumaric acid, hydroxytyrosol, hydroxytyrosol acetate, luteolin, (+)-pinoresinol, tyrosol and vanillic acid were measured in the 1/10 dilution, whereas caffeic acid, catechol, quercetin, salidroside and verbascoside were determined in non-diluted samples. Finally, *o*-coumaric acid, luteolin-7-O-glucoside, oleuropein and rutin were not detected either in the samples corresponding to the 1/10 dilution, nor in the non-diluted ones.

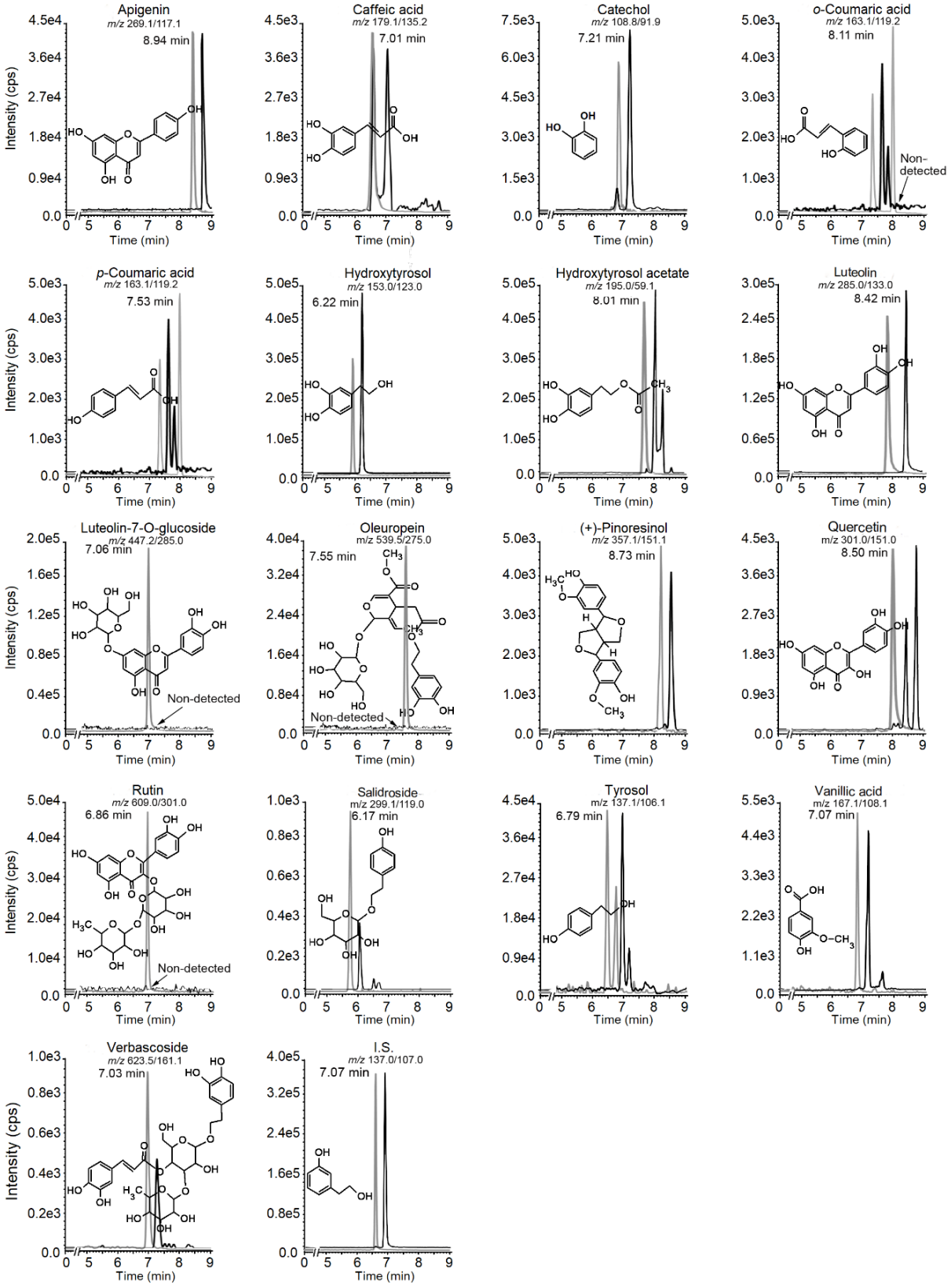

Figure 5. LC-MS/MS extracted ion chromatogram (XIC) of polyphenols at 1 µM and the internal standard, (2-(3-hyroxyphenyl)-ethanol) at 2 µM in a standard solution in methanol 80% (gray trace) and in EVOO of Arbequina variety (black trace).

The concentrations of polyphenols identified in Arbequina EVOO is represented in Figure 6. The most abundant polyphenol was hydroxytyrosol acetate (4.60 ± 0.19 mg/kg), followed by hydroxytyrosol and luteolin with similar concentrations of 3.68 ± 0.08 and 3.63 ± 0.11 mg/kg, respectively. Subsequently, tyrosol and apigenin were found at 2.19 ± 0.07 mg/kg and 1.61 ± 0.06 mg/kg. After these, polyphenols were found in concentrations lower than 0.5 mg/kg, since catechol was found at 0.43 ± 0.01 mg/kg and was ensued by vanillic acid (0.30 ± 0.01 mg/kg), (+)-pinoresinol (0.20 ± 0.004 mg/kg) and p-coumaric acid (0.14 ± 0.01 mg/kg).

Finally, quercetin, caffeic acid, verbascoside and salidroside yielded values inferior to 0.015 mg/kg. Then, the total amount of polyphenols that were found in EVOO samples of the Arbequina variety was 16.8 mg/kg.

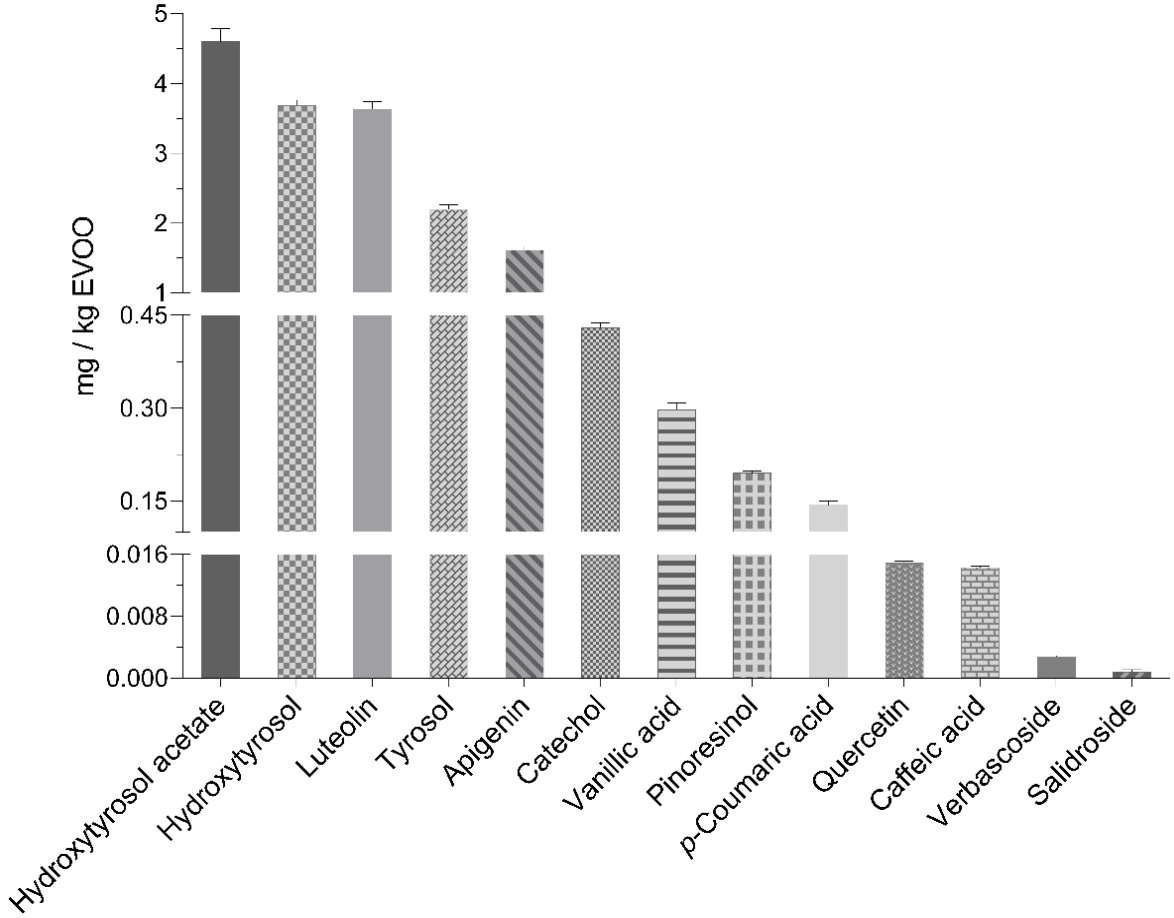

Figure 6. Concentrations of polyphenols in EVOO of the Arbequina variety. Results are expressed as means ± SEM of three independent samples extracted in duplicate.

3.2. Determination of pentacyclic triterpenes and polyphenols in rat feed

Pentacyclic triterpenes and polyphenols were analyzed on animal feed in order to confirm that the determination of each compound in plasma was only to due to ingestion of Arbequina EVOO. No pentacyclic triterpenes were found in the animal diet.

Concerning polyphenols, most of them were absent from the animal feed, however minor concentrations of apigenin (0.024 ± 0.001 mg/kg), caffeic acid (0.255 ± 0.001 mg/kg), *p*-coumaric acid (1.718 ± 0.004 mg/kg) and vanillic acid (0.542 ± 0.004 mg/kg) were found.

3.3. Analysis of the pentacyclic triterpenes and polyphenols in rat plasma

3.3.1. Validation of the method

After the oral administration of EVOO to Sprague-Dawley rats, plasma was assessed for its content of pentacyclic triterpenes and polyphenols.

Table 3. Validation parameters of the analytical methods employed for the analysis of pentacyclic triterpenes and polyphenols in plasma: sensitivity, linearity, accuracy and precision.

Compound	Sensitivity		Linearity			Accuracy	Precision
	LOD (nM)	LOQ (nM)	Range (nM)	Equations	R^2	(%)	(%)
Pentacyclic triterpenes analyzed by LC-APCI-QTRAP-MS							
Maslinic acid	0.05	0.17	0-200	$y = 0.0279x + 0.010$	0.9962	1.23	2.77
Oleanolic acid	0.46	1.53	0-200	$y = 0.0079x - 2.81e^{-5}$	0.9985	−0.35	1.16
Ursolic acid	0.10	0.30	0-200	$y = 0.0071x - 1.9e^{-7}$	0.9974	0.23	2.42
Erythrodiol	1.19	3.97	0-200	$y = 0.0071x - 0.011$	0.9988	2.53	0.50
Uvaol	2.76	9.20	0-200	$y = 0.0025x - 0.003$	0.9978	1.53	2.68
Polyphenols analyzed by LC-ESI-QTRAP-MS/MS							
Apigenin	0.19	0.64	0-150	$y = 0.0013x + 4.7e^{-7}$	0.9978	−2.15	4.90
Caffeic acid	0.05	0.16	0-150	$y = 0.0024x + 0.0022$	0.9996	−0.89	0.91
Catechol	0.51	1.71	0-150	$y = 0.00014x + 0.0006$	0.9960	−3.88	8.19
p-Coumaric acid	0.11	0.38	0-150	$y = 0.000603x + 0.001$	0.9981	−1.31	3.55
Hydroxytyrosol	0.38	1.09	0-150	$y = 0.0022x - 4.4e^{-8}$	0.9980	−1.43	3.57
Hydroxytyrosol acetate	0.62	2.06	0-150	$y = 0.00026x + 0,0005$	0.9975	−4.29	6.28
Luteolin	0.06	0.20	0-150	$y = 0.0077x – 1.67e^{-6}$	0.9983	−3.14	7.52
Luteolin-7-O-glucoside	0.03	0.09	0-150	$y = 0.0095x + 2.37e^{-7}$	0.9986	−1.29	2.7
Oleuropein	0.04	0.15	0-150	$y = 0.0023x + 0,004$	0.9983	−1.09	5.50
(+)-Pinoresinol	0.33	1.12	0-150	$y = 0,0014x - 2.7e^{-7}$	0.9972	−0.60	4.97
Quercetin	0.27	0.92	0-150	$y = 0,0031x + 0,001$	0.9984	−0.67	5.95
Rutin	0.04	0.14	0-150	$y = 0,0011x - 0,0003$	0.9975	0.41	6.75
Salidroside	0.94	3.13	0-150	$y = 0.00027x + 0.0004$	0.9990	−2.06	3.77
Tyrosol	1.05	3.50	0-150	$y = 7.36e^{-5}x + 7.36e^{-5}$	0.9931	−0.42	2.93
Vanillic acid	0.17	0.50	0-150	$y = 0.00065x + 3.2e^{-7}$	0.9984	−0.22	1.62
Verbascoside	0.06	0.21	0-150	$y = 0.0014x + 0,001$	0.9988	−1.28	4.46

The analytical methods were validated (Table 3) for the assessment of pentacyclic triterpenes by LC-APCI-QTRAP-MS and polyphenols by LC-ESI-QTRAP-MS/MS following the recommendations of the Food and Drug Administration (2018). The validation parameters evaluated were sensitivity, linearity, accuracy, precision and carry-over.

The sensitivity of the analytical methods was assessed by calculating the LOD as the concentration that had a signal-to-noise of 3:1 while the LOQ was considered as the concentration that fulfills the criteria of a signal-to-noise ratio of 10:1. The sensitivity was inferior to 2 nM for most of the analytes studied, around 3 nM for erythrodiol, salidroside and tyrosol and the highest LOQ corresponded to uvaol with a concentration of 9.20 nM. Linearity was evaluated at the expected concentrations to be found in the samples, so the standards added to blank plasma samples ranged from 0 to 200 nM for pentacyclic triterpenes, since they were expected to be present in higher concentrations than polyphenols that were evaluated up to 150 nM. The analytical method was linear over the concentrations assayed, with calibration coefficients (R^2) that were above 0.9961 for all the compounds except for tyrosol that gave a value of 0.9931 (Table 3).

Table 3 displays the mean results obtained for accuracy and precision that were lower than 10%, thus indicating that the methods were precise and accurate, since they were lower than the 15% established in the FDA guidelines (2018). Finally, the carry-over from one sample to another on the LC-QTRAP-MS instrument was evaluated as described above. Blank plasma samples, as well as blank reagents and methanol 80% did not show peaks at the same retention time of the compounds and IS.

3.3.2. Identification and quantification of pentacyclic triterpenes by LC-APCI-QTRAP-MS

Pentacyclic triterpenes were assessed at 0, 15, 30, 60, 90 and 120 min after administration of Arbequina EVOO at a dose of 6.16 mL/kg. Table 4 reports the dose of each pentacyclic triterpenes that the experimental animals received.

Table 4. Dose of pentacyclic triterpenes administered to Sprague-Dawley rats.

Compound	**µg/kg rat body weight[1]**	**µg in EVVO[2]**
Maslinic acid	659	165
Oleanolic acid	166	41.5
Ursolic acid	--	--
Erythrodiol	21.1	5.28
Uvaol	1.22	0.31

[1]Dose of pentacyclic triterpenes administered to Sprague-Dawley rats.

[2] Amount of pentacyclic triterpenes administered to Sprague-Dawley rats of 250 g of body weight.

Analysis of plasma samples revealed that concerning pentacyclic triterpenes of only maslinic acid was found in all the time points extracted. This compound appeared at the retention time of 22.68 min, and it can be seen in Figure 7, which depicts a representative chromatogram obtained 15 min after the oral administration of EVOO.

Figure 7 also depict the standards of oleanolic acid, ursolic acid, erythrodiol and uvaol that eluted at 31.01 min, 32.26 min, 37.11 min and 38.31 min. Ursolic acid was not detected in the EVOO administered, so it was not expected to be found in plasma. However, oleanolic acid that

was administered at a dose of 166 µg/kg was not found in any sample. Moreover, erythrodiol and uvaol that were present in minor amounts of EVOO were not present in any of the plasmas evaluated.

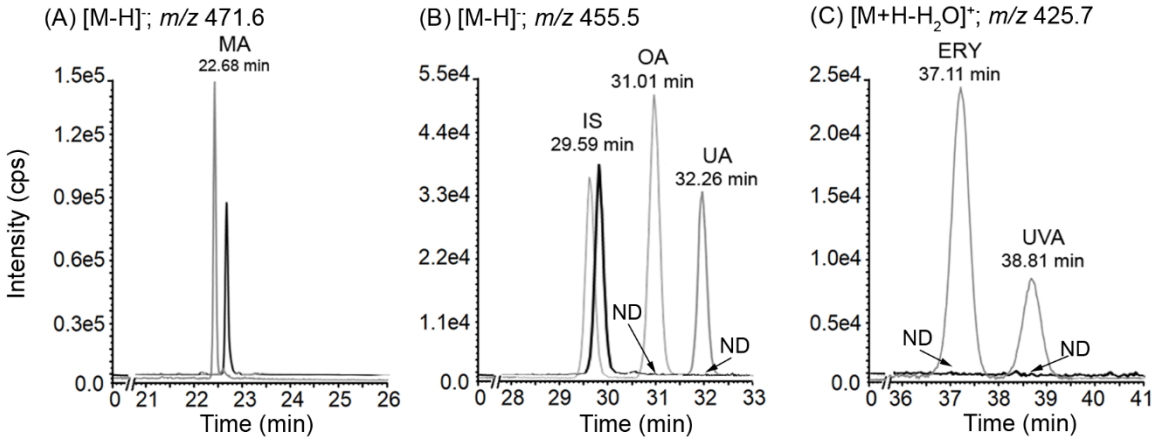

Figure 7. Extracted ion chromatograms (XIC) obtained by LC-QTRAP-MS of (A) maslinic acid; (B) betulinic acid (IS), oleanolic acid and ursolic acid and (C) erythrodiol and uvaol at blank plasma spiked with standards at 150 nM and the IS at 200 nM (gray trace) and of the pentacyclic triterpenes detected in rat plasma sample 15 min after oral intake of Arbequina EVOO (black trace). ND, not detected.

Quantification of the peaks obtained at the different sampling times allowed the establishment of the plasmatic concentrations of maslinic acid (Figure 8). The time at which the maximum concentration of 21.2 ± 5.13 nM appears is 15 min. Then, concentrations drop nearly to half, since at 30 min maslinic acid was found at 11.9 ± 0.20 nM. From that sampling time on, the plasmatic concentrations diminished but in a steadier way, being still found at 120 min with values of 5.62 ± 0.82 nM.

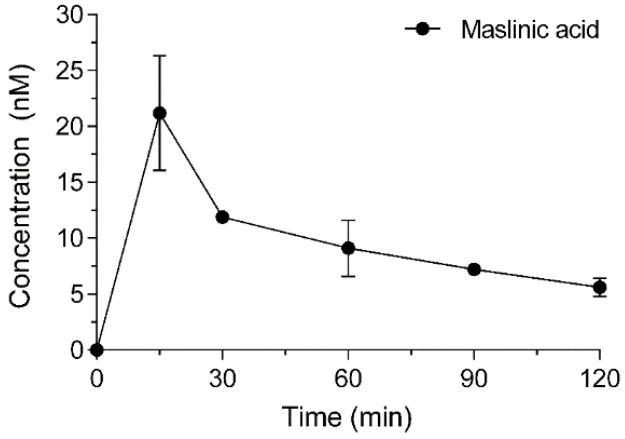

Figure 8. Plasmatic concentrations of maslinic acid after the oral administration of EVOO of the Arbequina variety. Values are represented as means ± S.E.M (n = 4).

Chromatograms were searched for phase I and II metabolites of maslinic acid and oleanolic acid by comparing the expected masses in the plasma samples corresponding to the animals that received EVOO in comparison to the chromatograms obtained from rats that never received this food. No metabolites from oleanolic acid were detected. However, phase I metabolites of maslinic acid corresponding to the addition of a hydroxyl group were found at concentrations below the LOQ.

3.3.3. Identification and quantification of polyphenols by LC-ESI-QTRAP-MS/MS

The administration of 6.16 mL/kg of EVOO of the Arbequina variety supplied a dose of polyphenols that is indicated in Table 5.

Table 5. Dose of polyphenols administered to Sprague-Dawley rats.

Compound	**µg/kg rat body weight[1]**	**µg in EVVO[1]**
Apigenin	9.12	2.28
Caffeic acid	0.081	0.020
Catechol	2.44	0.61
o-Coumaric acid	--	--
p-Coumaric acid	0.81	0.20
Hydroxytyrosol	20.9	5.22
Hydroxytyrosol acetate	26.0	6.51
Luteolin	20.6	5.15
Luteolin-7-O-glucoside	--	--
Oleuropein	--	--
(+)-Pinoresinol	1.11	0.28
Quercetin	0.084	0.021
Rutin	--	--
Salidroside	0.0047	0.0012
Tyrosol	12.4	3.11
Vanillic acid	1.69	0.42
Verbascoside	0.015	0.0039

[1]Dose of polyphenols administered to Sprague-Dawley rats.

[2] Amount of polyphenols administered to Sprague-Dawley rats of 250 g of body weight.

Hydroxytyrosol acetate, hydroxytyrosol and luteolin were the compounds with the higher doses being 26.0, 20.9 and 20.6 µg/kg of rat body weight. They were followed by tyrosol and apigenin with values of 12.4 and 9.12 µg/kg of rat body weight. Catechol, vanillic acid, pinoresinol and *p*-coumaric acid provided doses of 2.44, 1.69, 1.11 and 0.81 µg/kg of rat body weight, whereas quercetin, caffeic acid, verbascoside and salidroside brought doses inferior to 0.1 µg/kg of rat body weight. The assessment of the chromatograms obtained after the LC-QTRAP-MS/MS analysis of polyphenols in plasma showed that from the 13 polyphenols quantified in EVOO only 7 were detected in plasma sample after the administration of the oil (Figure 9).

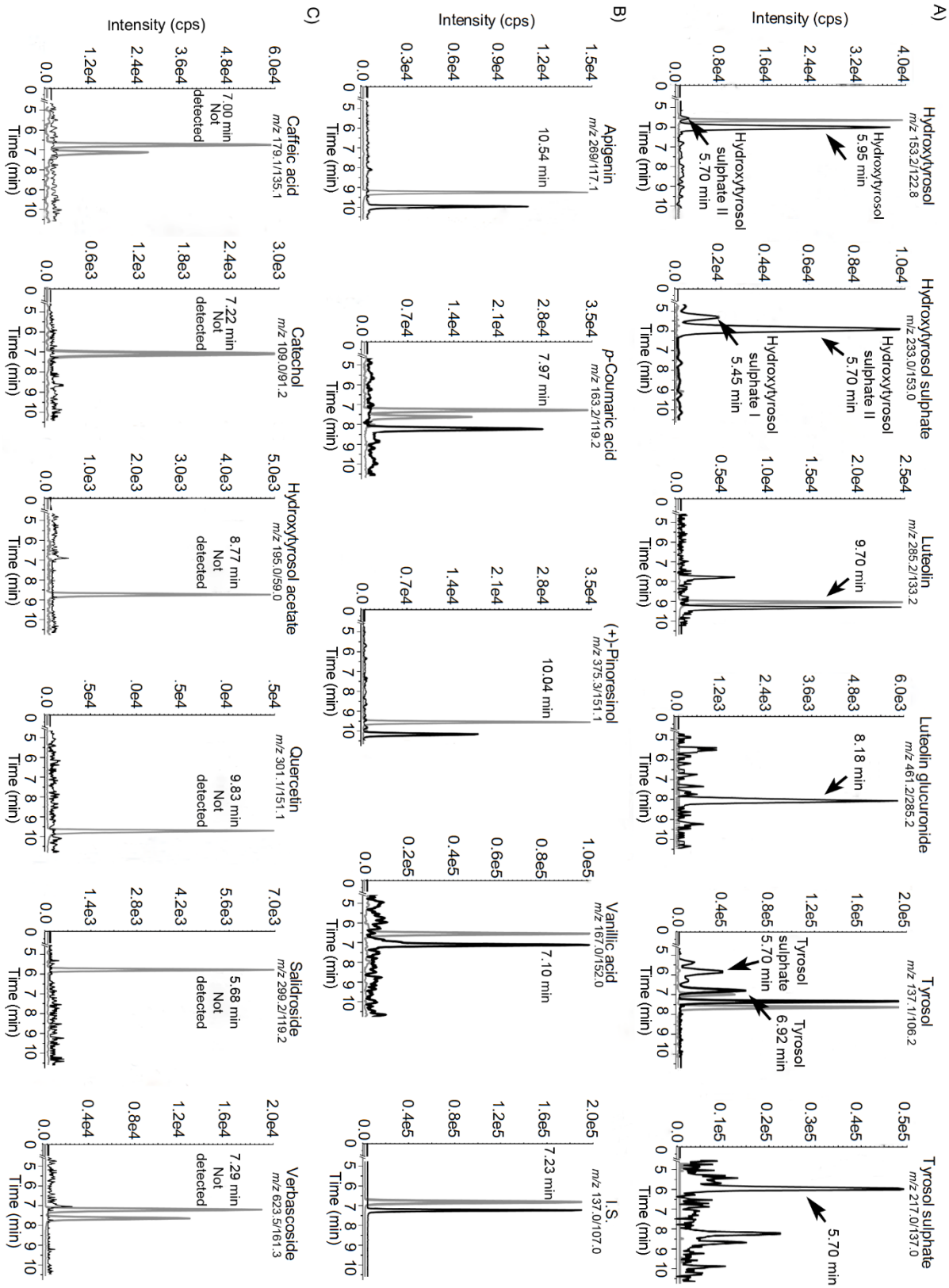

Figure 9. Extracted ion chromatograms (XIC) obtained by LC-QTRAP-MS/MS of rat blank plasma spiked with polyphenols at 150 nM (grey trace) and rat plasma obtained 15 min after oral intake of Arbequina EVOO (black trace). Polyphenols and its corresponding metabolites (A), polyphenols without metabolites (B) and non-detected analytes (C).

Caffeic acid, catechol, quercetin, verbascoside and salidroside that were present at minor concentrations in the EVOO of the Arbequina variety were not detected in plasma. Remarkably, hydroxytyrosol acetate that was the main polyphenol in EVOO was not found in any of the plasma samples evaluated. Figure 10 depicts the concentrations of polyphenols in plasma and the metabolites detected for the main compounds. All the analytes detected as well as the metabolites hold a similar pattern, with the highest concentration at 15 min, and a progressive decline over time.

The polyphenol that yielded the highest concentration in plasma was vanillic acid, with values of 59.8 ± 7.34 nM at 15 min which diminished to 28.0 ± 8.59 nM at 30 min and was still measured at 120 min (8.25 ± 3.03 nM). The following polyphenol was tyrosol that gave concentrations of 40.8 ± 4.02 nM at 15 min, decreasing continuously with time with values of 20.2 ± 5.1 nM at 30 min and 12.2 ± 4.8 nM at 60 min, and reached minor amounts of 4.68 ± 1.50 nM at 120 min. Tyrosol was searched for in phase II metabolites, specifically, glucuronide and sulfate, and only the later was detected. It is worth mentioning that tyrosol was largely converted to sulfate presenting the maximal concentration of 259.6 ± 26.7 nM at 15 min, ensuing reduction in the plasmatic concentrations of 135.9 ± 29.5 nM at 30 min and 87.0 ± 11.4 at 60 min. Tyrosol sulfate was still found at 120 min with concentrations of 38.1 ± 0.06 nM.

Hydroxytyrosol showed the maximal concentration of 28.8 ± 1.64 nM at 15 min. This compound followed the same pattern as the other polyphenols of a steady lessening of its presence in plasma, but being still detected at 120 min (5.12 ± 0.45 nM). Phase II metabolites of hydroxytyrosol were also investigated. No glucuronide was detected at any of the times evaluated. Conversely, two sulfate isomers were found, a small peak corresponding to hydroxytyrosol sulfate I, with retention time of 5.45 min followed by a large peak eluting at 5.70 min that was designated as hydroxytyrosol sulfate II (Figure 9). Hydroxytyrosol sulfate I was only identified in two rats at 15 min time with a concentration of 4.54 ± 0.01 nM, and was not detected at any other time studied. On the other hand, hydroxytyrosol sulfate II was detected at all the extraction times, and the plasmatic concentration was maximal at 15 min, with values of 17.4 ± 3.34 nM at 15 min which descended to 9.32 ± 0.01 nM at 30 min and was still detected at 120 min (1.07± 0.005 nM).

The phenolic *p*-coumaric acid reached a value of 16.1 ± 6.57 nM at 15 min and at 120 min the concentration was 0.35 ± 0.15 nM. Finally, the flavonoids luteolin and apigenin and the lignan pinoresinol were detected in plasma but in low concentrations. Luteolin achieved the highest value of 7.11 ± 1.11 nM at 15 min, and experienced a sharp drop at 30 min to 2.02 ± 0.12 nM. From this time and up to 120 min the values of luteolin in plasma were kept at concentrations that ranged from 2.5 to 1 nM. Phase II metabolites of luteolin were also screened and only a glucuronide was detected. This metabolite was present at small concentrations that still were above the LOQ (0.57 ± 0.10 nM at 15 min and 0.14 ± 0.005 nM at 120 min). Apigenin was detected in low concentrations at 15 min (3.21 ± 0.75 nM), although it still was detected at 120 min (0.72 ± 0.13 nM). Finally,

(+)-pinoresinol was only found at 15 and 30 min with values of 4.75 ± 1.29 nM and 1.01 ± 0.26 nM, respectively.

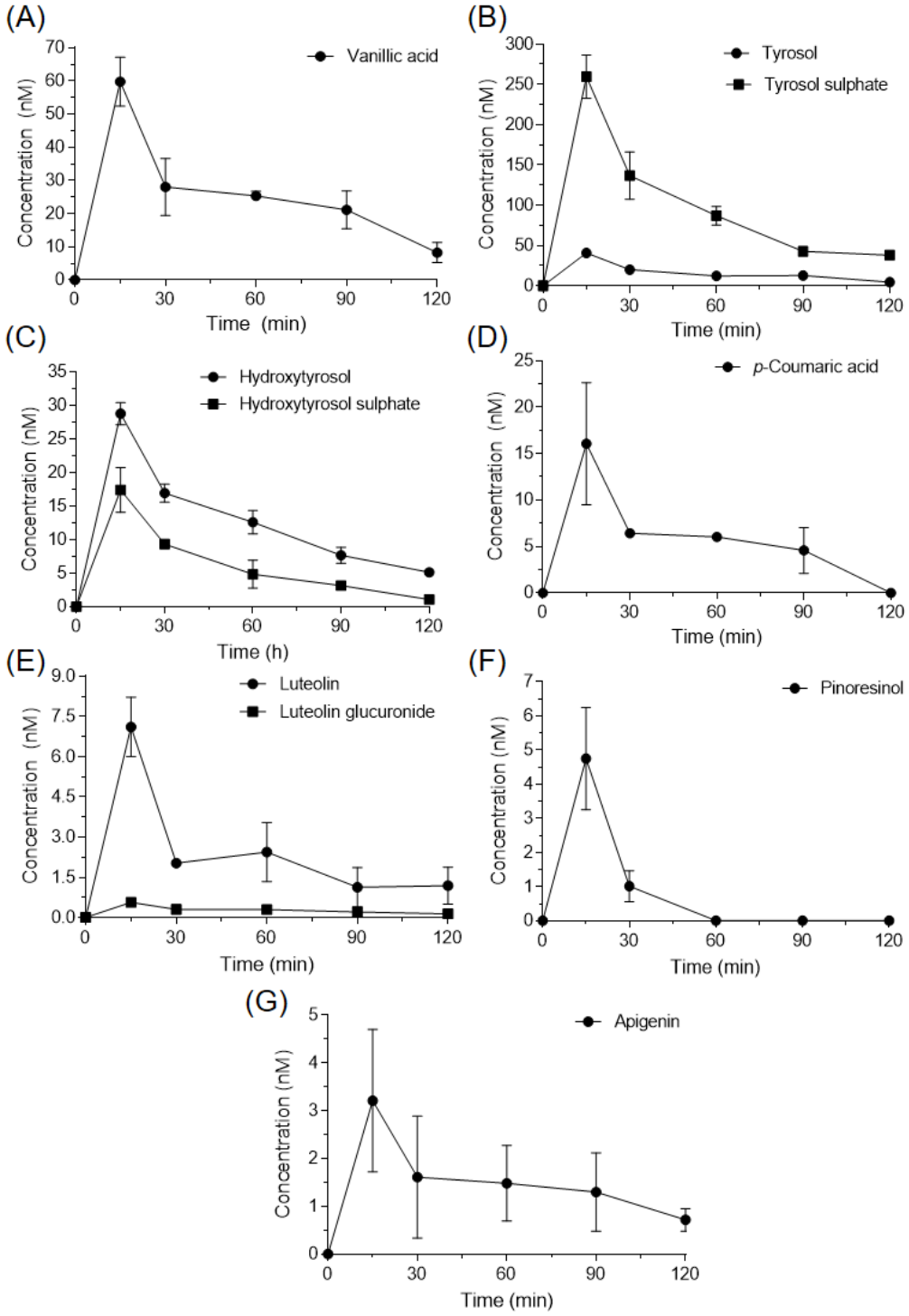

Figure 10. Plasmatic concentrations of (A) tyrosol, (B) hydroxytyrosol, (C) vanillic acid, (D) *p*-coumaric, (E) luteolin, (F) pinoresinol and (G) apigenin after the oral administration of EVOO of the Arbequina variety. Values are represented as means ± S.E.M (n = 4)

4. Discussion

The present study aims at deepening in the knowledge on the composition of pentacyclic triterpenes and polyphenols in EVOO olive oil, as well as the bioavailability of both groups of bioactive compounds after the consumption of this food. EVOO is characterized by the presence of high amounts of monounsaturated fatty acids, mostly oleic acid, as well as other valuable minor components such as phytosterols, tocopherols, squalene, pentacyclic triterpenes and phenolic compounds (Guo *et al*., 2018). Although all the components of EVOO could contribute to the prevention of chronic diseases, most of the beneficial effects on health have been attributed to their content in polyphenols. Consequently, research has been mainly devoted to the study of phenolic compounds, firstly, in the characterization of their content in the oil and secondly, in the assessment of the plasmatic concentrations after the intake of this food. Besides, pentacyclic triterpenes, which are a group of bioactive molecules highly present in the leaves and fruit of *Olea europaea* L., have been understudied in EVOO even though they hold relevant biological activities (Sánchez-Quesada *et al*., 2013). Bearing in mind all these asseverations, we have aimed at improving the knowledge, not only in the content of pentacyclic triterpenes in EVOO but also in assessing the concentrations of polyphenols in this food. To achieve this goal, we have established a new methodology that allows the concurrent extraction of both groups of compounds in the same oil sample. In second place, we wanted to evaluate the fate of pentacyclic triterpenes and polyphenols after the consumption of EVOO. For this reason, we have orally administered the EVOO by gavage to Sprague-Dawley rats and we have evaluated the plasmatic concentrations, of pentacyclic triterpenes and polyphenols, at different time points, from 15 min to 120 min. Also, the extraction process from plasma samples was performed simultaneously for both groups of compounds.

Olive oil is the main source of fat in the countries bordering the Mediterranean basin, and its regular consumption has been associated with a lower prevalence of cardiovascular disease and certain cancers, among other ailments (Ghanbari, 2019). According to the International Olive Council (IOC), the standard grades of olive oil currently available in the market can be grouped as EVOO, virgin olive oil (VOO), refined olive oil and pomace oil. This classification is based on the free acidity and the processing. EVOO consist on the first-press juice obtained from fresh olives, usually processed within the 24 hours after harvesting and fulfilling the criteria of an acidity lower than 0.8%, a peroxide content inferior to 20 meq O_2/kg and adequate organoleptic characteristics verified by professional tasters (Vossen, 2007). Although VOO also comes from the first pressing of the fruit of *Olea europaea* L., it has a lesser grade than EVOO. VOO has to accomplish an acidity of 2% as well as good taste, although it may contain some sensory flaws (Vossen, 2007). Refined olive oil is virgin oil that has been treated by means of chemical and physical filters to eliminate high acidity or organoleptic defects, thus rendering an acidity lower than 0.3%. This type is commercialized as “olive oil” and this primarily refined oil may be mixed with VOO to improve the organoleptic characteristics (Ucella *et al*., 2001). Finally, olive pomace oil is a by-product of

the VOO production that involves the extraction by chemical means of olive skins, seeds, and pulp, that has to be further refined to be fit for consumption (Vossen, 2007). From the different types of oils classified by the IOC, the one that has been associated with more beneficial effects on health is EVOO (Foscolou *et al*., 2018). Consequently, we have selected this grade for our study. In addition, we have chosen the Arbequina variety, one of the most appreciated in Spain, due to its high-quality oil (Bakhouche *et al*., 2013). This variety, named after the village of Arbeca in Lleida, accounts for a 50% of the Catalan olive grove, and its cultivation has spread not only within Spain but also worldwide due to its resistance to frost, low vigor and high productivity (Bakhouche *et al*., 2013). Although the fruit is characterized for its small size with a low flesh-to-stone ratio, the Arbequina variety is highly praised since the trees produce a large amount of fruits with a high oil yield of 20.5% (Franco *et al*., 2014).

The analysis of EVOO was performed with the use of ethanol-methanol (1:1; v/v) as extraction solvents before the analysis by means of LC-MS. The extraction procedure that allowed the concurrent assessment of pentacyclic triterpenes and polyphenols from EVOO was established, since none of the methods existing in the literature analyzed both groups of compounds. The analysis of bioactive compounds from olive oil is generally performed by liquid-liquid extraction, instead of solid-phase extraction, a more laborious procedure that also yields worse recoveries (Ricciutelli et 2017; Olmo-García *et al*., 2018). The solvents generally used were methanol 100% (Owen *et al*., 2000)), methanol 60% (Caporaso *et al*., 2015; Ricciutelli et 2017) or ethanol mixed with different percentages of water (Olmo-García *et al*., 2018). Generally speaking, the extraction processes reported in the literature consisted in vigorous shaking in the vortex followed by centrifugation and can be accompanied by the removal of the lipid fraction with n-hexane (Rigane *et al*., 2013; Caporaso *et al*., 2015; Bajoub *et al*., 2015). Therefore, in the development of the method for the simultaneous analysis of polyphenols and triterpenes from EVOO, we took into account the existing techniques previously established. Accordingly, the initial attempts consisted on employing ethyl acetate 100%, methanol 100% and 60%, as well as methanol:ethanol (1:1; v/v). In both cases a wash with n-hexane was attempted to remove the lipid fraction. Ethyl acetate was discarded, due to low recoveries, as well as the use of the wash with n-hexane that also eliminated part of pentacyclic triterpenes. The best recoveries were achieved using methanol:ethanol (1:1; v/v). Once the solvent was chosen, we also evaluated the best volume to be employed. Then, we proceeded to optimize its volume to quantitatively extract pentacyclic triterpenes and polyphenols. We assessed the process, with three extractions with a total volume of solvents of 3.0 mL, 2.25 mL or 1.5 mL. Given that, no improvement was observed with increasing the amount of solvent used since the three volumes yielded the same recoveries. Consequently, the inferior volume was selected for the sake of reducing the analysis time. Evaporation to dryness of 1.5 mL methanol:ethanol took place in a short time, and this fact could also involve a lesser degradation of unstable polyphenols. Once the method was established, it was validated for sensitivity, linearity, precision, accuracy and carry-

over. The results obtained in the validation process showed that the method was adequate for the analysis of pentacyclic triterpenes and polyphenols in EVOO.

The application of the developed method to the analysis of EVOO of the Arbequina variety revealed that pentacyclic triterpenes were the most abundant bioactive compounds with concentrations of 149 mg/kg, while polyphenols were found at 16.8 mg/kg. Although the presence of pentacyclic triterpenes have long been known in the fruits of *Olea europaea* L. (Vioque & Maza, 1963) the search for their content in olive oil have hardly been investigated, and only a few studies report their content in this food (Pérez-Camino & Cert, 1999; Allouche *et al*., 2009; Velasco *et al*., 2018; Olmo-García *et al*., 2019b). Among the scarce studies, only Pérez-Camino & Cert (1999) evaluated the content of pentacyclic triterpenic acids on EVOO. Our findings are in agreement with their values on the amounts of maslinic and oleanolic acids in EVOO of the Arbequina variety, and also in the fact that they don't detect any trace of ursolic acid. The authors did not provide any results on erythrodiol and uvaol. These pentacyclic triterpenic alcohols are used as a purity parameter to detect adulterations of EVOO with lower quality oils, especially pomace olive oil (EU, reg. 2568/91) and are expressed as the sum of percent of total sterols. Our results meet the criteria indicated in terms of content of pentacyclic alcohols since erythrodiol and uvaol were found at 3.95 mg/kg. Concerning the Arbequina variety, the content of pentacyclic triterpenes have only been described in VOO, with a content of maslinic and oleanolic acids of 33.58 ± 5.1 mg/kg (Allouche *et al*., 2009), which are much lower than the ones found for EVOO. A similar content of pentacyclic triterpenic acids was described for other varieties (Allouche *et al*., 2009; Olmo-García *et al*., 2019b). Noteworthy, that ursolic acid has only been described in some of them, and in the cases that it appears, their content never exceeded 2 mg/kg (Allouche *et al*., 2009). With regards to other pentacyclic triterpenic acids, only betulinic acid was found in VOO in the amount of 0.81 mg/kg by Olmo-García *et al*. (2019a).

Conversely, the phenolic fraction of olive oil is much more heterogeneous than the one described for pentacyclic triterpenes, with at least 36 structurally different polyphenols identified (Guo *et al*., 2018). These compounds can be classified into 5 categories, namely, phenolic alcohols, phenolic acids, secoiridoids, flavonoids and lignans (Bendini *et al*., 2007; Servili *et al*., 2009). Concerning the composition of polyphenols among olive oils, there is a great divergence between the results found, with concentrations ranging from 0.02 to 600 mg/kg (Tripoli *et al*., 2005; Cicerale *et al*., 2009). On the one hand, the content of polyphenols depends on several variables that, besides the variety of *Olea europaea* L. and the ripening stage of the fruit, could be grouped as environmental factors (altitude of the cultivar, soil, irrigation practices, temperature), processing conditions (heating, added water, extraction systems) along with storage conditions (Tripoli *et al*., 2005; Cicerale *et al*., 2009). On the other hand, the lack of a suitable analytical methodology may be another source of inaccuracies and discrepancies in the reported data (Tripoli *et al*., 2005). In that sense, the total content of polyphenols described for the Folin-Ciocalteau colorimetric test

yields imprecise values due to the low specificity towards phenolic compounds (Tripoli *et al*., 2005). The use of HPLC or LC analysis has improved the specificity; however, the methods require long analysis time and some of the methods reported do not have standards for all the polyphenols (Tripoli *et al*., 2005; Cicerale *et al*., 2009). Hence, the present study reports an analytical strategy that allows a rapid sample preparation, along with LC-MS with a short chromatographic run of 9 min, enabling the sensible and accurate identification of polyphenols as well as adequate quantification using calibration curves built with standards. Our results indicate that, from the 17 polyphenols that we had included in the establishment of the method, in EVOO of the Arbequina variety we have detected 13 analytes that yield a total value of 16.8 mg/kg. Of these, the most abundant group correspond to the phenolic alcohols (10.9 mg/kg), followed by flavonoids (5.3 mg/kg), phenolic acids (0.5 mg/kg) and the lignan, pinoresinol (0.20 mg/kg). No traces of oleuropein was found in the samples analyzed. Our results are in agreement with those in the literature for EVOO of the Arbequina variety, which indicate that phenolic alcohols, phenolic acids and flavonoids are present in small amounts, whereas secoiridoids and lignans are found in higher concentrations (García *et al*., 2003; Oliveras-López *et al*., 2007; Gómez-Rico *et al*., 2008; García-González *et al*., 2010; Sánchez de Medina *et al*., 2015; Monasterio *et al*., 2017). Bakhouche *et al*. (2013) that evaluated the content in Arbequina EVOO from Catalunya gave similar values for hydroxytyrosol, hydroxytyrosol acetate and tyrosol that were 3.2, 1.2 and 1.37 mg/kg, respectively, and similarly to our data, apigenin and luteolin were found at 0.79 and 2.18 mg/kg. Those authors reported that major compounds were secoiridoids with the decarboxymethylated form of oleuropein (94.1 mg/kg) and elenolic acid (12.6 mg/kg), whereas oleuropein aglycone and other ligstroside aglycone were found at low concentrations (Bakhouche *et al*., 2013). The content of oleuropein is not reported and it is in accordance with our study, in which from the group of secoiridoids, we only evaluated oleuropein that was not found in any of the samples analyzed. Hence, the results obtained for Arbequina EVOO support the existing data provided in the literature, and expand the knowledge on content of polyphenols to salidroside, catechol and verbascoside, that are scarcely reported.

Information on the content of pentacyclic triterpenes and polyphenols in EVOO of the Arbequina variety constitutes a first step for establishing the biofunctionality of this food. However, for achieving this goal, it is essential to dispose of accurate information on the oral bioavailability of these compounds. Although pentacyclic triterpenes were the group of bioactive compounds with the higher concentrations in EVOO, they were not the most abundant in plasma samples. Of the 4 pentacyclic triterpenes, that is, maslinic acid, oleanolic acid, erythrodiol and uvaol, only maslinic acid was found. The latter was administered at a dose of 659 µg/kg and the peak plasmatic concentrations at 30 min were 21.2 nM. No data has been reported concerning the plasmatic concentrations of pentacyclic triterpenes after the oral administration of olive oil. However, there is information in this regard after the intake of table olives of the Marfil variety (Giménez *et al*., 2017). This variety of table olives is especially rich in these compounds and maslinic acid was

administered to Sprague-Dawley rats at the dose of 4.57 mg/kg, which is 7 times higher than the one that we have administered in Arbequina EVOO, and was found in plasma at 23 nM at 120 min (Giménez *et al*., 2017). Hence, our results suggest that maslinic acid in EVOO is more bioavailable than in table olives, and this can be attributed to the fact than in EVOO bioactive compounds avoid the step of releasing from the food matrix post-ingestion to be available for intestinal absorption at the enterocytes (Rein *et al*., 2012). Concerning oleanolic acid, the fact that this compound could not be detected in plasma could be due to not only to the fact that it is present at lower concentrations in EVOO, and the dose administered was 166 μg/kg, but also to the low bioavailability of this compound. Experiments performed in rats indicated that after the administration of 25 and 50 mg/kg, the bioavailability was found to be 0.7%. Concerning polyphenols, there is much information after the intake of olive oil, but it has been restricted mostly to the phenolic alcohols hydroxytyrosol and tyrosol (Parkinson & Cicerale, 2016; Crespo *et al*., 2018). The methodology that we have employed was sensible with LOQs lower than other authors (Suárez *et al*., 2009). In this sense, we were able to find hydroxytyrosol, tyrosol, luteolin, vanillic acid, apigenin, *p*-coumaric acid and pinoresinol in rat plasma after the administration of Arbequina EVOO. These polyphenols were able to pass into the circulatory system after a simple oral dose of oil. In the process of crossing epithelial cells of the gastrointestinal tract, these polyphenols could suffer a biotransformation, starting the first metabolic pass. In this way, bioactive compounds can be subjected to phase I reactions (hydrogenation, hydroxylation, hydration and methylation) and subsequently, to phase II reactions (glucuronidation and sulfation), being this second type the ones which occur more frequently for polyphenols (Gómez-Romero *et al*., 2012). Glucuronidation and sulfation by glucotransferase and sulfotransferase enzymatic activity possibly takes place in the enterocytes as well as in the liver, resulting in the conjugated forms of hydroxytyrosol, tyrosol and luteolin as we have observed in our study. Our results are in agreement with other findings that indicate that the phenolic alcohols are relatively well absorbed in the small intestine compared to flavonoids (Corona *et al*., 2006; Parkinson & Cicerale, 2016).

Overall, it could be stated that the present study provides more comprehensive information not only on the concentrations of pentacyclic triterpenes and polyphenols in EVOO of the Arbequina variety, but more importantly, on the plasmatic concentrations achieved after the ingestion of this food. The outcome of both the assessment of the content of relevant bioactive compounds in the food and its fate on the organism after consumption expands the knowledge and provides a convincing support for EVOO as a relevant dietetic source of polyphenols, but more importantly, pentacyclic triterpenes.

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Conflicts of Interest: The authors declare no conflict of interest.

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III. Resultados ~ Capítulo 4

3.4. CAPÍTULO 4. EFECTO QUIMIOPREVENTIVO DE LA INGESTA DE ACEITUNAS DE MESA DE LA VARIEDAD ARBEQUINA SOBRE LESIONES PRENEOPLÁSICAS EN COLON DE RATA INDUCIDAS POR 1,2-DIMETILHIDRAZINA (DMH)

Los resultados presentados en este capítulo se enucentran recogidos en el artículo 4:

The daily consumption of table olives reduces preneoplastic in the colon of rats treated with 1,2-dimethylhydrazine

Moreno-González, R., Juan, M.E., Planas, J.M.

Manuscrito en preparación

Los resultados obtenidos han dado lugar a las siguientes comunicaciones a congresos:

- *The fruit of Olea europaea* L. *as a dietary source of bioactive compounds*
  Kundisová, I., Moreno-González, R., Franco-Ávila, T., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  III Workshop Anual INSA-UB. La salut de la microbiota. Prebiòtics i Probiòtics en nutrició animal i humana
  Santa Coloma de Gramanet, España, 16 de noviembre de 2017

- *The daily intake of Arbequina table olives reduces the preneoplastic morphological markers in colon induced by 1,2-dimethylhydrazyne in rats*
  Moreno-González, R., Juan, M.E., Planas, J.M.

  Comunicación presentada como póster en el congreso:

  XVII Congreso de la SEÑ y X Jornada de l'Associació Catalana de Ciències de l'Alimentació (ACCA)
  Barcelona, España, 27 - 29 de junio de 2018

- *Matrix effects on the intestinal concentration of maslinic acid and its effects on colonic preneoplastic lesions induced by 1,2-dimethylhydrazyne in rats*
  Moreno-González R., Juan M.E., Planas J.M.

  Comunicación presentada como póster y publicada en el libro de actas del congreso:

  Europhysiology 2018
  Londres, Reino Unido, 14 - 16 de septiembre de 2018

- *Table olives as a dietary source of pentacyclic triterpenes with antitumoral activity on colon cancer*

  Juan M.E., Moreno-González, R., Planas, J.M.

  Comunicación presentada como póster en el congreso:

  2nd International Yale Symposium on Olive Oil and Health
  Delfos, Grecia, 1- 4 de diciembre de 2019

El consumo diario de aceitunas de mesa reduce las lesiones preneoplásicas en el colon de ratas tratadas con 1,2-dimetilhidrazina

Moreno-González, R., Juan, M.E., Planas, J.M.

Manuscrito en preparación

3.4.1. Resumen del artículo 4

Objetivos: El árbol del olivo se ha utilizado desde hace siglos por todas las culturas que se han desarrollado en la cuenca mediterránea, tanto con fines sagrados como en la preparación de alimentos, cosméticos o para tratar diversas enfermedades. Hoy día se conoce que los productos derivados de *Olea europaea* L. contienen una amplia variedad de compuestos bioactivos que incluyen los triterpenos pentacíclicos y los polifenoles, a los cuales se les atribuye propiedades protectoras, destacando la actividad contra diferentes tipos de tumores como el cáncer de colon. El objetivo del siguiente estudio fue investigar *in vivo* el efecto antitumoral que produce la administración oral de aceitunas de mesa a una dosis de 3,85 g/kg durante 49 días, sobre las lesiones preneoplásicas inducidas en el colon de rata por la administración de 1,2-dimetilhidrazina (DMH). Además, se han cuantificado los triterpenos pentacíclicos y polifenoles, así como sus metabolitos, en el plasma y en el contenido de colon.

Material y métodos: Las ratas Sprague-Dawley, se dividieron en un grupo DMH que recibió 3 inyecciones subcutáneas del carcinógeno (20 mg/kg) los días 8, 15 y 22 y un grupo control al que se le inyectó sólo solvente. A su vez, los animales se distribuyeron en 2 subgrupos y fueron administrados oralmente por sonda con agua o aceitunas durante 49 días. Las aceitunas de mesa de la variedad Arbequina (Cooperativa del Camp Foment Maialenc SCCL, Maials, Lleida) correspondientes a la cosecha 2016/2017, se prepararon como una suspensión finamente triturada a una dosis de 3,85 g/kg (equivalente al consumo diario de 30 aceitunas por una persona de 60 kg). Durante todo el experimento se llevó a cabo el control del peso corporal, del pienso y del agua. Al final del estudio, se extrajo sangre (punción cardíaca) y contenido intestinal del colon para la determinación de los compuestos bioactivos triterpenos pentacíclicos y polifenoles, así como de sus metabolitos por LC-MS. Las concentraciones de estos compuestos en las aceitunas de mesa administradas a ratas también fueron analizadas. Asimismo, el colon se retiró, se dividió en tres segmentos (proximal, medial y distal) y se colocaron abiertos sobre placas de poliestireno con formalina neutra tamponada al 10% para su fijación. Los focos de criptas aberrantes (ACF) y las criptas aberrantes (AC) se contaron por microscopía óptica (10x) después de teñir el tejido con azul de metileno 0,2%, mientras que la tinción de diamina con alto contenido en hierro/azul alcián /rojo neutro (HID-AB) sirvió para cuantificar (20x) el número de focos con depleción de mucina (MDF) y las criptas aberrantes con depleción de mucina (MDAC).

Resultados: El tratamiento tanto con DMH como con aceitunas, no afectó ni al peso corporal ni al consumo de pienso y agua. La observación óptica de los tejidos de la pared del colon permitió

observar que las ratas inyectadas con DMH desarrollaron ACF siguiendo un patrón regionalizado, con lesiones en el segmento proximal (11,5 ± 1,06), un mayor número en el medial (34,2 ± 4,09) y abundantes en el distal (68,0 ± 10,8). La administración de 3,85 g/kg de aceitunas de mesa Arbequina, disminuyó los ACF en un 43,5%, 45,9% y 60,0% en los segmentos proximal, medial y distal, respectivamente. Además, la suma total de AC en todo el tejido de colon decreció un 54,1%. Los MDF fueron solo detectados en los segmentos medial (14,3 ± 3,69) y distal (27,8 ± 4,78), cuya reducción fue de un 20,9% y 43,7% respectivamente. Asimismo, el número total de MDAC en el colon, descendió un 43,9%.

El análisis del plasma permitió detectar los compuestos tales como el ácido maslínico, ácido oleanólico e hidroxitirosol en cantidades ≤ 10 nM. Además, también se cuantificaron metabolitos de estos tres componentes, encontrándose derivados "monohidroxilado", "monohidroxilado y deshidrogenado" y "sulfatado" en concentraciones similares al compuesto original.

La determinación en contenido intestinal requirió la puesta a punto de un método que fue validado según las directrices de la guía *Food and Drug Administration* (FDA, 2018). Los parámetros analizados fueron: efecto matriz, sensibilidad, linealidad, precisión, exactitud y arrastre. Los resultados de la validación mostraron la ausencia de efecto matriz, ya que al comparar las pendientes de las rectas elaboradas con extracto de contenido de colon con concentraciones crecientes (0,05-3 μM) de triterpenos pentacíclicos y polifenoles añadidos y aquellas rectas construidas con metanol 80% a la misma concentración (Matuszewski, 2006), dieron valores en el rango de 80-120%. La sensibilidad fue evaluada de acuerdo al límite de cuantificación, cuyos resultados mostraron unos valores para los triterpenos pentacíclicos de 0,33 nM en el ácido maslínico a 3,09 nM en el uvaol. Los polifenoles obtuvieron un LLOQ en el rango de 0,16 nM para luteolina-7-O-glucósido a 6,73 nM para tirosol, excepto para el catecol, que fue de 16,2 nM. El LLOQ se validó mediante el análisis de seis muestras de extracto de contenido de colon enriquecido con soluciones de trabajo de triterpenos pentacíclicos y polifenoles, mostrando unos resultados de precisión y exactitud inferiores al 20% aceptado. Las curvas de calibración presentaron buena linealidad ($R^2 > 0,996$), con un rango desde LLOQ a 3 μM para los triterpenos pentacíclicos y de LLOQ a 2 μM para polifenoles. Además, la precisión y exactitud en las diferentes concentraciones estudiadas, fue inferior al 15% recomendado. No se detectó arrastre, ya que las soluciones blanco y de metanol 80% interpuestas a lo largo de todo el proceso, no mostraron picos al mismo tiempo de retención que los compuestos estudiados. El método validado permitió cuantificar los triterpenos pentacíclicos y los polifenoles, así como sus derivados metabólicos.

El análisis de los triterpenos pentacíclicos en el contenido de colon determinó que el compuesto mayoritario fue el ácido maslínico, seguido del ácido oleanólico y en menor concentración el eritrodiol, detectándose en ratas DMH+/Olives+ a concentraciones de 321 ± 67,0 nmol/g, 84,8 ± 14,3 nmol/g y 1,19 ± 0,27 nmol/g, respectivamente. Los polifenoles fueron medidos en cantidades menores, alcanzando sus máximos valores el hidroxitirosol (3,31 ± 0,24 nmol/g), el ácido *p*-

cumárico (1,83 ± 0,67 nmol/g) y el tirosol (1,46 ± 0,19 nmol/g). Los metabolitos encontrados derivaron del ácido maslínico, ácido oleanólico, hidroxitirosol y luteolina, los cuales, mostraron concentraciones menores que el compuesto de origen.

Las aceitunas de mesa Arbequina también se analizaron de acuerdo a los procedimientos de Moreno-González *et al*. (2020a, 2020b), presentando las mayores concentraciones el ácido maslínico (2,34 ± 0,08 g/kg), el ácido oleanólico (0,86 ± 0,04 g/kg), el hidroxitirosol (0,48 ± 0,01 g/kg), el verbascósido (0,33 ± 0,03 g/kg) y la luteolina (0,09 ± 0,004 g/kg).

Conclusiones: En vista de los resultados, se puede concluir que la administración de aceitunas de mesa a una dosis de 3,85 g/kg durante 49 días, redujo las lesiones preneoplásicas inducidas en el colon de rata con DMH, sin causar efectos tóxicos.

El análisis de los triterpenos pentacíclicos en aceitunas de mesa Arbequina mostró que los principales compuestos son el ácido maslínico, seguido del ácido oleanólico, mientras que el eritrodiol se encontró a baja concentración. Los polifenoles más representativos fueron el hidroxitirosol, seguido de verbascósido y luteolina.

El análisis del plasma permitió determinar los triterpenos pentacíclicos, ácido maslínico y ácido oleanólico y el polifenol hidroxitirosol. Asimismo, estos tres compuestos mostraron metabolitos derivados también a baja concentración.

En cuanto al contenido intestinal, tanto los analitos que llegan al colon como sus metabolitos, fueron analizados en mayor concentración que en plasma. Los triterpenos pentacíclicos determinados fueron el ácido maslínico, ácido oleanólico y el eritrodiol, siendo los dos primeros los mayoritarios. El análisis de los polifenoles mostró que el principal compuesto fue el hidroxitirosol y en concentraciones ligeramente inferiores la apigenina, ácido cafeico, ácido *p*-cumárico, luteolina, quercetina, tirosol, ácido vanílico y verbascósido.

Por tanto, los resultados de las concentraciones superiores de estos compuestos bioactivos en el contenido de colon respecto a la cantidad medida en el plasma, permite sugerir que poseen una baja biodisponibilidad. Este hecho podría facilitar la actividad quimiopreventiva en la aparición de lesiones preneoplásicas en la mucosa del colon.

The daily consumption of table olives reduces preneoplastic lesions in the colon of rats treated with 1,2-dimethylhydrazine

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Abstract: Table olives constitute an important edible fruit of the Mediterranean diet and a source of bioactive compounds with protective activities against several types of tumors, including colorectal cancer (CRC). To investigate the effect of table olives in early stages of colon carcinogenesis induced by 1,2-dimethylhydrazine (DMH), Sprague-Dawley rats were distributed in a DMH group that received 3 weekly subcutaneous injections of the carcinogen and a control group given only the solvent. Animals were further divided in two subgroups administered orally by gavage with either water or olives for 49 days. Arbequina table olives were prepared as a grinded suspension at a dose of 3.85 g/kg (equivalent to the ingestion of 30 olives by a 60 kg-human). At the end of the experimental period, rats were killed, colon was excised and fixed with formalin. Aberrant crypt foci (ACF) were counted after staining with methylene blue, while the high-iron diamine/alcian blue/neutral red staining (HID-AB) was used to evaluate mucin depleted foci (MDF). No preneoplastic lesions were found in the control groups administered with water or olives. DMH-injected rats developed ACF following a regionalized pattern with 11.5 ± 1.06, 34.2 ± 4.09 and 68.0 ± 10.8 in the proximal, medial and distal colon. However, consumption of olives reduced ACF by a 43.5%, 45.9% and 60.0% in the same segments. Moreover, olives decreased ACF with 1, 2, 3 and ≥ 4 crypts in total colon in a 52.0%, 56.1%, 63.0% and 38.5%, respectively. MDF were not detected in the proximal segments of both DMH groups. The MDF lesions observed in the medial and distal segments, diminished by a 20.9% and 43.7%, respectively. The reduction of the preneoplastic lesions induced by DMH in rat colon by table olives suggests that the daily consumption of the fruit of *Olea europaea* L. could exert a cancer chemopreventive activity.

Keywords *Olea europaea* L.; Arbequina variety; 1,2-dimethylhydrazine; colon cancer; aberrant crypt foci; mucin depleted foci; table olives

1. Introduction

Cancer is one of the leading causes of deaths worldwide, representing an important public health issue. Among the different types, one of the most frequent cancer is colorectal being third in incidence and second in mortality in both genders (Ferlay *et al*., 2018). Around 5 - 15% of patients show a genetic predisposition to developing the disease due to inherited gene mutations, namely

the familial adenomatous polyposis syndrome or the hereditary nonpolyposis colorectal cancer (Vasen *et al*., 2015). However, most cases are consequence of sporadic gene mutations linked to environmental risk factors, such as tobacco, alcohol, diet or physical exercise (Parkin *et al*., 2011), meaning that they might be partly prevented by way of adequate lifestyle and dietary habits. In this context, there is convincing evidence that several dietary factors common in Western society, such as high intake of fat, red meat, refined sugars and alcohol are linked with the risk of developing cancers in the gastrointestinal tract (Ratjen *et al*., 2017). In contrast with the populations living along the Mediterranean Sea shows a protective association against the development of colorectal cancer (Farinetti *et al*., 2017; Ratjen *et al*., 2017). In this sense, the healthfulness of the Mediterranean diet cannot be restricted to the countries bordering the Mediterranean Sea, since it has recently been stated that a greater adherence to this type of diet is associated with a lower prevalence of colorectal adenomas in men of all races (Haslam *et al*., 2017). One of the main features of this dietary pattern is the high consumption of fresh vegetables and fruits, legumes, a low intake of red meat, fish and beneficial fatty acids that are provided by the consumption of olive oil as a main dietary fat (Farinetti *et al*., 2017). Many *in vivo* and *in vitro* studies indicate that a regular consumption of olive oil exerts a protective effect in reducing the risk for colorectal cancer (Farinetti *et al*., 2017; Borzì *et al*., 2019). This activity has been associated with its high content of monounsaturated fats, mainly oleic acid, however olive oil is a complex food containing also other bioactive compounds that could contribute to this healthy effect such as tocopherols, squalene, polyphenols and pentacyclic triterpenes (Borzì *et al*., 2019). Phenolic compounds have been highly evaluated on their antitumoral activities, especially the antiproliferative effects of hydroxytyrosol (Juan *et al*., 2010) and hydroxytyrosol acetate (Mateos *et al*., 2013) in human adenocarcinoma cell lines. In addition to polyphenols, pentacyclic triterpenes have raised numerous research that demonstrate the antitumoral activities of maslinic and oleanolic acids in human adenocarcinoma cell lines (Juan *et al*., 2008; Reyes-Zurita *et al*., 2016), as well as in the prevention of colorectal cancer *in vivo*, since both compounds reduced the appearance of pre-neoplastic lesions induced in the colon of rats by 1,2-dimethylhydrazine (DMH) (Anderson *et al*., 2008; Juan *et al*., 2019). In addition, the antitumoral effect of maslinic acid have been substantiated in male $Apc^{Min/+}$ mice which is a genetic model of familiar cancers such as human familial adenomatous polyposis (FAP) and hereditary non-polyposis colon cancer (Sánchez-Tena *et al*., 2013).

It is noteworthy that despite the numerous studies pointing out an inverse correlation between a regular ingestion of olive oil and colorectal cancer risk, there is no information regarding table olives on this malignancy, although it has a similar nutritional profile and a higher content of polyphenols and pentacyclic triterpenes that have been demonstrated to exert an antitumoral activities both in *in vitro* an *in vivo* experiments. Consequently, here we aimed at evaluating whether the daily intake of Arbequina table olives at the dose of 3.85 g destoned olives/kg body weight elicits a chemopreventive effect on colonic preneoplastic lesions induced by the administration of 1,2-dimethylhydrazine (DMH) to Sprague-Dawleyr rats. DMH is a carcinogen that induces in

animal models lesions like those of the most common intestinal neoplasia, which is the non-familial colorectal type and occurs sporadically (Perše & Cerar, 2010). The treatment with DMH induced the appearance of aberrant crypt foci (ACF) which were measured as markers of cancer risk and mucin depleted foci (MDF) that are lesions that harbor characteristics comparable to microadenomas (Femia *et al*., 2008c). In addition, we wanted to establish the relationship between effect on preneoplastic markers and concentrations of polyphenols and pentacyclic triterpenes commensurate for efficacy. To that end, both groups of compounds were quantified in the plasma and colon content after liquid extraction prior to LC-QTRAP-MS analysis. The knowledge of the bioactive compounds from table olives in contact with the epithelial cells of the colon may partly explain their activity on colonocytes and could shed light on their influence on gut health.

2. Materials and Methods

2.1. Chemicals

Hydroxytyrosol and hydroxytyrosol acetate were bought at Seprox BIOTECH (Madrid, Spain). L-ascorbic acid, caffeic acid, catechol, *o*-coumaric acid, *p*-coumaric acid, 2-(3-hydroxyphenyl)-ethanol (IS of polyphenols), erythrodiol, maslinic acid, oleuropein, (+)-pinoresinol, quercetin, rutin, salidroside, vanillic acid and verbascoside were from Sigma-Aldrich (St. Louis, USA). Extrasynthèse (Genay, France) was the supplier of apigenin, betulinic acid (internal standard (IS) of pentacyclic triterpenes), luteolin, luteolin-7-O-glucoside, oleanolic acid, tyrosol, ursolic acid and uvaol. All solvents used were LC-MS grade. Acetone, acetonitrile, methanol, 2-propanol and tetrahydrofuran were purchased from Panreac Química SLU (Castellar del Vallés, Spain). Acetic acid was acquired from Merck (Darmstadt, Germany) while ethyl acetate and ethanol were from J. T. Baker (Deventer, Holland). Phosphate-buffered solution and 10% buffered formalin (pH 7.4) were bought at Sigma-Aldrich. All other chemicals used in the preparation of solutions were of analytical grade and purchased at Sigma-Aldrich. Ultrapure water obtained from a Milli-Q water purification system (Millipore, St. Louis, Missouri, USA), was employed in all the experiments.

2.2. Animals and Diets

Male adult Sprague–Dawley rats (7-8 weeks-old) came from the Animal House Facility of the Facultat de Farmàcia i Ciències de l'Alimentació (Universitat de Barcelona, UB). Animals were housed in cages (n = 2/cage) and maintained under controlled conditions of a dark-light cycle of 12 h at a temperature of 22 ± 2˚C and a relative humidity between 40% and 70%. Rats had free access to water and food (2014 Teklad Global 14%, Harlan, Barcelona, Spain). All animal manipulations were performed in the morning to minimize the effects of circadian rhythm. Handing and killing of rats were in full accordance with the ethical requirements stablished by the Guide for the Care and Use of Laboratory Animals. The experimental protocols were reviewed and approved by the Ethics Committee of Animal Experimentation of the Universitat de Barcelona and the Generalitat de Catalunya and received the reference number 9468.

2.3. Table olives from the Arbequina variety

The chemopreventive activities on pre-neoplastic lesions was evaluated with table olives of Arbequina variety (Cooperativa del Camp Foment Maialenc SSCL, Maials, Lleida, Spain) from the 2016/2017 season. The trees of *Olea europaea* L. were cultivated in Ribera d'Ebre (Tarragona, Spain) in orchards submitted to drip irrigation. Olives were picked in the green-yellow stage of maturation in perfect sanitary conditions, and were processed following the Greek-style, as natural olives in brine. Olives from the Arbequina variety are characterized for its small size, with a mean weight per olive of 1.55 ± 0.03 g (n = 83) with a percentage of stone corresponding to 29.4 ± 0.32%.

Arbequina table olives were administered to the experimental animals as a finely grinded suspension. Sprague-Dawley rats received a dose of 3.85 g of destoned olives/kg of body weight, that was administered at a constant volume of 10 mL/kg. Thus, 10 g of destoned olives (approximately 8 olives) were mixed with 25 mL of Milli-Q water and carefully triturated with a Polytron homogenizer (PTA 20 TS rotor; setting 5; Kinematica AG, Lucerne, Switzerland) by means 6 short pulses of 30 s. The animal dose was equivalent to the ingestion of 30 Arbequina table olives by a 60 kg persona was calculated according to Reagan-Shaw *et al*. (2017) study for dose translation from animal to human based on the body surface area. This olive suspension was prepared every two days and was kept at 4˚C in a 50 mL falcon tube tightly closed with parafilm and protected from light by an aluminum foil.

2.4. Experimental design

Sprague-Dawley rats were weighed and randomly distributed into 4 groups: Group DMH-/Olives- (negative control: no carcinogen, no test agent; n = 2), Group DMH-/Olives+ (no carcinogen, Arbequina table olives; n = 2), Group DMH+/Olives- (positive control: DMH, no test agent; n = 6) and Group DMH+/Olives+ (DMH, Arbequina table olives; n = 6). Arbequina table olives were orally administered through a stainless-steel animal feeding tube (18 gauge × 76 mm, ref. FTSS-18S-76, Instech Laboratories, Inc., PA, USA) for 49 days. Animals in the groups Olives- were orally administered water during the same period. At days 8, 15 and 22 of the experimental period, rats in the DMH+ groups received a subcutaneous injection of the carcinogen (20 mg DMH/kg dissolved in EDTA 1 mmol/L, pH 6.5; volume of 1 mL/kg), whereas those in the DMH- were given a subcutaneous injection of the solvent. Carcinogen was freshly prepared immediately before each administration. Oral and subcutaneous doses were adjusted according to the rat weight.

Body weight was recorded daily, and food and water consumption once per week. Food conversion efficiency (FCE) was calculated, as a percentage, dividing the weekly body weight gain by the weekly food consumption.

2.5. Sample collection

At the end of the experimental period, rats were deprived of food overnight and anesthetized with 90 mg/kg of ketamine (Imalgene 100 mg/mL, Merial Laboratorios S.A., Barcelona, Spain) and

10 mg/kg of xylacine (Rompun 20 mg/mL, Bayer Hispania S.L., Barcelona, Spain). Blood samples were collected by cardiac puncture, transferred into 4 mL EDTA-K_3 tubes (Sarstedt, Nümbrecht, Germany) to avoid coagulation and centrifuged at 3345 × *g* for 15 min at 4˚C (Megafuge 1.0R, Heraeus Instruments GmbH, Hanau, Germany). Plasma was subsequently separated and stored at −80˚C until analysis.

Subsequent to blood withdrawal, the abdomen was opened by a midline longitudinal incision and a gross necropsy was performed. Therefore, liver, brain, lungs, kidneys, testicles, spleen, heart and thymus were excised, separated of any adherent mesenteric tissues and their wet weights were immediately recorded. Results are expressed as organ weight relative to 100 g of body weight (%). Then, the colon was removed, and the intestinal lumen was rinsed with 1 mL of ice-cold phosphate-buffered solution (PBS) (pH 7.4) to obtain the intestinal content that was stored at −80˚C until the analysis of pentacyclic triterpenes and polyphenols by LC-MS. The colonic tissue was washed in PBS, separated from the surrounding mesenteric tissue, and its wet weight was registered. Then, was divided into three segments of similar length: proximal (close to the cecum), medial, and distal (close to the rectum). Each segment was opened along the longitudinal median, was pinned flat onto a polystyrene board and was fixed in 10% buffered formalin (pH 7.4) for a minimum of 24 h.

2.6. Aberrant Crypt Foci

After being fixed, colon segments were washed in PBS and stained with methylene blue following the protocol described by Bird (1987) for the assessment of aberrant crypt foci (ACF). Briefly, colon segments were dyed with methylene blue 0.2% for 8 min (proximal) or 10 min (medial and distal). The excess of dye was removed by rinsing the tissues with PBS, then each segment was placed with the mucosal side up on a microscopic slide prior to examination at the light microscope at 10x magnification (BX41, Olympus Corporation, Tokyo, Japan). ACF are crypts characterized by a larger size (2-3 times that of normal surrounding crypts), and by displaying a more intense stain, distortion of the opening of the lumen and elevation above the surface of the mucosa (Bird, 1987). The number of ACF were determined in the proximal, medial and distal segments of each rat. Multiplicity was assessed by counting the number of aberrant crypts (AC) forming each focus (AC/ACF). Finally, the total of ACF and AC in total colon were also calculated. The scores were checked by two independent observers who were blinded to the treatments. All of the images of the mucosal surface were captured with a color camera XC 50-UTV1X-2 (Olympus Corporation, Tokyo, Japan).

2.7. Mucin Depleted Foci

Once ACF were counted, colon segments were kept in PBS at 4˚C until being dyed with the high-iron diamine/alcian blue/neutral red staining (HID-AB) that allows the determination of mucin production. First, segments were washed with PBS and dyed in the high-iron diamine solution for 18-24 h protected from the light. Then, tissues were rinsed in PBS prior to being stained with alcian

blue 1% in acetic acid 3% for 5 min. Subsequently, were washed again, and finally stained for 2 min in neutral red (neutral red 0.1% with acetic acid 0.002%). Tissues were mounted on microscopic slides and examined under a light microscope (BX41, Olympus Corporation) at 20x magnification. The total number of mucin depleted foci (MDF), multiplicity expressed as mucin-depleted aberrant crypts per aberrant focus (MDAC/MDF) and total mucin-depleted aberrant crypts (MDAC) per focus in colon were assessed as indicated Caderni *et al*. (2003). MDF are characterized by absence or little production of mucins, distortion of the opening of the lumen compared with normal surrounding crypts and elevation of the lesion above the surface of the colon. As stated above, the scores were evaluated by two independent observers blinded to the groups. Images were taken with a color camera (XC50-UTV1X-2, Olympus Corporation).

2.8. Determination of pentacyclic triterpenes and polyphenols in table olives, plasma and colon content

The content of pentacyclic triterpenes and polyphenols in Arbequina table olives were determined by liquid extraction followed by LC-MS analysis as previously described (Moreno-González *et al*., 2020a; Moreno-González *et al*., 2020b).

The concentrations of pentacyclic triterpenes and polyphenols in plasma and colon content were determined in the DMH-/Olive+ and DMH+/Olive+ groups in samples obtained between 17-19 h after the last oral administration. Pentacyclic triterpenes and polyphenols were concurrently extracted from plasma by liquid-liquid extraction with ethyl acetate as a solvent prior to LC-QTRAP-MS analysis as previously described (Moreno-González *et al*., 2020c). In addition, the colon content was extracted following the validated method of Lozano-Mena *et al*. (2016) and pentacyclic triterpenes and polyphenols were analyzed by LC-QTRAP-MS as reported by Moreno-González *et al*. (2020c).

2.9. Statistical analysis

The results are presented as the mean ± standard error of the means (SEM). Analyte concentrations in Arbequina table olives are presented in mg/kg of destoned olive, in plasma results are given in nM and in colon content as nmol/g of colon content. Outliers were rejected following the Chauvenet's criterion. Data evaluation, statistical analysis and elaboration of graphs were performed with a commercially available package (Prism version 6; GraphPad Software Inc., San Diego, CA, USA). Kolmogorov-Smirnov test was used for the assessment of normality and depending on the significance, a parametric or non-parametric analysis was performed. Differences between groups in the weights of the vital organs, as well as the number of ACF and MDF per segment were compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Body weight, FCE and multiplicity of crypts were analyzed by two-way ANOVA followed by Tukey's multiple comparison test. The total number of AC, ACF, MDF, and MDAC were compared using Student´s unpaired *t* test. Differences in the concentration of

pentacyclic triterpenes and polyphenols and its metabolites were also analyzed by Student´s unpaired *t* test. For all tests three levels were considered as significant, $p < 0.05$, $p < 0.01$ and $p < 0.001$.

3. Results

3.1. Body weight, food and water consumption, and food conversion efficiency

No mortality or adverse effects occurred during the experiment. The consistency of stools was pelleted and firm, with no visible differences between groups.

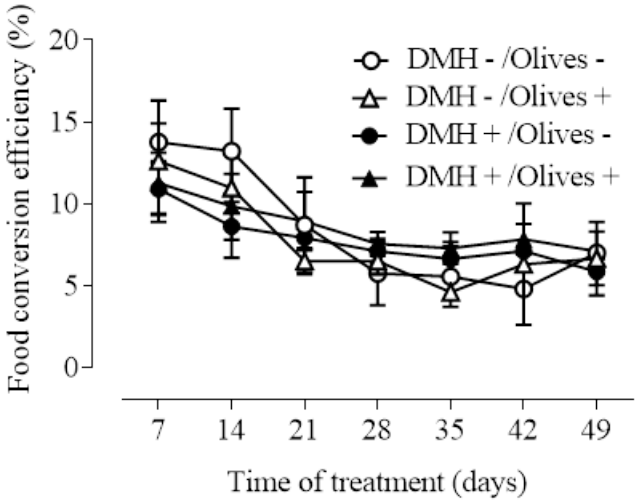

Figure 1. Food conversion efficiency of male Sprague-Dawley rats. Results are expressed as means ± SEM (n=2-6) and were analyzed by two-way ANOVA, followed by Tukey's multiple comparisons test. No differences between groups or over time were found, $p > 0.05$.

Body weight showed no statistically significant differences between the four groups by the administration of olives or DMH with respect to the control group DMH-/Olives-. Thus, the body weight of the negative control group increased from 244.0 ± 12.0 g on day 1 to 335.5 ± 22.5 g on 49 day, whereas DMH-/Olives+ rats showed 246.0 ± 2.00 g on day 1 and 315.5 ± 11.5 g on day 49. The DMH+/Olives- group increased from 253.3 ± 8.78 g on day 1 to 337.5 ± 9.19 g on day 49 and the DMH+/Olives+ group presented 250.5 ± 6.89 g on day 1 and 311.8 ± 7.20 g on day 49. There were no significant differences in water or food consumption between the four groups. Feed conversion efficiency (FCE), that showed no significant differences between groups, was highest during the first week, decreased during the second and third week, and remained constant until the end of the experiment (Figure 1).

3.2. Gross Necropsy

At the end of study, animals were subjected to a detailed postmostern examination of the vital organs. No mascrocopic differences of the size color or texture were observed in any of the groups studied. In addition, the weights of the liver, brain, lungs, kidneys, testicles, spleen, heart and

thymus were recorded and the relative weight was calculated, showing no differences between the groups studied.

3.3. Aberrant Crypt Foci

The Sprague-Dawley rats in the two DMH- groups, that is, the ones that did not received the carcinogen and were injected subcutanously with EDTA 1 mmol/L (pH 6.5) and orally administered with either water (DMH-/Olives-) or 3.85 g/kg of Arbequina table olives (DMH-/Olives+), did not present any lesion compatible with AC in the colon mucosa. Since no preneoplastic lesions were found in the DMH-/Olives- and DMH-/Olives+ groups, the subsequent results involve only the DMH+/Olives- and DMH+/Olives+ groups (Figure 2).

ACF followed a regional distribution along the colon that followed a similar pattern in all the groups treated with DMH, with a low number of lesions in the proximal colon, which increased in the medial segments and they reached their highest value in distal colon (Table 1).

Table 1. Effect of Arbequina table olives on the development of aberrant crypt foci (ACF) on the different segments of the colon of Sprague-Dawley rats.

	DMH+/Olives- Group	**DMH+/Olives+ Group**
ACF/proximal	11.5 ± 1.06	6.50 ± 1.48*
ACF/medial	34.2 ± 4.09	18.5 ± 3.33*
ACF/distal	68.0 ± 10.8	27.2 ± 5.51**

Values are means ± SEMs, n=6. Significantly different from DMH+/Olives- group, *$p < 0.05$, **$p < 0.01$.

The oral administration of table olives at a dose of 3.85 g/kg for 49 days induced a reduction in the proximal, medial and distal segments of 43.5% ($p < 0.05$), 45.9% ($p < 0.05$) and 60.0% ($p < 0.01$), respectively (Table 1). The ACF in total colon experienced a decrease of 54.1% ($p < 0.01$), since the number of crypts experinced a reduction from 114 in the DMH+/Olives- group to 52 in the DMH+/Olives+ group (Figure 2D).

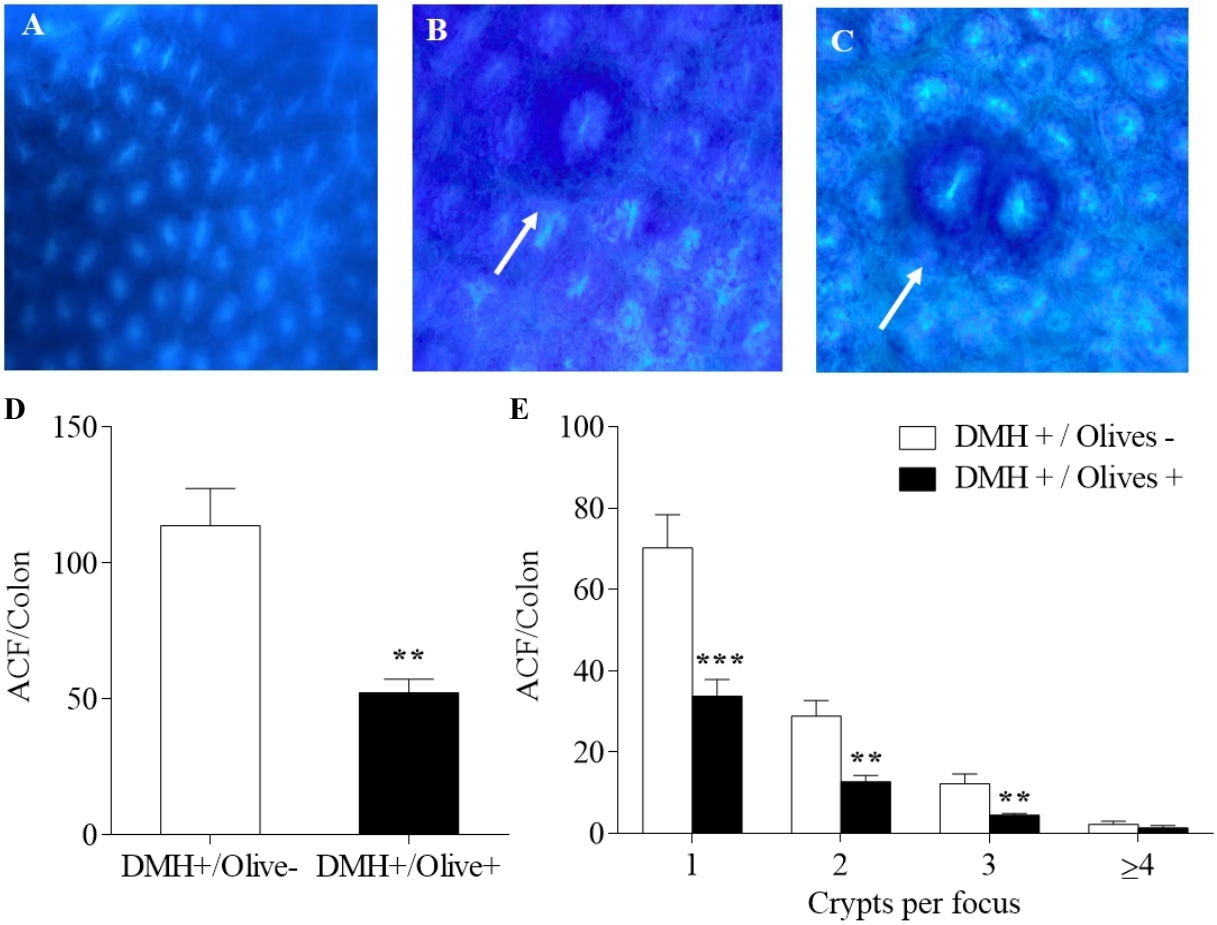

Figure 2. Aberrant crypt foci (ACF) observed under a light microscope after staining of the colon with methylene blue (magnification x 10). The images show the whole mount colon of animals in (A) the negative control (DMH-/Olives-) and DMH-treated animals depicting a topographic view of ACF with 1 crypt (B) and 2 crypts (C). Effects of table olives on the number of ACF in total colon (D) and crypt multiplicity of ACF (E). Results are expressed as means + SEMs (n = 6). Asterisks indicate differences vs DMH+/olives-; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Crypt multiplicity which is the number of AC per focus was also counted (Figure 2E) and showed more singlets than 2, 3, or ≥ 4 AC in both groups administered with DMH. The oral administration of Arbequina table olives at a dose of 3.85 g/kg decreased foci with 1, 2, 3 and 4 crypts in total colon in a 52.0% ($p < 0.001$), 56.1% ($p < 0.01$), 63.0% ($p < 0.01$) and 38.5% ($p > 0.05$), respectively. Treatment with DMH also induced a total number of aberrant crypts (AC) of 174 ± 23.2 in the colon, that were reduced to 78 ± 5.8 by the consumption of Arbequina table olives, which represents a decreased 55.3% ($p < 0.01$).

3.4. Mucin depleted foci

Mucin depleted foci (MDF) consists on aberrant crypts characterized by the loss of mucin production which is a biomarker of dysplastic lesions (Figure 3C).

Table 2. Effect of Arbequina table olives on the development of mucin depleted foci (MDF) on the different segments of the colon of Sprague-Dawley rats.

	DMH+/Olives- Group	DMH+/Olives+ Group
MDF/proximal	0.33 ± 0.21	0.33 ± 0.21
MDF/medial	14.3 ± 3.69	11.3 ± 2.20
MDF/distal	27.8 ± 4.78	15.7 ± 2.58*

Values are means ± SEMs, n=6. Significantly different from DMH+/Olives- group, *$p < 0.05$.

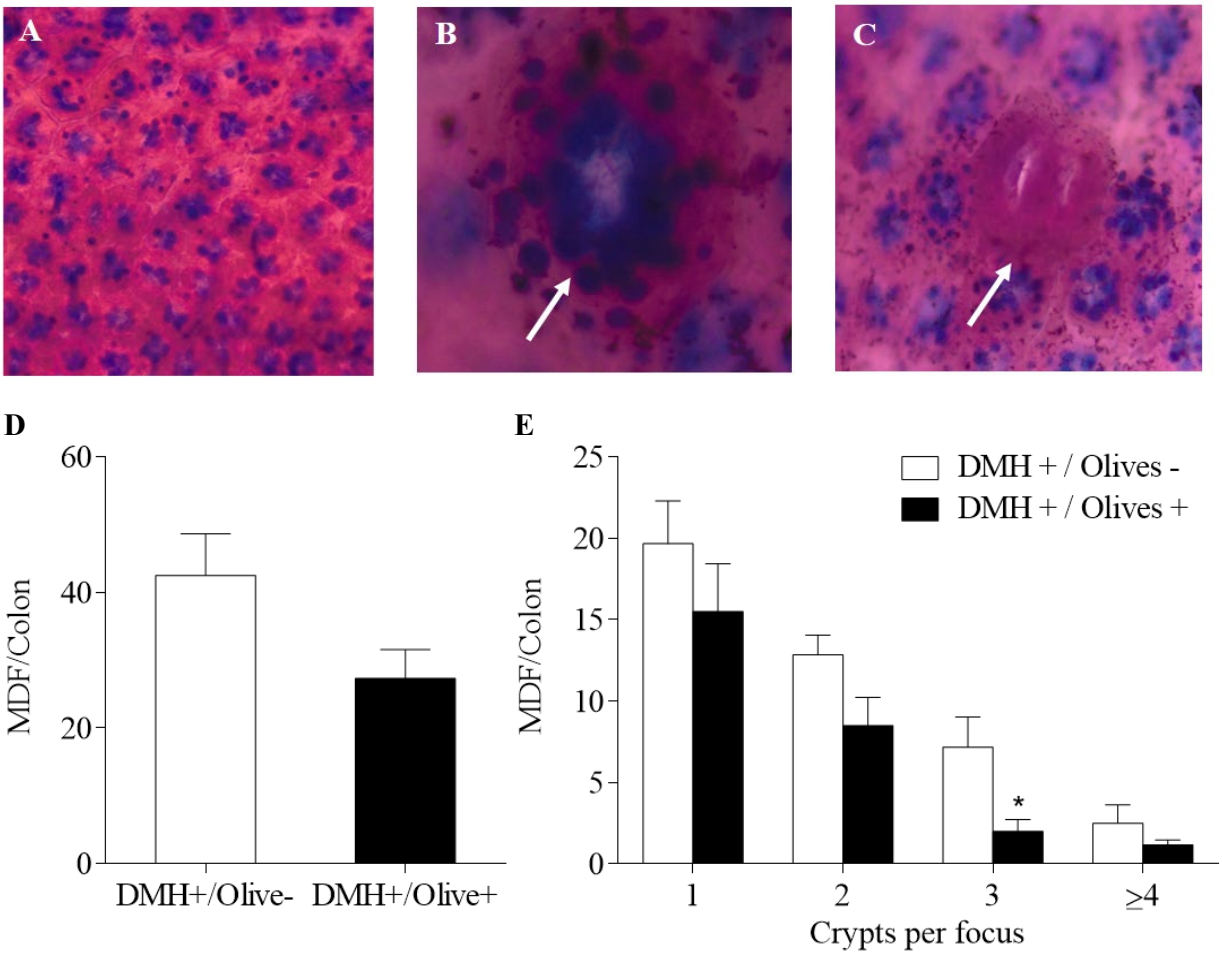

Figure 3. Mucin depleted foci (MDF) observed under a light microscope after staining of the colon with HID-AB (magnification x 20). The images show the whole mount colon of animals in (**A**) the negative control (DMH-/Olives-) and the positive control (DMH+/Olives+) depicting a topographic view of one mucinous ACF (**B**) and one MDF (**C**). Effects of table olives on the total number of MDF (**D**) and number of crypt devoid of mucin per focus in total colon (**E**). Asterisks indicate differences vs DMH+/Olives-; *$p < 0.05$.

The distribution of MDF followed the same pattern as ACF but they were present in inferior amounts. The consumption of Arbequina table olives reduced MDF in a 20.9% ($p > 0.05$) and 43.7%

($p < 0.05$) in the medial and distal segments, respectively (Table 2). Then, the total number of MDF was 42.4 ± 2.3 in the DMH+/Olives- group that was reduced to 27.3 ± 5.0 in the DMH+/Olives+, which represented a reduction of a 35.7% in the appearance of these dysplastic lesions ($p > 0.05$) (Figure 3D).

Multiplicity was also considered for these lesions devoid of mucins (Figure 3E). The number of foci with 1, 2, 3 and ≥ 4 AC was also slightly reduced in rats administered with olives, with a decrease of 21.2% ($p > 0.05$), 33.8%, ($p > 0.05$), 72.1% ($p < 0.05$) and 53.3% ($p > 0.05$), respectively. Moreover, the total number of crypt devoids of mucin (MDAC) was 78.5 ± 4.04 in the DMH-/Olives- group that was reduced to 44.0 ± 6.93 in the DMH+/Olives+, which represented a reduction of a 43.9% in the appearance of these dysplastic lesions ($p < 0.05$).

3.5. Determination of pentacyclic triterpenes and polyphenols in olives

Table 3 shows the concentrations of pentacyclic triterpenes and polyphenols identified in Arbequina table olives of the 2016/2017 season, which were administered to rats. The total content of pentacyclic triterpenes was 3214 ± 123 mg/kg of flesh, whereas polyphenols reached an amount of 1043 ± 46.9 mg/kg. Maslinic acid and oleanolic acid were the main pentacyclic triterpenes found with concentrations of 2342 ± 82.2 and 862 ± 44.0 mg/kg, respectively, whereas erythrodiol counted only with 10.4 ± 0.11 mg/kg. Ursolic acid and uvaol were detected in concentrations below of LOD. Regarding to polyphenols, hydroxytyrosol and verbascoside were the most concentrates with values of 475 ± 11.8 and 334 ± 30.8 mg/kg, respectively, followed by luteolin with 89.6 ± 2.97 mg/kg. Hydroxytyrosol acetate, rutin and tyrosol reached similar concentrations with 26.9 ± 0.71, 26.0 ± 3.14 and 23.1 ± 0.58 mg/kg, whereas salidroside gave a slightly lower value with 17.4 ± 0.98 mg/kg. Oleuropein and luteolin-7-O-glucoside showed concentrations with values that were half of the ones observed for tyrosol, with 12.6 ± 0.24 and 11.1 ± 1.74 mg/kg, respectively. Quercetin, *p*-coumaric acid, caffeic acid and apigenin presented a like amounts ranged from 4.52 to 6.49 mg/kg. Finally, vanillic acid and pinoresinol, had the lower content with 3.56 ± 0.06 and 3.08 ± 0.22 mg/kg. Catechol and *o*-coumaric acid were not detected.

3.6. Quantification of pentacyclic triterpenes, polyphenols and its metabolites in rat plasma

The method utilised to analyse plasma was validated following the recommendations of the FDA (2018). The parameters evaluated were matrix effect, sensitivity, linearity, precision, accuracy and carry-over. Matrix effect was determined following the method described by Matuszewski (2006) in terms of relative standard line slope.

Then, the slope of calibration curves prepared with post-extraction spike method were compared to those prepared with calibrations standards in methanol 80% at the same concentration. The values obtained were within of the range 80-120%. The LLOQ was suitable for the 5 pentacyclic triterpenes, since the values was between 0.11 (oleanolic acid) to 4.60 nM (uvaol),

whereas the polyphenols were found in the interval of 0.09 (luteolin-7-O-glucoside) to 4.66 nM (hydroxytyrosol acetate), except catechol which obtained a value of 9.71 nM.

Table 3. Concentration of pentacyclic triterpenes and polyphenols in the drupe of table olives of the Arbequina variety and in plasma and colon content collected between 17-19 h after the last oral administration of 3.85 g of destoned olives/kg of body weight.

Analyte	Arbequina table olives (mg/kg)	Plasma (nM)		Colon content (nmol/g)	
		DMH-/ olives+	DMH+/ olives+	DMH-/ olives+	DMH+/ olives+
Pentacyclic triterpenes					
Maslinic acid	2342 ± 82.2	3.64 ± 0.27	10.1 ± 2.74	55.5 ± 5.22	321 ± 67.0
Oleanolic acid	862 ± 44.0	2.25 ± 0.17	3.19 ± 0.58	22.8 ± 1.30	84.8 ± 14.3
Ursolic acid	< LOD	φ	φ	φ	φ
Erythrodiol	10.4 ± 0.11	φ	φ	0.13 ± 0.01	1.19 ± 0.27
Uvaol	< LOD	φ	φ	φ	φ
Total	**3214 ± 123**	**5.89 ± 0.10**	**13.2 ± 3.32**	**78.4 ± 6.52**	**407 ± 81.2**
Polyphenols					
Apigenin	4.52 ± 0.17	φ	φ	0.47 ± 0.07	0.37 ± 0.02
Caffeic acid	4.64 ± 0.14	φ	φ	0.76 ± 0.23	0.73 ± 0.19
Catechol	φ	φ	φ	φ	φ
o-Coumaric acid	< LOD	φ	φ	φ	φ
p-Coumaric acid	5.65 ± 0.10	φ	φ	0.92 ± 0.20	1.83 ± 0.67
Hydroxytyrosol	475 ± 11.8	2.11 ± 0.02	2.18 ± 0.54	1.36 ± 0.30	3.31 ± 0.24
Hydroxytyrosol acetate	26.9 ± 0.71	φ	φ	φ	φ
Luteolin	89.6 ± 2.97	φ	φ	0.49 ± 0.19	0.43 ± 0.26
Luteolin-7-O-glucoside	11.1 ± 1.74	φ	φ	<LLOQ	<LLOQ
Oleuropein	12.6 ± 0.24	φ	φ	φ	φ
(+)-Pinoresinol	3.08 ± 0.22	φ	φ	<LLOQ	<LLOQ
Quercetin	6.49 ± 0.16	φ	φ	0.23 ± 0.03	0.23 ± 0.07
Rutin	26.0 ± 3.14	φ	φ	<LLOQ	<LLOQ
Salidroside	17.4 ± 0.98	φ	φ	φ	φ
Tyrosol	23.1 ± 0.58	φ	φ	1.17 ± 0.10	1.46 ± 0.19
Vanillic acid	3.56 ± 0.06	φ	φ	1.00 ± 0.13	1.12 ± 0.11
Verbascoside	334 ± 30.8	φ	φ	0.16 ± 0.002	0.20 ± 0.01
Total	**1043 ± 46.9**	**2.11 ± 0.02**	**2.18 ± 0.54**	**6.56 ± 0.08**	**9.68 ± 0.77**

Results are expressed as means ± SEM (n=2-6). Pentacyclic triterpenes and polyphenols in plasma and colon content in both groups were compared by Student's unpaired *t* test ($p < 0.05$). φ, not detected; LOD, limit of detection (signal-to-noise ratio of 3:1); LLOQ, lower limit of quantification (signal-to-noise ratio of 5:1).

LLOQ in both class of compounds meet the criteria of accuracy within the ± 20% limit and precision (%RSD; coefficient of variation) no greater than 20%. Calibration curves kept the linearity within range 5 to 100 nM for the pentacyclic triterpenes and from 2.5 to 25 nM for the

polyphenols, with correlation coefficients (R^2) above 0.996 for both groups of compounds. The precision (%RSD; coefficient of variation) and accuracy for all the concentration levels were below to 15% limit established. The absence of carry-over on the LC-MS instrument was verify by injection of the highest calibration standard followed by a blank reagent. Moreover, as a precautionary measure, 6 injections of methanol 80% were programmed along the bach. None of the blank reagent or methanol 80% allowed the detection of peaks with the same retention time of pentacyclic triterpenes, polyphenols or their internal standards.

The results in plasma obtained between 17-19 h after the last administration of table olives showed three bioactive compunds (Table 3). Maslinic acid was the main analyte, with a concentration of 3.64 ± 0.27 nM in DMH-/olive+ group and 10.1 ± 2.74 nM in DMH+/Olives+ rats, followed by oleanolic acid with values of 2.25 ± 0.17 and 3.19 ± 0.58 nM in the same way. Hydroxytyrosol was the unique polyphenol found in plasma and presented a concentration of 2.11 ± 0.02 nM in the DMH-/Olives+ and 2.18 ± 0.54 nM in DMH+/Olives+ group. The results of the plasma analytes of both DMH+/Olives+ and DMH-/Olives+ groups did not differ statistically, $p > 0.05$.

Table 4 displays the metabolites quantified in plasma between 17 and 19 h after the last administration of table olives. Maslinic acid, the main compound determined in plasma, showed only one metabolite, the monohydroxylated derivative M1 (*m/z* 487.3) that represented for 35.5% in DMH-/Olives+ rats and 26.9% in DMH+/Olives+ group. Oleanolic acid presented two derivatives in DMH+/Olives+ rats, a monohydroxylated metabolite M1 (*m/z* 471.5) and monohydroxylated and dehydrogenated M2 (*m/z* 469.5) which accounted with a 50.0% and 5.24%, respectively. Hydroxytyrosol showed only a sulfated metabolite in concentrations higher than the parent compound. The derivate was detected as two isomers M1 (*m/z* 233.0/153.0) which represented a 22.7% (M1a) and 46.4% (M1b) in DMH-/Olives+ group and 21.1% (M1a) and 57.4% (M1b) in DMH+/Olives+ rats. The results of the plasma metabolites in both groups did not differ statistically, $p > 0.05$.

3.7. Quantification of pentacyclic triterpenes, polyphenols and its metabolites in colon content

The method employed to determine the compounds in colon content was validated in accordance with the FDA (2018). Matrix effect, was assessed following the method described by Matuszewski (2006) at the concentration levels of 50, 1000 and 2000 nM of pentacyclic triterpenes and polyphenols. The results showed absence of matrix effect, with values within the range of 80-120%. The method provided an adequate sensitivity, with LLOQ for pentacyclic triterpenes ranged from 0.33 nM for maslinic acid to 3.09 nM for alcohol uvaol, whereas the polyphenols were within 0.16 (luteolin-7-O-glucoside) to 2.85 nM (*p*-cumaric acid), except to hydroxytyrosol acetate, tyrosol and catechol that presented values of 4.28, 6.73 and 16.2 nM, respectively. LLOQ was validated by means of the analyses of six samples of colon content extract enriched with working

solutions of triterpenes and pentacyclic polyphenols. The results showed values of precision (%RSD; coefficient of variation) and accuracy whitin the ± 20%. The linearity was observed over the concentration ranges assayed for pentacyclic triterpenes (LLOQ-3000 nM) and polyphenols (LLOQ-2000 nM), with correlation coefficients (R^2) above to 0.996 in both groups of compounds.

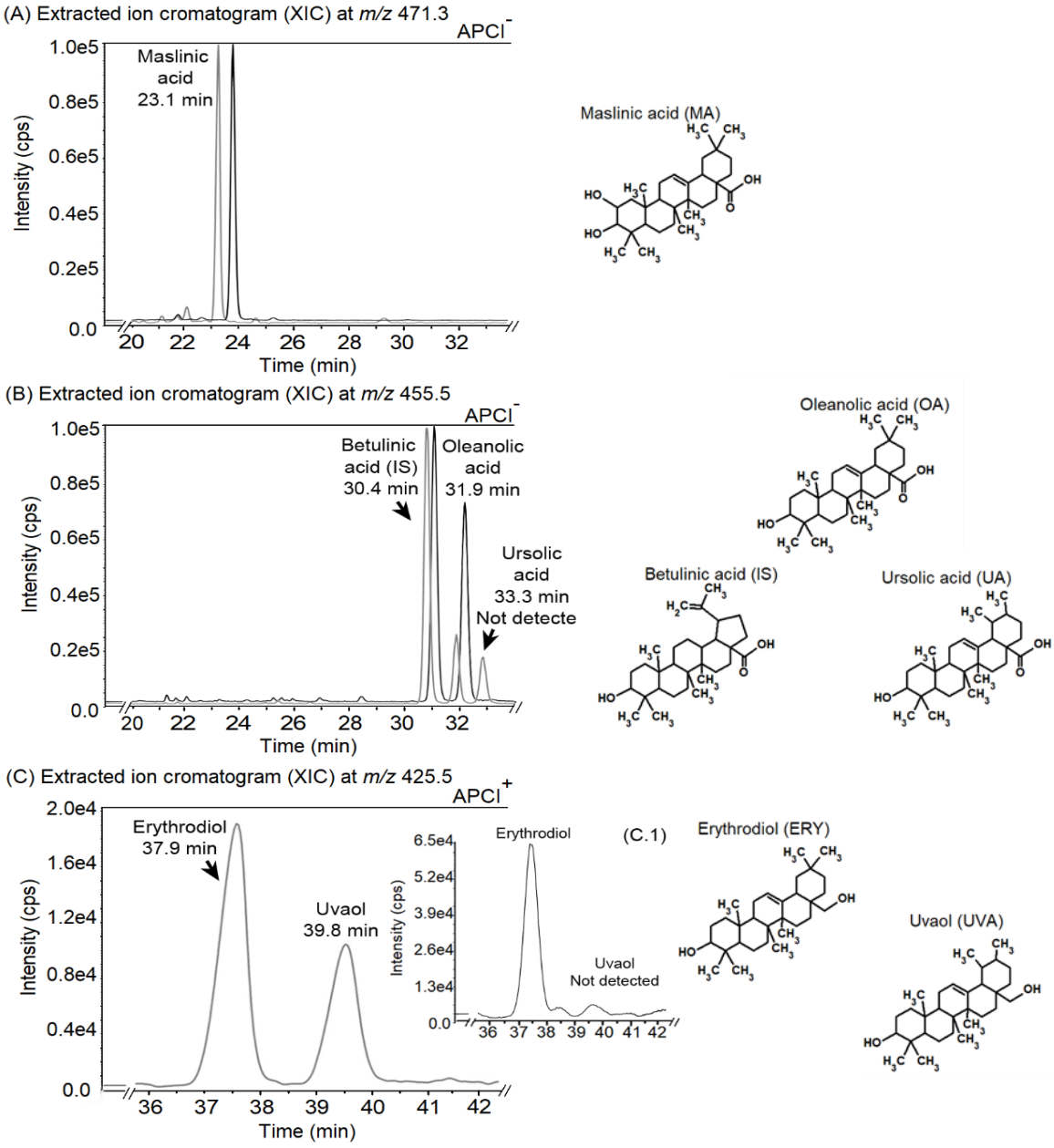

Figure 4. Extracted ion chromatograms (XIC) obtained by LC-APCI-QTRAP-MS of the rat blank colon content spiked with a pentacyclic triterpenes mixture of 50 nM and betulinic acid (IS) at 200 nM (trace gray) and of the colon content of a rat from the DMH+/olives+ group (black trace). The insert (**C1**) depicts the chromatogram of not diluted sample extract. Ionization in negative (**A** and **B**) and positive (**C**) APCI modes.

The precision (%RSD; coefficient of variation) and accuracy at different concentrations levels was acceptable, with values inferior to 15% recommended by the FDA (2018). Carry-over was

controlled as has been described above. The results did not detected any peaks with the same retention time of pentacyclic triterpenes, polyphenols or internal standard in the blank colon content samples, blank reagents and methanol 80%.

Figure 4 depicts the representative chromatograms obtained by LC-APCI-QTRAP-MS of rat blank colon contain spiked with a 50 nM mixture of pentacyclic triterpenes and betulinic acid at 200 nM and a sample of colon content from DMH+/Olives+ group. Maslinic acid and oleanolic acid were quantified at the dilution of 1/25, whereas erythrodiol, which was not detected in this dilution, it was analyzed in samples without to dilute by means of the direct injection of the supernatants obtained after the extraction in the LC-APCI-QTRAP-MS. The total concentration of pentacyclic triterpenes quantified in rats DMH-/Olives+ between 17 and 19 h after the last administration of olives at a dose of 3.85 g/kg was of 78.4 ± 6.52 nmol/g (Table 3). The quantity of each triterpene showed a proportional profile to found in olives, with maslinic acid as main compound with a concentration of 55.5 ± 5.22 nmol/g, followed by oleanolic acid with 22.8 ± 1.30 nmol/g and in minor amount, erythrodiol with 0.13 ± 0.01 nmol/g. Rats from DMH+/Olives+ group displayed higuer concentrations, counting with a total of 407 ± 81.2 nmol/g. The pattern was similar with a higher concentration for maslinic acid (321 ± 67.0 nmol/g), followed oleanolic acid (84.8 ± 14.3 nmol/g) and in minor amount, erythrodiol (1.19 ± 0.27 nmol/g). However, the amount of each compound in both group of rats did not showed statistically significant differences, $p > 0.05$.

Figure 5 shows the representative chromatograms procured by LC-ESI-QTRAP-MS/MS of rat blank colon content spiked with a 50 nM mixture of polyphenols and 2-(3-hydroxyphenyl)-ethanol as internal standard at 500 nM and a sample of colon content from the DMH+/Olives+ rats. The polyphenols were analyzed in the not diluted extract, since in other dilutions as 1:25 and 1:5 the compounds were not detected.

The total concentration of polyphenols in colon content reached 6.56 ± 0.08 nmol/g in the group DMH-/Olives+ and 9.68 ± 0.77 nmol/g in rats DMH+/Olives+ (Table 3). The main phenol found was the alcohol hydroxytyrosol with amounts of 1.36 ± 0.30 and 3.31 ± 0.24 nmol/g in the DMH-/Olives+ and DMH+/Olives+ respectively. In contrast, the rest of polyphenols detected followed a different pattern respect to olives. The next compounds found in both groups were *p*-coumaric acid, tyrosol and vanillic acid, although the trend was different between them. Then, in rats from the DMH-/Olives+ group tyrosol presented the higher concentration (1.17 ± 0.10 nmol/g), followed by vanillic acid (1.00 ± 0.13 nmol/g) and *p*-coumaric acid (0.92 ± 0.20 nmol/g), whereas in DMH+/Olives+ rats, *p*-coumaric acid was superior (1.83 ± 0.67 nmol/g) than tyrosol (1.46 ± 0.19 nmol/g) and vanillic acid (1.12 ± 0.11 nmol/g). The follow compounds detected (from 0.73 ± 0.19 to 0.20 ± 0.01 nmol/g) were caffeic acid, luteolin, apigenin, quercetin and verbascoside, which presented similar concentration in both rat groups. Luteolin-7-*O*-glucoside, (+)-pinoresinol and

rutin showed amounts inferior to LLOQ, whereas hydroxytyrosol acetate, oleuropein and salidroside were not detected. Both groups did not present differences statistical, $p > 0.05$.

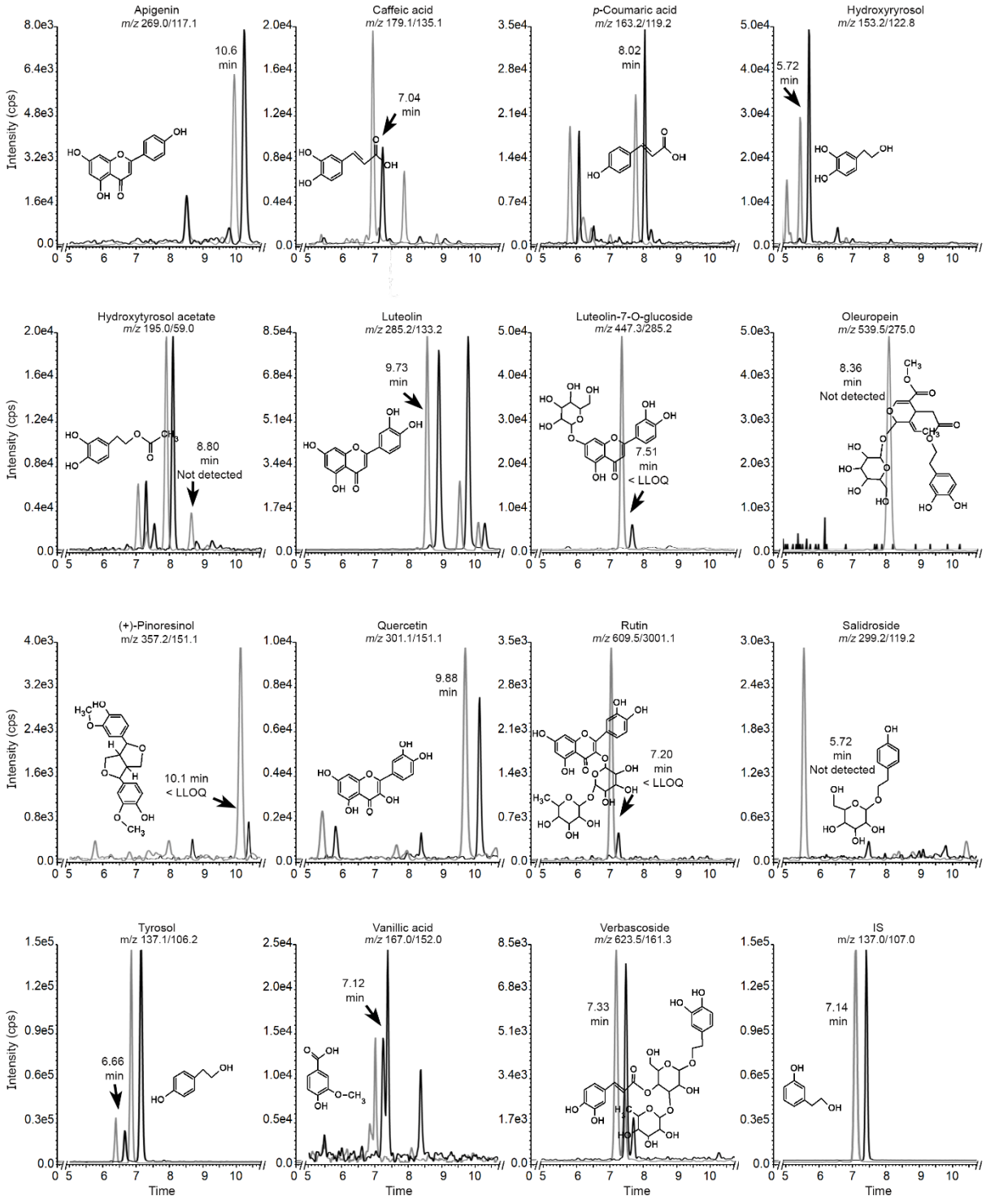

Figure 5. Extracted ion chromatograms (XIC) obtained by LC-ESI-QTRAP-MS of the rat blank colon content spiked with a mixture of 50 nM of polyphenols and 2-(3-hydroxyphenyl)-ethanol (IS) at 5000 nM (gray trace) and of the colon content of a rat from DMH+/olives+ group (black trace). Ionization in negative ESI mode for all of compounds.

Table 4. Concentrations of metabolites from maslinic acid, oleanolic acid, hidroxytyrosol and luteolin detected in plasma and colon content collected between 17 to 19 h after the last oral administration of 3.85 g of destoned olives/kg of body weight.

Metabolite		Plasma (nM)			Colon content (nmol/g)		
		RT (min)	DMH-/ olives+	DMH+/ olives+	RT (min)	DMH-/ olives+	DMH+/ olives+
Maslinic acid *m/z* 471.3		**23.2**	**3.64 ± 0.27**	**10.1 ± 2.74**	**23.3**	**55.5 ± 5.22**	**321 ± 67.0**
Monohydroxylation							
M1a	*m/z* 487.3	13.8	2.01 ± 0.65	3.71 ± 1.33	14.0	17.2 ± 1.48	35.1 ± 2.38
M1b		--	φ	φ	15.0	12.1 ± 0.31	18.3 ± 1.05
Monohydroxylation and dehydrogenation							
M2	*m/z* 485.3	--	φ	φ	16.7	14.0 ± 2.05	15.6 ± 0.63
Dihydroxylation							
M3	*m/z* 503.3	--	φ	φ	10.8	10.6 ± 0.22	11.1 ± 0.08
Dihydroxylation and dehydrogenation							
M4	*m/z* 501.3	--	φ	φ	13.8	10.6± 0.32	11.1 ± 0.16
Oleanolic acid *m/z* 455.5		**31.7**	**2.25 ± 0.17**	**3.19 ± 0.58**	**31.7**	**22.8 ± 1.30**	**84.8 ± 14.3**
Monohydroxylation							
M1	*m/z* 471.5	17.0	φ	3.56 ± 0.78	--	φ	φ
Monohydroxylation and dehydrogenation							
M2	*m/z* 469.5	13.8	φ	0.37 ± 0.05	14.0	5.54 ± 0.32	7.32 ± 0.09
Sulfation							
M3a	*m/z* 535.5	--	φ	φ	13.6	4.40 ± 0.01	4.55 ± 0.04
M3b		--	φ	φ	14.3	4.43 ± 0.04	4.53 ± 0.04
Hydroxytyrosol *m/z* 153.2/123.8		**5.76**	**2.11 ± 0.02**	**2.18 ± 0.54**	**5.73**	**1.36 ± 0.30**	**3.31 ± 0.24**
Sulfation							
M1a	*m/z* 233.0/153.0	5.50	1.55 ± 0.1	2.15 ± 0.66	5.40	0.22 ± 0.05	0.33 ± 0.04
M1b		5.65	3.17 ± 0.2	5.84 ± 2.46	--	φ	φ
Glucuronidation							
M2	*m/z* 329.0/153.0	--	φ	φ	6.64	0.06 ± 0.001	0.06 ± 0.005
Luteolin *m/z* 285.2/133.3		**--**	**φ**	**φ**	**9.74**	**0.49 ± 0.19**	**0.43 ± 0.26**
Sulfation							
M1	*m/z* 365.0/285.2	--	φ	φ	10.8	0.04 ± 0.02	0.05 ± 0.01
Glucuronidation							
M2	*m/z* 461.2/285.2	--	φ	φ	--	0.007 ± 0.001	0.009 ± 0.001

Results are expressed as means ± SEM (n=2-6). Pentacyclic triterpenes and polyphenols in plasma and colon content in both groups were compared by Student's unpaired *t* test ($p < 0.05$). φ, not detected.

Table 4 shows the metabolites identified in the colon at a concentration minor than the original compound. The most abundant metabolites were derivatives from maslinic acid, highlighting metabolite monohydroxylated M1 (*m/z* 487.3) present in two isomers which accounted with 14.4% (M1a) and 10.1% (M1b) in rats DMH-/olives+ and 8.52% (M1a) and 4.44% (M1b) in the

DMH+/olives+g roup. The next metabolite from maslinic acid was a monohydroxylated and dehydrogenated M2 (*m/z* 485.3) that represented a 11.7% and 3.79% in the DMH-/Olives+ and DMH+/Olives+ groups, respectively. The metabolites dihydroxylated M3 (*m/z* 503.3) and dehydroxylated and dehydrogenated M4 (*m/z* 501.3) presented similar values within the same group of rats, being around a 8.8% in DMH-/Olives+ rats and a 2.7% in DMH+/Olives+. Oleanolic acid presented a monohydroxylated and dehydrogenated metabolite M2 (*m/z* 469.5) with a percentage of 14.9% in rats DMH-/Olives+ and 7.23% in DMH+/Olives+, whereas the sulfated metabolite M3 (*m/z* 535.5), presented in two sulfated isomers, accounted for 11.8% (M1a) and 11.9% (M1b) in rats DMH-/Olives+ and 4.50% (M1a) and 4.48% (M1b) in the DMH+/Olives+ group. The polyphenols hydroxytyrosol and luteolin despite to present a low concentration in colon content, they showed sulfated and glucuronidated derivatives. Hydroxytyrosol sulfate M1 (*m/z* 233.0/153.0) represented a 13.4% in rats DMH-/Olives+ and 8.82% in the DMH+/Olives+ group, whereas hydroxytyrosol glucuronized M2 (*m/z* 329.0/153.9) accounted only with 3.90% and 1.62% in the same way. Luteolin and its sulfate M1 (*m/z* 365.0/285.2) and glucuronide M2 (*m/z* 461.2/285.2) reached around of 8% and 2%, respectively in both rat groups. The metabolites did not show statistical differences between groups ($p > 0.05$).

4. Discussion

Colorectal cancer (CRC) is the third most common cancer per incidence worldwide, the third in men (746,000) and the second in women (614,000 cases), counting with more than half of the cases of the industrialized regions in the world (Borzì *et al.*, 2019). Colorectal cancer risk can be reduced through a healthy lifestyle including good eating habits (O'Keefe, 2016; Torre *et al.*, 2016).

The main function of the large intestine or colon is the absorption of water and salt from feces and the propulsion of these to the rectum for the defecation. The colon is covered by an absorptive epithelium formed by crypts. These structures contain stem cells in the bottom that give rise to progenitor cells with capacities of differentiating into different lineages (enterocytes, enteroendocrine and globet cells). The stages of proliferation, differentiation, migration toward the large intestinal lumen and apoptosis keep the epithelium renewed in the crypts. The cells with damaged DNA do not cause apoptosis and, when they reach the top of the crypt they continue proliferating (Tanaka, 2009). This pre-cancerous change produces aberrant crypt foci (ACF) and in a more advanced stage, mucin depleted foci (MDF), which nowadays are used as biomarkers in the chemoprevention of colon carcinogenesis (Femia *et al*., 2008b; Tanaka, 2009; Sakai *et al*., 2012). CRC possess mainly genetic alterations in the WTN pathway. In normal conditions, WNT ligands bind FZD and LRP receptor complexes, initiating membrane recruitment of scaffold proteins (AXIN, DVL), and inactivation of the β-CATENIN destruction complex (composed mainly by AXIN, APC, CK1, GSK3β). When this complex is inactive, β-CATENIN is accumulated in the cytosol and subsequently is translocated into the nucleus where it bound with TCF family transcription factors and to other co-activators to carry out transcription of target genes (Schatoff *et*

al., 2017). However, it has been suggested that the APC protein involved in the regulation of WNT activity, could be altered in the majority of colon cancer, giving place to a high activity of WNT pathway. Other pathways that could be affected in CRC are RAS-MAPK, PI3K, -β and p53. Moreover, independent of origin or mutation, nearly 100% of these tumors have alterations in MYC transcriptional (Cancer Genome Atlas Network, 2012).

In our research, DMH carcinogen has been used to induce preneoplastic lesions in rats. DMH is an alkylating agent with a mode of action involving the methylation in the position O^6 or N^7 of the DNA guanine base. Then, DMH is metabolized through intermediates azoxymethane (AOM) and methylazoxymethanol (MAM), to the methyldiazonium ion metabolite highly reactive. Previous researched demonstrated that both DMH and its AOM intermediary to induce colorectal cancer in rodents via subcutaneous or intraperitoneal administration (Rosenberg *et al*., 2009; Perše & Cerar, 2011), developing preneoplastic lesions, such as aberrant crypt foci (ACF) and mucin depleted foci (MDF) (Caderni *et al*., 1995). ACF are hyperplastic lesions which have mutations frequently in *K-ras*, whereas MDF are dysplastic lesions with mutations mainly in *β-catenin* and *Apc* genes. Mutations in *K-ras* gives to rise to the constitutive activation of RAS and its down-stream signaling pathways (Raf/MEK/MAPK and PI3K/Akt/PKB), which may be involved in the cell proliferation, whereas *β-catenin* and *Apc* gene mutations, cause stabilization of β-CATENIN in the cytoplasm and its association with TCF-4 in the nucleus. This could produce the induction of constitutive transcriptional activation which may play important roles in the dysplastic changes that present in the MDF (Takahashi & Wakabayashi, 2004; Femia *et al*., 2007; Chen & Huang, 2009). In addition, alterations in *K-ras* or *β-catenin* are related with an increasing of expression of *iNOS*, *cox-2, cyclin-D1, c-myc* (Takahashi & Wakabayashi, 2004; Perše & Cerar, 2011) and *PPAR-δ and mmp-7* (Perše & Cerar, 2011).

In the present study, it has been demonstrated the protective effect of table olives over the early carcinogenic lesions in colon of DMH-treated rats where carcinogenesis develops through a series of stages as it occurs in humans (Femia & Caderni, 2008a). Likewise, the concentrations of pentacyclic triterpenes and polyphenols in colon content and in plasma have been determined. The method employed for the extraction, identification, and quantification of the pentacyclic triterpenes and polyphenols and its metabolites both colon content and plasma, presented a suitable precision and sensitivity. The procedures were applied to the samples obtained 17-19 h after of the last oral administration (3.85 g/kg) of Arbequina table olives. Rats were induced through three subcutaneous injections of 20 mg/kg of DMH that permitted to develop preneoplastic lesions, such ACF and MDF within a period of 4 weeks, without to manifest signs of toxicity (Alfaras *et al.*, 2010). The number of ACF and MDF in the DMH+/olives- control group to be appropriate for the tested of chemopreventive activity of natural products and was in agreement with previous studies (Bird & Good, 2000; Paulsen *et al.*, 2000; Suzui *et al.*, 2013). In this way, the ACF and MDF assays can be carry out with a small number of animals and a short experimental time as compared with others

carcinogenesis studies, being widely used as a short-term test to determination of the effects of dietetic patterns on experimental colon carcinogenesis. Thus, after to the administration of table olives during 49-days, it was confirmed a protective effect, since ACF and MDF in total colon were reduced by a 54.1% and 35.7%, respectively. Multiplicity (the number of crypts present in each focus) of the ACF and MDF also was affected by table olives, since the number of focus with 1, 2, 3 and ≥ 4 AC was reduced. Moreover, vital organs did not showed evidence of toxicity. Therefore, the consumption of table olives could have a chemopreventive role in the initiation phase of CRC. Analysis of pentacyclic triterpenes and polyphenols in colon content confirmed that maslinic acid, oleanolic acid, and hydroxytyrosol were the compounds with the highest concentrations. Conversely, both pentacyclic triterpenes and polyphenols were measured in plasma at very low concentration or were not detected. In this way, the low bioavailability of these bioactive compounds could favor to attain higher concentrations in the colon which could be exert a higher chemopreventive activity over intestine walls. Derivative metabolites from these compounds also were detected in very low concentration, both in colon content and in plasma.

Previous studies *in vitro* have demonstrated the antiproliferative and apoptotic effects of maslinic acid (Reyes-Zurita *et al.*, 2011; Reyes-Zurita *et al*., 2016), oleanolic acid (Janakiram *et al.*, 2008) and ursolic acid (Andersson *et al.*, 2003) over HT-29 or Caco-2 human colon cells. Besides, studies *in vivo* confirmed the chemopreventive activity of these compounds, since preneoplastic lesions such ACF induced by DMH or AOM in rat colon, were reduced (Kawamori *et al.*, 1995; Andersson *et al*., 2008; Furtado *et al*., 2008; Janakiram *et al*., 2008; Juan *et al*., 2019). In this way, maslinic acid and its precursor, oleanolic acid could be involved in the diminution of the inflammation characteristic of the lesions, since would inhibit the NF-κβ transcription factor involved in the regulation of expression of *iNOS* (Janakiram *et al*., 2008) and *cox-2* (Janakiram *et al*., 2008; Hsum *et al*., 2011).

The low bioavailability of maslinic acid from Arbequina table olives agreed with previous studies carried out in our group, where this compound presented a low oral bioavailability of about 5% (Lozano-Mena *et al*., 2016). The preventive action of polyphenols over CRC also has been suggested. Several studies have demonstrated that both hydroxytyrosol and tyrosol and its precursor oleuropein, the main polyphenols found in *Olea europaea* L., possess antiproliferative and apoptotic activities over HT-29 or Caco-2 colon cells (Giovannini *et al*., 1999; Corona *et al.*, 2009; Cárdeno *et al.*, 2013; Terzuoli *et al*., 2016; López de Las Hazas *et al.*, 2017). Hydroxytyrosol and tyrosol could have been described to be absorbed in the intestinal tract (Corona *et al.*, 2006). In contrast, oleuropein would not absorb in the small intestine and would reach large intestine where could be subject to degradation by microflora giving place mainly to hydroxytyrosol (Corona *et al.*, 2006). Then, the metabolism of colon bacteria might exert an increase in the bioavailability of hydroxytyrosol (Corona *et al.*, 2006). Our results show similar concentrations of hydroxytyrosol both colon content and plasma, demonstrating that its bioavailability is higher than of pentacyclic

triterpenes. Hydroxytyrosol acts diminishing the proliferation of cell since this polyphenol have been described to arrest cell cycle in the G1 stage and to promote apoptosis through activation of caspases. Moreover, hydroxytyrosol phase II metabolites (sulfate and glucuronide) also could be involved in the apoptosis mediated by caspases (López de las Hazas *et al.*, 2017). Atzeri *et al*., (2016), also demonstrated the high antioxidant power of sulfate metabolites from hydroxytyrosol and tyrosol in Caco-2 human against pro-oxidant dietary agents.

On the other hand, there are a controversy with respect to cancer preventive activity of the main monounsaturated fat (oleic acid) found in oil olive and olives. The main benefits of oleic acid are widely related with its cardioprotective action (Bermudez *et al*., 2011), although there are studies that supported its protective effect against colon cancer (Bartolí *et al*., 2000; Berger *et al.*, 2017). On the contrary, other researches carried out with foods rich in oleic acid, such the beef and poultry, or oils of corn, palm, peanut, soybean and sunflower seed, have been associated with a major incidence of colon and breast cancer in humans (Rao *et al.*, 1998). Storniolo, et al., 2019 observed that oleic acid increased Caco-2 cell growth, but that this effect was significantly reverted when cells were grown with minor compounds form EVOO such as hydroxytyrosol, oleuropein, pinoresinol, squalene and maslinic acid. Others studies recognized that oleic acid could to induce apoptosis in HT-29 cells with a comparable effect than olive oil but would be ineffective in Caco-2 cells, suggesting that it would be cell line-specific (Llor *et al*., 2003).Therefore, it is very likely that the consistent chemopreventive effect of table olives and EVOO could be due to the minor components, such as pentacyclic triterpenes and polyphenols.

In conclusion, the present study demonstrated that a diary doses of 3.85 mg/kg of table olives diminished the early preneoplastic lesions, such ACF and MDF in rat colon. The high concentrations of constituents such as pentacyclic triterpenes and polyphenols present in table olives may have a potentially protective role on early stages of colon cancer. Nevertheless, table olives are formed by different bioactive compounds that could exert simultaneously a protective effect. Thus, the present results suggest that the diary intake of the fruit from *Olea europaea* L. would exert healthy properties.

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Conflicts of Interest: The authors declare no conflict of interest.

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IV. DISCUSIÓN GENERAL

El olivo (*Olea europaea* L.), es uno de los árboles cultivados más antiguos del mundo cuyo fruto, la aceituna, es económicamente importante, ya que de él se extrae el aceite de oliva y se producen las aceitunas de mesa. El fruto de *Olea europaea* L. tiene un importante valor nutracéutico, debido a su composición en ácidos grasos insaturados, vitamina E, carotenoides, minerales, triterpenos pentacíclicos y polifenoles (Ghanbari *et al*., 2012). Estos dos últimos grupos representan metabolitos secundarios de la planta que se sintetizan y acumulan en el fruto y en las hojas contribuyendo a su protección (Charoenprasert y Mitchell, 2012; Szakiel *et al*., 2012; Guo *et al*., 2018; Martín-García *et al*., 2019). Los triterpenos pentacíclicos se encuentran principalmente en la piel la planta (Diarte *et al*., 2019), mientras que la pulpa del fruto es rica en polifenoles (Boskou, 2017). Diversos estudios sugieren que los triterpenos pentacíclicos poseen propiedades beneficiosas para la salud que incluyen el efecto antiviral, antidiabético, cardioprotector y antitumoral (Sánchez-Quesada *et al*., 2013; Lozano-Mena *et al*., 2014), mientras que los polifenoles destacan por su actividad antioxidante, antiinflamatoria, antimicrobiana y antitumoral (Malheiro *et al*., 2014; Pedret *et al*., 2018). Los beneficios cardioprotectores de los polifenoles quedan corroborados de acuerdo a la declaración de propiedades beneficiosas para la salud (Reg. EU nº 432/2012), donde se afirma que el consumo diario de 5 mg de hidroxitirosol y sus derivados (por ejemplo, oleuropeína y tirosol), contribuirían a la protección de los lípidos de la sangre frente al estrés oxidativo. Sin embargo, las concentraciones en algunos aceites de oliva pueden ser bastante inferiores a las recomendadas para alcanzar dicho efecto (Pedret *et al*., 2018). En cambio, las aceitunas de mesa aportan cantidades más elevadas (Giménez *et al.*, 2015; Cabrera-Bañegil *et al.*, 2017) que las encontradas en el aceite (Olmo-García *et al*., 2019a; Olmo-García *et al.*, 2019b).

La composición de las aceitunas de mesa está influenciada por un conjunto de factores que incluyen la variedad de aceituna, el grado de madurez del fruto, la calidad del suelo, las condiciones ambientales y el procesamiento llevado a cabo para hacerlas comestibles (Boskou, 2017; Guo *et al*., 2018). Los principales métodos comerciales de elaboración de aceitunas de mesa son el estilo Español, el Californiano y el Griego. Este último, que es el que han seguido las aceitunas que se han analizado en esta tesis, consiste en la inmersión del fruto en NaCl (4-15%; p/v) donde se produce la fermentación espontánea y finalmente el envasado en salmuera fresca (Kailis y Kiritsakis, 2017).

En la presente tesis se ha comenzado con el análisis el contenido de polifenoles en aceitunas de mesa. Hasta la fecha, se han identificado al menos 36 polifenoles de cinco clases diferentes en los frutos y hojas de *Olea europaea* L. (Charoenpraset y Mitchell 2012). Sin embargo, muchas de las investigaciones se han focalizado en el aceite de oliva y, las que existen para las aceitunas de mesa, determinan pocos compuestos, mayoritariamente polifenoles y centrados en una clase (Boskou *et al*., 2005; Ghanbari *et al*., 2012; Guo *et al*., 2018).

De acuerdo a la bibliografía, los estudios de los polifenoles en las hojas del olivo, el fruto y el aceite utiliza una variedad de métodos analíticos (Gómez-Caravaca *et al.*, 2015), siendo los más utilizados LC acoplada a detectores de ultravioleta (UV) o de diodos (DAD) (Romani *et al*., 1999; Obied *et al*., 2007; IOC, 2009; Kanakis *et al*., 2013; Melliou *et al*., 2015; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017; Romero *et al*., 2017; Selli *et al*., 2018). A pesar de ello, estos métodos tienen los inconvenientes de tener tiempos de análisis largos (de 30 a 100 min) y límites de cuantificación elevados, no permitiendo la distinción de los diferentes polifenoles de *Olea europaea* L. y, especialmente, los que se encuentran a baja concentración (Romani *et al*., 1999; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017; Romero *et al*., 2017). Cuando la LC se combina con la detección por MS, aumenta la sensibilidad junto con la selectividad. Por ello, el primer objetivo de esta tesis se basó en el desarrollo de un procedimiento analítico rápido y de alta resolución para el análisis rutinario de los polifenoles en las aceitunas de mesa, lo cual, permite a su vez determinar la calidad de las mismas. El método se ha llevado a cabo mediante extracción líquida seguido de identificación y cuantificación por LC-ESI-MS/MS. Tras ser validado siguiendo las directrices de la EMA (2011), se ha aplicado a las aceitunas de mesa Marfil, una variedad originaria de la región catalana de Montsià.

Para establecer el método, se tuvieron en cuenta diversos puntos importantes. Uno de ellos fue la preparación de la muestra, que se llevó a cabo mediante un procedimiento directo que se estableció previamente para el análisis de triterpenos pentacíclicos en aceitunas de mesa (Giménez *et al*., 2015). Esto tiene lugar mediante la elaboración de una suspensión de aceitunas deshuesadas finamente triturada que se somete a tres extracciones consecutivas con metanol:etanol (1:1; v/v). Aunque se han descrito numerosos métodos (Brenes *et al*., 1995; Romani *et al*., 1999; Servili *et al*., 1999; Blekas *et al*., 2002; Ryan *et al*., 2003; Sivakumar *et al*., 2005; Obied *et al*., 2007; Rodríguez *et al*., 2009; Gutierrez-Rosales *et al*., 2010; Jerman *et al*., 2010; Zoidou *et al*., 2010; Kanakis *et al*., 2013; Malheiro *et al*., 2014; Melliou *et al*., 2015; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017; Romero *et al*., 2017; Crawford *et al*., 2018; D'Antuono *et al*., 2018; Johnson *et al*., 2018; Selli *et al*., 2018), en pocos de ellos se ha evaluado el rendimiento utilizando diferentes solventes de extracción (Jerman *et al*., 2010; Cabrera-Bañegil *et al*., 2017; Crawford *et al*., 2018). En este estudio se evaluó el uso de metanol:etanol (1:1; v/v) dando buenas recuperaciones, tal y como muestran los resultados obtenidos en la validación. Además, el tratamiento de la muestra es mínimo, ya que, a diferencia de otros estudios, no incluye pasos de evaporación (Brenes *et al*., 1995; Blekas *et al*., 2002; Sivakumar *et al*., 2005; Gutierrez-Rosales *et al*., 2010; Selli *et al*., 2018), ultrasonido (Jerman *et al*., 2010; Melliou *et al*., 2015; Cabrera-Bañegil *et al*., 2017) o extracción en fase sólida (Romani *et al*., 1999; Crawford *et al*., 2018). Otro punto importante fue la elección de la columna y la fase móvil, los cuales se evaluaron paralelamente. La mayoría de los análisis de polifenoles en aceitunas de mesa utilizan la columna Spherisorb ODS-2C18, que produce una buena separación, aunque con tiempos de análisis que van de 60 a 80 min en LC (Blekas *et al*., 2002; Morelló *et al*

2004; Rodríguez *et al*., 2009; Jerman *et al*., 2010; Malheiro *et al*., 2014; Romero *et al*., 2017) y de 50 min en LC-MS (Selli *et al*., 2018). Los disolventes comúnmente utilizados para el análisis por LC-MS de polifenoles en aceitunas de mesa son el agua Milli-Q y el metanol o acetonitrilo en combinación con ácido fórmico o acético (Ryan *et al*., 2002; Sivakumar *et al*., 2005; Obied *et al*., 2007; Gutierrez-Rosales *et al*., 2010; Kanakis *et al*., 2013; Melliou *et al*., 2015; D'Antuono *et al*., 2016; Johnson *et al*., 2018; Selli *et al*., 2018). Así pues, se observó que la mejor intensidad de señal, separación y resolución de picos se obtiene con la columna Zorbax Eclipse XDB C-18, y la fase móvil formada por agua Milli-Q acidificada con ácido acético al 0,025% y acetonitrilo con acetona al 5%. Las condiciones de cromatografía y espectrometría de masas optimizadas, permitieron la detección selectiva de 17 polifenoles de diferentes clases en menos de 9 minutos.

Seguidamente, se realizó la validación del proceso de extracción y del análisis LC-ESI-MS/MS. La recuperación del método de extracción de todos los polifenoles fue superior al 95%, que a excepción del método de Kanakis *et al*. (2013), fue mayor que lo que han conseguido otros estudios (Zoidou *et al*., 2010; Melliou *et al*., 2015). También se determinó el efecto matriz, dado que las aceitunas de mesa contienen una mezcla compleja de compuestos que podrían suprimir o aumentar la señal en la fuente de ESI. Los resultados mostraron ausencia de dicho efecto, ya que las relaciones de pendiente se encontraron en el rango de 0,92 a 1,13, con una RSD (%) inferior al 15%. Además, la sensibilidad (LLOQ) alcanzada (por ejemplo, hidroxitirosol, 2,8 nM; oleuropeína, 2,7 nM y tirosol, 28 nM) fue superior a la descrita en la literatura para el análisis de polifenoles en aceitunas de mesa por LC (Zoidou *et al*., 2010; Cabrera-Bañegil *et al*., 2017) o LC-MS (Kanakis *et al*., 2013; Melliou *et al*., 2015; Johnson *et al*., 2018). Por lo tanto, los resultados de la validación indicaron que el método es exacto, preciso, lineal y selectivo. Teniendo en cuenta los resultados de validación, éste se podría aplicar al análisis de estos compuestos en estudios de biodisponibilidad, donde la detección de polifenoles en plasma u orina requieren una alta sensibilidad.

El procedimiento validado se aplicó al estudio de la composición fenólica de las aceitunas Marfil. El análisis permitió identificar 15 polifenoles con un contenido total que representa aproximadamente el 1% del peso de la pulpa fresca. Los principales polifenoles fueron el hidroxitirosol (44,3%) y el tirosol (23,2%). La concentración del primero concuerda con los valores obtenidos por otros autores para aceitunas de mesa de diferentes variedades procesadas de manera natural (Blekas *et al*., 2002; Boskou *et al*., 2006; Rodríguez *et al*., 2009; Zoidou *et al*., 2010; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017; D'Antuono *et al*., 2018). Sin embargo, el valor de tirosol es casi el doble de los aportados por otros autores (Blekas *et al*., 2002; Boskou *et al*., 2006; Rodríguez *et al*., 2009; Zoidou *et al*., 2010; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017), a excepción de la variedad italiana *Cellina di Nardò*, donde la concentración es mayor (D'Antuono *et al*., 2018). La luteolina en aceitunas Marfil representó el 10,2% de todos los polifenoles cuantificados y mostró valores más elevados que en otras variedades (Blekas *et al*., 2002; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017), salvo para las aceitunas negras saladas

y secas (Selli *et al*., 2018) y las *Cellina di Nardò* (D'Antuono *et al*., 2018). La salidrosida, que se ha descrito en aceitunas procesadas de forma natural como Kalamata y Thassos (Romero *et al*., 2004) y en las variedades Manzanilla y Hojiblanca (Ramírez *et al*., 2016), también se cuantificó en Marfil, representando el 9,9% de los polifenoles. Asimismo, se detectaron los ácidos fenólicos, vanílico (3,6 %), *p*-cumárico (2,2 %) y cafeico (1,8%), obteniéndose concentraciones mayores a las descritas en otras investigaciones (Boskou *et al*., 2006; Cabrera-Bañegil *et al*., 2017; D'Antuono *et al*., 2018; Selli *et al*., 2018) y el lignano pinoresinol (1,7%), que solo se ha descrito en aceitunas italianas (Perpetuini *et al*., 2018). La apigenina y quercetina representaron en torno al 1%, concordando los valores con los obtenidos en otras variedades (D'Antuono *et al*., 2016; Selli *et al*., 2018). Finalmente, el acetato de hidroxitirosol, la oleuropeína, la luteolina-7-*O*-glucósido, el verbascósido y la rutina, representaron menos del 1% de los polifenoles en Marfil, siendo sus valores más bajos que los encontrados en otras variedades (Melliou *et al*., 2015; D'Antuono *et al*., 2016; Cabrera-Bañegil *et al*., 2017; D'Antuono *et al*., 2018; Selli *et al*., 2018).

Teniendo en cuenta nuestros resultados, esta variedad contiene un alto rango de polifenoles que representan un total de 0,87 ± 0,08 g/kg. Destacan la cantidad de hidroxitirosol, tirosol y oleuropeína, que suman un total de 675,4 mg/kg, lo que significa que una aceituna puede contener 0,774 mg de estos componentes. Por lo tanto, el consumo diario de 7 aceitunas (8 g de porción comestible) de la variedad Marfil, proporciona 5,41 mg de estos compuestos, que es mayor que la cantidad de hidroxitirosol, tirosol y oleuropeína que según la EFSA sería necesaria para ejercer efectos beneficiosos (Reg. EU nº 432/2012). A pesar de que las aceitunas se conservan en salmuera, la cantidad de sodio proporcionada por 7 aceitunas (8 g de pulpa) de la variedad Marfil sería de 107 mg. La FDA considera 2300 mg como la ingesta de sodio diaria recomendada (FDA, HHS, Food labeling, 2016). Por todo esto, se podría afirmar que las aceitunas de mesa y, en particular la variedad Marfil, son una fuente saludable de polifenoles.

Las aceitunas de mesa también contienen triterpenos pentacíclicos que incrementan sus beneficios (Sánchez-Quesada *et al*., 2013; Giménez *et al*., 2015). Sin embargo, las investigaciones se suelen centrar en la determinación de uno de estos dos grupos de compuestos (Blekas, *et al*., 2002; Romero *et al*., 2010; Alexandraki *et al*., 2014; Cabrera-Bañegil *et al*., 2017; D'Antuono *et al*., 2018), mientras que los estudios que realizan un análisis completo de ambos son minoritarios (Durante *et al*., 2018; García *et al*., 2018; Medina *et al*., 2019). Por consiguiente, se puso a punto un método de extracción simultánea de triterpenos pentacíclicos y polifenoles a partir de una misma muestra de aceitunas para el análisis por LC-MS. Las principales ventajas respecto a otras metodologías aportadas en la bibliografía, son la rapidez, la rentabilidad y el respeto por el medio ambiente, ya que, al realizar la extracción de ambos grupos en un único proceso se generan menos residuos de solventes. El método diseñado se validó siguiendo las recomendaciones de la Guía Eurachem (2014) y se aplicó a las aceitunas de mesa de la variedad Empeltre y Arbequina procedentes de Cataluña y correspondientes a dos cosechas diferentes (2014/2015 y 2015/2016). Además, para

completar el análisis de compuestos bioactivos en las aceitunas Marfil, se determinaron los triterpenos pentacíclicos correspondientes a las cosechas 2013/2014 y 2014/2015.

Tras extraer todos los compuestos, los triterpenos pentacíclicos se analizaron mediante LC-APCI-MS donde no solo se evalúan simultáneamente los ácidos y los alcoholes, sino que también se separan isómeros. El método llevado a cabo fue establecido por Giménez *et al*. (2015), pero con algunas modificaciones. De este modo, los ácidos oleanólico y ursólico, así como el eritrodiol y el uvaol, que solo difieren en la posición de un grupo metilo, pueden ser cromatográficamente separados y detectados por MS. Respecto a los polifenoles, se determinaron 17 de diferentes clases (alcoholes fenólicos, ácidos fenólicos, secoiridoides, flavonoides y lignanos) por LC-ESI-MS/MS (Moreno-González *et al*. 2020a). La validación del método presentó resultados tales como ausencia de efecto matriz, buena linealidad, sensibilidad, así como precisión y exactitud.

Los resultados de triterpenos pentacíclicos en Arbequina alcanzaron un valor total alrededor de 3,30 g/kg en ambas cosechas, siendo los compuestos mayoritarios, el ácido maslínico (2,51 ± 0,07 g/kg) seguido del oleanólico (0,78 ± 0,04 g/kg). A pesar de que no hay evidencias bibliográficas en aceitunas de mesa Arbequina, estos valores coinciden con los obtenidos en el fruto sin procesar, donde las concentraciones de ácidos triterpénicos se encuentran entre 2,40 g/kg y 3,10 g/kg (Guinda *et al*., 2010; Romero *et al*., 2010; Romero *et al*. 2017). El hecho de que los valores de las aceitunas procesadas y crudas sean parecidos, quizá tenga su explicación en que la elaboración tuvo lugar de forma natural, lo que permite preservar los ácidos maslínico y oleanólico, a diferencia de otras metodologías como el estilo-Español, donde se observa una disminución de los mismos (Alexandraki *et al*., 2014). Con respecto a la variedad Empeltre, nuestros resultados muestran valores totales de 2,77 ± 0,197 g/kg y 2,29 ± 0,07 g/kg en las cosechas 2014/2015 y 2015/2016, respectivamente, coincidiendo con los resultados de la bibliografía para aceitunas de mesa de la misma variedad (Medina *et al*., 2019). Los resultados de triterpenos pentacíclicos en Marfil mostraron una concentración total de 3,77 ± 0,15 g/kg en la cosecha 2013/2014 y de 3,62 ± 0,29 g/kg en la 2014/2015. Esta concentración coincide con la obtenida por Giménez *et al.* (2015) para la misma variedad de aceitunas de mesa. La diferencia más notable de la variedad Marfil se encontró en el ácido oleanólico (1,39 ± 0,06 g/kg), cuya concentración superó a Arbequina y Empeltre. El eritrodiol se cuantificó en las tres variedades a una concentración alrededor de los 0,01 g/kg, aunque la variedad Marfil mostró una fluctuación entre ambas cosechas. De este compuesto no se aportan resultados por parte de otros autores. Esto quizá se deba al uso de LC-DAD (Romero *et al*., 2010; Romero *et al*., 2017; Durante *et al*., 2018; Medina *et al*., 2019) o de cromatografía de gases con detector de ionización de llama (GC- FID) (Guinda *et al*., 2010), que son menos sensibles que el detector de MS empleado en nuestro estudio y/o al hecho de que los métodos utilizados no se validaron para este alcohol.

En cuanto a los polifenoles, los valores de la variedad Marfil se obtuvieron en el primer objetivo, siendo su cantidad total próxima a los valores de Arbequina y Empeltre. Los resultados en las cosechas 2014/2015 y 2015/2016 en Arbequina fueron de 1,04 ± 0,03 g/kg y 0,67 ± 0,03 g/kg, mientras que en Empeltre fueron de 0,48 ± 0,05 g/kg y 0,83 ± 0,05 g/kg. El tratamiento de las aceitunas crudas para eliminar el amargor puede afectar al perfil de polifenoles finales. Aunque el estilo-Griego retiene más polifenoles en el fruto que el estilo-Español o estilo-Californiano (Boskou, 2017; D'Antuono *et al*., 2018), la hidrólisis de los glucósidos hace que disminuya la oleuropeína, el ligstrósido, el verbascósido, así como los glucósidos de hidroxitirosol y tirosol, lo que conduce a un aumento en el contenido de las agliconas (Boskou, 2017). Además, los polifenoles son compuestos solubles en agua, pudiendo pasar a la salmuera y perderse en los lavados (Romero *et al*., 2004). Las concentraciones obtenidas en este estudio para Arbequina, son superiores a las encontradas por Cabrera-Bañegil *et al*. (2017) para la misma variedad siguiendo el procesamiento de estilo-Español (0,19 g/kg). En las aceitunas Empeltre, los resultados alcanzados (0,48 ± 0,05 g/kg y 0,83 ± 0,05 g/kg en las cosechas 2014/2015 y 2016/2017, respectivamente) son consistentes con los aportados por Medina *et al.* (2019) para la misma variedad procesada al estilo-Griego. Además, nuestros resultados para polifenoles coinciden con los indicados para otras variedades tratadas al estilo-Griego (Romero *et al*., 2004; D'Antuono *et al*., 2018). Por consiguiente, el presente estudio indica que el consumo de 8 aceitunas Arbequinas o 5 Empeltre conferirían la cantidad necesaria de polifenoles para ejercer un efecto beneficioso (Reg. EU nº 432/2012). Además, la ingesta de 8 aceitunas Arbequina contribuirían con 25,0 mg de ácido maslínico y 7,20 mg de ácido oleanólico, mientras que 7 aceitunas Marfil, aportarían 19,2 mg y 10,4 mg de estos mismos componentes. Las aceitunas Empeltre tienen un tamaño superior y 5 unidades proporcionarían 19,0 mg de ácido maslínico y 9,0 mg de ácido oleanólico. Estas concentraciones aportan la cantidad necesaria de triterpenos pentacíclicos que se ha visto que puede tener un efecto positivo sobre la salud (Sánchez-Quesada *et al*., 2013).

Una vez se estableció el método de análisis de triterpenos pentacíclicos y polifenoles en aceitunas de mesa y que se conocieron sus concentraciones en las variedades Arbequina, Empeltre y Marfil, se estableció y validó un procedimiento para determinar dichos componentes en el AOVE elaborado con aceitunas de la variedad Arbequina. Asimismo, se administró el aceite por vía oral a ratas y se midieron las concentraciones plasmáticas a diferentes tiempos (0, 15, 30, 60, 90 y 120 min). El AOVE se caracteriza por la presencia de elevadas cantidades de ácidos grasos monoinsaturados, principalmente ácido oleico, así como de importantes componentes minoritarios como fitoesteroles, tocoferoles, escualeno, triterpenos pentacíclicos y polifenoles (Guo *et al*., 2018). Los fenoles son los antioxidantes más abundantes del AOVE y se agrupan en las diferentes clases que incluyen los alcoholes fenólicos, ácidos fenólicos, flavonoides, secoiridoides y lignanos. Además, estos componentes también contribuyen a las propiedades organolépticas como el amargor, la astringencia y la acidez (Vitaglione *et al*., 2015). En cuanto a los triterpenos pentacíclicos, sus

estudios en el aceite de oliva son escasos, aunque se conoce la presencia de los ácidos maslínico y oleanólico así como pequeñas cantidades de eritrodiol y uvaol (Pérez-Camino y Cert, 1999; Allouche *et al*., 2009; Allouche *et al*., 2010).

De acuerdo al Consejo Oleícola Internacional (IOC), los tipos de aceite disponibles en el mercado se agrupan en AOVE, aceite de oliva virgen (AOV), aceite de oliva refinado y aceite de orujo. Esta clasificación se basa en la acidez libre y en el procesamiento. El aceite que posee mayores beneficios para la salud es el AOVE (Foscolou *et al*., 2018), el cual se seleccionó para este estudio. Además, se ha escogido la variedad Arbequina, que es una de las más apreciadas en España por la calidad de su aceite (Bakhouche *et al*., 2013).

La extracción de triterpenos pentacíclicos y polifenoles en el AOVE se realizó de manera simultánea utilizando etanol:metanol (1:1; v/v) como solvente. De acuerdo a la bibliografía, la extracción líquido-líquido produce mayores recuperaciones que la extracción en fase sólida (Ricciutelli *et al*., 2017; Olmo-García *et al*., 2018b). El solvente que se suele utilizar es el metanol 100% (Owen *et al*., 2000), metanol 60% (Caporaso *et al*., 2015; Ricciutelli *et al*., 2017) o etanol mezclado con diferentes porcentajes de agua (Olmo-García *et al*., 2018b). En el presente estudio se realizaron pruebas con acetato de etilo 100%, metanol 100%, metanol 60%, así como metanol:etanol (1:1; v/v), y, además, se llevaron a cabo lavados con n-hexano para eliminar la fracción lipídica. El acetato de etilo se descartó debido a las bajas recuperaciones y el n-hexano tampoco se tuvo en cuenta debido a que eliminó parte de los triterpenos pentacíclicos. Las mejores recuperaciones se consiguieron usando metanol:etanol (1:1; v/v). Posteriormente, se evaluó el proceso con los volúmenes de solvente 3,0 mL, 2,25 mL y 1,5 mL, dando las mismas recuperaciones. Por consiguiente, se seleccionó el volumen de 1,5 mL, ya que conllevó a un reducido tiempo de evaporación a sequedad y a una menor degradación de los polifenoles. Una vez se estableció el método, se validó la sensibilidad, linealidad, precisión y exactitud. Los resultados obtenidos en el proceso de validación mostraron que el método era adecuado para el análisis de triterpenos pentacíclicos y polifenoles en AOVE.

El análisis de AOVE de la variedad Arbequina determinó una concentración total de triterpenos pentacíclicos de 149 ± 30 mg/kg. Tan solo Pérez-Camino y Cert (1999) evaluaron el contenido de ácidos triterpénicos pentacíclicos en diferentes AOVE. Nuestros valores para el ácido maslínico (116 ± 9,83 mg/kg) y el oleanólico (29,3 ± 0,83 mg/kg) son del orden a los indicados por Pérez-Camino y Cert (1999)–para los mismos ácidos (98 mg/kg y 85 mg/kg, respectivamente) en la variedad Arbequina y, además, estos autores tampoco detectan ácido ursólico y no proporcionan resultados eritrodiol y uvaol. Estos alcoholes triterpénicos se utilizan como parámetro de pureza para inspeccionar adulteraciones de AOVE con aceites de menor calidad, especialmente con aceite de orujo de oliva (UE, reg. 2568/91). Nuestro resultado para eritrodiol (3,73 ± 0,35mg/kg) y uvaol (0,22 ± 0,02 mg/kg) cumplen con los criterios indicados en términos de contenido de alcoholes

pentacíclicos. Con respecto a las diferencias entre AOV y AVOE de la variedad Arbequina, el contenido de los ácidos maslínico y oleanólico descrito en AOV (Allouche *et al*., 2010) es bastante menor que el que encontramos en el AOVE. De igual manera, otras variedades de AOV también contienen estos ácidos triterpénicos en una concentración inferior a la que cuantificamos con nuestro método (Allouche *et al*., 2009; Olmo-García *et al*., 2019b).

Por el contrario, la fracción fenólica del aceite de oliva es mucho más heterogénea que la descrita para los triterpenos pentacíclicos, con al menos 36 polifenoles identificados estructuralmente diferentes (Guo *et al*., 2018). Las concentraciones de polifenoles totales en los aceites de oliva, pueden variar de 0,02 a 600 mg/kg (Tripoli *et al*., 2005; Cicerale *et al*., 2009). Al igual que para las aceitunas de mesa, esta diferencia puede estar influenciada por factores como la variedad de aceituna, las técnicas de cultivo, la etapa de maduración del fruto, las condiciones ambientales, y la metodología de extracción, procesamiento y almacenamiento (Tripoli *et al*., 2005; Cicerale *et al*., 2009). Además, la falta de una metodología analítica adecuada puede generar también diferencias (Tripoli *et al*., 2005). Por ejemplo, la prueba colorimétrica Folin-Ciocalteu, contiene una baja especificidad hacia los compuestos fenólicos (Tripoli *et al*., 2005). El uso de la LC ha mejorado la especificidad, sin embargo, los tiempos de análisis son largos y no siempre se dispone de todos los estándares de polifenoles (Tripoli *et al*., 2005; Cicerale *et al*., 2009). En este estudio se presenta un método de LC-MS con un tiempo cromatográfico inferior a 9 min que permite la identificación sensible y precisa de polifenoles, así como una cuantificación adecuada utilizando curvas de calibración construidas con estándares.

Nuestros resultados indican que de los 17 polifenoles que se habían incluido en el establecimiento del método, se han detectado 13 en el AOVE a una concentración total de 16,8 ± 0,3 mg/kg, concordando con los valores de la bibliografía (Silva *et al*., 2018; Olmo-García *et al*., 2019b). El acetato de hidroxitirosol (4,60 ± 0,19 mg/kg), hidroxitirosol (3,68 ± 0,08 mg/kg), luteolina (3,63 ± 0,10 mg/kg), tirosol (2,19 ± 0,06 mg/kg) y apigenina (1,61 ± 0,05 mg/kg), fueron los polifenoles mayoritarios que se determinaron. El predominio de estos compuestos podría deberse al hecho de que en las hojas y los frutos del olivo, los compuestos fenólicos glucosilados, principalmente los secoiridoides y flavonoides glucosídicos, están presentes en alta concentración, pero durante la maduración del fruto o en el proceso de extracción del aceite, la actividad de la enzima endógena *β*-glucosidasa provoca la hidrólisis de estos polifenoles a sus formas agliconas que pasarían a la fase oleosa (Kanakis *et al*., 2013; Clodoveo *et al*., 2014). Bakhouche *et al*. (2013) que evaluaron el contenido en AOVE Arbequina de Cataluña, aportaron valores similares a los que encontramos para los alcoholes fenólicos acetato de hidroxitirosol (1,2 mg/kg), hidroxitirosol (3,2 mg/kg) y tirosol (1,37 mg/kg), así como para los flavonoides luteolina (2,14 mg/kg) y apigenina (0,79 mg/kg). En cuanto al contenido de secoiridoides, estos autores detectan diferentes, pero no aportan resultados de la oleuropeína quizá porque al igual que nosotros no lo detecten. Por lo tanto, los resultados obtenidos para AOVE de Arbequina, respaldan los datos existentes proporcionados en la literatura,

y aportan resultados del contenido de polifenoles tales como salidrosida, catecol y verbascósido, de los que apenas hay información.

La información sobre el contenido de triterpenos y polifenoles pentacíclicos en el AOVE de la variedad Arbequina da información sobre la biofuncionalidad de este alimento. Sin embargo, para lograr este objetivo, es importante conocer la biodisponibilidad oral de estos compuestos. Aunque los triterpenos pentacíclicos fueron el grupo de compuestos bioactivos más concentrado en el AOVE, en el plasma no fueron los mayoritarios. De los 4 triterpenos pentacíclicos detectados en el aceite, solo se encontró ácido maslínico en plasma. Este triterpeno se administró a una dosis de 659 µg/kg y alcanzó la concentración plasmática máxima (Cmax) a los 15 minutos, con un valor de 21,2 ± 5,13 nM. No hay bibliografía de las concentraciones plasmáticas de triterpenos pentacíclicos tras la administración oral de aceite de oliva, sin embargo, hay información de la ingesta de aceitunas de mesa de la variedad Marfil (Giménez *et al*., 2017). La administración de esta variedad de aceitunas a ratas Sprague-Dawley a una dosis de ácido maslínico de 4,57 mg/kg, que es 7 veces mayor que la que se ha administrado con AOVE de Arbequina, se midió en plasma a una concentración de 23 nM a los 120 min (Giménez *et al*., 2017). Por tanto, nuestros resultados sugieren que el ácido maslínico en el AOVE tiene una mayor biodisponibilidad que en las aceitunas de mesa. En cuanto al ácido oleanólico, el hecho de que no se detecte en el plasma podría deberse no solo a que su concentración es más baja en el AOVE, con lo que la dosis administrada es de 166 µg/kg, sino a su baja biodisponibilidad que según los experimentos realizados en ratas parece que presenta (Giménez *et al*., 2017).

Con respecto a los polifenoles, hay bastantes estudios de la ingesta de aceite de oliva, pero principalmente se centran en el hidroxitirosol y tirosol (Parkinson y Cicerale, 2016; Crespo *et al*., 2018). El procedimiento que nosotros empleamos presentó una mayor sensibilidad que otros estudios realizados (Suárez *et al*., 2009). De este modo, los polifenoles encontrados en plasma de ratas tras la administración de AOVE de Arbequina fueron el hidroxitirosol, tirosol, luteolina, ácido vanílico, apigenina, ácido *p*-cumarico y pinoresinol. Este hecho demuestra que los compuestos pasan al sistema circulatorio sistémico después de una simple dosis oral de aceite. Además, en las células epiteliales del tracto gastrointestinal, podrían sufrir una biotransformación, comenzando el metabolismo de primer paso. De esta forma, los compuestos bioactivos pueden someterse a reacciones de fase I (hidrogenación, hidroxilación, hidratación y metilación) y posteriormente a reacciones de fase II (glucuronidación y sulfatación), siendo este segundo tipo las que ocurren con mayor frecuencia para los polifenoles (Gómez-Romero *et al*., 2012). La glucuronidación y sulfatación por la actividad enzimática glucotransferasa y sulfotransferasa posiblemente tiene lugar en los enterocitos, así como en el hígado, dando como resultado las formas conjugadas de hidroxitirosol, tirosol y luteolina, como se ha observado en nuestro estudio. Los resultados obtenidos están de acuerdo con otros hallazgos que indican que los alcoholes fenólicos se absorben

relativamente bien en el intestino delgado en comparación con los flavonoides (Corona *et al*., 2006; Parkinson y Cicerale, 2016).

En cuanto a los ácidos fenólicos, se encontraron el ácido vanílico y el *p*-cumárico. El lignano pinoresinol se cuantificó solo en los tiempos 15 y 30 minutos. El metabolismo de este compuesto no se ha investigado ampliamente. Tan solo se conoce el estudio de Soler *et al*. (2010), que identificaron sulfato de pinoresinol y, en menor concentración, glucurónido de pinoresinol después de la incubación de pinoresinol libre con células Caco-2/TC7.

Por tanto, este estudio ha servido para aportar una información más completa de las concentraciones de triterpenos pentacíclicos y polifenoles en el AOVE de la variedad Arbequina y de los valores plasmáticos alcanzados después de su consumo. Los resultados confirman que el AOVE no solo es una fuente de polifenoles, sino lo más importante, de triterpenos pentacíclicos. Además, las concentraciones plasmáticas nos han permitido conocer el destino de estos componentes en el organismo y proporciona un apoyo convincente de la composición saludable de los alimentos derivados de *Olea europaea* L.

Por último, se realizó un estudio para determinar los efectos quimiopreventivos de las aceitunas de mesa en la dieta. Existen diversos factores que podrían prevenir el cáncer colorrectal y que incluyen una dieta baja en carnes rojas y procesadas, alta en fibra, una actividad física regular, la prevención del aumento de peso, restringir el tabaco y minimizar el consumo de alcohol (Torre, *et al.,* 2016). Otros hábitos alimentarios como el consumo de frutas, verduras, aceites de pescado y calcio, también podrían influir de manera positiva en la prevención (O'Keefe, 2016). En esta investigación, se evaluó el efecto de la administración oral de las aceitunas de mesa de la variedad Arbequina en ratas Sprague-Dawley a las que se les había inducido lesiones preneoplásicas en el colon con DMH. La dosis administrada fue de 3,85 g/kg, que equivale al consumo diario de 30 aceitunas por una persona de 60 kg. Además, para completar el estudio se llevó a cabo el análisis por LC-MS de los triterpenos pentacíclicos y polifenoles en el plasma y el contenido intestinal.

El colon está cubierto por un epitelio absorbente formado por criptas. Estas estructuras contienen células madre en el fondo de las mismas que darían lugar a células progenitoras con capacidad de diferenciarse en linajes que producen enterocitos, células enteroendocrinas y células caliciformes (Tanaka, 2009). Las etapas de proliferación, diferenciación, migración hacia la luz del intestino grueso y apoptosis, mantienen el epitelio renovado en las criptas. Las células con el ADN dañado no causan apoptosis y cuando alcanzan la parte superior de la cripta, continúan proliferando (Tanaka, 2009). Este cambio precanceroso produce ACF y en una etapa más avanzada MDF, ambos usados hoy día como biomarcadores en los estudios de prevención del cáncer de colon (Femia y Caderni, 2008a; Femia *et al*., 2008c; Tanaka, 2009; Sakai *et al*., 2012). El origen de estas lesiones tempranas, así como de las anomalías más avanzadas hasta llegar a cáncer, está asociado con mutaciones génicas en diferentes vías metabólicas que en la presente tesis no se han llegado a

estudiar. Sin embargo, de acuerdo a la información bibliográfica, se sugiere que el cáncer de colon posee principalmente alteraciones en la vía WTN (Schatoff, *et al*., 2017). En condiciones normales en las que debe haber un mantenimiento y recambio de células madre epiteliales ubicadas en la base de las criptas intestinales, los ligandos extracelulares WNT se unen al complejo receptor FZD/LRP situado en la membrana plasmática, el cual inicia el reclutamiento de las proteínas intracelulares AXIN1, DVL a la membrana plasmática. Este acontecimiento hace que se inactive el complejo compuesto por AXIN1, APC, CK1, GSK3β, el cual se encarga de fosforilar a la β-CATENINA para su destrucción en el proteosoma. Cuando este complejo está inactivo, la β-CATENINA se acumula en el citosol y posteriormente se trastoca al núcleo, donde se une a los factores de transcripción de la familia TCF y a otros coactivadores, llevando a cabo la transcripción de genes diana implicados en la proliferación (Schatoff, *et al*., 2017). Existen estudios que sugieren que la proteína APC que está involucrada en la regulación de la actividad de la vía WNT, estaría alterada en aproximadamente el 93% de los cánceres de colon (Cancer Genome Atlas Network, 2012), lo que llevaría a una gran actividad de esta vía que desembocaría en una proliferación celular (Schatoff, *et al*., 2017). Hay otras cuatro vías que también podrían estar afectadas en esta enfermedad y que son: RAS-MAPK, PI3K, TGF-β y p53 (Cancer Genome Atlas Network, 2012).

En nuestra investigación, el carcinógeno DMH se ha utilizado para inducir lesiones preneoplásicas espontáneas en ratas. La DMH es un agente alquilante que su modo de acción implica la metilación en la posición O^6 o N^7 de la base guanina del ADN. La metabolización de la DMH tiene lugar a través de los intermediarios AOM y MAM, hasta el metabolito de iones metildiazonio que es altamente reactivo. Se ha demostrado que tanto la DMH como su intermediario AOM inducen cáncer colorectal en roedores (Rosenberg, *et al*., 2009; Perše y Cerar, 2011), desarrollando lesiones preneoplásicas tales como ACF y MDF (Caderni *et al*., 1995). Las ACF son lesiones hiperplásicas que frecuentemente portan mutaciones en los genes *K-ras* (Takahashi y Wakabayashi, 2004), mientras que las MDF son lesiones más avanzadas o displásicas con mutaciones principalmente en los genes de β-*catenina* y *Apc* (Femia *et al*., 2008b). Las mutaciones en *K-ras* dan lugar a la activación constitutiva de RAS y sus vías posteriores (Raf/MEK/MAPK y PI3K/Akt/PKB), las cuales pueden estar involucradas en la proliferación celular (Takahashi y Wakabayashi, 2004; Chen y Huang, 2009). Las mutaciones en los genes *β-catenina* y *Apc*, causan la estabilización de β-CATENINA en el citoplasma e inducen la activación transcripcional de genes que desempeñan un papel importante en los cambios displásicos que se presentan en los MDF (Chen y Huang, 2009).

Para realizar el estudio de la actividad quimiopreventiva, las ratas se dividieron en cuatro grupos: DMH-/Aceitunas- (control negativo: sin carcinógeno, sin aceitunas de mesa Arbequina), DMH-/Aceitunas+ (sin carcinógeno, con aceitunas de mesa Arbequina), DMH+/aceitunas- (con carcinógeno, sin aceitunas de mesa Arbequina) y DMH+/aceitunas+ (con carcinógeno, con aceitunas de mesa Arbequina). La administración diaria de aceitunas a la dosis de 3,85 g/kg se llevó a cabo durante 49 días y la inducción de las lesiones con DMH tuvo lugar a través de una inyección

subcutánea los días 8, 15 y 22 del periodo experimental. La dosis de carcinógeno de 20 mg/kg permitió desarrollar ACF y MDF en un período de 4 semanas, después de la última inyección de carcinógeno, sin manifestar signos de toxicidad que pudieran afectar al estudio (Alfaras *et al*., 2010). Además, el número de ambos tipos de lesiones en el grupo control DMH+/aceitunas- fue apropiado para dicha prueba dietética y estuvo de acuerdo con estudios previos (Bird y Good, 2000; Paulsen *et al*., 2000; Suzui *et al*., 2013). Una de las ventajas de estudiar las lesiones de ACF y MDF, es que pueden llevarse a cabo con un número reducido de animales y en un tiempo corto experimental, a diferencia de otros estudios de carcinogénesis que requieren periodos más largos.

El desarrollo de ACF siguió un patrón regionalizado, con un bajo número de focos en el colon proximal, un incremento en el medial y una mayor cantidad en el distal, coincidiendo estos resultados con estudios anteriores (Alfaras *et al*., 2010). La administración de aceitunas consiguió reducir los ACF en un 43,5%, 45,9% y 60,0% en los segmentos proximal, medial y distal, respectivamente. Además, las aceitunas disminuyeron los ACF con 1, 2, 3 y ≥ 4 criptas en un 52,0%, 56,1%, 63,0% y 38,5%, correspondientemente. Los MDF no se detectaron en el colon proximal, aunque sí en el medial y el distal, obteniéndose una reducción del 20,9% y 43,7%. Del mismo modo, el número de MDF con 1, 2, 3 y ≥ 4, decreció en un 21,2%, 33,8%, 72,1% y 53,3%, respectivamente. Asimismo, el peso corporal de los diferentes grupos de animales no se vio afectado, ni tampoco se observaron cambios anormales en los órganos vitales, lo que sugiere la ausencia de toxicidad de las aceitunas de mesa.

Para poder relacionar la presencia de estos compuestos bioactivos con su efecto quimiopreventivo en el colon, se llevó a cabo el análisis de muestras de plasma y contenido de colon obtenidos entre 17-19 h después de la última administración oral de las aceitunas. El método empleado para la extracción, identificación y cuantificación por LC-QTRAP-MS de los triterpenos pentacíclicos y polifenoles, se validó siguiendo las recomendaciones de la FDA (2018). Los resultados mostraron la ausencia de efecto matriz, así como de arrastre y una elevada sensibilidad, linealidad, precisión y exactitud.

La concentración total de triterpenos pentacíclicos en el plasma fue bastante inferior a la determinada en el contenido de colon 17-19 h después de la última administración de aceitunas. En el plasma se detectó ácido maslínico y en menor concentración ácido oleanólico, cuya suma de los dos fue de 5,89 nM en el grupo DMH-/Aceitunas+ y de 13,2 nM en DMH+/Aceitunas+. En el contenido de colon se encontró ácido maslínico, seguido de ácido oleanólico y en menor cantidad, eritrodiol. Estos tres triterpenos pentacíclicos sumaron un total de 78,4 nM en el grupo DMH-/Aceitunas+ y de 407 nM en DMH+/Aceitunas+. En cuanto a los polifenoles, se detectó 1 en el plasma, mientras que en el contenido intestinal se detectaron 9. El polifenol hidroxitirosol fue el único en plasma encontrándose a una concentración de 2,11 ± 0,02 nM en el grupo DMH-/Aceitunas+ y a 2,18 ± 0,54 nM en DMH+/Aceitunas+. Este mismo polifenol fue el mayoritario en

el contenido de colon, obteniendo un valor muy parecido al cuantificado en plasma (1,36 ± 0,30 nM en el grupo DMH-/Aceitunas+ y 3,31 ± 0,24 nM en DMH+/Aceitunas+). Los otros polifenoles detectados en el contenido intestinal fueron la apigenina, ácido cafeico, ácido *p*-cumárico, luteolina, quercetina, tirosol, ácido vanílico y verbascósido, en el rango de concentraciones de 0,16 a 1,83 nM.

Asimismo, se midieron los metabolitos provenientes de los triterpenos pentacíclicos y los polifenoles para comprender la biotransformación que tiene lugar en estos compuestos bioactivos y aumentar el conocimiento de la biodisponibilidad. De acuerdo a los metabolitos de los triterpenos pentacíclicos, en el plasma se encontró 1 derivado del ácido maslínico (≤ 3,71 nM) y 2 del ácido oleanólico (≤ 3,56 nM), mientras que en el contenido de colon se encontraron 4 metabolitos derivados del ácido maslínico (≤ 35,1 nmol/g) y 2 del ácido oleanólico (≤ 7,32 nmol/g).

En cuanto a los metabolitos de los polifenoles, en el plasma se determinó 1 derivado del hidroxitirosol en forma de isómeros (≤ 5,84 nM) y en el contenido de colon 2 metabolitos del hidroxitirosol (≤ 0,33 nmol/g) y 2 de la luteolina (≤ 0,05 nmol/g).

Los resultados del plasma y el contenido intestinal nos sugieren que la biodisponibilidad de estos compuestos bioactivos sería bastante menor en los triterpenos pentacíclicos que en los polifenoles. Este hecho limitaría su paso a plasma, permitiendo alcanzar concentraciones más altas en el colon que podrían facilitar la actividad quimiopreventiva sobre las paredes intestinales. La capacidad protectora de los triterpenos pentacíclicos ya había sido previamente estudiado *in vitro*, donde se demostró los efectos antiproliferativos y apoptóticos del ácido maslínico (Reyes-Zurita *et al*., 2011; Reyes-Zurita *et al*., 2016) y del ácido oleanólico (Janakiram *et al*., 2008) sobre las células de colon humano HT-29 o Caco-2. Además, otros experimentos *in vivo* confirmaron la actividad quimiopreventiva de estos compuestos, dado que las lesiones preneoplásicas tales como los ACF inducidas por DMH o AOM en el colon de ratas se redujeron (Kawamori *et al*., 1995; Furtado *et al*., 2008; Janakiram *et al*., 2008; Juan *et al*., 2019). Se ha sugerido que el ácido maslínico y el ácido oleanólico podrían estar implicados en la disminución de la inflamación característica de las lesiones, ya que inhiben el factor de transcripción NF-κβ involucrado en la regulación de la expresión de *iNOS* (Janakiram *et al.*, 2008) y *cox-2* (Janakiram *et al.*, 2008; Hsum *et al*., 2011). También se ha investigado la acción preventiva de los polifenoles sobre el carcinoma de colon, demostrándose que tanto el tirosol como el hidroxitirosol y su precursor oleuropeína, los principales polifenoles encontrados en *Olea europaea* L., poseen actividades antiproliferativas y apoptóticas sobre las células de colon HT-29 o Caco-2 (Giovannini *et al*., 1999; Corona *et al*., 2009; Cárdeno *et al*., 2013; Terzuoli *et al*., 2016; López de las Hazas *et al*., 2017).

Por otro lado, existe una controversia con respecto a la actividad anticancerígena del ácido oleico, el principal ácido graso monoinsaturado que se encuentra en el aceite de oliva y las aceitunas. Aunque los beneficios de este ácido graso están ampliamente relacionados con su efecto

cardioprotector (Bermudez *et al*., 2011), hay estudios que respaldan su acción preventiva del cáncer de colon (Bartolí *et al*., 2000; Berger *et al*., 2017). Sin embargo, otras investigaciones realizadas con alimentos ricos en ácido oleico, como la carne de res y de ave, los aceites de maíz, palma, cacahuete, soja y semillas de girasol, han asociado este ácido con una mayor incidencia de cáncer de colon y mama en humanos (Rao *et al*., 1998). Storniolo *et al*. (2019) observaron que el ácido oleico aumentaba el crecimiento de células Caco-2, pero que dicho era efecto era revertido significativamente cuando las células crecían con compuestos minoritarios de AOVE tales como el hidroxitirosol, oleuropeína, pinoresinol, escualeno y ácido maslínico. Otros estudios reconocieron que el ácido oleico podría inducir la apoptosis en las células HT-29 con un efecto comparable al del aceite de oliva, pero con ineficacia en las células Caco-2, lo que sugiere que su acción sería específica de la línea celular (Llor *et al*., 2003). Por tanto, es muy probable que el efecto quimiopreventivo de las aceitunas de mesa y del AOVE se deba a los componentes minoritarios tales como los triterpenos pentacíclicos y los polifenoles.

En resumen, el análisis de las aceitunas de mesa de la variedad Arbequina, Empeltre y Marfil, así como del AOVE de Arbequina, permitió confirmar que estos alimentos son una fuente de compuestos bioactivos tales como los triterpenos pentacíclicos y polifenoles. No obstante, las concentraciones en las aceitunas de mesa son bastante superiores a las detectadas en el AOVE. Sin embargo, los triterpenos pentacíclicos son más biodisponibles en el aceite que en las aceitunas de mesa. Aunque la baja biodisponibilidad observada en las aceitunas también es un factor positivo, ya que los componentes bioactivos alcanzarían el intestino grueso lo que favorecería su contacto con las paredes del colon permitiendo realizar la acción quimioprotectora. Por tanto, tanto las aceitunas de mesa como el AOVE son alimentos saludables y su consumo diario podrían ejercer un efecto preventivo de enfermedades como el cáncer.

V. CONCLUSIONES

El análisis de los resultados obtenidos en esta tesis permite establecer las siguientes conclusiones de acuerdo con los objetivos planteados:

1. Puesta a punto de un método de extracción líquida seguido de análisis por cromatografía líquida acoplada a espectrometría de masas para determinar los polifenoles representativos en aceituna de mesa.

- Se ha desarrollado y validado un método de extracción líquida seguido de la identificación y cuantificación por LC-ESI-MS/MS, para el análisis de 17 polifenoles contenidos en las aceitunas de mesa.
- El método permite la extracción de los compuestos fenólicos con un mínimo tratamiento de la muestra y un tiempo de análisis cromatográfico inferior a 9 minutos. La validación del método mostró ausencia de efecto matriz, así como recuperación, linealidad, sensibilidad, exactitud y precisión adecuadas.
- El análisis de las aceitunas de mesa de la variedad Marfil puso de manifiesto la presencia de 15 polifenoles. La cantidad total de polifenoles fue de 866 mg/kg, destacando el hidroxitirosol (384 mg/kg), el tirosol (201 mg/kg), la luteolina (88,0 mg/kg) y la salidrosida (85,9 mg/kg).
- Este análisis indica que el consumo diario de solo 7 aceitunas Marfil, proporciona 5 mg de hidroxitirosol y derivados, que es la cantidad indicada por la EFSA (Reg. EU n° 432/2012) para ejercer una protección de los lípidos de la sangre frente al estrés oxidativo.

2. Estudio del perfil de triterpenos pentacíclicos y polifenoles en las aceitunas de mesa por cromatografía líquida acoplada a espectrometría de masas.

- Se ha validado un nuevo método para el análisis en aceitunas de mesa de triterpenos pentacíclicos y polifenoles por LC-APCI-MS y LC-ESI-MS/MS, respectivamente. El procedimiento permitió extraer de forma conjunta ambos grupos de compuestos mediante una técnica que según los resultados obtenidos en la validación es lineal, sensible, selectiva, exacta y precisa.
- El método validado se aplicó al estudio de las aceitunas de mesa de la variedad Arbequina y Empeltre. La cantidad de triterpenos pentacíclicos fue de 3,28 g/kg en Arbequina y 2,77 g/kg en Empeltre. En ambas variedades, se encontraron 3 triterpenos pentacíclicos, siendo el ácido maslínico el mayoritario (2,51 mg/kg en Arbequina y 1,86 mg/kg en Empeltre), seguido del ácido oleanólico y en menor concentración el eritrodiol. Referente a los polifenoles, se identificaron y cuantificaron 16 compuestos con un total de 1,04 g/kg y 0,83 g/kg, para las variedades Arbequina y Empeltre. Los polifenoles encontrados en mayor proporción fueron el hidroxitirosol (0,51 g/kg en Arbequina y 0,39 g/kg en Empeltre), seguido de luteolina y verbascósido.

- Los resultados indican que el consumo de 8 aceitunas de la variedad Arbequina aportan los 5 mg de hidroxitirosol que ejercerían una actividad cardioprotectora (Reg. EU n° 432/2012), así como 25 mg de ácido maslínico y 7,20 mg de ácido oleanólico que también pueden aportar efectos beneficiosos para la salud. Con respecto a la variedad Empeltre, la ingesta de 5 aceitunas aportaría la cantidad de polifenoles indicadas por la EFSA, así como 19,0 mg y 9 mg de ácidos maslínico y oleanólico, respectivamente.

- También se midieron los triterpenos pentacíclicos de las aceitunas Marfil, que mostraron una concentración total de 3,77 ± 0,15 g/kg, siendo el mayoritario el ácido maslínico (2,53 g/kg), seguido del ácido oleanólico (1,22 g/kg) y en menor concentración el eritrodiol (0,02 g/kg).

3. Determinación por cromatografía líquida acoplada a espectrometría de masas de los triterpenos pentacíclicos y polifenoles contenidos en aceite de oliva virgen extra de la variedad Arbequina y en plasma tras su administración oral en rata.

- Se ha establecido un método que permite la extracción conjunta de los triterpenos pentacíclicos y polifenoles en aceite de oliva virgen extra (AOVE) seguidos de su análisis por LC-MS. Los resultados de validación indican que el método es lineal, sensible, selectivo, preciso y exacto.

- El análisis de AOVE de la variedad Arbequina permitió la identificación y cuantificación de 4 triterpenos pentacíclicos en un total de 149 mg/kg. El ácido maslínico (116 mg/kg), fue el triterpeno pentacíclico encontrado en mayor concentración, seguido por el ácido oleanólico, el eritrodiol y, en menor cantidad, el uvaol. En relación a los polifenoles se determinaron 13 compuestos, con un total de 16,8 mg/kg, siendo el mayoritario el acetato de hidroxitirosol (4,60 mg/kg), seguido del hidroxitirosol, la luteolina y el tirosol.

- El AOVE de la variedad Arbequina se administró a ratas Sprague-Dawley macho a una dosis de 6,16 mL (equivalente al consumo de 60 mL por una persona de 60 kg) y se analizó el plasma a los 0, 15, 30, 60, 90 y 120 min. Para ello, se utilizó un método que permitió la extracción conjunta de triterpenos pentacíclicos y polifenoles desde una misma muestra de plasma y su posterior identificación y cuantificación por LC-QTRAP-MS.

- Los resultados en el plasma permitieron detectar 1 triterpeno pentacíclico (ácido maslínico) y 7 polifenoles (ácido vanílico, tirosol, hidroxitirosol, ácido *p*-cumárico, luteolina, apigenina y pinoresinol). Todos los compuestos siguieron un patrón similar, alcanzando la máxima concentración (Cmax) a los 15 minutos (tmax) y, a excepción del pinoresinol, aún se detectaron a los 120 min. La Cmax osciló entre 59,8 nM (ácido vanílico) a 3,21 nM (apigenina).

4. Estudio quimiopreventivo de la ingesta de aceitunas de mesa de la variedad Arbequina sobre lesiones preneoplásicas en colon de rata inducidas con 1,2-dimetilhidrazina (DMH).

- La administración diaria de aceitunas de mesa de la variedad Arbequina a ratas Sprague-Dawley a una dosis de 3,85 g/kg durante 49 días no afecta el peso corporal, al consumo de pienso y agua, ni a la eficiencia en la conversión de los alimentos.
- No se encontraron focos de criptas aberrantes (ACF) en los grupos que no recibieron el carcinógeno y que fueron administrados con agua o con aceitunas.
- Las ratas tratadas con el carcinógeno DMH desarrollaron ACF siguiendo un patrón regionalizado con 12 ± 1; 34 ± 4 y 68 ± 11 en el colon proximal, medial y distal. Sin embargo, el tratamiento con aceitunas redujo los ACF en un 44%, 46% y 60%, respectivamente. Además, el consumo de aceitunas también disminuyó los ACF con 1, 2, 3 y ≥ 4 criptas en el colon total en un 52%, 56%, 63% y 39%, respectivamente.
- La administración de DMH indujo la aparición de focos de criptas con depleción de mucinas (MDF), observándose, que en el control positivo apenas se detectaron en el segmento proximal, mientras que, en los segmentos medial y distal, se cuantificaron 14 ± 4 y 28 ± 5, respectivamente. La administración de 3,85 g/kg de aceitunas durante 49 días disminuyó el recuento de MDF en un 21% y 44% en los segmentos medial y distal. Del mismo modo, el consumo de aceitunas disminuyó el MDF con 1, 2, 3 y ≥ 4 criptas en el colon total, respecto al grupo control positivo.
- El análisis del contenido de colon obtenido 17-19 horas después de la última administración de aceitunas identificó 3 triterpenos pentacíclicos, el ácido maslínico (321 nmol/g) seguido del ácido oleanólico y el eritrodiol. Concerniente a los polifenoles, se encontraron 9, de los cuales, los más abundantes fueron el hidroxitirosol (3,31 nmol/g), el ácido *p*-cumárico (1,83 nmol/g) y el tirosol (1,46 nmol/g).
- La determinación de las concentraciones plasmáticas al final del experimento puso de manifiesto que solo había ácido maslínico (10,1 nM), ácido oleanólico (3,19 nM) e hidroxitirosol (2,18 nM).
- La reducción de las lesiones preneoplásicas ACF y MDF inducidas por DMH en el colon de rata tras la ingesta de aceitunas de mesa tiene un efecto quimiopreventivo. Las concentraciones de los triterpenos pentacíclicos y los polifenoles en el contenido de colon favorecen la actividad quimiopreventiva de las aceitunas en el cáncer colorectal.

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ANEXO I

ANEXO I: ESTUDIO DEL PERFIL DE TRITERPENOS PENTACÍCLICOS EN ACEITUNAS DE MESA Y EN PLASMA DE RATAS POR CROMATOGRAFÍA LÍQUIDA ACOPLADA A ESPECTROMETRÍA DE MASAS

Los resultados presentados en este anexo se encuentran recogidos en un capítulo de libro:

Pentacyclic triterpenes in table olives: determination of their composition and bioavailability by LC-MS

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Open Access Journal of Pharmaceutical Research (2020), 000eB-001

Determinación de la composición de los triterpenos pentacíclicos de aceitunas de mesa y de su biodisponibilidad por LC-MS

Juan, M.E., Moreno-González, R., Planas, J.M.

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3.2.1. Capítulo de libro

Introducción: El fruto de *Olea europaea* L. es especialmente rico en triterpenos pentacíclicos como el ácido maslínico y el ácido oleanólico, y en menor cantidad, el eritrodiol. Estos productos son metabolitos secundarios de la planta que forman parte de la composición química de las ceras epicuticulares del fruto (Bianchi *et al*., 1992). La cutícula es una estructura protectora que recubre las partes aéreas de la planta y se compone principalmente de cutina (polímeros de hidroxi e hidroxiepoxi ácidos grasos) y de ceras, las cuales se encuentran en la parte más externa formando las capas intracuticulares y epicuticulares (Szakiel *et al*., 2012).

La síntesis de los triterpenos tiene lugar en el citosol celular de la planta a través de la vía del MVA. Para ello, el sustrato endógeno celular acetil-CoA, se transforma en HMG-CoA, que mediante la enzima HMG-CoA reductasa forma el MVA. La doble fosforilación de este último genera IPP, que es isomerizado a DMAPP. La formación de triterpenos tiene lugar mediante la condensación de 2 unidades de IPP y 1 unidad de DMAPP, que darán lugar a GPP y seguidamente a FPP. Posteriormente, dos unidades de este compuesto se fusionan para formar escualeno y más adelante 2,3-oxidosqualeno, que es el punto clave para sintetizar metabolitos primarios y secundarios. La actividad de las oxidosculeno ciclasas, cataliza las reacciones de formación de anillos monocíclicos, bicíclicos, tricíclicos, tetracíclicos y pentacíclicos.

Los triterpenos pentacíclicos comprenden diferentes miembros, destacando los grupos lupano, oleanano y ursano por sus actividades biológicas. Los componentes del esqueleto oleanano son los más abundantes en *Olea europea* L. (y en otras plantas superiores) y surgen de la ß-amirina, que en primer lugar forma eritrodiol y posteriormente se transforma en los ácidos oleanólico y maslínico. Otros triterpenos pentacíclicos detectados, aunque en menor abundancia, son los componentes de las clases ursano (uvaol y ácido ursólico) y lupanos (ácido betulínico) Debido a las propiedades beneficiosas atribuidas a estos compuestos, el presente capítulo resume dos procedimientos analíticos desarrollados en nuestro grupo para determinar estos componentes en aceitunas de mesa y en plasma de rata después de la administración oral de aceitunas de mesa a ratas Sprague-Dawley.

Material y métodos: Para determinar los triterpenos pentacíclicos en aceitunas de mesa, se ha tenido en cuenta la complejidad de la matriz de la aceituna, así como la posible presencia en las muestras de dos pares de isómeros posicionales, tales como el ácido oleanólico y ácido ursólico y los alcoholes eritrodiol y uvaol. En consecuencia, se desarrolló un método selectivo y sensible de LC-MS que permitió la determinación simultánea de los compuestos ácidos maslínico, oleanólico

y ursólico, y de los alcoholes eritrodiol y uvaol. Para ello, se utilizó la columna Zorbax Eclipse PAH (Agilent Technologies) y una fase móvil isocrática compuesta de metanol y agua. Posteriormente, se ajustaron las condiciones de MS, seleccionándose la fuente APCI. Se analizó la fragmentación de los compuestos, pero se observó una intensidad baja de las porciones resultantes, por lo que se eligió el modo sin fragmentar de SIM. El método se validó utilizando estándares de calibración y siguiendo los criterios de la guía EMA (2011).

La extracción de triterpenos pentacíclicos de las aceitunas de mesa también se puso a punto. Para ello, la pulpa se trituró hasta obtener una suspensión fina que se sometió a una triple extracción con metanol:etanol (1:1; v/v). Los sobrenadantes se unieron y se diluyeron para su análisis por LC-MS. La precisión del proceso de extracción fue evaluada añadiendo al triturado de aceitunas una cantidad considerada de los ácidos maslínico y oleanólico y procediento a la recuperación. El método desarrollado se aplicó al análisis de las aceitunas de mesa Marfil.

La determinación por LC-MS en plasma se llevó a cabo utilizando las condiciones fijadas para el análisis de las aceitunas de mesa, ya que proporcionaron suficiente sensibilidad para la detección precisa de estos compuestos. La extracción se realizó siguiendo un método previamente establecido para el análisis de ácido maslínico (Sánchez-González *et al*., 2013). No obstante, éste fue validado para evaluar si las condiciones eran adecuadas para la determinación del ácido oleanólico, el ácido el eritrodiol y el uvaol. Por consiguiente, se añadieron triterpenos pentacíclicos a muestras de plasma blanco de rata y se realizó la validación siguiendo las directrices de la EMA (2011). El método analítico fue verificado tras la administración de aceitunas Marfil a ratas Sprague-Dawley a una dosis de 3 g de pulpa/kg de peso corporal (dosis ajustada mediante el método descrito por Reagan-Shaw *et al*., 2007), que equivale al consumo de 28 aceitunas de la variedad Marfil por una persona de 60 kg de peso corporal.

Resultados: El método de análisis de triterpenos pentacíclicos en aceitunas de mesa por LC-MS mostró una sensibilidad excelente, con un LOD para los ácidos triterpénicos inferior a 1 nM, mientras que para el eritrodiol y uvaol fueron 4,5 y 7,5 nM, respectivamente. El método fue lineal para analitos ácidos en el rango de concentraciones de 0,005 a 15 µM, y para los alcoholes desde 0,005 a 15 µM, obteniéndose coeficientes de correlación superiores a 0,99. La precisión (intradía e interdía) y la exactitud fueron ≤ 9,90% y ≤ 9,57%, respectivamente.

Los resultados en las aceitunas Marfil confirmaron que el ácido maslínico era el mayoritario (1740 ± 60 mg/kg), seguido del ácido oleanólico (1380 ± 100 mg/kg) y en concentración inferior, el eritrodiol (18,1 ± 1 mg/kg). No se detectó ácido ursólico y uvaol.

Tras confirmar que las aceitunas de mesa contienen una elevada proporción de triterpenos pentacíclcios, se estableció un método analítico capaz de determinarlos en plasma después de consumir aceitunas. La validación mostró curvas de calibración lineales con coeficientes de correlación superiores a 0,99. La precisión intradía e interdía presentó valores adecuados (0,22–

9,93%). No se observó efecto matriz, ya que la relación de pendientes de curvas preparadas con plasma blanco de rata con los analitos añadidos y aquellas elaboradas con metanol 80% a los mismos niveles de concentración, mostraron resultados dentro del rango del 80-120%, lo que indica la ausencia de interferencias entre los analitos y los componentes de la matriz. Las recuperaciones fueron de alrededor del 100% para los compuestos evaluados. El límite de cuantificación (LOQ) varió de 1 nM para el ácido maslínico a 10 nM para uvaol, confirmando una sensibilidad correcta.

Las muestras de plasma obtenidas 120 minutos después de la administración oral de aceitunas de la variedad Marfil indicaron la presencia de ácido maslínico a 23,1 ± 5,3 nM y de ácido oleanólico a 4,32 ± 0,20 nM, mientras que el eritrodiol no se detectó.

Conclusiones: Los resultados obtenidos en las aceitunas de mesa corroboran que este alimento es una fuente prominente de ácido maslínico y ácido oleanólico. La administración de aceitunas a ratas y su posterior análisis plasmático permiten confirmar la bioaccesibilidad de estos compuestos a partir de las aceitunas de mesa. La aplicación de los métodos analíticos desarrollados podría extenderse a otros alimentos o plantas usadas en medicina tradicional, permitiendo ampliar el conocimiento de estos triterpenoides bioactivos

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Chapter 7: Pentacyclic Triterpenes in Table Olives: Determination of Their Composition and Bioavailability by LC-MS

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Abstract

The fruit of *Olea europaea* L. is particularly rich in pentacyclic triterpenes, being maslinic and oleanolic acid the most prevalent compounds with minor amounts of erythrodiol. These secondary plant metabolites have been described to exert beneficial effects on health, such as hepatoprotective, anti-diabetic, antiviral, cardioprotective and antitumor, among other activities. The present review summarizes our results on the content of pentacyclic triterpenes in table olives analyzed by LC-MS, and their bioavailability after the oral administration of this food to Sprague-Dawley rats.

Keywords: Maslinic acid; Oleanolic acid; Erythrodiol; *Olea europaea* L.; LC-MS; Bioavailability

Abbreviations: MVA: Mevalonic Acid; HPCL: High-Performance Liquid Chromatography; LC-MS: Liquid Chromatography Coupled To Mass Spectrometry; ESI: Electrospray Ionization; APCI: Atmospheric Pressure Chemical Ionization; MRM: Multiple Reaction Monitoring; TIC: Total Ion Chromatogram; MA: Maslinic Acid, OA: Oleanolic Acid; UA: Ursolic Acid.

Introduction

Triterpenes are one of the major classes of natural products that contain six isoprene units with the basic molecular formula of $C_{30}H_{48}$. According to the structures of their backbones, triterpenes are classified into acyclic, monocyclic, bicyclic, tricyclic, tetracyclic, and pentacyclic triterpenes [1]. Many of them occur in their free form, as well as glycosides (saponins) or other combinations [1]. with more than 20000 different compounds identified in nature to date [2]. The majority of these natural products are biosynthesized and accumulated in plants as secondary metabolites that contribute to their protection [3]. From ancient times, plants containing these secondary metabolites have been used in traditional medicine to treat different human diseases [4]. In the last decades, numerous studies have been carried out in order to elucidate the beneficial effects on health and their potential pharmacological use. Recently, numerous biological activities, such as antitumor, anti-inflammatory, anti-diabetic, antiviral and hepatoprotective, among others were attributed to these compounds [5-11].

Triterpenes

Triterpenes are formed in higher plants predomintantly, through the mevalonate pathway, named after its key intermediate, mevalonic acid (MVA) (Figure 1). The cellular endogenous substrate, acetyl-CoA, is transformed to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Then, HMG-CoA reductase, one of the most highly regulated enzymes in nature, catalyzes the synthesis of MVA, which is the precursor of the pathway [12,13]. The involvement of two kinases leads to the generation of isopentenyl diphosphate (IPP) that undergoes isomerization to dimethylallyl diphosphate (DMAPP) by the enzyme isopentenyl diphosphate isomerase. IPP and DMAPP are two key isoprene units given to their role as universal 5-carbon building blocks in the synthesis of terpenes [13].

For many years, IPP and DMAPP were thought to be exclusively formed via the MVA pathway. However, in the 1990s a species-specific second route was described and termed the 2-C-methyl-D-erythtritol-4-phosphate (MEP) pathway because MEP was formed from pyruvic acid and glyceraldehyde-3-phosphate as initial substrates [14]. Recent research demonstrated that the MVA pathway is solely involved in the synthesis of IPP and DMAPP in the cytosol of animals, fungi, archaea as well as a few bacteria, whereas the MEP route is exclusive of most other bacteria and parasitic apicomplexa [14].

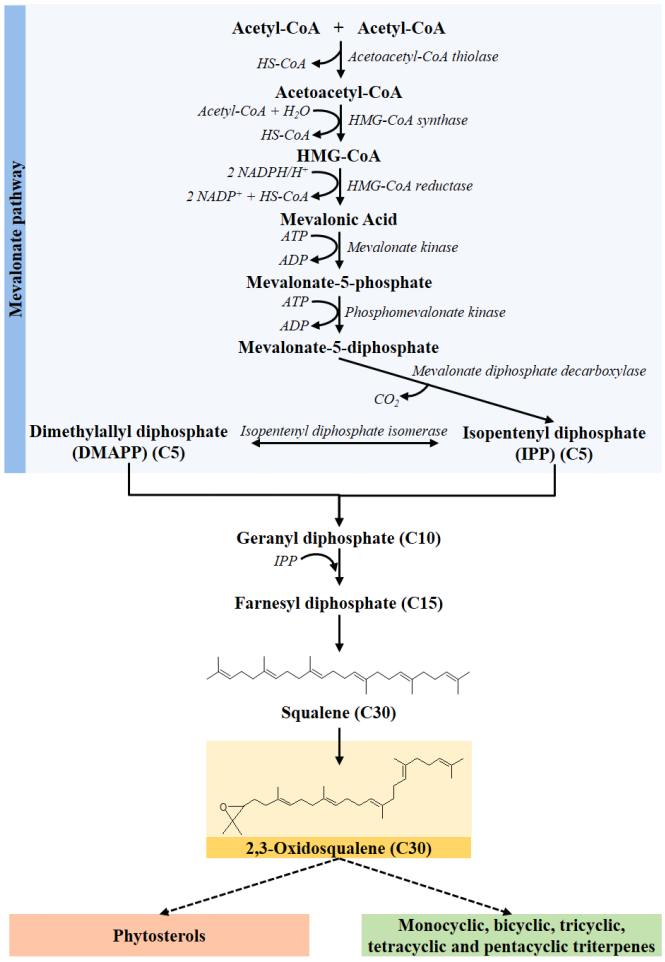

Figure 1: Biosynthesis of triterpenes through the mevalonate pathway in the cytosol of higher plants. Single arrows represent one step conversion, dashed arrows represent multiple steps.

In plants, both pathways have been described, but in independent compartments. The enzymes for the MVA pathway are located in the cytosol, whereas the ones for the MEP pathway are found in the plastids [13]. This pathway has been reported to synthesize the building blocks, mainly for the generation of monoterpenes (C10), diterpenes (C20), sesterpenes (C25), carotenoids (C40) and long-chain phytol [4]. Conversely, the cytosolic MVA pathway have been described to be responsible for the production of IPP and DMAPP mainly used in the formation of sesquiterpenes (C15), triterpenes (C30) and polyterpenes (>45) [4]. Although each route supplies IPP and DMAPP for the synthesis of the different terpenes, a metabolic crosstalk takes place between them [14].

The synthesis of triterpenes in plants takes place by a condensation reaction of 2 units of IPP and 1 unit of DMAPP to produce farnesyl diphosphate (C15) in a two-step process including the formation of the intermediate geranyl diphosphate Then, two units of farnesyl diphosphate merge to form squalene (C30) which serves as the precursor of acyclic triterpenes in plants [4].

Squalene undergoes an epoxidation reaction that enables the synthesis of 2,3-oxidosqualene (C30) which is the branch point between the pathway for the biosynthesis of primary

and secondary metabolites. The activity of oxidosqualene cyclases, also known as triterpenes synthases, catalyzes the ring forming reactions producing the diverse triterpenoids scaffolds, namely monocyclic, bicyclic, tricyclic, tetracyclic, and pentacyclic triterpenes [4]. Otherwise, the cyclization of 2,3-oxidosqualene to cycloartenol via cycloartenol synthase serves to the formation of membrane phytosterols and steroid hormones [4].

Pentacyclic Triterpenes

Pentacyclic triterpenes comprise different members, among which the oleanane, ursane and lupane groups are the most relevant due to their numerous biological activities, such as antitumor, anti-inflammatory, anti-diabetic, antiviral and hepatoprotective, among others [5-11]. Of them, the oleanane skeleton is the most abundant in higher plants, being oleanolic acid one of the most widely distributed triterpene in nature, and together with maslinic acid and in lesser amounts [1]. These compounds arises from ß-amyrin, leading to the formation, in first place, to the precursor erythrodiol, that is subsequently transformed to oleanolic and maslinic acids.

Erythrodiol or 3ß-olean-12-en-3,28-diol (Figure 2) has been found in very few species aside from *Olea europaea* L. where it has been detected in the fruit and oil in low amounts [3]. Despite the scarce information about this compound, erythrodiol has been reported to exert antitumor [15-17], anti-inflammatory [18] and cardioprotective activities [19,20].

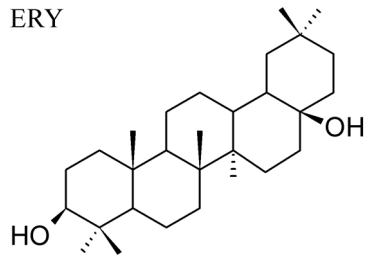

Figure 2: Chemical structure of erythrodiol (ERY).

Oleanolic acid or 3ß-hydroxy-olean-12-en-28-oic acid Figure 3. is formed after the oxidation of alcohol in the C28 position of erythrodiol. This compound has been isolated from more than 1600 species, including edible foods and medicinal plants [1,5]. Worth mentioning is the fact that this pentacyclic triterpene is prevalent in the Oleaceae family, especially in *Olea europaea* L., the plant species from which this compound has been named [21]. Oleanolic acid possesses prominent pharmacological activities, being the hepatoprotective, anti-inflammatory, antioxidant, anti-diabetic, and antitumor activities the most outstanding ones [22].

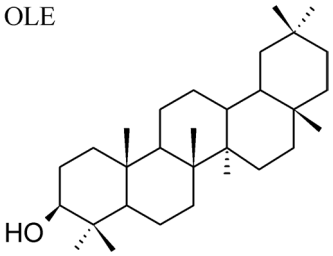

Figure 3: Chemical structure of oleanolic acid (OA).

Maslinic acid or 2α,3ß-hydroxy-olean-12-en-28-oic acid Figure 4. is synthesized from oleanolic acid differing only in an additional hydroxyl group at the 2-carbon position. Maslinic acid was isolated in 1927 from the leaves of Crataegus oxycantha L. and was named "crategolic acid" [23]. This compound has been detected in 30 species worldwide and it is one of the main pentacyclic triterpenes found in *Olea europaea* L. [24,25]. This compound has received less attention compared to the numerous studies devoted to its precursor oleanolic acid, despite exerting promising health-protecting properties, such as antitumor, anti-diabetic, antioxidant, cardioprotective, neuroprotective, antiparasitic and growth-stimulating [7].

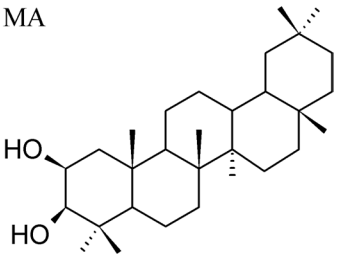

Figure 4: Chemical structure of maslinic acid (MA).

Natural Sources of Pentacyclic Triterpenes

Pentacyclic triterpenes from the oleanane type, namely maslinic and oleanolic acids, are ubiquitously distributed in higher plants Table 1.

Maslinic and oleanolic acids are located mainly in the intracuticular wax compartment of the cells [3]. The wax layer is one of the two main components of the cuticle that covers the surfaces of aerial parts, namely, leaves, flowers, fruits and non-woody stems, of all terrestrial plants forming the first protective barrier against abiotic and biotic environmental stresses [3]. Therefore, these compounds would be incorporated in the diet, principally with the ingestion of foods with edible peel [3] and have been described in numerous vegetables, legumes, species and fruits (Table 1). Maslinic and oleanolic acids have been

found in eggplants and spinaches [26], in legumes such as chickpeas and lentils [27] or in species such as basil [26] and rosemary [33]. These compounds are also present in fruits, such as grapes [3,30] or apples [28], the latter being one of the fruits most consumed worldwide and its antitumor effects have been correlated with its peel which contains both, oleanolic and maslinic acids [35].

Food	Maslinic acid	Oleanolic acid	References
Vegetables (mg/kg dry weight)			
Eggplant	840 ± 70	530 ± 40	[26]
Spinach	1260 ± 110	1670 ± 130	[26]
Carrot	n.d.	250 ± 40	[26]
Celery	n.d.	170 ± 20	[26]
Cooked legumes (mg/kg fresh weight)			
Chickpeas	61.9	3.56	[27]
Lentils	26.3-38.5	4.2-5.3	[27]
Pinto beans	n.d.	25.9	[27]
Fruits (mg/kg dry weight)			
Apple (fruit peel)	0.96 ± 0.03	3.18 ± 0.10	[28]
Apple pomace	10	139	[29]
Grapes (seeds)	10 ± 2	42 ± 3	[30]
Grapes (mg/kg fresh weight)	n.d.	30 – 160	[31]
Kiwi	17.3 ± 0.3	3.1 ± 0.1	[32]
Pomegranate	10.7 ± 0.4	n.d.	[32]
Lemon	3.4 ± 0.07	n.d.	[32]
Bilberry	n.d.	5.8-9.7	[3]
Aromatic herbs (mg/kg dry weight)			
Basil	350 ± 40	960 ± 70	[26]
Fennel	n.d.	540 ± 80	[26]
Rosemary	n.d.	31.6 ± 4.0	[33]
Brown mustard	330 ± 80	n.d.	[34]

Table 1: Content of maslinic and oleanolic acids in edible plants.

Consequently, in view of their wide distribution in edible plants (Table 1) the consumption of a diet rich in vegetables and fruits, such as the dietary pattern followed along the Mediterranean basin that has been associated with a lower incidence of cancer along with other chronic diseases [36] could provide a constant supply of these phytochemicals, besides other nutraceuticals with health protecting activities.

Plants used in traditional medicine to treat diverse ailments contain also these pentacyclic triterpenic acids. *Lagerstroemia speciose* or banaba has been widely employed as tea and herbal remedy since ancient times for the treatment of diabetes [37]. The leaves are specially rich in maslinic acid with concentrations of 4.96 ± 0.13 mg/g followed by oleanolic acid with 0.82 ± 0.03 mg/g [30]. Maslinic and oleanolic acids isolated from this specie acted as α-glucosidase inhibitors with IC_{50} of 5.52 ± 0.19 and 6.29 ± 0.37 μg/mL, respectively. *Syzygium aromaticum* or clove, also used for its hypoglycemic activities, contains both compounds that were demonstrated to down-regulate the increase of SGLT1 and GLUT2 expressions in the small intestine of STZ-induced diabetic rats, and also inhibited small intestine α-amylase, sucrase and α-glucosidase activity [38]. *Crataegus monogyna* L., commonly known as hawthorn, contain 0.93 ± 0.01 mg/g and 2.34 ± 0.11 mg/g of maslinic and oleanolic acids, respectively [30]. This plant exerts hypotensive, antioxidant, anti-inflammatory, and vasodilating effects, and has been traditionally used to strengthen cardiovascular function.

Ortosiphon stamineus L. possesses several pharmacological activities such as diuretic, hepatoprotective,

anti-diabetic, antihyperlipidemic, and has been described to contain 0.84 ± 0.06 mg/g and 2.77 ± 0.19 mg/g of maslinic and oleanolic acids, respectively [30]. Moreover, these pentacyclic triterpenes have been reported in *Eriobotrya japonica*, employed as antitussive and anti-inflammatory for chronic bronquitis [39], in *Geum japonicum* utilized as diuretic [40], and *Agastache rugose* applied in the treatment of intestinal disorders [41].

Synthesis of Pentacyclic Triterpenes in *Olea europaea* L.

Olea europaea L. is a prevalent species in human nourishment that has been cultivated for more than 5000 years in the countries bordering the Mediterranean Sea. The olive tree has been highly appreciated since ancient times [42]. This plant is characterized for being resistant and productive thus providing a versatile and valuable crop. For centuries, table olives and olive oil have been a pivotal component of the diet consumed along the Mediterranean shore [42,43]. Consequently, *Olea europaea* L. has traditionally provided important economic and dietetic benefits for the people of the region. Moreover, olive oil and leaves have been widely used in traditional medicine, standing out the use of the olive leaves as hypoglycemic, antihypertensive, antimicrobial, and antiatherosclerotic among others [43]. The by-products of *Olea europaea* L. have long been known to contain a wide range of bioactive compounds, including high amounts of pentacyclic triterpenes that were first identified in olive pomace [44], and later in the fruit [45]. The synthesis of this group of compouds has been comprehensively studied in *Olea europaea* L. by Stiti, et al. [46] which has postulated a biosynthetic pathway from 2,3-oxidosqualene.

In the olive tree, the production of pentacyclic triterpenes requires the generation of different carbocationic intermediates. Therefore, the cyclization of 2,3-oxidosqualene leads to the formation of the tetracyclic dammarenyl cation that is further transformed to the pentacyclics baccherenyl and lupenyl intermediates prior to the formation of the oleanyl and ursanyl cations (Figure 5) [46]. The final products are due to the stabilization of specific carbocationic intermediates which are further metabolized into more-oxygenated compounds mainly through reactions catalyzed by cytochrome (CYP) 450 enzymes [47].

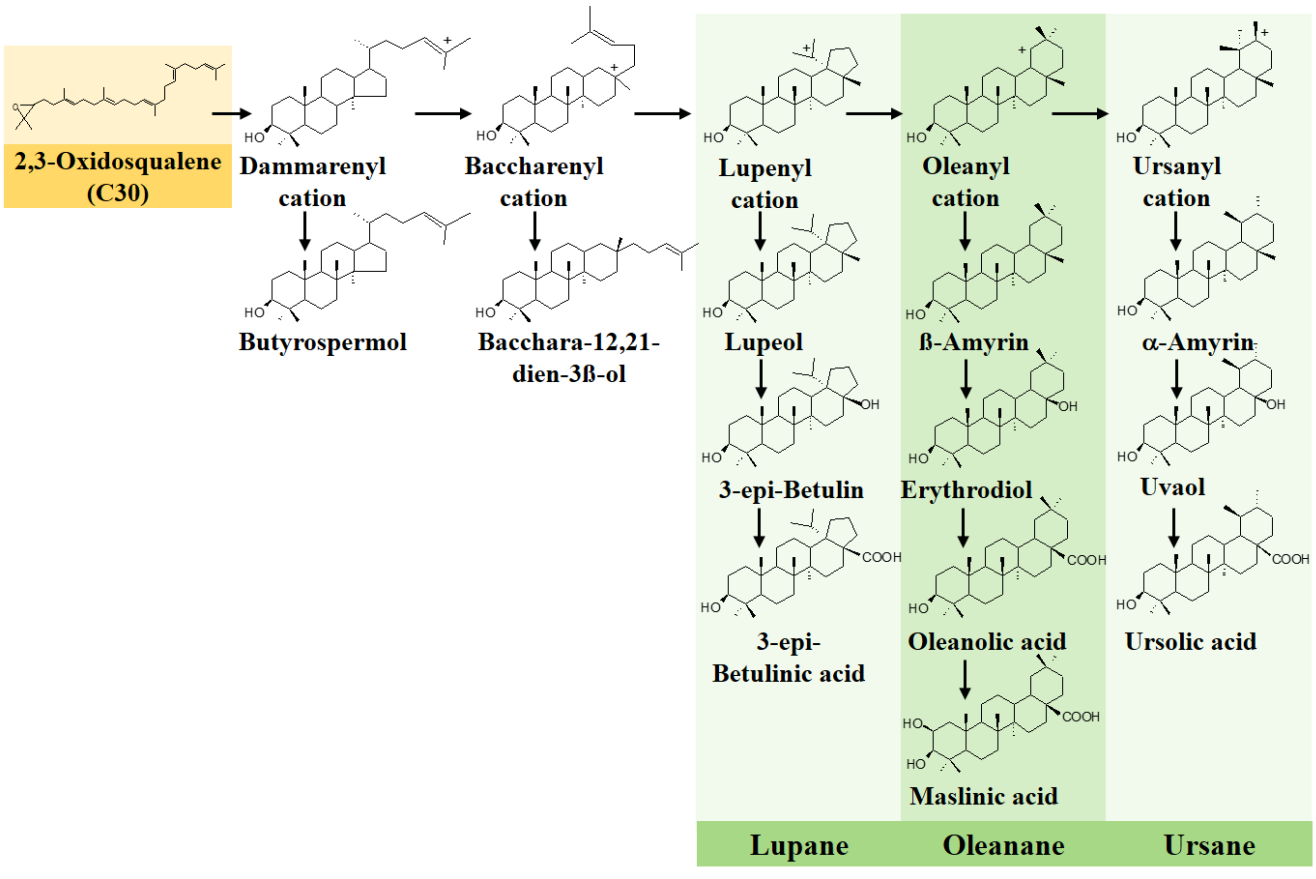

Figure 5: Synthesis of pentacyclic triterpenes in *Olea europaea* L.

In *Olea europaea* L., the main pentacyclic triterpenes arises from the stabilization of the oleanyl cation to ß-amyrin (olean-12-en-3ß-ol). This compound is sequentially oxidized at the C_{28} position by a cytochrome (CYP) P450 enzyme to yield in first place the alcohol erythrodiol and in second place oleanolic acid [4]. Finally, a different CYP P450 catalyzes the addition of a hydroxyl group forming maslinic acid [47]. The pentacyclic triterpenes from the oleanane group are produced in higher amounts than those of the ursane or the lupane classes [46]. Stiti, et al. [46] analyzed the pentacyclic triterpenes in the olive, and evaluated their content throughout fruit ontogeny (Table 2). Although these authors identified 19 pentacyclic triterpenes arising from different carbon skeletons, they demonstrated that oleanane triterpenoids were largerly predominant, representing the 99.2% of the different triterpenoids in olives [46].

Pentacyclic triterpenes	mg/Kg	%	Reference
Oleanane group			
ß-Amyrin	4	0.16	[46]
Erythrodiol	13	0.52	[46]
Oleanolic acid	946	38.08	[46]
Maslinic acid	1502	60.46	[46]
Ursane group			
α-Amyrin	n.d.	--	[46]
Uvaol	0.4	0.02	[46]
Ursolic acid	4	0.16	[46]
Lupane group			
epi-Betulin	0.8	0.03	[46]
epi-Betulinic acid	14	0.56	[46]

Table 2: Pentacyclic triterpenes in olives of the Chemlali variety harvested 30 weeks after flowering and analyzed by gas chromatography - mass spectrometry.

Distribution of Pentacyclic Triterpenes in *Olea europaea* L.

Pentacyclic triterpenes are synthesed in *Olea europaea* L. as secondary metabolites required for the plant survival in its environment [3]. These compounds have been described in the epicuticular waxes of olive fruits [45] and leaves [48] and their protective role has been associated with the development of a physical barrier that prevents of water loss and acts as first defense against pathogens [3].

Food	Maslinic acid	Oleanolic acid	References
Table olives (mg/kg fresh weight)			
Manzanilla, plain black	287.1 ± 66.6	178.8 ± 43.7	[49]
Manzanilla, plain green	384.1 ± 50.0	202.6 ± 57.3	[49]
Hojiblanca, plain green	904.7 ± 259.6	565.2 ± 107.1	[49]
Gordal, plain green	414.2 ± 89.3	294.3 ± 4.5	[49]
Kalamata, plain natural black	1318.4 ± 401.0	841.4 ± 162.9	[49]
Conservolea	1349 ± 123	536 ± 82	[50]
Olive oil (mg/kg)			
Olive oil, extra virgin	19-98	17-85	[51]
Olive oil, virgin	145-251	167-356	[51]
Pomace oil	575-698	405-703	[52]

Table 3: Content of maslinic and oleanolic acids in *Olea europaea* L.

In the fruits of *Olea europaea* L. the formation of the oleanane type of pentacyclic triterpenes is predominant with respect to the ursane group. Up to now, the studies in the literature had only measured pentacyclic triterpenic acids in table olives, being maslinic acid found in higher concentrations than oleanolic acid. However, the content depends on different factors, such as the variety, cultivar, climate, degree of ripening on the time of harvesting but also on the method of elaboration of olives and post-fermentation conditions.

Virgin olive oil is obtained in a process involving pressing, which may disrupt the surface waxes on the fruit. Therefore, part of maslinic acid contained in the olive may be transferred to the oil. However, the amount of maslinic and oleanolic acids in the oil is much lower than in the fruit and depends on the oil quality (Table 3).

Lately, olive leaves have raised much attention due to the high presence of different families of bioactive compounds that could be used as raw material for the obtainment of high added value compounds of use as functional foods, drugs or cosmetics. Therefore, recent research have reported that the leaves contain pentacyclic triterpenes arising from both ß-amyrin and α-amyrin [24,48,53], being especially rich in oleanolic acid that accounts for a 54-76% of all the triterpenes depending on the cultivar [24,48,53]. The concentrations in Arbequina, Hojiblanca and Picual have been reported to range from 29.2 mg/kg in Arbequina [24] to 39.8 mg/kg in the Picual variety [53]. Maslinic acid is the second compound in terms of concentration, followed by ursolic acid and the dialcohols, erythrodiol and uvaol (Table 4).

Pentacyclic triterpenes	g/kg dry weight	References
Oleanane group		
Erythrodiol	1.86-4.39	[24,48,53]
Oleanolic acid	13.0-39.8	[24,48,53]
Maslinic acid	1.91-7.30	[24,48,53]
Ursane group		
Uvaol	1.81-5.15	[24,48,53]
Ursolic acid	1.99-4.90	[24,48,53]

Table 4: Pentacyclic triterpenes in olive leaves from different cultivars.

Analysis of Pentacyclic Triterpenes

Pentacyclic triterpenes from the oleanane group have been reported to possess important beneficial effects on health [5-11] and their content in table olives cannot be disregarded. This food is regularly consumed not only in the countries where the cultivation of *Olea europaea* L. has been performed from ancient times, but also worldwide, due to the increasing interest in healthy eating to improve health and quality of life. However, one of the drawbacks in recommending the intake of a precise number of olives is the lack of knowledge on their content of pentacyclic triterpenes.

Pentacyclic triterpenes have been traditionally analyzed by gas chromatography although their high molecular weight along with low volatility require a derivatization step prior to its determination in table olives [24], olive oil [51] and commercial botanicals and food supplements [30]. To avoid the preliminary derivatization of analytes and a lower laboriousness of sample preparation and analysis, more recently, high-performance liquid chromatography (HPLC) was introduced in the determination of these compounds from olives [49,54]. However, HPLC coupled to UV or diode-array detectors holds the disadvantage of low UV absorption provided by their saturated skeleton, which leads to high limits of quantification. Sensitivity was improved by derivatization as performed for the analysis of pentacyclic triterpenic acids in fruits [26] or the analysis of food samples [55]. In addition, HPLC leads to long chromatographic runs, broadening chromatographic peaks and, as a result it generates an additional loss of sensitivity. The use of gradient elution does not completely resolve the problem, given that on the one hand, it could reduce retention times, but on the other, a loss of resolution for structurally close isomers is observed. Therefore, the methods used to analyze pentacyclic triterpenes hold several weaknesses that difficult the accurate analysis of pentacyclic triterpenes in olives, none of them allow the simultaneous analysis of the acids (maslinic and oleanolic acids) with the alcohol (erythrodiol), as well as the concurrent determination of the pentacyclic triterpenes from both, the ß-amyrin and α-amyrin classes.

Determination of Pentacyclic Triterpenes in Table Olives by LC-MS

The determination of pentacyciclic triterpenes in table olives was a challenging task, not only for being contained in a complex matrix, but also, for the possible presence in the

samples of two pairs of positional isomers, namely, oleanolic acid and ursolic acid as well as erythrodiol and uvaol.

Although in *Olea europaea* L. the predominant pathway in the formation of pentacyclic triterpenes is via ß-amyrin formation, the fact that derivatives from α-amyrin could be present cannot be underestimated. Hence, the most important problem in existing approaches to the determination of pentacyclic triterpenes is the separation of analytes. To overcome these shortcomings, liquid chromatography coupled to mass spectrometry detection (LC–MS) has become a powerful hyphenated technique that enables the separation, unambiguous detection and characterization of bioactive compounds in complex samples. Therefore, we developed a selective and sensitive LC–MS method for the simultaneous determination of maslinic, oleanolic and ursolic acids, as well as erythrodiol and uvaol, the main triterpenic compounds present in *Olea europaea* L. [25].

The separation of the isomers was attempted instead of using a traditional octadecyl silica column, by the use of the stationary phase designed for the analysis of polycyclic aromatic hydrocarbons with polymeric C18 bonding. This Zorbax Eclipse PAH (Agilent Technologies) column was used due to its well-known resolution power towards geometric isomers. The combination of these novelty stationary phase along with the use of an isocratic mobile phase consisting of methanol 83% and water 17% allowed the adequate separation of oleanolic acid from ursolic acid, and erythrodiol from uvaol. Moreover, the simplicity of the mobile phase, that did not use any modifier avoided the formation of adducts when coupled to mass spectrometry.

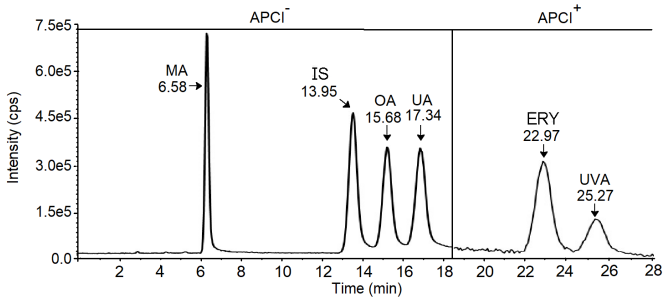

Figure 6: Representative total ion chromatogram (TIC) obtained by LC-APCI-MS of maslinic acid (MA), oleanolic acid (OA), ursolic acid (UA), erythrodiol (ERY) and uvaol (UVA) dissolved in methanol 80% at 2.5 µM and betulinic acid (IS) at 2 µM.

Once the chromatographic separation was established, we optimized the conditions for the detection of these compounds by MS. Several authors had proposed electrospray ionization (ESI) for the analysis of pentacyclic triterpenes [48,56,57], however although triterpenic acids were ionized in negative mode, this ionization source proved to be completely inadequate for the alcohols, erythrodiol and uvaol, that failed to be detected neither in positive or in negative polarity. Hence, atmospheric pressure chemical ionization (APCI) that has been described to provide a more effective ionization of low polarity compounds was evaluated. APCI in negative mode was selected for the analysis of triterpenic acids, since it gave a sensitivity two order of magnitude higher than negative ESI or positive APCI (Figure 6). On the other hand, erythrodiol and uvaol were only detected with the APCI source set in positive mode Figure. 6.

Fragmentation of pentacyclic triterpenes was evaluated in the LC–QqQ-MS to perform multiple reactions monitoring (MRM) analysis. The obtained MS/MS transitions were as follows: maslinic acid 471.3 → 393.3 and 471.3 → 377.3; oleanolic and ursolic acids 455.3 → 407.3, as well as erythrodiol and uvaol, 425.3 → 191.3. These transitions are consistent with the ones indicated by Paragón [54] in table olives and Sánchez-Avila, et al. [48] in olive leaves. Nevertheless, pentacyclic triterpenes were poorly fragmented even at high values of collision energy, resulting in peaks of low intensity. Then, the limits of quantification (LOQ) in MRM mode ranged from 125 to 650 nM for the five analytes and were two orders of magnitude higher than those achieved in single ion monitoring mode (SIM). The low sensitivity observed in MRM detection was in agreement with the valued obtained for Sánchez-Ávila, et al. [48] that gave values ranging from 190 to 650 nM. Although MRM mode allows a reliable identification of analytes, the fact that the isomers hold the same molecular weight, along with the same MS/MS transitions does not improve the selectivity already accomplished in the chromatographic separation. Therefore, pentacyclic triterpenes were detected using SIM mode in order to achieve the highest sensitivity in the analysis [25].

Once the LC-MS conditions were established, the developed method was validated using calibration standards following the EMA Guidelines on Bioanalytical Method Validation [58]. Excellent sensitivity was achieved with limits of detection for the triterpenic acids lower than 1 nM, whereas for erythrodiol and uvaol were 4.5 and 7.5 nM, respectively. The method was linear for the five analytes in the range of concentrations from 0.005 to 15 µM with correlation coefficients exceeding 0.99. The precision and accuracy were ≤ 9.90% and ≤ 9.57%, respectively [25].

The validated method was applied to the determination of pentacyclic triterpenes in table olives, since the fruit of *Olea europaea* L. constitutes an example of food rich in those bioactive molecules. However, prior to the analysis, the extraction process was optimized in terms of type of solvent, by spiking olive samples with MA and OA and evaluated their

recovery. The best recovery was achieved when a mixture of ethanol:methanol (50:50%) was employed

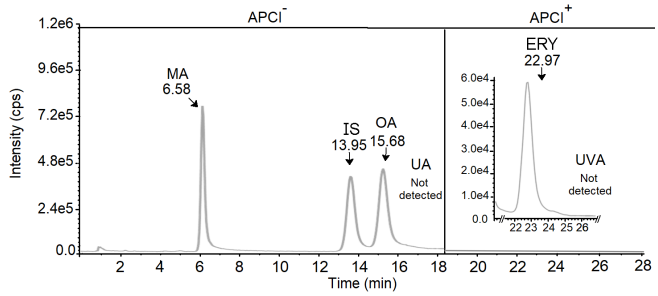

Figure 7: Representative total ion chromatogram (TIC) obtained by LC-APCI-MS of the pentacyclic triterpenes detected in table olives from the Marfil variety. MA, maslinic acid; OA, oleanolic acid; UA, ursolic acid; ERY, erythrodiol; UVA, uvaol; IS, internal standard. Erythrodiol was detected in non-diluted samples and is depicted in an insert.

Overall, the extraction process established for the analysis of pentacyclic triterpenes in table olives was quite straightforward, holding minimal sample pre-treatment. In first place, olives were pitted, and the destoned fruit was grinded in order to break down the tissue and yield a fine and consistent suspension. This homogenization facilitated the second step, which consisted on three cycles of extraction, with the first one with 12 mL of ethanol:methanol and the repetitions only involving 6 mL of solvent. Finally, the pooled supernatants were diluted and directly analyzed by LC-MS [25].

The developed method was applied to the analysis of Marfil table olives which are a scarcely studied variety distinguished by an ivory hue from which it takes its names and native to the Montsià region (Tarragona, Spain). These olives were processed following the Greek-style, which consist in a natural fermentation in brine. The developed method enabled the identification and quantification analytes derived only from the ß-amyrin, namely, maslinic and oleanolic acids, as the main pentacyclic triterpenes and erythrodiol in minor amounds (Figure 7). Ursolic acid and uvaol were not found Marfil table olives.

The content of maslinic and oleanolic acids found in the Marfil variety, processed as natural green olives (Table 5), is consistent with those described by Peragón [54] and Romero, et al. [49] also for fruits that followed a natural fermentation process, such as Kalamata and Hojiblanca. These results agree with other authors which reported the presence of the derivatives from α-amyrin in olive leaves but not in the fruit [24,54].

Pentacyclic Triterpenes	mg/kg	%
Oleanane group		
Maslinic acid	1740 ± 60	55.4
Oleanolic acid	1380 ± 100	44
Erythrodiol	18.0 ± 1	0.6
Ursane group		
Ursolic acid	n.d.[1]	0
Uvaol	n.d.	0

Table 5: Pentacyclic triterpenes in Marfil table olives.
[1] n.d. Not detected

Determination of Pentacyclic Triterpenes in Plasma by LC-MS

Based on our results, the scarcely known Marfil variety of table olives emerges as an important source of bioactive compounds [25]. However, there was a lack of knowledge on the absorption, metabolism and distribution either in humans or in animals of pentacyclic triterpenes after the consumption of table olives. To carry out these bioavailability studies, the first step consists in the development of an analytical method able to detect maslinic acid, oleanolic acid and erythrodiol that could reach the blood after the oral intake of olives. For the LC-MS determination of pentacyclic triterpenes, the conditions previously developed for the analysis of table olives were used since they provided enough sensitivity for the accurate detection of the compounds in plasma [25]. However, a different extraction process should be applied, given that plasma and table olives are quite different matrixes. Consequently, the extraction of pentacyclic triterpenes from plasma was attempted using a previously developed method for the analysis of maslinic acid [59]. The method consisted on two consecutive extractions with ethyl acetate followed by evaporation to dryness and reconstitution with methanol 80% prior to LC-

MS analysis. Previous to the implementation of the method, it was validated in order to evaluate if the conditions were adequate for the extraction of oleanolic and ursolic acids as well as erythrodiol and uvaol. Consequently, blank rat plasma samples were spiked using three concentration levels in order to validate the analytical performance following the EMA Guidelines [58]. Linearity was confirmed with calibration curves that gave correlation coefficients above 0.99. Regarding accuracy and precision, evaluated as intra-day and inter-day reproducibility, the method showed adequate RSD (%) values (0.22–9.93%) lower than others [60-62]. In addition, no significant matrix effect for analytes and IS were observed as values were around 91–117%, being within the 80-120% range that indicates that the ionization competition between the analyte and endogenous co-elutions was negligible and the LC–MS method was robust. Recoveries were around 100% for all the evaluated analytes. Moreover, the LOQ was adequate since it ranged from 1 nM for maslinic acid to 10 nM for uvaol, thus providing similar [60-63] or higher sensitivity [56,64] in comparison with the published methods. Therefore, the proposed methodology represents an important achievement and opens the possibility to bioavailability studies after consumption of different foods, or administration of plants widely used in traditional medicine, with the aim of studying in depth the beneficial effects of these compounds in human beings [65].

The analytical performance of the method was verified by administering a finely grinded suspension of Marfil olives to Sprague-Dawly rats. The dose to be administered to the experimental animals was established bearing in mind that it should be kept within a nutritional range, and olives should be finely grinded so that they could be administered intragastrically with a cannula. For this reason, approximately 12 g of destoned fruit was grinded with 40 mL of water, and the experimental animals were administered with this finely minced suspension at the volume of administration of 10 mL/kg. The dose that the rats received corresponded to 3 g of destoned olives/kg body weight. This dose was translated to the equivalent to be ingested by a person with the body surface area normalization method described by Reagan-Shaw, et al. [66]. The equivalent dose for a human corresponded to 0.48 g/kg, which means that a person of 70 kg of body weight would consume 28 olives of the Marfil variety to accomplish the dose administered to rats. Although this dose does not correspond to the amount of olives eaten in a meal or as appetizer, it is not so different from the one usually consumed and would be compatible with the dose to be administered in future pharmacokinetic studies using olives.

Plasma samples obtained 120 min after the oral administration of olives from the Marfil variety indicated the presence of maslinic acid at 23.1 ± 5.3 nM and oleanolic acid at 4.32 ± 0.20 without traces of erythrodiol. The fact that the latter could not be found in plasma, can be attributed to its low content in the fruits of *Olea europaea* L. that was two orders of magnitude lower than maslinic acid and oleanolic acid. The relatively low concentration obtained for maslinic acid (~ 25 nM) after the administration of the suspension of olives that contained an approximate dose of 4.57 mg maslinic acid/kg of rat body weight, could be explained by the described oral bioavailability of approximately 5% obtained in rats [67]. On the other hand, oleanolic acid was administered in the form of a suspension at a dose of 3 g of destoned olive/kg which contained 3.60 mg of oleanolic acid/kg of body weight was detected at concentrations around 5 nM. Previous studies in the literature that administered oleanolic acid to rats, either as a single compound or in part of an extract, indicates the poor oral bioavailability of this pentacyclic triterpene [63,68,69] that has been described to be 0.7% for oral doses of 25 and 50 mg/kg [68]. Therefore, the lowest bioavailability described for oleanolic acid could explain our results, in which this pentacyclic triterpene was barely detected in comparison to maslinic acid. Hence, the oral administration of olives to rats and its determination in plasma verified that the established methodology is appropriate for bioavailability studies.

Conclusion

Pentacyclic triterpenes from the oleanane group, mainly maslinic and oleanolic acids, have received much attention in the recent years due to their numerous biological activities, such as antitumor, anti-inflammatory, anti-diabetic, antiviral and hepatoprotective, among others. These compounds are widely distributed in nature, in both medicinal species and edible plants, especially in vegetables, legumes and fruits regularly consumed following a Mediterranean dietary pattern. Among the foods rich in pentacyclic triterpenes from the oleanane group stands out the fruit of *Olea europaea* L. The present chapter presents the optimization and validation of two analytical strategies that allows the determination of these bioactive compounds in table olives and in plasma samples after the administration of this food. Remarkably, both extraction processes allowed a fast sample treatment prior to LC–MS analysis that allows a sensitive and reliable detection of pentacyclic triterpenes from the oleanane and ursane family as indicated in the validation of the method. The results obtained in table olives confirms this food as a prominent source of maslinic and oleanolic acids. Moreover, the administration of this food to rats and its subsequent plasmatic analysis allows the confirmation of the bioaccessibility of these compounds from table olives. Furthermore, the application of the analytical methods could be extended to other foods or plants used in traditional medicine, thus broadening the knowledge of these bioactive triterpenoids.

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