UNIVERSIDAD DE CÓRDOBA

FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

NUEVOS ESTUDIOS SOBRE LA MEJORA DEL ACEITE DE OLIVA Y EL APROVECHAMIENTO DE RESIDUOS DEL OLIVAR

Verónica Sánchez de Medina Baena

Córdoba, mayo de 2014

TITULO: *Nuevos estudios sobre la mejora del aceite de oliva y el aprovechamiento de residuos del olivar*

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TÍTULO DE LA TESIS: NUEVOS ESTUDIOS SOBRE LA MEJORA DEL ACEITE DE OLIVA Y EL APROVECHAMIENTO DE RESIDUOS DEL OLIVAR

DOCTORANDA: Verónica Sánchez de Medina Baena

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda Verónica Sánchez de Medina Baena cursó de forma brillante el máster en Química Fina Avanzada, en el que obtuvo excelentes calificaciones. El trabajo fin de máster dio lugar a dos publicaciones en la revista Journal of Agricultural and Food Chemistry (en posición 1 de 57 en el área multidisciplinar de agricultura y 12 de 71 en la de química aplicada), que constituyen los capítulos 11 y 12 de la Memoria de su Tesis.

El tema de la Tesis se encuadra en una de las líneas de investigación del Grupo al que pertenece la doctoranda (aprovechamiento de residuos de la industria agroalimentaria) y se concreta en el olivar/aceite de oliva, su mejora y sus características, de tanto interés en la Comunidad Andaluza en particular y en la cuenca mediterránea en general.

La realización de la investigación recogida en la Memoria ha proporcionado a la doctoranda una formación analítica sólida, que ha abarcado: (a) La preparación de la muestra (extracción acelerada de los compuestos de interés de matrices sólidas con auxilio de energías tales como microondas, ultrasonidos o presión+ temperatura altas — extracción con líquidos sobrecalentados). (b) La identificación de compuestos, llevada a cabo tras la separación mediante cromatografía de líquidos o de gases utilizando espectrómetría de masas de alta resolución en tándem (detector híbrido de cuadrupolo–tiempo de vuelo). (c) El análisis cuantitativo se ha realizado mediante cromatografía de líquidos acoplada a espectrometría de masas con analizador de triple cuadrupolo o mediante cromatografía de gases con detección por ionización en llama. (d) El tratamiento de los datos proporcionados por estos equipos mediante softwares adecuados e identificación de los compuestos desconocidos mediante bases de datos públicas o conformadas en el propio laboratorio en el que se integra la doctoranda.

La investigación ha estado dirigida a: (i) Mejorar los métodos de preparación de muestra para el análisis de la fracción insaponificable del aceite y estudiar sus diferentes familias. (ii) Desarrollar estrategias de análisis metabolómico global y orientado para el estudio de las fracciones fenólica y de ácidos grasos en programas de mejora de olivo. (iii) Enriquecer aceites de diferentes naturaleza para mejorar sus propiedades nutracéuticas y su estabilidad (mejora de su comportamiento en condiciones de calentamiento).

Todo ello ha dado lugar a un total de 12 artículos, de los cuales 6 están publicados, otros en fase de revisión y alguno enviado recientemente.

Por todo ello, consideramos que la investigación desarrollada y recogida en esta Memoria reúne los requisitos de originalidad, innovación y calidad, y autorizamos la presentación de la Tesis Doctoral de Doña Verónica Sánchez de Medina Baena.

Córdoba, 21 de abril de 2014

Firma de los directores

Prof. Dr. M.D. Luque de Castro

Fdo.: M. D. Luque de Castro

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NUEVOS ESTUDIOS SOBRE LA MEJORA DEL ACEITE DE OLIVA Y EL APROVECHAMIENTO DE RESIDUOS DEL OLIVAR

Los directores,

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Trabajo presentado para optar al grado de
Doctora en Ciencias, Sección Químicas

Fdo.: Verónica Sánchez de Medina Baena
Licenciada en Química

María Dolores Luque de Castro y Feliciano Priego Capote, en calidad de directores de la Tesis Doctoral presentada por la Licenciada en Química, Verónica Sánchez de Medina Baena, con el título **"Nuevos estudios sobre la mejora del aceite de oliva y el aprovechamiento de residuos del olivar"**

CERTIFICAN:

Que la citada Tesis Doctoral se ha realizado en los laboratorios del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajos.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, a 21 de abril 2014

Fdo.: María Dolores Luque de Castro Fdo.: Feliciano Priego Capote

AGRADECIMIENTOS

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ÍNDICE

OBJETIVOS

El *objetivo global* de la investigación desarrollada fue aportar nuevos conocimientos sobre el cultivo del olivo, sobre su producto estrella, el aceite de oliva, y sobre los desechos generados en su producción, alperujo y hojas de olivo. De esta forma se cierra el ciclo formado por el cultivo, la producción de aceite y la gestión de residuos generados Este objetivo se dividió a su vez en dos *objetivos generales* en función del objeto del estudio en cuestión: Objetivo 1, desarrollar y aplicar herramientas analíticas y metabolómicas a la monitorización de familias de compuestos que influyen en la calidad del aceite de oliva (tanto de la fracción mayoritaria como de la minoritaria, principalmente los componentes de carácter fenólico) para su implementación en programas de mejora del olivo. Objetivo 2, demostrar que los desechos del olivo y de la industria aceitera contienen componentes que pueden mejorar la calidad de cualquier tipo de aceite, especialmente de los refinados.

De estas premisas derivaron los siguientes *objetivos concretos*:

i) Adquirir una información exhaustiva y crítica sobre la llamada fracción minoritaria del aceite de oliva virgen (VOO), tanto de sus características químicas y de sus difundidas y comprobadas propiedades saludables, como de los métodos analíticos (preparación de muestra y análisis) para la determinación de cada una de las familias que componen esta fracción. Resultado de este estudio bibliográfico ha sido la publicación de dos revisiones (Capítulos 1 y 2) en las que se abarcan estos aspectos no considerados hasta ahora de forma exhaustiva y conjunta para todos los componentes de la fracción.

ii) Comparar métodos de preparación la muestra de la fracción insaponificable para establecer las ventajas de cada uno de ellos mediante LC–MS/análisis quimiométrico y seleccionar el más adecuado en cada caso, según recoge el Capítulo 3.

iii) Aplicar el método seleccionado en ii) para el estudio de la fracción insaponificable en VOOs monovarietales y en diferentes

aceites refinados para poner de manifiesto las diferencias y semejanzas entre ellos en lo que se refiere a esta fracción usualmente olvidada por los programas de mejora, tal como muestra el Capítulo 4.

Un objetivo concreto tan amplio como es la aplicación de herramientas analíticas y metabolómicas a la monitorización de familias de compuestos que influyen en la calidad del aceite de oliva para su implementación en programas de mejora de olivo se ha dividido en función de las fracciones que se pretende estudiar: La fracción de ácidos grasos (fracción mayoritaria) y la fenólica (perteneciente a la fracción polar), lo que ha dado lugar a la siguiente división de los objetivos:

iv) Estudiar, mediante análisis metabolómico global y orientado —LC–MS/MS y LC–QTOF, respectivamente—, a) la caracterización de diferentes VOOs monovarietales, analizando sus semejanzas y diferencias (Capítulo 5); b) la influencia de la maduración del fruto en el perfil fenólico del VOO obtenido de 12 genotipos, tal como recoge el Capítulo 6; c) la discriminación entre perfiles fenólicos asociados al genotipo comparando entre sí los aceites procedentes de tres cruces y con los de los genitores en todos los casos (Capítulo 7).

v) Conocer el comportamiento de la fracción de ácidos grasos (tanto los esterificados como los no esterificados) del VOO obtenido, a) en función del binomio cruce genético–grado de maduración (Capítulo 8); b) en función del cruzamiento y en comparación con la de los genitores (Capítulo 9).

vi) Relacionar de forma conjunta los cambios producidos en ambas fracciones del VOO por las dos variables en estudio, cruce genético y grado de maduración del fruto, ha dado como resultado la información recogida en el Capítulo 10.

El objetivo general 2, entroncado directamente con una de las líneas de investigación del grupo en el que se integra la doctoranda (el aprovecha-

miento de residuos de la industria agroalimentaria) pretendió realizar una nueva aportación para reforzar las aproximaciones a la explotación de los residuos y desechos del olivo/VOO. Para esta investigación se utilizaron dos tipos de desechos del olivo/aceite: Las hojas y el alperujo, para conseguir extractos de los que se conocía su semejanza en composición de compuestos antioxidantes característicos y su discordancia en la concentración relativa de estos compuestos. Con estas premisas, los objetivos en este caso fueron:

vii) Enriquecer diferentes aceites con ambos tipos de extractos y estudiar la transferencia que se produce de cada uno de los fenoles característicos del olivo, en cada aceite y desde cada tipo de extracto, utilizando parámetros típicos de cada aceite (índices de acidez y de peróxidos y estabilidad según el método Rancimat), según se muestra en el Capítulo 11.

viii) Estudiar el perfil fenólico de cada uno de los aceites enriquecidos en comparación con el VOO mediante LC–QTOF y realizar el tratamiento quimiométrico apropiado de los perfiles obtenidos para poner de manifiesto el grado de semejanza del aceite resultante de cada enriquecimiento con el patrón al que pretende aproximarse: El VOO (Capítulo 12).

La formación de la futura doctora, que constituye el *objetivo último* de toda tesis doctoral, ha incluido el máster en "Química Fina" con el número de créditos correspondientes. En paralelo con el máster y con la investigación recogida en la parte principal de la Memoria, se ha pretendido una formación más amplia de la doctoranda mediante la realización de otras actividades que se recogen como anexos, tales como:

Investigación simultánea con la de la tesis realizada en colaboración con otros grupos de la UCO (Departamento de Bioquímica) lo que ha dado lugar a un artículo científico enviado para su publicación (Anexo I).

Comunicaciones en conferencias nacionales e internacionales (Anexo II).

INTRODUCCIÓN

INTRODUCCIÓN

1 El aceite de oliva

La agricultura de los países mediterráneos está dominada por un cultivo: El olivo, cuyo producto estrella —el aceite de oliva virgen— es uno de los principales contribuyentes al reconocimiento por la UNESCO de la dieta mediterránea como patrimonio de la humanidad [1,2]. El cultivo milenario del olivo en la cuenca mediterránea abarca actualmente un área de 9 millones de hectáreas, con una producción aproximada de 15 millones de toneladas de aceitunas/año, a la que España contribuye mayoritariamente, según se muestra en la Tabla I.1 [3], y en la que España, Italia y Grecia producen el 70% de toda la cuenca [4].

El fruto del olivo (aceituna u oliva) es tradicionalmente reconocido por su alto contenido en ácidos grasos monoinsaturados (MUFAs), principalmente ácido oleico, y en fenoles, razones por las que el aceite de oliva es considerado el aceite comestible con mejores propiedades nutricionales y al que se atribuyen muchas de las características saludables de la dieta mediterránea (e.g. baja incidencia de la aterosclerosis, de ciertos tipos de cáncer y de enfermedades cardiovasculares y neurodegenerativas) [1]. Estas probadas cualidades han propiciado su reconocimiento por organismos oficiales [5] y el incremento del consumo de aceite de oliva a nivel mundial en un 40% [6].

2 Clasificación de la calidad del aceite de oliva

El aceite de oliva se clasifica en nueve tipos en función de sus características físico-químicas y del método utilizado para su producción: 1) Aceite de oliva virgen extra (EVOO); 2) aceite de oliva virgen (VOO): 3) aceite de oliva corriente; 4) aceite de oliva virgen lampante; 5) aceite de oliva refinado; 6) aceite de oliva, mezcla de aceites refinado y de oliva virgen; 7) aceite de orujo crudo; 8) aceite de orujo refinado; y 9) aceite de orujo de oliva. La Tabla I.2 resume las características de cada uno de estos aceites.

Tabla I.1. Producción total de aceituna y aceite de oliva en los países mediterráneos.

País	Aceituna/Olivar		Producción aceite de oliva (toneladas)
	Producción (toneladas)	Área cultivada (Ha)	
España	3 626 600	2 300 000	992 000
Italia	2 992 330	1 056 005	570 000
Grecia	2 100 000	900 000	351 800
Turquía	1 820 000	805 500	206 300
Siria	1 095 043	700 000	200 000
Túnez	963 000	1 800 000	192 600
Marruecos	1 315 794	968 123	130 000
Argelia	39 3840	328 884	32 000
Libia	139 091	216 013	15 000
Israel	63 000	33 700	12 300
Líbano	90 307	56 529	11 300
Egipto	465 000	55 000	8 800
Francia	27 969	17 055	3 600
Chipre	14 865	10 852	2 400
Albania	12 5000	48 000	800
Croacia	50 900	19 000	600
Eslovenia	2 000	1 000	600
Montenegro	2 888	2 350	187
Malta	8	8	3
Bosnia y Herzegovina	160	110	**(1)**
Producción total	**15 287 795**	**9 318 129**	**2 730 290**

(1) Dato no proporcionado por la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO).

Tabla I.2. Criterios de calidad

Parámetros		Aceite de oliva virgen extra	Aceite de oliva virgen	Aceite de oliva virgen corriente	Aceite de oliva virgen lampante (1)	Aceite de oliva refinado	Aceite de oliva	Aceite de orujo de oliva crudo	Aceite de orujo de oliva refinado	Aceite de orujo de oliva
Propiedades sensoriales (2)	Olor y sabor	Md=0	Md≤3.5	3.5 < Md≤6 (3)	Md>6	Aceptable	Bueno	-	Aceptable	Bueno
		Mf>0	Mf>0							
	Color	-	-	-		Amarillo	Claro, amrillo a verde	-	Claro, amrillo a amarillo oscuro	Claro, amarillo a verde
	Aspecto a 20 ºC durante 24 horas	-	-	-	-	Límpido	Límpido	-	Límpido	Límpido
Acidez libre, % m/m expresada en ácido oleico		≤ 0.8	≤2.0	≤3.3	> 3.3	≤ 0.3	≤ 1.0	No limitada	≤ 0.3	≤ 1.0
Índice de peróxidos en meq. de oxígeno de los peróxidos por kg de aceite		≤ 20	≤ 20	≤ 20	No limitado	≤ 5	≤ 15	No limitado	≤ 5	≤ 15
Absorbancia en el UV (K)	270 nm (ciclohexano) / 268 nm (isoctano)	≤ 0.22	≤ 0.25	≤ 0.30	-	≤ 1.10	≤ 0.9	-	≤ 2.0	≤ 1.7
	ΔK	≤ 0.01	≤ 0.01	≤ 0.01	-	≤ 0.16	0.15	-	≤ 0.20	≤ 0.18
	232 nm (4)	≤ 2.50 (5)	≤ 2.60 (5)	-	-	-	-	-	-	-

Tabla I.2. Criterios de calidad (cont.)

Parámetros		Aceite de oliva virgen extra	Aceite de oliva virgen	Aceite de oliva virgen corriente	Aceite de oliva virgen lampante	Aceite de oliva refinado	Aceite de oliva	Aceite de orujo de oliva crudo	Aceite de orujo de oliva refinado	Aceite de orujo de oliva
Contenido en agua y en materias volátiles, % m/m		≤ 0.2	≤ 0.2	≤ 0.2	> 0.3	≤ 0.1	≤ 0.1	≤ 1.5	≤ 0.1	≤ 0.1
Contenido en impurezas insolubles en el éter de petróleo, % m/m		≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.2	≤ 0.05	≤ 0.05		≤ 0.05	≤ 0.05
Punto de inflamación		-	-	-	-	-	-	≥ 120ºC	-	-
Trazas metálicas mg/kg	Hierro	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0	-	≤ 3.0	≤ 3.0
	Cobre	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	-	≤ 0.1	≤ 0.1

Tabla I.2. Criterios de calidad

Parámetros	Aceite de oliva virgen extra	Aceite de oliva virgen	Aceite de oliva virgen corriente	Aceite de oliva virgen lampante	Aceite de oliva refinado	Aceite de oliva	Aceite de orujo de oliva crudo	Aceite de orujo de oliva refinado	Aceite de orujo de oliva
Ésteres metílicos de ácidos grasos (FAME, y ésteres etílicos de ácidos grasos (FAEE)	Σ FAME + FAEE ≤75 mg/kg o 75 mg/kg < Σ FAME + FAEE ≤150 mg/kg y (FAEE/FAME) ≤1,5								
Contenido en ceras	≤ 150	≤ 150	≤ 250	≤ 300	≤1 50	≤ 350	> 350(5)	> 350	> 350
Diferenica máxima entre el contenido real y el contenido teórico en triglicéridos con ECN 42	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.3	≤ 0.3	≤ 0.3	≤ 0.6	≤ 0.5	≤ 0.5
Contenido de monopalmitato de glicerilo (%)	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 1.4	≤ 1.4	≤ 1.2
	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %	≤ 0.9 si el total de ácido palmítico % ≤ 14 %			
Contenido en estigmastadienos	≤ 0.05	≤ 0.05	≤ 0.1	≤ 0.5	-	-	-	-	-

(1)La simultaneidad de los criterios 1, 2 y 3 no es obligatoria; puede bastar uno sólo.
(2) Sobre una escala continua Md=mediana del defecto; Mf= mediana del frutado.
(3) O cuando la mediana del defecto sea inferior o igual a 3,5 y la mediana del frutado sea igual a 0.
(4) Esta determinación está destinada a ser aplicada únicamente por los socios comerciales y con carácter facultativo.
(5) Los socios comerciales del país en que se venda al por menor podrán exigir que se respeten estos límites cuando el aceite se ponga a disposición del consumidor final.

Los aceites de oliva vírgenes, EVOO y VOO, son los más valiosos y su obtención a partir del fruto del olivo (*Olea europaea L.*) se realiza sólo mediante procesos mecánicos (ver sección siguiente). Quedan, por tanto, excluidos de ellos los aceites que se obtienen mediante el uso de disolventes, por re-esterificación o por cualquier mezcla de otros aceites [7].

Los aceites de oliva vírgenes contienen un 98% de compuestos saponificables (mayoritariamente tri-, di- y monoacilgliceroles) y un 2% de compuestos insaponificables (principalmente alcoholes alifáticos y triterpénicos, esteroles, hidrocarburos, compuestos volátiles, carotenos, tocoferoles y fenoles) [8]. Las características de estos compuestos y los métodos para su determinación se recogen en las revisiones que constituyen los Capítulos 1 y 2, respectivamente, de esta Memoria. Tal como muestra la Tabla I.2, la diferencia entre EVOO y el VOO viene definida por dos aspectos fundamentales: su valoración organoléptica (obtenida por las pruebas del panel de cata) y las características físico-químicas, especialmente la menor acidez del EVOO. La calidad del aceite de oliva está influenciada por diferentes factores. El cultivar, las condiciones ambientales y las prácticas agronómicas afectan a la fisiología del fruto, mientras que las condiciones de almacenamiento y el procesado alteran la composición del aceite [9]. Por otra parte, el índice de maduración (RI) es un factor clave en la composición del aceite de oliva, que pone de manifiesto, entre otros, que la relación de ácidos oleico/linoleico es inversamente proporcional al grado de maduración. Existen diferentes formas de determinar el índice de madurez; en esta Memoria se ha utilizado la propuesta por Rallo y Barranco [10]. El RI viene determinado por el color del fruto, clasificado en las siguientes categorías: 0= verde oscuro o vivo; 1= amarillo o amarillo verdoso; 2= enverado; 3= rojizo o violeta pálido; 4= negro [11].

Tabla I.3. Características químicas de las aguas residuales de la molienda de la aceituna (OMWW), de los residuos generados en el sistema de extracción de aceite en dos fases (TPOMW) y residuos sólidos de la producción de aceite de oliva.

Parámetro	**Productos de desecho**		
	OMWW	**Residuo sólido (orujo)**	**TPOMW**
Pulpa (%)		12–35	10–15
Hueso (%)		15–45	12–18
Materia seca (%)	6.33–7.19	87.1–94.4	
Cenizas (%)	1	1.7–4	1.42–4
pH	2.24–5.9		4.9–6.8
Conductividad eléctrica (dS/m)	5.5–10		1.78–5.24
Carbono total (%)	2–3.3	29.03–42.9	25.37
Materia orgánica (%)	57.2–62.1	85	60.3–98.5
Carbono orgánico total (g/L)	20.19–39.8		
Sólidos suspendidos totales (g/L)	25–30		
Sólidos suspendidos minerales (g/L)	1.5–1.9		
Sólidos suspendidos volátiles (g/L)	13.5–22.9		
Sólidos volátiles (g/L)	41.9		
Sólidos minerales (g/L)	6.7		
Acidez volátil (g/L)	0.64		
Carbono inorgánico (g/L)	0.2		
Nitrógeno total (%)	0.63	0.2–0.3	0.25–1.85
P (%)	0.19	0.03–0.06	0.03–0.14
K (%)	0.44–5.24	0.1–0.2	0.63–2.9
Na (%)	0.15		0.02–0.1
Ca (%)	0.42–1.15		0.23–1.2
Mg (%)	0.11–0.18		0.05–0.17
Fe (%)	0.26 ± 0.03		0.0526–0.26
Cu (%)	0.0021		0.0013–0.0138
Mn (%)	0.0015		0.0013–0.0067
Zn (%)	0.0057		0.0010–0.0027
Lípidos (%)	0.03–4.25	3.5–8.72	3.76–18.0
Fenoles totales (%)	0.63–5.45	0.2–1.146	0.4–2.43
Azúcares totales (%)	1.5–12.22	0.99–1.38	0.83–19.3
Proteínas totales (%)		3.43–7.26	2.87–7.2
Demanda de oxígeno biológica (g/L)	35–132		
Celulosa (%)		17.37–24.14	14.54
Hemicelulosa (%)		7.92–11.00	6.63
Lignina (%)		0.21–14.18	8.54

3 Obtención del aceite de oliva virgen

El proceso mecánico global para la extracción del VOO de la aceituna incluye tres etapas, tal como muestra la Figura I.1: i) Molturación del fruto para obtener una pasta homogénea; ii) batido para incrementar la cantidad de aceite libre y ayudar a la coalescencia y aglomeración de las gotas de aceite, facilitando así la separación de las fases acuosa y lipídica; iii) separación de esta última por prensado (proceso discontinuo) o por centrifugación (proceso continuo).

La separación por prensado es el método más antiguo de obtención de VOO y da como resultado la presencia de 3 fases: La fase lipídica, como fase deseable, pero también otras dos; una fase sólida constituida por partículas de hueso, pulpa y piel —que recibe el nombre de orujo— y una fase acuosa —conocida como alpechín—, o agua residual de la decantación del aceite, formada por el contenido acuoso de la aceituna más el agua añadida durante el proceso para facilitar la separación de las fases líquidas inmiscibles [12]. El alpechín es la fase más indeseable por su alta carga contaminante, con valores de demanda química de oxígeno (COD) de hasta 220 g/L y de demanda bioquímica de oxígeno (BOD) de hasta 100 g/L. Esta fracción está formada principalmente por azúcares, polialcoholes, pectinas, lípidos y cantidades significativas de taninos y fenoles, responsables de su actividad antimicrobiana y fitotóxica [6,12,13].

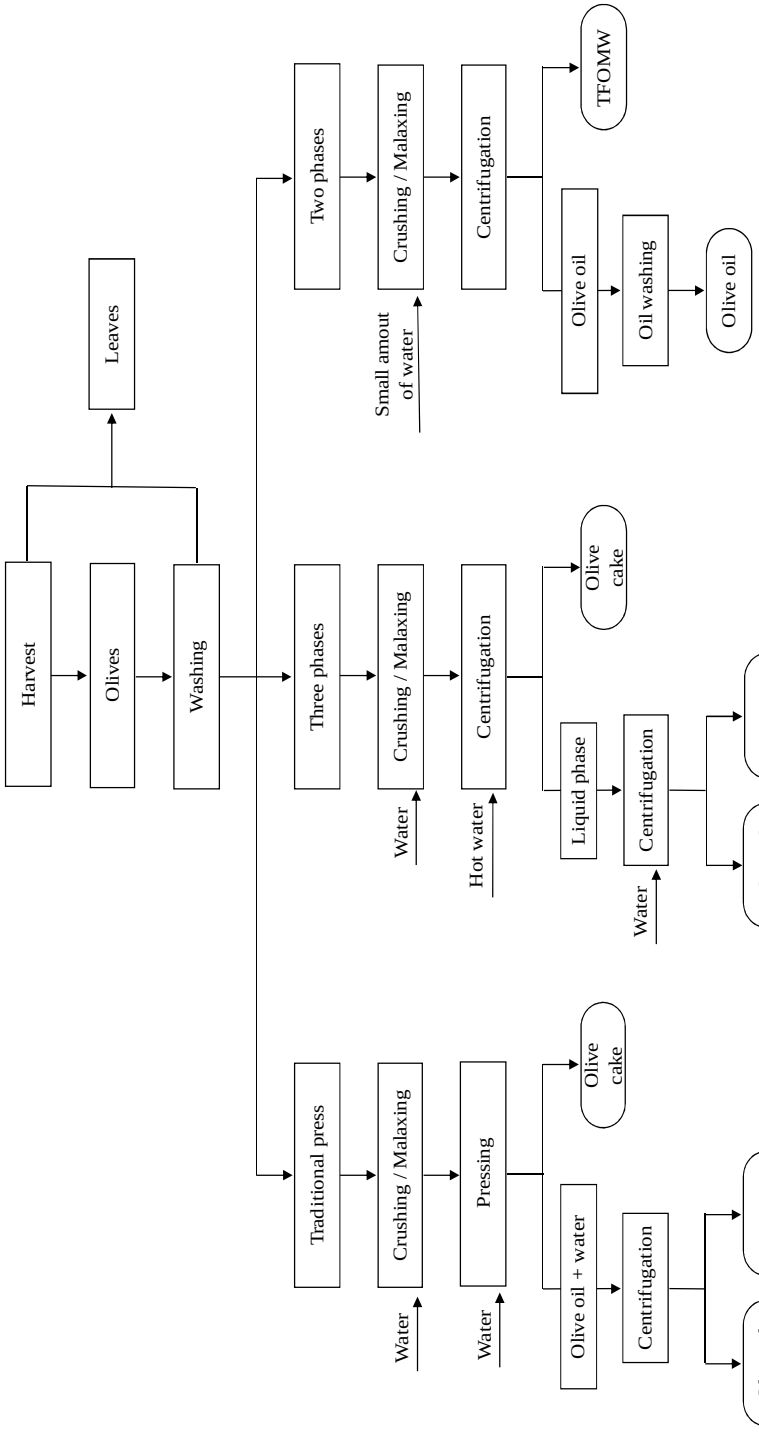

Figura I.1. Proceso mecánico global para la extracción del VOO de la aceituna.

La separación por centrifugación puede dar como resultado tres fases similares a las obtenidas mediante prensado, o dos; en ambos casos con el uso de decantadores para separar las fases como etapa previa a la centrifugación. El sistema en tres fases, con decantación y centrifugado, requiere mayor adición de agua que el sistema de prensado (por lo que el aceite queda más empobrecido en compuestos hidrofílicos). El consumo de agua se reduce con el sistema en dos fases, aparecido en los años 90 del pasado siglo y que redujo el 75% de los desechos de las almazaras [12].

Además de un aceite más enriquecido en compuestos hidrofílicos, el sistema de dos fases produce un residuo semisólido que es la combinación del residuo sólido (orujo) y el líquido (alpechín) del sistema de tres fases, por lo que recibe el nombre de alperujo. Este residuo puede reprocesarse para la extracción del aceite remanente, bien mediante una extracción con disolventes orgánicos o mediante una segunda centrifugación.

El alperujo contiene una gran cantidad de agua (entre el 56 y el 75% de su peso) y una fracción orgánica constituida por lignina (48%), hemicelulosa (37.7%), celulosa (20.8%), carbohidratos solubles en agua (10.1%), grasas (13.0%), proteínas (7.7%) y fenoles (1.5%), como principales componentes [14]. La Tabla I.3 resume las características de los diferentes desechos de la producción de aceite de oliva.

La Tabla I.2 muestra los parámetros que caracterizan los diferentes tipos de aceite de oliva. Esta caracterización abarca entre otros: (i) La medida de la suma de los ésteres metílicos y etílicos de los ácidos grasos; (ii) la acidez; (iii) el índice de peróxidos; (iv) el contenido en ceras; (v) el porcentaje de 2-glicerilmonopalmitato; (vi) la cantidad de stigmatadieno; (vii) la diferencia entre el ECN42 obtenido mediante HPLC y el calculado teóricamente; (viii) los valores de K (absorbancia a 2 longitudes de onda en la región UV, que pueden variar ligeramente dependiendo del disolvente orgánico), y su relación; (ix) la evaluación organoléptica.

En el Capítulo 1 de esta Memoria se recogen las características y propiedades de los compuestos que constituyen la fracción minoritaria, mientras que el Capítulo 2 se dedica a los métodos analíticos para la determinación de cada una de los grupos que la componen.

4 Las ómicas en la mejora de la calidad del aceite de oliva

La imparable aplicación de las disciplinas ómicas en todas las áreas de interés social, y en el área vegetal en particular, no podía soslayar, ni siquiera realizar una aplicación poco profunda en el caso del olivo (*Olea europaea* L.). Un cierto retraso en estos estudios con respecto a los de otros vegetales se explica por la complejidad de este cultivo, con un variado patrimonio genético resultado de la enorme longevidad de la planta y del escaso cambio de su genotipo a lo largo de los siglos de cultivo. Su presencia milenaria en nuestra cultura ha dado lugar a que sólo en la cuenca mediterránea existan más de 1.200 cultivares diferentes. Se desarrolla a continuación una breve discusión de las aportaciones realizadas en este cultivo por las diferentes ómicas, comentadas en el orden correspondiente a su antigüedad.

La *genómica* se ha considerado desde hace más de dos décadas una herramienta insustituible en el estudio de las complejas bases moleculares que sustentan la calidad del fruto y del aceite de oliva. También el desarrollo de nuevos marcadores moleculares, pilares básicos de la selección en programas de cruzamiento para la obtención de nuevos cultivares con los fenotipos deseados, se ha considerado que debía tener el soporte de aproximaciones genómicas. No obstante, a pesar de su relevancia económica, la investigación sobre la genómica del olivo o de especies relacionadas ha permanecido muy olvidada si se compara con la de otras especies de plantas, tales como *Vitis vinifera* [15,16] o *Populus trichocarpa* [17]. Las secuencias genómicas completas de estas últimas son actualmente asequibles, aunque los primeros estudios de secuenciación de todas ellas empezaron simultáneamente o incluso más tarde que los relacionados con el olivo [18]. Entre los grupos pioneros en la secuenciación del genoma del olivo se encuentran la Fundación Genoma, que

desarrolló el proyecto OLEAGEN [www.chirimoyo. ac.uma.es/oleagen/] y los implicados en el proyecto OLEA auspiciado por el Ministerio de Agricultura de Italia [19]. Algunos estudios recientes en esta área son los siguientes:

(i) El aislamiento y análisis de la expresión del ADN complementario (cDNA) y la identificación de un número de estas moléculas asociadas a la alternancia de las cosechas de aceituna abundantes y escasas. En la cascada de procesos biológicos, la acción de estas moléculas en las correspondientes secuencias de ARN, la presencia de una potencial monooxigenasa expresada cinco veces más en las hojas de olivo correspondientes a años de buena cosecha y que se correlacionan con metabolismos esenciales tales como el de la galactosa, abre una vía para establecer las bases genéticas de esta alternancia que causa grandes pérdidas a los productores de aceite [20].

(ii) Una aplicación de esta ómica de enorme trascendencia económica es la de la búsqueda de marcadores de trazabilidad del VOO. El análisis del ADN es la forma más efectiva de identificación de cultivares sin influencia de las condiciones ambientales y sin interferencia de la variedad del polinizador. Puesto que la variación del ADN plastidial (cpDNA) es pequeña, se ha estudiado el uso de haplotipos de cpDNA para discriminar entre variedades y establecer los aspectos comunes a todas ellas que permitan diferenciar los aceites de otros provenientes de diferentes frutos o semillas [21,22].

(iii) Un reciente estudio de 23 variedades del banco de germoplasma del olivo ha tenido como objetivo validar la diversidad genética, la estructura de la población y los flujos de genes como núcleo para la creación de una colección de germoplasma [23].

La *transcriptómica* y el olivo. Teniendo en cuenta la complejidad e incompleta secuenciación del genoma, la secuenciación del transcriptoma se ha considerado una vía efectiva para el descubrimiento de genes y la identificación de transcriptos implicados en procesos biológicos específicos.

Un estudio interdisciplinar desarrollado por investigadores de la Universidad de Málaga, Jaén, Sevilla, Valencia, Madrid y Córdoba ha proporcionado la información más amplia, hasta la fecha, de la secuencia de este cultivo, aplicando tecnologías de pirosecuenciación que han permitido generar más de 2 millones de lecturas de doce bibliotecas de ADNc obtenidas de cultivares de Picual, Arbequina y Lechín de Sevilla y de una progenie segregada a partir de un cruce de Picual y Arbequina. Las bibliotecas incluyen el mesocarpo y las semillas en tres estados de maduración, ramas jóvenes y hojas, brotes activos e inactivos de olivos jóvenes y adultos, y raíces también de árboles jóvenes y adultos. La información sobre este nuevo conjunto y su anotación funcional sobre el transcriptoma del olivo se ha publicado recientemente [21]. Otras aportaciones recientes sobre el binomio transcriptoma–olivo han sido:

i) El estudio comparativo de la respuesta al estrés por salinidad del agua de riego, para conocer la tolerancia al NaCl, que ha puesto de manifiesto una expresión diferencial de transcriptos en presencia y ausencia de la sal, la presencia de interacciones regulatorias y una similaridad manifiesta entre el olivo y la especie *Arabidopsis* en las mismas condiciones de estrés [24].

ii) La alternancia entre cosechas abundantes y escasas (estudiada en frutos verdes y maduros y en hojas recogidas en julio y en noviembre en años consecutivos), en la que han resultado ser claves los microARN (miRNA en inglés) como reguladores de la expresión génica [25]. La diferente expresión de transcritos en estas muestras y su protagonismo en procesos tales como el crecimiento, la diferenciación, la regulación hormonal, el estrés y el metabolismo de proteínas, lípidos y carbohidratos proporciona una explicación a este comportamiento alternante de las cosechas de aceitunas [26].

La *proteómica* ha abierto nuevas vías de conocimiento del olivo y su producto estrella. No obstante, mientras que otros componentes, especialmente del fruto, han sido objeto de una amplia investigación,

comparativamente la fracción proteica ha sido menos estudiada. Las proteínas están distribuidas en las diferentes partes del fruto del olivo y se ha demostrado que algunas de ellas están también presentes en el VOO. El mayor contenido de proteínas lo ostenta la semilla de la aceituna, lo que ha permitido una mejor caracterización de ellas en comparación con las existentes en la pulpa y en el aceite. Entre las proteínas del olivo, las que han despertado un mayor interés han sido las enzimas, ya que juegan un papel esencial en las características y la estabilidad del VOO. Algunas de las investigaciones recientes en proteómica se comentan a continuación de forma sucinta.

i) En la investigación sobre el proteoma del olivo, el desarrollo de nuevas plataformas analíticas en las que se mejoran las etapas de preparación de la muestra y la ampliación de las bases de datos han contribuido de forma decisiva a la obtención de una información más amplia y precisa sobre este tema [27]. La conjunción de diferentes protocolos de extracción de proteínas del hueso y de la pulpa de la aceituna, la captura de los datos generados mediante nLC–MS/MS utilizando un programa adecuado (ProteoMiner) y una biblioteca propia combinatoria de péptidos ha permitido ampliar enormemente la señal correspondiente a especies poco abundantes. De esta forma ha sido posible la identificación de 61 proteínas en la semilla del fruto (frente a las 4 previamente descritas), y 231 en la pulpa (frente a las 56 previas) [28]; lo que supone un avance notable en la interpretación del proteoma del olivo.

ii) La obtención de perfiles protéicos para clasificar las hojas de olivo y la pulpa de aceitunas desarrollado recientemente ha estado basado en el uso de la electroforesis capilar. Se utilizaron 12 cultivares de diferentes regiones españolas, que se clasificaron correctamente con una excelente resolución entre categorías; lo que pone de manifiesto que estos perfiles son característicos de cada cultivar [29].

iii) Las proteínas de la pulpa de la aceituna constituyen una fracción minoritaria que había sido escasamente estudiada a pesar de su papel, considerado importante, en la estabilidad del aceite y en la alergenicidad del olivo. Un estudio reciente de este material ha estado basado en la extracción de las proteínas mediante electroforesis en gel previa al análisis, tanto por MALDI–TOF como por LC–MS/MS, que ha permitido identificar una serie de proteínas, mientras la imposibilidad de la identificación de otras ha sido en gran parte debida a la escasa presencia de entradas de la *Olea europaea* en las bases de datos de proteínas [30].

iv) Los cambios del proteoma durante la inflorescencia del olivo, trascendentales en la formación de los metabolitos característicos del VOO, han sido estudiados recientemente de forma exhaustiva [31]. También el proteoma del hueso de la aceituna se ha estudiado para la obtención de una huella dactilar que permita la evaluación del desarrollo del cultivar [32].

v) La investigación de Bianco y col. ha establecido un nexo entre el proteoma y el metaboloma del olivo al estudiar la regulación del primero durante el periodo de maduración de la aceituna. Estos autores identifican un número de proteínas, especialmente las asociadas con el metabolismo de ácidos grasos, fenoles y compuestos aromáticos, pero también las implicadas en la fotosíntesis en el fruto y en la oleogénesis. Para evaluar si los cambios observados en las proteínas durante la maduración eran consistentes con los producidos en los ARNm, los datos proteómicos obtenidos se compararon con los transcriptómicos correspondientes a estudios previos [33]. Por tanto, la investigación conjunta constituye un excelente ejemplo de la aplicación de la biología de sistemas.

La *metabolómica*, como la más reciente de las grandes ómicas, ha sido la última en aplicarse para obtener información sobre el olivo en general y sobre el VOO en particular, si bien los estudios sobre proteómica, como se ha comentado, ya habían arrojado luz sobre los metabolitos y su dependencia de la acción enzimática. Uno de los grupos más activos en la caracterización

de metabolitos del olivo ha sido el grupo en el que se integra la doctoranda, en la que se establecieron:

i) El perfil de ácidos grasos proporcionado por cada tejido del fruto en función del método de extracción del aceite (VOO o aceite de oliva refinado) y del cultivar (frantoio, arbequina, hojiblanca, manzanilla, ocal, picual, picudo y lechín de Sevilla; pertenecientes a la misma área geográfica y con recolección del fruto en la misma etapa de maduración). Las semejanzas y diferencias entre los perfiles obtenidos se estudiaron mediante técnicas quimiométricas [34]. Un posterior estudio sobre la evolución del perfil de ácidos grasos en función del grado de maduración del fruto y del cultivar permitió establecer cuándo se alcanza el máximo rendimiento en ácidos grasos en función del cultivar, así como el intervalo de concentraciones de cada familia de ácidos grasos en el aceite [35].

ii) El perfil cualitativo y cuantitativo de los azúcares en diferentes partes del olivo (frutos, hojas y ramas) condujo a la caracterización de 22 compuestos correspondientes a mono-, di- y trisacáridos, azúcares de ácidos carboxílicos y alcoholes, polioles cíclicos y compuestos derivados. La preparación de la muestra (acelerada por ultrasonidos) y el método de análisis basado en GC–MS/MS proporcionaron una plataforma adecuada para este tipo de estudios [36].

iii) Los compuestos fenólicos (y en menor proporción los terpenos y esteroles) han sido el objetivo de numerosos estudios, aún cuando los términos metabolito o metabolómica no aparecen en la mayor parte de las publicaciones. La abundancia de estos estudios es debida especialmente a las propiedades nutracéuticas de la mayoría de estos compuestos. En un estudio para la caracterización de VOOs provenientes de diferentes cultivares (Arbequina, Picual y Hojiblanca) llevado a cabo mediante la combinación de CE y HPLC–TOF se consiguió determinar más de 45 compuestos fenólicos pertenecientes a importantes familias, como alcoholes y ácidos fenólicos, lignanos, flavonoides y secoiridoides, así como nuevos compuestos que hasta

el momento no se habían descrito. Durante la optimización del método encontraron diferencias significativas en el análisis de las diferentes variedades utilizadas, por lo que estos compuestos podrían usarse como marcadores geográficos. [37].

iv) Reciente y amplio es el estudio de Segura-Carretero y col. sobre hojas de olivo en el que realizaron la identificación de 38 fenoles, entre los que se incluían ácidos fenólicos, secoiridoides, derivados del ácido hidroxicinámico, flavonoles y flavonas. Puesto que la identificación no fue confirmada con estándares, los autores consideraron que esta etapa sólo alcanzó el grado de tentativa [36].

v) Un estudio más reciente, también sobre la identificación de compuestos fenólicos, pero en este caso en alperujo y con el propósito de proporcionar una base sólida para la valorización de este residuo, se ha llevado a cabo por el grupo en el que se integra la doctoranda. El uso de LC–QqTOF tras la extracción de los compuestos de interés mediante líquidos sobrecalentados ha permitido la identificación y confirmación de diferentes familias de especies, tales como hidroxitirosol, tirosol y derivados (6 compuestos); precursores de iridoides (15 compuestos); secoiridoides y derivados (10 compuestos); 8 flavonoides; 3 lignanos y 9 ácidos fenólicos [39].

Con esta sección se ha pretendido dar una ligera noción de la importancia que actualmente tienen las ómicas en la búsqueda de un mejor conocimiento del olivo, y especialmente del VOO, considerando las aportaciones más recientes, que ponen de manifiesto el interés que despiertan las investigaciones en esta área.

5 Aprovechamiento de los residuos del olivo y de la producción del aceite de oliva

Existe una diferencia fundamental entre los residuos del olivo (ramas y hojas) y los provenientes de la producción de aceite: Los primeros carecen de impacto ambiental y pueden permanecer en el lugar en el que se producen

(durante la tala del olivo) o en las proximidades de la almazara una vez que se separan del fruto con el que han caído y se han mezclado como consecuencia del vareo. Los segundos son más variados, ya que dependen del proceso de producción del aceite, pero tienen la característica común de su alta carga contaminante, en gran parte debida a su contenido en compuestos antioxidantes.

I.5.1 Aprovechamiento de las hojas de olivo

Tradicionalmente las hojas de olivo se han utilizado sobre todo en compostaje. Más reciente ha sido la investigación sobre las características de este material y la demostración de su alto contenido en fenoles hidrofílicos, mayor que en cualquier otra parte del olivo. Como ejemplo, tomando la oleuropeína como fenol modelo, su contenido en hojas es cien veces mayor que en el VOO y diez veces mayor que en el alperujo. Demostrada la gran capacidad antioxidante y alto poder como captador de radicales libres de los fenoles hidrofílicos, los extractos de hojas de olivo se vienen utilizando en la industria cosmética para enriquecer sus productos [40,41]. También diferentes productos farmacéuticos en fase de desarrollo avanzado incluyen estos extractos como principio activo, refiriendo su contenido a la oleuropeína en sus diferentes formas como componente principal y que en su degradación da como producto de hidrólisis el hidroxitirosol, al que se atribuyen las principales bondades de estos compuestos fenólicos [42]. Muchas de las industrias españolas que utilizan estos extractos (tanto sólidos como líquidos) se surten de ellos a través de una empresa española especializada en su obtención [43]. Se han realizado muchos intentos para demostrar la idoneidad del uso de los extractos de hojas de olivo para el enriquecimiento/suplementación de alimentos [44–46]. Uno de los estudios más recientes y que muestran de forma indiscutible la influencia de estos compuestos en la resistencia a la degradación de los aceites sometidos a calentamiento lo ha realizado la autora de esta Memoria. Puesto que los extractos utilizados se han obtenido tanto de hojas como de alperujo, se

comentarán en la siguiente sub-sección y en la sección dedicada al enriquecimiento.

Son destacables los esfuerzos que se realizan en la actualidad por numerosos grupos de investigación y desarrollo, así como por organismos nacionales e internacionales, para el aprovechamiento de éste y otros residuos del olivo/producción de aceite. El grupo en el que se integra la autora de esta Memoria orienta parte de sus esfuerzos en esta línea, como lo demuestra sus contactos con diversas almazaras y la preparación de un proyecto enmarcado en el programa Horizonte 2020, Waste 7.

I.5.2 Aprovechamiento de los desechos de la producción de aceite

El aprovechamiento de los residuos de la producción de aceite de oliva es, lógicamente, función de sus características derivadas del método de obtención utilizado, por lo que en algunos casos requiere discusión separada.

El *alpechín* ha constituido tradicionalmente un enorme problema ambiental en los países productores de VOO por la gran cantidad de este residuo que resulta durante los aproximadamente 3 meses/año en los que se genera. En la cuenca mediterránea se producen más de 30 millones de metros cúbicos de alpechín en el tratamiento de cada cosecha.

El aprovechamiento tradicional más abundante de este residuo ha sido como fertilizante. El riego directo de los campos de cultivo con alpechín ha sido una práctica común en la región mediterránea, proporcionando una fuente barata de nutrientes [47], con el doble beneficio de eliminar un desecho indeseable y contribuir a la agricultura sostenible [48]. No obstante, las propiedades químicas del alpechín dependen de la maduración del fruto en el momento de la producción del VOO y de la presencia de aceite en este desecho [49]. Por tanto, las zonas irrigadas se han visto afectadas por la presencia en el alpechín de grasa, su salinidad generalmente alta, la acidez y la concentración de fenoles —estos últimos son los principales responsables de acciones fitotóxicas y antimicrobianas que claramente van en detrimento de la calidad del suelo para el cultivo [50]. Los resultados de las investigaciones

de estos aspectos han conducido a una drástica disminución del uso del alpechín como fertilizante [51].

El aprovechamiento tanto del *orujo* como del *alperujo* se ha realizado bien tras un proceso de biorremediación, o directamente para la fabricación de materiales de construcción (ladrillos).

La biorremediación mediante el uso de microorganismos para eliminar los contaminantes de estos residuos y reducir su demanda química de oxígeno (COD) se ha considerado una forma de hacerlos compatibles con el medio ambiente con un coste proporcionado. La degradación de su biomasa produce una mezcla de gases y un fertilizante libre de fenoles y, por tanto, sin peligro para las comunidades de bacterias. La degradación puede llevarse a cabo en condiciones aeróbicas, con una producción mayoritaria de CO_2 en lugar de CH_4. Este último es característico de la degradación en condiciones anaeróbicas y se conoce comúnmente como biogás. En estas últimas condiciones, la máxima producción de biogás a partir de orujo que se ha documentado ha sido de aproximadamente 0.70 L de biogás por L de volumen de digestor por día durante un periodo de 20 días. El contenido de metano del biogás fue del 80%, el resto estaba principalmente constituido por dióxido de carbono [52].

Uno de los usos más prometedores del orujo o del alperujo es su inclusión en la composición de materiales de construcción, tal como ladrillos. Los estudios sobre esta aplicación, además de la contribución a la reducción de la contaminación, es que el uso de estas materias primas disminuye la cantidad de energía requerida para la fabricación de estos materiales de construcción. Al reemplazar hasta un 12% de arcilla por alperujo los materiales obtenidos muestran: i) una menor densidad (1710 kg/m^3 frente a 1850 kg/m^3) que los fabricados sólo con arcilla (la menor densidad es debida a una mayor absorción de agua y permite la fabricación de bloques del mismo tamaño, pero más ligeros que los tradicionales, lo que facilita el manejo y reduce los costes de transporte) [53]. ii) Aislamiento térmico más efectivo

(disminución respecto a la conductividad de la arcilla en un 18%). iii) Resistencia de aproximadamente 14 N/mm^2, suficientemente grande para este tipo de unidades. iv) Reducción de la temperatura de cocido de 920 a 880 ºC cuando se utiliza la mezcla al 12% de alperujo con la arcilla; lo que reduce los costes del calentamiento del horno entre un 2.4 y un 7.3% [54]. El apoyo a este uso con sus consiguientes ventajas está en manos de las empresas del sector, en general reacias a cambios.

El *uso de parte de los componentes del alpechín o del alperujo* es, posiblemente la alternativa más importante actualmente, susceptible incluso de mejora si se encuentra aplicación de los componentes restantes para los que no existe una utilización masiva en la actualidad.

Una característica del *alpechín* que lo hace tremendamente interesante para la obtención de fenoles hidrofílicos es que aproximadamente el 53% de estos compuestos permanecen en este desecho después de la separación del aceite [55]. Como consecuencia de los procesos de hidrólisis, el fenol más abundante en el alpechín es el hidroxitirosol, que representa alrededor del 70% del total de fenoles, seguido por el tirosol, el ácido *p*-cumárico, la oleuropeína y el ácido caféico [56,57]. El rango en el que se encuentra una serie de compuestos inorgánicos (principalmente cationes metálicos, pero también alcalinos y alcalino-térreos y algunos aniones) también se ha estudiado [12].

La posibilidad de utilizar membranas de filtración para la purificación de aguas presenta en el caso del alpechín la doble vertiente favorable de conseguir un vertido poco o nada contaminante y obtener compuestos de interés sin necesidad de utilizar disolventes de ningún tipo.

El binomio membranas de filtración–alpechín ha proporcionado sus mejores resultados en un sistema integrado constituido por una serie de membranas de alta a baja porosidad que ha permitido un fraccionamiento en 3 fases: i) Compuestos de alto peso molecular que pueden someterse a digestión anaeróbica para la producción de biogás; ii) compuestos bioactivos

como fenoles, utilizables en las industrias de cosmética, alimentación y farmacéuticas; iii) agua purificada, que puede reutilizarse en el proceso de extracción de aceite [58]. Otros sistemas basados en ultrafiltración–nanofiltlración [58] o ultrafiltración–destilación por ósmosis [59] permiten recuperar el 96% o el 78%, respectivamente, del total de los fenoles del alpechín.

También el *alperujo* es una excelente fuente de antioxidantes naturales, ya que se estima que el 45% del contenido fenólico total de las aceitunas permanece en el alperujo tras la separación del aceite [55]. La extracción de fenoles del alperujo se basa en el contacto entre el residuo semisólido con el extractante (generalmente agua o, mejor, una mezcla etanol–agua si el extracto se pretende utilizar en alimentación; o una mezcla metanol–agua si sólo se pretende obtener información analítica del extracto). La aceleración, incluso la automatización, del proceso de extracción puede conseguirse mediante la asistencia de energías auxiliares tales como ultrasonidos [60], microondas [61] o presión+temperatura altas [39], tal como se muestra en la investigación recogida en el Capítulo 3. Los extractos obtenidos de esta materia prima tienen como ventaja respecto a los procedentes de hojas de olivo el que la aceituna es un fruto comestible que se ha venido utilizando como tal desde tiempos inmemoriales; por tanto, no existe restricción respecto a su uso en alimentación.

Estudios realizados con el uso de estos extractos para conocer su efecto en individuos obesos han puesto de manifiesto su acción en las rutas metabólicas de los ácidos grasos esenciales omega-3 y omega-6 y su influencia en la producción de metabolitos con características antiinflamatorias en detrimento de los favorecen la inflamación [62,63]. Los estudios recogidos en los Capítulos 11 y 12 de esta Memoria constituyen una clara evidencia de su acción protectora frente a la degradación de los aceites por calentamiento y abre una amplia vía de utilización de estos extractos para mejorar el tiempo de vida de los aceites de fritura [39,64,65]. La EBT en la

que está implicado el grupo en el que se integra la doctoranda se dedica a la investigación de estos extractos y a su aplicación en cosmética y en alimentación [40].

6 Enriquecimiento de aceites en compuestos antioxidantes

La investigación sobre el enriquecimiento de alimentos en general ha estado mayoritariamente dirigida al uso de antioxidantes (e.g. suplementación de yogur y leche [66], embutidos [67], galletas [68], pescado [69] o zumos de frutas [70]), aunque también el incremento del contenido de fibra ha sido objeto de investigación [68]; en todos los casos con el propósito último de mejorar las cualidades saludables del alimento en cuestión.

En la tendencia general al enriquecimiento o suplementación de alimentos, el referido a aceites ocupa un lugar amplio y variado ya que, además del objetivo común de obtener un alimento más saludable para el consumidor, se pretende conservar las características del aceite en cuestión durante un tiempo más largo, bien de almacenaje, bien de uso en condiciones de calentamiento. El objetivo de conservación se ha conseguido tradicionalmente mediante la adición de antioxidantes artificiales, tales como butilhidroxitolueno (BHT), hidroxibutilanisol (BHA) o terbutilhidroxiquinona (TBHQ). Los comprobados beneficios inherentes a los antioxidantes naturales y el rechazo cada vez mayor a los productos artificiales (colorantes, antioxidantes o cualquier otro aditivo) han dado lugar a una investigación extensa en esta área y a una mayor presencia en el mercado de colorantes naturales [71] y de antioxidantes individuales (e.g. ácido ascórbico, licopeno, oleuropeína, hidroxitirosol), familias (e.g. tocoferoles, flavonoides) o extractos genéricos (e.g. de hojas de olivo, de alperujo, de romero, o de uvas) [72]. El enriquecimiento de aceites vegetales con extractos de plantas aromáticas tales como orégano, salvia, melisa, nébeda, hisopo o tomillo [73,74] ha tenido como objetivos mejorar la estabilidad oxidativa y el sabor del aceite. La utilización de estos extractos siempre ha resultado favorable en el caso de una comparación con la adición de antioxidantes artificiales.

Dado el probado efecto beneficioso de los compuestos de la fracción minoritaria del VOO y la alta concentración de estos compuestos en las hojas del olivo y en los desechos de la producción de este aceite, no resulta extraño que la investigación sobre el uso de estos antioxidantes en el enriquecimiento de aceites haya sido muy amplia. Establecido como seguro el efecto saludable, los estudios se han orientado en unos casos a conocer el aumento de la estabilidad en el almacenamiento de los aceites enriquecidos y en otros la resistencia a la degradación en el calentamiento (aumento del tiempo de uso en fritura).

La utilización individual de compuestos de la fracción insaponificable para el enriquecimiento de aceites sólo se justifica con fines de investigación y no como búsqueda de un procedimiento aplicable finalmente a escala industrial, dado el coste de estos compuestos. Estudios de este tipo han implicado compuestos comerciales e incluso el aislamiento a partir de VOO mediante cromatografía preparativa para su posterior adición al aceite refinado [75]. No obstante, más común ha sido el uso directo de hojas de olivo o de aceitunas y, especialmente, el de extractos líquidos o sólidos de hojas de olivo o de alperujo, que finalmente puede conducir a una aplicación industrial.

I.6.1 Formas de enriquecimiento de aceites a partir de antioxidantes del olivo

El material para el enriquecimiento puede ser sólido o líquido.

El uso de un material sólido para el enriquecimiento da lugar a tres situaciones:

i) Transferencia de masa sólido–líquido con posterior separación del material sólido y establecimiento o no del equilibrio de extracción. Éste es el caso del uso de hojas de olivo, que pueden ponerse en contacto con el aceite durante un tiempo más o menos largo para favorecer un determinado grado de transferencia de masa [76], o acelerarse ésta de forma drástica si se auxilia mediante ultrasonidos [65]. Un enriquecimiento global de unos 400 mg/L puede conseguirse en 20 min de tratamiento, con una transferencia relativa

de cada fenol función de su afinidad por la fase lipídica. Teniendo en cuenta que los ultrasonidos de baja frecuencia favorecen la oxidación de los lípidos [77], deberá adoptarse una solución de compromiso en la potencia de esta energía auxiliar de forma que se consiga una máxima transferencia de masa con mínimo o nulo deterioro de la fracción lipídica.

ii) El enriquecimiento con extractos sólidos de hojas o de alperujo puede dar lugar a dos situaciones dependiendo de la cantidad de sólido puesta en contacto con el aceite. a) Uso de la cantidad exacta que se pretende aplicar, con lo cual se trata de una disolución propiamente dicha. b) Uso de una cantidad en exceso con transferencia de masa hasta alcanzar el enriquecimiento adecuado y con posterior separación del sólido en exceso mediante filtración.

iii) El enriquecimiento por adición de trozos de aceituna, por contacto prolongado con los sólidos de la base de los tanques de almacenamiento del aceite y por las partículas suspendidas que proceden de la pasta inicial del proceso ha sido materia de una patente [78]. La estabilidad de esta materia sólida a tiempos de almacenamiento largos no ha sido estudiada, si bien los resultados a corto plazo muestran un mejor comportamiento de las aceitunas verdes frente a las de mayor grado de maduración.

El uso de una disolución de los antioxidantes puede también dar lugar a tres situaciones:

i) Que el disolvente sea miscible con el aceite a enriquecer, con lo cual se tratará de una mezcla propiamente dicha. Tal es el caso del enriquecimiento de aceite refinado de oliva con disoluciones etanólicas de extractos de hojas de olivo [79].

ii) Que el disolvente no sea miscible con el aceite, con lo que se tratará de una extracción líquido–líquido que establecerá una transferencia de cada compuesto en función de su coeficiente de reparto entre las dos fases inmiscibles.

iii) Que se utilice un disolvente (o mejor una mezcla de disolventes) no miscibles con el aceite, con desarrollo del proceso de transferencia en un rotavapor, de forma que durante la transferencia se va concentrando la fase donadora a medida que se produce la transferencia a la fase aceptora. Éste ha sido el procedimiento utilizado cuando se ha requerido enriquecimiento de aceites en la investigación que se recoge en esta Memoria (Capítulos 11 y 12).

Es necesario destacar el hecho de que cualquier tipo de enriquecimiento de los VOOs les haría perder este estatus, que implica que resultan sólo del zumo de la aceituna, libre de cualquier aditivo, incluso de aquéllos que podría considerarse que les proporcionan una mejora en sus cualidades.

I.6.2 Ventajas del enriquecimiento de aceites

A excepción de los ejemplos ya comentados en los que los aceites se han enriquecido con extractos de antioxidantes procedentes de diferentes plantas [73,74] o con antioxidantes individuales, generalmente del olivo [69,75], la mayor parte de las investigaciones hacen uso de los extractos de hojas [64,65] y en menor proporción del alperujo [55].

Aunque lo deseable es utilizar un extractante no tóxico (preferentemente agua o mezclas etanol–agua), algunos autores han extraído los antioxidantes de las hojas de olivo mediante metanol y han llevado el extracto a sequedad para después reconstituirlo en etanol [79,80]. El grupo en el que se integra la doctoranda ha demostrado en repetidas ocasiones que las mezclas etanol–agua constituyen excelentes extractantes para estos compuestos, tanto cuando proceden de hojas como cuando provienen de alperujo [39,44,65,81–83].

El estudio de las mejoras derivadas del enriquecimiento con antioxidantes ha tenido diferentes orientaciones y, generalmente, cada grupo de investigación ha adoptado una o varias de ellas. Así, mientras unos autores han considerado exclusivamente el nivel de antioxidantes antes y después del enriquecimiento [65,78], la actividad antioxidante [80], o esta actividad más el efecto en el índice de amargor [75]; otros han comparado la estabilidad que

confiere a los aceites la adición de un antioxidante artificial y un extracto de antioxidantes naturales [79], o la cantidad de extracto de hojas necesaria para conseguir una estabilidad en el almacenamiento del aceite de oliva refinado similar a la del VOO con un contenido dado de antioxidantes intrínsecos [84].

Un estudio de la dependencia del grado de enriquecimiento de la composición en ácidos grasos de diferentes aceites (de orujo, girasol, girasol con alto contenido en oleico, coco y linaza) ha puesto de manifiesto que los aceites con mayor concentración de ácidos grasos poliinsaturados tienen un factor de distribución de los antioxidantes más favorable que los de alta concentración de ácidos grasos saturados [85].

Los estudios de estabilidad frente al calentamiento del aceite de girasol mejorada por la presencia de un inhibidor de la oxidación (BHT) o un extracto de hojas de olivo han mostrado la superioridad de este último [86]; lo que apoya la tendencia actual del uso de antioxidantes naturales.

Los Capítulos 11 y 12 de esta Memoria recogen los estudios realizados por la doctoranda en este ámbito, en los que se utilizaron tanto extractos de hojas como de alperujo para enriquecimientos en 200 y en 400 mg/L de fenoles totales de aceites de maíz, soja, girasol, girasol con alto contenido en oleico, oliva refinado y colza. La comparación del comportamiento de cada aceite a cada enriquecimiento con el del VOO con un contenido de fenoles totales de 400 mg/L [61,87] se documenta ampliamente en la sección de discusión de esta Memoria.

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MATERIALES Y HERRAMIENTAS ANALÍTICAS

En este apartado de la Memoria se describen someramente las diferentes muestras así como los instrumentos y aparatos usados durante el desarrollo experimental de la Tesis. En los diferentes capítulos se incluye una explicación más detallada de los que se han utilizado en cada uno de ellos.

MUESTRAS

En la investigación realizada se han utilizado dos tipos de muestras: Aceites de diferentes variedades de aceituna y de diferentes procedencias, y desechos de la industria aceitera, como hojas y alperujo.

En los estudios de comparación de métodos de extracción de la fracción insaponificable, así como en los de caracterización de fenoles (Capítulos 3 y 5, respectivamente) se utilizaron muestras de VOO monovarietales provenientes de diferentes áreas geográficas, especificadas con más detalle en cada capítulo. Para la comparación de la fracción insaponificable de VOO y aceites refinados (Capítulo 4) se utilizaron, además de VOO, cuatro tipos de aceites refinados (de girasol, soja, orujo y oliva), seleccionados en función de la frecuencia de consumo.

Para los Capítulos del 6 al 10 se usaron muestras obtenidas a partir de los cruces procedentes de variedades de olivo del programa de mejora varietal establecido por la cooperación de la Universidad de Córdoba (UCO) y el IFAPA (Centro "Alameda del Obispo") desde 1991 en Córdoba. El esquema del programa de mejora se describe con más detalle en cada capítulo.

En el último bloque de esta Tesis, dedicado al enriquecimiento de aceites, se usaron muestras de alperujo proporcionadas por Núñez de Prado, C.B. (de Baena, Córdoba), obtenidas durante la cosecha 2009/2010 y hojas provenientes de olivos de las variedades Picual, Picudo y Hojiblanca. Los aceites que se enriquecieron fueron aceites refinados, con un coste inferior a 1 €/L, de maíz, soja, girasol, girasol alto oleico, colza, oliva y orujo.

EXTRACCIÓN DEL ACEITE DE OLIVA

La extracción del aceite de oliva en el caso de las muestras obtenidas de variedades procedentes del programa de mejora se llevó a cabo por el método Abencor, que requiere la utilización de un sistema mecánico que simula el proceso industrial. El sistema consiste en triturar las aceitunas para romper las estructuras vegetales de las células que retienen el aceite, batir la pasta lentamente y separar las fases líquida y sólida mediante centrifugación. La fase líquida se somete a decantación para separar el aceite de la fracción acuosa. El sistema consta de tres elementos fundamentales: molino, termobatidora y centrífuga.

PREPARACIÓN DE MUESTRA

En los aceites se llevó a cabo el estudio de la fracción fenólica, la fracción insaponificable y la fracción de ácidos grasos. El pretratamiento de muestra para la determinación de compuestos fenólicos se basó en extracciones líquido–líquido. Los ácidos grasos requirieron una etapa de transesterificación en frío previa al análisis de los ácidos grasos esterificados (EFAs) y una metilación en caliente en medio ácido previa al de los ácidos grasos no esterificados (NEFAs). Para el análisis de compuestos insaponificables se llevó a cabo una saponificación de los aceites, haciendo uso o no de energías auxiliares como calentamiento a reflujo, ultrasonidos o

microondas. Esta última energía auxiliar también se utilizó para la extracción de los compuestos fenólicos presentes en los desechos de la industria aceitera (hojas y alperujo). Un paso previo en caso de las hojas fue su secado y triturado. Los extractos obtenidos de las muestras en cada caso se centrifugaron y se evaporaron mediante el uso de un rotavapor para preconcentrar los fenoles extraídos.

SISTEMAS NO CONTINUOS PARA LA PREPARACIÓN DE MUESTRA

Se describen a continuación los sistemas de tipo no continuo utilizados para la preparación de la muestra:

Dispositivo de microondas. Se utilizó un digestor comercial Microdigest 301 fabricado por Prolabo, basado en microondas focalizadas, para la extracción de los compuestos fenólicos de las hojas y alperujo del (Capítulo 11), y como energía auxiliar durante la saponificación de los aceites (Capítulo 3).

Sonda de ultrasonidos. La sonda de ultrasonidos utilizada fue una Branson 450 digital, que permite la selección de la amplitud de la radiación así como el modo de irradiación, continuo o discontinuo. Esta sonda se utilizó para acelerar el proceso de saponificación. Para ello la sonda se introdujo directamente en un recipiente que contenía el aceite y los reactivos, tal como se detalla en el Capítulo 3.

Molino ciclónico. El molino, un Tecator Cyclotec, se usó para triturar las hojas de olivo hasta conseguir un tamaño homogéneo de partícula (Capítulo 11).

Agitador eléctrico. Se utilizó un agitador eléctrico Selecta Vibromatic para favorecer la extracción líquido–líquido en el enriquecimiento de los aceites (Capítulo 11).

Los distintos extractos obtenidos con los dispositivos anteriores se centrifugaron mediante una Centrífuga Selecta Mixtasel-BL para separar los residuos sólidos en cada extracto, y se concentraron en un Rotavapor Büchi R-200 equipado con un baño termostatizado B-490, que se utilizó también para asistir el proceso de derivatización en el análisis de ácidos grasos. Se utilizó también un agitador MS2 de IKA para favorecer los procesos de extracción y saponificación.

INSTRUMENTACIÓN ANALÍTICA

Durante el desarrollo experimental de esta Tesis Doctoral se han empleado cromatógrafos de líquidos y de gases, así como detectores basados en ionización en llama, en absorción molecular (generalmente detectores de diodos en fila), así como espectrómetros de masas de diferente naturaleza.

Así, en el Capítulo 11, dedicado al análisis de aceites enriquecidos con extractos fenólicos de hojas de olivo y de alperujo se utilizó un cromatógrafo de líquidos Varian ProStar, equipado con una bomba ProStar 240, un detector de diodos en fila ProStar 330 y un automuestreador ProStar 410, para la determinación individual de compuestos fenólicos. La columna analítica fue una C18 Inerstil ODS-2 (250 mm de longitud × 4.6 mm de diámetro interno, 5 micras de tamaño de partícula).

En los Capítulos 8 y 9, dedicados al estudio de los cambios producidos en la composición de ácidos grasos, debidos tanto a la influencia de la maduración como al efecto de genotipo, se empleó un cromatógrafo de gases (GC) Agilent 7820A, equipado con un automuestreador, un inyector con/sin división del flujo (split/splitless) y un detector de ionización de llama

(FID). La columna analítica utilizada fue de tipo SPTM-2380 (60 × 0.25 mm, 0.2 μm).

En el Capítulo 5 se recoge la investigación dedicada a la caracterización de VOOs monovarietales, en la que la separación por cromatografía líquida y posterior detección por espectrometría de masas en tándem por triple cuadrupolo se llevó a cabo con un cromatógrafo Agilent 1200 Series LC equipado con una bomba binaria, un desgasificador, un automuestreador, un compartimento de columna termostatizado, y un espectrómetro de masas Agilent 6410 con una fuente de ionización por electrospray (ESI). Para la toma de datos y el análisis cualitativo y cuantitativo se usó el software Agilent MassHunter Workstation.

Para los estudios de la influencia de la maduración y el genotipo en el perfil fenólico (Capítulos 6 y 7), para el análisis de compuestos insaponificables (Capítulos 3 y 4), así como para la caracterización de aceites enriquecidos (Capítulo 6), se usó un equipo de cromatografía líquida (LC) acoplado a un detector de masas en tándem de cuadrupolo–tiempo de vuelo (QTOF) de alta resolución, Agilent 6540, tras la separación cromatográfica en fase reversa (C-18). En todos los casos la adquisición de espectros, el análisis cualitativo y semicuantitativo se realizaron mediante el software MassHunter.

En cuanto a los equipos no cromatográficos se utilizó un espectrofotómetro UV/vis de ThermoSpectronic Helios para la cuantificación de fenoles totales mediante el método de Folin-Ciocalteu, tal como se recoge en los Capítulo 10 y 11. Los coeficientes de extinción específica K232 y K270 se monitorizaron con un espectrofotómetro UV/vis Lambda 25 Perkin-Elmer, y se empleó un equipo Rancimat para medir la estabilidad oxidativa de aceites en condiciones drásticas (elevadas temperaturas y con corriente de aire).

TÉCNICAS QUIMIOMÉTRICAS

De acuerdo con la importancia de la quimiometría en química analítica, en esta Tesis Doctoral se han utilizado extensamente herramientas quimiométricas para el tratamiento de datos, que implicaron distintos programas informáticos: lenguaje estadístico (R), Statgraphics, the Unscrambler y Mass Profiler Professional. Con ellos se realizaron:

- El alineamiento de picos cromatográficos.
- La normalización de matrices de datos por transformaciones logarítmicas.
- El análisis de varianza (ANOVA) para evaluar la influencia de variables de estudio sobre las muestras.
- El análisis no supervisado mediante componentes principales (PCA), que permitió detectar agrupamientos entre muestras.
- El análisis supervisado por regresión parcial por mínimos cuadrados (PLS) para el desarrollo de modelos de predicción/discriminación.
- El análisis de clúster mediante mapas auto-organizativos (SOM) o mediante mapas de calor, para encontrar similitudes entre muestras teniendo en cuenta la abundancia de sus perfiles.
- El análisis de Pearson para evaluar correlaciones entre pares de variables.
- La evaluación de cambios de concentración relativa mediante algoritmos para identificar aquellos compuestos que cambian su concentración de forma relativa debido a la influencia de

un factor o variable. La utilización de estos algoritmos está destinada a detectar los compuestos que pueden contribuir a explicar una determinada fuente de variabilidad.

PARTE EXPERIMENTAL

SECCIÓN A: ESTUDIO DE LA FRACCIÓN INSAPONIFICABLE DEL VOO Y DESARROLLO DE MÉTODOS ANALÍTICOS PARA SU CARACTERIZACIÓN

En esta Sección A de la parte experimental de esta Memoria se recogen en primer lugar las publicaciones —revisiones bibliográficas— que han contribuido a la formación teórica de la doctoranda en dos aspectos fundamentales: i) En la interpretación crítica de la investigación publicada por otros autores, tanto en lo que se refiere a la lectura de publicaciones individuales, como a la comparación de resultados obtenidos por diferentes autores, e incluso con los propios que han resultado de la investigación en el tema de las revisiones. ii) En la puesta al día sobre lo que se ha publicado en la materia en cuestión, de forma se adquiera una base sólida sobre la que soportar la investigación a desarrollar, con la que se dé un paso más, se ratifique una aportación ya realizada o se pongan de manifiesto unos resultados erróneos —deseablemente demostrando las causas del error. Las dos publicaciones, que se recogen en los Capítulos 1 y 2, tienen una temática común —la fracción minoritaria del VOO—, pero abarcan dos aspectos distintos, ambos tan amplios que han justificado el desdoblamiento. En la primera se describen y discuten las características y propiedades de las diferentes familias que componen esta fracción; la segunda se dedica a los métodos analíticos para cada familia; lo que da lugar a un amplio abanico de procedimientos de tratamiento de la muestra, de separación de los analitos —individual o no, según se requiera— y de su determinación.

La experiencia adquirida en la revisión sobre la fracción minoritaria del VOO dio lugar a la investigación que se recoge en los Capítulos 3 y 4. En el primero de ellos se comparan cuatro métodos para la separación de la fracción insaponificable, que en todos los casos se analizó mediante LC–MS de alta resolución y se realizó el correspondiente tratamiento quimiométrico de los datos obtenidos. El método de saponificación más adecuado se

seleccionó para el estudio que se recoge en el Capítulo 4, aplicándolo a la obtención de esta fracción en VOOs monovarietales y en diferentes aceites refinados para poner de manifiesto las diferencias y semejanzas existentes entre ellos en lo que se refiere a esta fracción.

CAPÍTULO 1:

Nonsaponifiable compounds in virgin olive oil. Part 1. Chemistry and properties

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Nonsaponifiable compounds in virgin olive oil. Part 1. Chemistry and properties

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Nonsaponifiable compounds in virgin olive oil. Part 1. Chemistry and properties

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Abstract

BACKGROUND: Minor components in virgin olive oil (VOO) seem to play a key role in the beneficial effects of this oil on several diseases; effects that stand from a prominent and well-balanced chemical composition, which is a blend of major compounds (98% of total oil weight) and minor compounds, which have not previously been reviewed jointly.

RESULTS: The minor compounds of VOO (or nonsaponifiable fraction), divided into the two characteristic groups have been reviewed. Therefore, the chemical characteristics and healthy properties of both compounds no chemically related to fatty acids such as hydrocarbons, alcohols, sterols, phenols, pigments or volatiles; and fatty acid derivatives such as phospholipids, waxes and sterol esters, have been discussed and critically compared when required. It has been emphasized that the nature of some of the minor compounds no chemically related to fatty acids (*e.g.* tocopherols and sterols) can be found in other vegetable oils; on the contrary, others are exclusive of *Olea europaea* species endowing it with chemotaxonomic interest.

CONCLUSION: The knowledge of the characteristics and properties of the VOO minor fraction may lead to a better exploitation of these exceptional metabolites.

INTRODUCTION

The Mediterranean diet includes virgin olive oil (VOO) as primary source of fat intake replacing commonly used animal fats, which seem to be detrimental to human health.[1,2] VOO, obtained from mechanical pressing of olive drupes, is considered one of the most priced vegetable oils thanks to its organoleptical properties and benefits on human health.[3,4] Its chemical composition is characterized by two main groups of components (Figure 1): major components (≈98%), and minor components (≈2%).[5] The first fraction is mainly constituted by esterified fatty acids (FA), predominantly in the form of triacylglycerides (TAGs) and small concentrations of diacylglycerides (DAGs), monoacylglycerides (MAGs), free fatty acids (FFA) and phospholipids, and characterized by a high concentration of monounsaturated FA (MUFA), especially oleic acid, to the detriment of polyunsaturated FA (PUFA), aspects that seem to contribute to reduce the risk of atherosclerosis,[6] and protect from different kinds of cancer.[7]

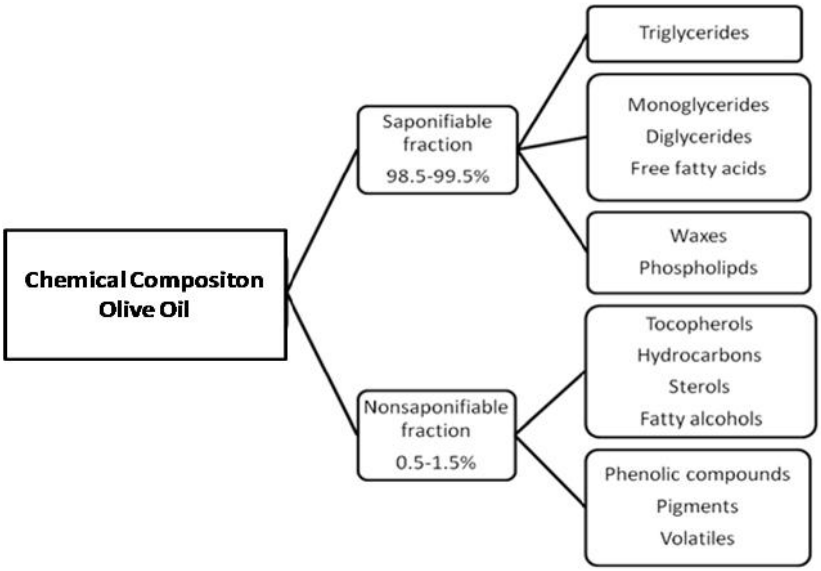

Figure 1. Scheme of chemical composition of olive oil (saponifiable and non-saponifiable fractions).

There are two types of minor compounds soluble in the lipid fraction: glycerolipids and nonglycerolipids. Upon reaction with alkali, the latter subfraction or nonsaponifiable fraction of VOO does not form soap and can be extracted from the saponified mixture with lipophilic solvents such as diethyl ether, hexane, or cyclohexane in the case of low-polar compounds; while separation from VOO of polar compounds such as phenols does not require saponification as they remain in the polar phase when nonpolar VOO components are removed by a nonpolar extractant such as hexane. Over the past decades, nonsaponifiable compounds have aroused the interest of researchers after observation that many of these compounds are endowed with antioxidant properties, the key support of the high oxidative stability of VOO. This is the reason why much research is currently focused on the biological and physiological activities of various nonsaponifiable constituents and their possible contribution to oil quality, with proved effects on improvement of users health.[8,9]

The aim of this article was to review the knowledge about VOO by providing updated information on the composition of the nonsaponifiable fraction and chemical characteristics of its components as well as on recent studies concerning its biological properties, and effects on humans.

CHEMICAL STRUCTURES AND HEALTHY PROPERTIES OF THE MAIN SECONDARY METABOLITES IN THE NON-SAPONIFIABLE FRACTION

Sterols

Sterols are among the most abundant compounds of the nonsaponifiable fraction, with a known wide range of biological activities; therefore, they constitute one of the most studied classes of this fraction, derivated from hydroxylated polycyclic isopentenoids. These compounds

contain a total of 27–30 C atoms with a side chain of 7 or higher number of C atoms attached to the carbon at position 17. Sterols, plant sterols or phytosterols, can be classified on a structural or biosynthetic basis as 4-desmethyl sterols, 4α-monomethyl sterols and 4,4-dimethyl sterols. In addition, 4-desmethyl sterols may be subdivided into Δ5-sterols, Δ7-sterols and Δ5,7-sterols, depending on the position of the double bonds in the β ring.[10]

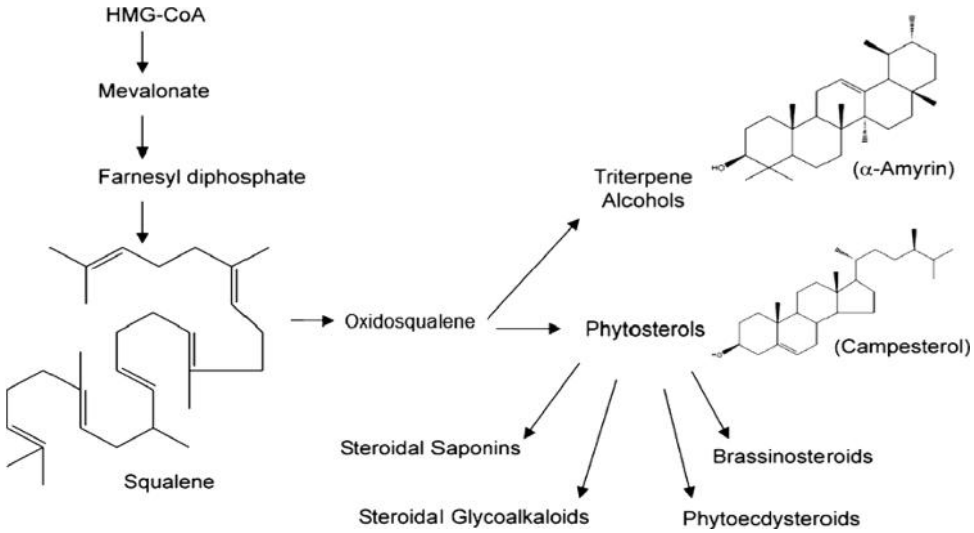

Figure 2. Biosynthesis of sterols and triterpenoids via the acetate–mevalonate pathway.

Phytosterols can be present either in free form or esterified by FA. The main VOO sterols are β-sitosterol (75–90% of sterols fraction), Δ-5-avenasterol and campesterol that, after saponification, might be present within the range 1000–2500 mg kg^{-1} oil.[11] The concentration of these and other sterols in VOO strongly depends on the olive tree cultivar.[12] Compositional analysis of the sterol fraction in VOO can be used to assess the quality of the oil and the absence of other vegetal oils.[13,14] Nevertheless, sterols are not only in VOO; in fact, natural sources of these compounds in the human diet, in addition to other oils and margarines, are a range of seeds

and legumes.[15] The composition of phytosterols in plants depends on the plant species,[16] and, in oils, it may vary according to agronomic and climatic conditions, the quality of the fruits or seeds, extraction and refining procedures, and storage conditions.[17] Sterols, including precursors of cholesterol and their metabolites, seem to be biologically active, although many of their biological effects require to be assessed.

Sterols are membrane components and, as such, they are involved in the regulation of its fluidity and permeability. In plants, where they are always present in mixtures, sterols act as substrates for the production of a wide variety of secondary metabolites such as cardenalids, glycoalcaloids, pregnane derivatives, and saponins (see Figure 2).[18]

The similar structure of plant sterols and cholesterol led Beneke to an erroneous identification of sterols as cholesterol in peas.[19] This error was extensively demonstrated as such by the research developed on the subject since the early fifties of the last century. The nutritional interest of sterols comes from their structure, similar to that of cholesterol, and their capacity to lower plasma cholesterol, mainly LDL cholesterol, by reducing the absorption of cholesterol from the gut by competing for the limited space for cholesterol in mixed micelles.[20,21] The similarity of sterols and cholesterol can be visualized in Figure 3 (in fact, cholesterol is a specific type of sterol that contains 27 C atoms, 46 hydrogen atoms and one oxygen atom in a ring structure). The existence of a Δ5 double bond and a 3β-hydroxyl group is common to cholesterol and sterols, being the differences in structural modifications of the side chain. Campesterol and sitosterol, 24-methyl and 24-ethyl cholesterol analogues, are the most abundant phytosterols in human plasma. They are a part of the diet and are exclusively taken up from the intestine. Although the dietary intake of plant sterols is usually high, their levels in plasma are usually lower than 1 μg L^{-1}; the reasons for this low concentration being poor intestinal absorption rates and high biliary removal

rates as compared to cholesterol.[22] The morbidity and mortality owing to cardiovascular diseases have been dramatically reduced using cholesterol lowering drugs (statins); therefore, the interest in sterols lies in their potential to act as a natural preventive dietary product.[23,24] Also, phytosterols are recognized as biologically active substances in cancer prevention, although this effect has still not been assessed by epidemiological studies.[25] Also the availability of sterols to bind to nuclear receptors and activate target gene transcription has encouraged much new research in this area.[26]

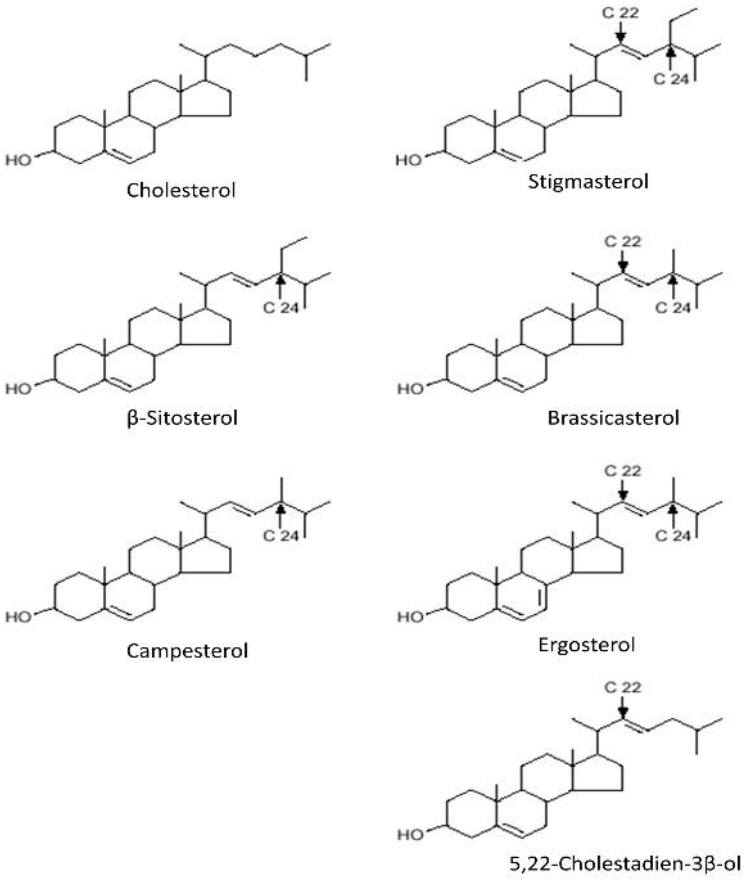

Figure 3. Chemical structure of main plant sterols.

Esterification of sterols increases their solubility.[27] In this regard, phytosterols are more efficient when mixed with fats than in isolated intake.[28] The suggestion that small amounts of fat (yogurt with 0.7% fat) can be effective to lower cholesterol[29] is supported by authors who have found that solubilization of sterols in phospholipids and diacylglycerol increases bioavailability.[30,31]

Moreover, phytosterols are useful emulsifiers in cosmetics manufacture and supply the majority of steroidal intermediates and precursors for the production of pharmaceutical hormones.[32] Sterols with specific structures inhibit oxidative deterioration of oils acting as potential antipolymerization agents for frying oils.[33]

Table 1. Main sterols present in VOO and pomace oil, and percentage of each, as analyzed by GG–FID

Compound	**VOO**	**Pomace oil**
Cholesterol	Trace	Trace
Brassicasterol	—	Trace
24-Methylene cholesterol	—	—
Campesterol	3.2	3
Campestanol	—	0.2
Stigmasterol	0.7	1.6
Δ7-Campesterol	—	—
Δ5,23-Stigmastadienol	—	1.4
Clerosterol	0.8	1
b-Sitosterol	86.8	73.2
Sitostanol	0.6	2.8
Δ5-Avenasterol	6.7	0.7
Δ5,24-Stigmastadienol	0.3	1.4
Δ7-Stigmastenol	0.1	0.2
Δ7-Avenasterol	0.4	0.2

Table 1 lists the main sterols present in VOO and pomace oil showing the highest concentration in the latter, except for β-sitosterol and Δ5-avenasterol.

Fatty alcohols

The most significant classes of fatty alcohols (aliphatic alcohols, triterpene alcohols and triterpene dialcohols, and diterpenoids) in VOO are discussed below.

Aliphatic alcohols

These compounds consist of a linear hydrocarbon chain containing a hydroxyl group, usually at the terminal position. The major mechanism of fatty alcohols formation is reduction of FA through aldehyde intermediates; finally, the formed alcohols can esterify FA to form waxes. These alcohols are mainly originated in plants, but also synthesized by animals and algae.

The concentration of these alcohols in VOO (but also in refined olive oils) may not surpass the legal limit of 350 mg kg^{-1} oil, the most abundant being hexacosanol (C26), octacosanol (C28) and tetracosanol (C24); while tricosanol (C23), pentacosanol (C25) and heptacosanol (C27) may be present at trace levels;[34] nevertheless, the olive tree cultivar seems to play a key role in the concentration of this class in the nonsaponifiable fraction.[35]

The significance of these alcohols in nutrition and health is at present recognized, as they are closely related to fatty acids. Research on the use of long-chain fatty alcohols from pomace olive oil has shown the ability of these alcohols to inhibit the in vitro release of different proinflammatory mediators by cells involved in inflammatory processes, and also the significant and dose dependent decrease of nitric oxide production by murine macrophages stimulated by liposaccharides.[36] Reduction of tumor necrosis factor-α, prostaglandin E_2 production and thromboxane A_2 production by these

alcohols suggests their protective effect and potential role as putative functional components.[36] Medium and long chain fatty alcohols have shown to be endowed with anticancer, antiviral, and antifungal properties, and thus, with potential application either in medicine and/or as health supplements.[37]

Octacosanol, present in carrots and ginseng, was found in 1994 to lower cholesterol levels.[38] Also, very long chain fatty alcohols (VLCFA) obtained from plant waxes and beeswax have been reported to lower plasma cholesterol in humans.[39]

Triterpene alcohols and triterpene dialcohols

Triterpene alcohols, also known as 4,4-dimethylsterols, possess a steroid structure and are present in all fatty vegetables at different levels. Their content in olive oil is within the range 1000–1500 mg kg^{-1} oil, the most abundant being α-amyrin, β-amyrin, cycloartenol, butyrospermol and 24-methylencycloartanol.[40]

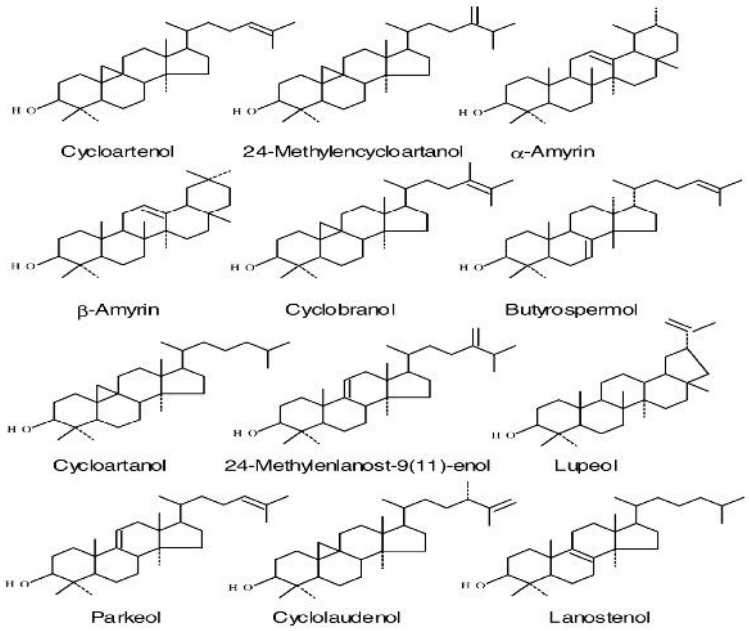

Figure 4. Chemical structure of main plant triterpenic alcohols.

Triterpene alcohols are common components of natural herbal preparations used for treatment of human diseases. Chemically, all pentacyclic triterpenes are based on a 30 C skeleton comprising five six-membered rings (ursanes and oleananes) or four six-membered rings and one five-membered ring (lupanes) (see Figure 4). As lipophilic molecules, they may penetrate the blood brain barrier and exert a neuroprotective effect.[41,42]

Concerning triterpene dialcohols, the most abundant in VOO are erythrodiol (homo-olestranol, 5α-olean-12-en-3β, 28-diol) and uvaol (Δ-12-ursen-3β, 28-diol), mainly from the olive fruit epicarp; therefore, while solvent extraction leads to high amounts of these compounds in refined olive oil, they are absent or present at low levels (within the range 10–200 mg kg^{-1} oil) in VOO. These dialcohols are present in pomace oil at about 116 g kg^{-1} oil.[43]

Triterpenes, mono and dialcohols are bioactive secondary metabolites with a wide range of biological activity, present in olives, but also are constituents of other plants, fruits and vegetables. They occur widely in the plant kingdom both as free acids and aglycones of triterpenoid saponins. Remarkably, the literature contains relatively sparse information on the distribution of triterpenic compounds in plants, despite their pharmacological properties that include anti-inflammatory, hepatoprotective, antitumor, antiviral, antimicrobial,[44] antifungal, antidiabetic, gastroprotective, and antihyperlipidemic effects, antifeedant, and insecticidal activities.[45–48] Other studies have also shown a relationship between triterpenes and vaso-relaxation in the aorta of hypertensive rats.[49] Recently, the action of erythrodiol derivatives to prevent skin aging or skin cancer promoted by ultraviolet radiation has been suggested.[50] Both acidic and alcoholic tri-terpenes seem to possess antitumor activities, although the different authors markedly differ in the cytotoxic activity of these compounds, and the precise mechanism of action is still unclear, especially for triterpenic diols.

Oxidized triterpenic dialcohols such as uvaol and also oleanolic acid and the maslinic acid derivative methyl maslinate have shown vasodepressor, cardiotonic, and antidysrhythmic properties.[51] Oleanolic and maslinic acids are also powerful proapoptotic agents in human colon cancer cells, while uvaol shows weak activity against an array of human cancer cell lines from different tissues.[52–54]

Diterpenoids

The VOO alcoholic fraction also includes acyclic diterpenoids, among which the most important are phytol and geranylgeraniol —the latter generated from chlorophylls. These compounds, endowed with antibacterial properties,[55] are present in VOO at concentrations around 25–595 mg kg^{-1} and 50 mg kg^{-1} for phytol and geranylgeraniol, respectively.

Tocopherols and tocotrienols

Tocopherols are constituted by 6-OH-chromane ring and a lateral chain of 16 C atoms. The carbon chain in tocopherol structure exists in two forms; the saturated form characterizes tocopherol and three insaturations characterize tocotrienols. Tocopherols and tocotrienols exist in forms α, β, γ, δ, according to the position of the methyl groups. These compounds (see Table 2)[56] display antioxidant properties and are active as vitamins (vitamin E), thus making them of especial interest for human health.[57,58] The antioxidant properties of tocopherols are attributed to α-tocopherol as it represents 90–95% of them.[57] Olive oils contain a higher percentage of β-tocopherol than of γ-tocopherol and only traces of δ-tocopherol. Tocotrienols are present in small amounts in food lipids, except in palm and grapeseed oils, and the annatto lipid fraction, which contain a relatively high content of these active components.[59,60] Concentrations of tocopherols and tocotrienols have been reported to be within the range 12–400 mg kg^{-1} in high quality oils; while the refination process causes loss of them.

Table 2. Main tocopherols in olive oil

Trivial name	IUPAC name	Abbreviation
7-Methyltocol	2,7-Dimethyl-2-(4',8',12'-trimethyltridecy]-6-chromanol	7-T
δ-Tocopherol (8-Methyltocol)	2,8-Dimethyl-2-(4',8',12'-trimethyltridecyl)- 6-chromanol	δ-T
5,7-Dimethyltocol	2,5,7-Trimethyl-2-(4',8',12' trimethyltridecyl)-6-chromanol	5,7-T
β-Tocopherol (5,8-Dimethyltocol)	2,5,8-Trimethyl-2-(49,89,129-trimethyltridecyl)-6-chromanol	β-T
γ-Tocopherol (7,8-Dimethyltocol)	2,7,8-Trimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol	γ-T
α-Tocopherol (5,7,8-Trimethyltocol)	2,5,7,8-Tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol	α-T

Reported rates of absorption of tocopherols and tocotrienols following food intake vary from a high range (51–86%) to a low range (21–29%). The forms of vitamin E, including all tocopherol and tocotrienol homologues, are similarly absorbed.[61] Tocopherols contribute to the stability of VOO, have a key biological role as antioxidants, and preserve oil quality during storage.[62] Some authors have suggested tocopherols as tracers for identification and differentiation of vegetable oils.[63] Moreover, tocotrienols are potential markers of the presence of palm and/or grapeseed oils in VOO.[64]

Hydrocarbons

Hydrocarbons are formed by homologous series of linear compounds that are mainly saturated chains of 15–33 C atoms (in food matrices, most hydrocarbons have an odd number of carbon atoms). Small amounts of ramified isomers are also present.[65]

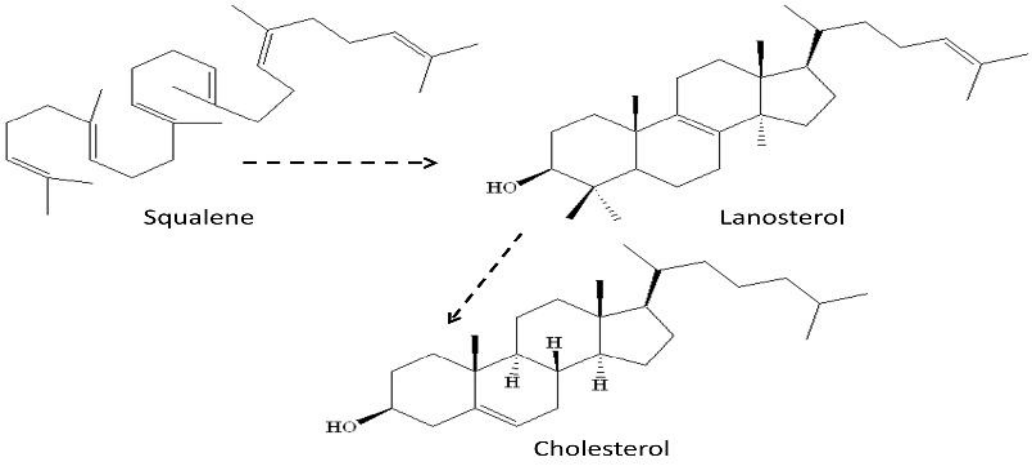

Figure 5. Chemical structure of squalene and biochemical pathway of cholesterol synthesis.

Hydrocarbons are in natural lipids at very small amounts (≤0.2% of the total lipids), the only exception being VOO that contains about 0.5%, mainly constituted by squalene. This compound, a biochemical precursor of sterols (see Figure 5), is a key hydrocarbon in VOO and refined olive oil. While VOO contains around 4000 mg squalene kg^{-1} oil, and in refined olive oil the content is close to 3000 g g^{-1}, other edible vegetal oils do not surpass 240 g squalene kg^{-1} oil.[66] Squalene, a member of the terpenoid family and a precursor of cholesterol biosynthesis, is a metabolite of great present interest.[67,68] It is synthesized by humans[69] and also by a wide number of organisms and substances; from sharks to olives and even it exists in bran, among others.[70,71] Because of its significant dietary benefits, biocompatibility, inertness, and other advantageous properties, squalene is extensively used as

an excipient in pharmaceutical formulations for disease management and therapy.[72–74] In addition, this terpenoid acts as a protective agent by decreasing side effects induced by chemotherapy, it has chemopreventive activity,[75] and despite its evidenced action as weak inhibitor of tumor cell proliferation, it contributes either directly or indirectly to the treatment of cancer due to its effect as inhibition potentiator.[76,77] In addition, squalene enhances the immune response associated to various antigens, and is frequently used for preparation of stable emulsions as either the main ingredient or a secondary oil.[78] These emulsions have been used for various applications, especially to deliver vaccines, drugs, and other medicinal substances.[79] A review on squalene benefits in the different areas of application was published in 2009.[80]

Small amounts of other hydrocarbons such as polycyclic aromatic hydrocarbons (*e.g.* PAHs such as phenanthrene or pyrene) or benzene have also been found in VOO —the last as a contaminant from gasoline.[81,82]

Pigments

The color of olive oil is due to 2 types of natural pigments, chlorophylls and carotenoids.[83] Chlorophyll pigments account for the greenness of the oils, while carotenoids account for their yellowness. Pigment richness in oils is characteristic of the olive tree cultivar, giving place to great differences or similarities between cultivars. Ripening of the fruit involves pigment loss, disappearance of chlorophylls always being slightly greater than that of carotenoids. However, independently of the cultivar and the different pigment content of the fruit, the ratio between chlorophylls and carotenoids remains within 2.5 to 3.7 mg total chlorophylls per mg total carotenoids.[84] Nevertheless, the interest in the color of olive oil, as that of many other products, goes beyond its relationship with consumer choices and commercial practices. The raising interest in oil pigments is due to their likely health benefits, the fact that color can be used for the rapid assessment of

the levels of these health promoting compounds, and for quality control in the food industry. In this regard, there is in the literature a number of multivariate statistical methods to correlate color parameters with pigment contents in olive oils.[85–87]

Carotenoids

Carotenoids are isoprenoid compounds that have a hydrocarbon structure with several conjugated double bonds (CDBs) (Figure 6) that accounts for many of their properties and the actions they are involved in.[88]

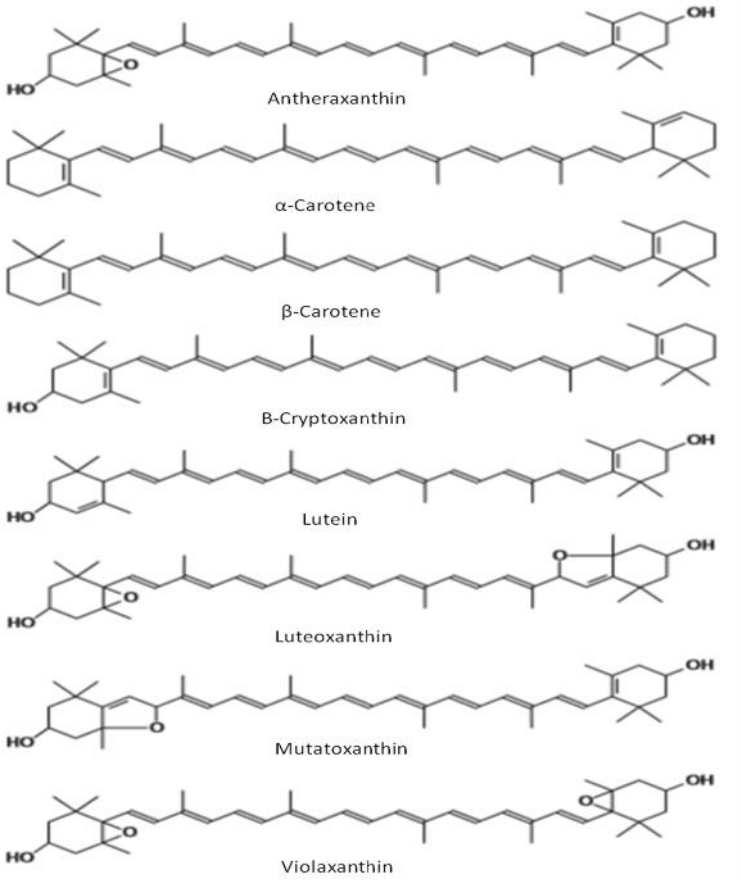

Figure 6. Chemical structure of some carotenoids reported in olive oil.

Most of the described carotenoids have 40 C atoms, although there are carotenoids with shorter and longer number of carbons. Depending on the presence or absence of rings in the molecule, they can be classified into cyclic or acyclic carotenoids. Likewise, they are divided into carotenes (carotenoids containing only carbon and hydrogen) and xanthophylls (carotenoids that also contain oxygenated functions, like epoxide, hydroxyl, acetate, carbonyl, and carboxylic groups, among others). In any case, carotenoids in natural structures can be free or associated with other compounds, such as fatty acids, sugars, and proteins.[83,89–91]

The color of carotenoids is due to the chromophore of CDBs, with high electrons delocalization (phytoene and phytofluene, with 3 and 5 CDBs, respectively, are colorless). Carotenoids with 7 or more CDBs absorb light maximally between 400 and 500 nm.[90,92] Although carotenoids are rather stable in their natural environment, they are very labile once extracted, as the loss of cellular compartmentation can promote isomerization or degradation, particularly by oxidation.[93,94] This fact explains why some pigments such as pheophytins, mutatoxanthin, luteoxanthin, which are not present in recent harvested olive drupes, can be found at a long storage stage or in the oil itself.[95–97]

Apart from their functions in the plant,[98] carotenoids have attracted the attention of scientists for decades due to their nutritional importance. Thus, some of them such as *β*-carotene, *α*-carotene, and *β*-cryptoxanthin are precursors of vitamin A, and very effective antioxidants.[99–101] Concerning major carotenoids in olive oil both *β*-carotene and lutein are thought to provide several health benefits. Vitamin A activity of *β*-carotene has for long been known; however, the interest in this compound was revived about 25 year ago as a result of its likely antioxidant activity, a line of research of present interest.[102] Likewise, much attention is being paid to its probable beneficial effects on cancer prevention.[102–104] However, there is still certain controversy about the goodness of *β*-carotene,[105,106] in most cases owing to

an inappropriate design of the experiments. Lutein is also receiving much attention because its nutritional relevance[107,108] that has resulted in its addition to animal feeds and use as human supplement.[109,110] This pigment is being extensively studied as it seems to accumulate in the macula lutea of the fovea together with zeaxanthin, and both are thought to be beneficial for prevention of cataracts and age-related macular degeneration.[111–113] Furthermore, lutein exhibits antioxidant activity,[113] and exerts a protective effect against DNA damage;[114,115] thus resulting in increased interest in lutein related to its status, bioaccessibility,[116] and bioavailability.[117] The high lipophilic character of carotenoids makes complex their absorption, a process that requires release from the food, incorporation into micelles, subsequent uptake for enterocytes and release to the circulation system. Researchers in this field consider that absorption of these compounds could be improved by the presence of oil, which means that olive oil can be a perfect vehicle for absorption of native carotenoids and those present in other foods simultaneously consumed.[118,119]

Chlorophylls

The structure of chlorophyll pigments consists of tetrapyrrole macrocycles (one of which is reduced), which contain an additional isocyclic ring. The macrocycle is coordinated to one Mg^{2+} ion to form a very stable planar complex, as shown in Figure 7. This structure contains a chromophore of several CDBs, responsible for absorption of these pigments in the visible region of the spectrum. Both the blueish–green chlorophyll *a* and the yellowish–green chloprophyll *b* can be found in olive oils, in which the ratio between both pigments could be in the range 6–8.[120] The fundamental difference between both chlorophylls relies on C3 substituent: a methyl group in the former and a formyl group in chlorophyll *b* (Figure 7). The hydrophobic nature of these pigments is due to esterification with a phytol group (R1 in Figure 7). Other chlorophyll pigments are pheophytins, chlorophyllides, pheophorbides, pyropheophytins, chlorines, rhodins, and

purpurins.[83] Pheophytins form by replacement of the magnesium ion in the chlorophyll molecules by 2 protons.

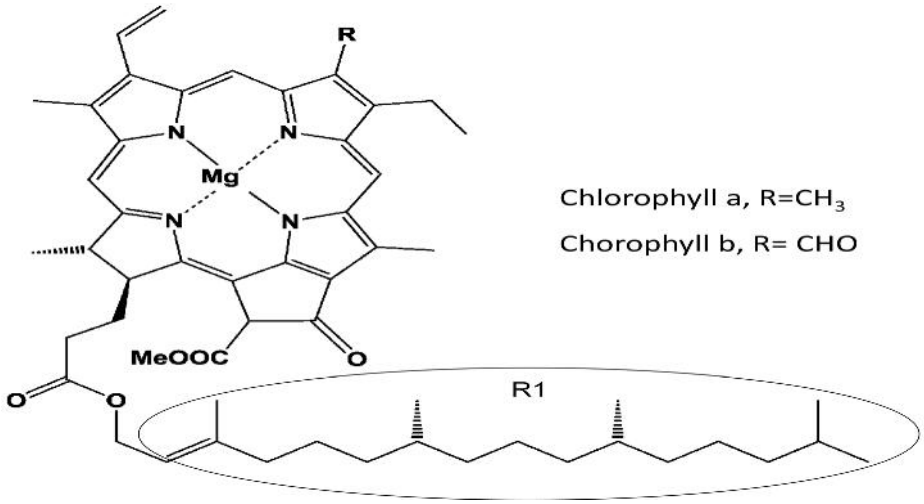

Figure 7. Chemical structure of chlorophylls a and b.

The result of this change is the grey–brown color of pheophytin *a*, whereas that of pheophytin *b* is olive green. On the other hand, the chlorophyllides are derivatives of the chlorophylls, formed by action of the enzyme chlorophyllase. Chlorophyll derivatives such as pheophorbides can be formed either by enzymatic action on the corresponding pheophytin or by loss of the magnesium ion of chlorophyllides in acidic medium. Pyropheophytins, in which the $COOCH_3$ group has been replaced by a hydrogen atom, are formed from the corresponding pheophytins by prolonged heating. The isocyclic ring is open in chlorophyll derivatives such as chlorines, rhodins, and purpurins, which are the result of oxidative reactions in either acidic or alkaline medium.[83] The reactions to form the derivatives occur when the cellular compartmentation of chlorophyll containing tissues is lost and/or as a result of common senescence metabolism in the plant.[121] During VOO production, the crushing, milling, beating, and heating processes, and pH changes favor the formation of

chlorophyll derivatives; in fact, chlorophyll *a* (the main chlorophyll in olive drupes) changes to its pheophytin *a* derivative when in contact with the acid medium created in VOO production. Visually, the transformation of chlorophyll *a* into its pheophytin derivative involves a shift of color from greenish to brownish hue.

The role of chlorophylls as natural pigments accounts for greenish colors and their involvement in photosynthesis is well known; however, there are only some reports hypothesizing that chlorophyll pigments and related compounds may be beneficial to human health. Studies in the 1980s decade evidenced that some of these pigments seemed to exhibit antioxidant activity under certain conditions,[122,123] although the same authors also observed that these compounds could also act as prooxidants.[124] The interest in the possible beneficial effects of chlorophylls and related compounds has re-emerged in the current decade and new evidences on their antioxidant characteristics have appeared.[125,126] More importantly, some authors have reported that they may also be beneficial to cancer prevention.[127,128] The renewed interest in chlorophyll pigments in connection to potential health benefits, their bioaccessibility (release from the foodstuff and processing into a form that can be absorbed) and uptake by human intestinal cultured cells have been evaluated.[129,130] Their role as bioactive compounds requires absorption and introduction into the circulation system to be distributed to the tissues where they could exert their functions or actions. The contribution of these compounds to the healthy characteristics of VOO could be sparse because their low concentration.

Volatiles

The complex flavor of VOO is mainly produced by volatile and phenol compounds. Most of the formers are low molecular weight compounds (lower than 300 Da), endowed with high volatility, sufficient hydrosolubility, fair liposolubility and chemical features to bond with specific

proteins.[131] These compounds have been identified and quantified in VOO more than 40 year ago,[132] and their formation is related to olive fruit cell destruction.

Table 3. Composition of the volatile fraction of virgin olive oils extracted by HS–SPME.

Compound	**LR1**
Hexanal	792
(E)-2-Hexenal	851
Heptanal	898
1-Heptanol	965
6-Methyl-5-hepten-2-one	985
(E,Z)-2,4-Heptadienal	999
Octanal	1001
(Z)-3-Hexenyl acetate	1004
1-Hexyl acetate	1007
Limonene	1032
3-Octen-2-one	1043
4-Methyl benzaldehyde	1070
Nonanal	1104
Phenylethyl alcohol	1108
Decanal	1205
Citronellol	1229
(E)-2-Decenal	1265
Cyclosativene	1370
Longicyclene	1372
α-Copaene	1382
β-Gurjunene	1431
trans-α-Bergamotene	1439
Aromadendrene	1441
(Z)-α-Farnesene	1445
(E)-Geranylacetone	1456
α-Muurolene	1502
(E,E)-α-Farnesene	1506

The wide research on this fraction has shown that the main components contributing to the global flavor of VOO are aldehydes, alcohols, esters, and ketones. Most of these volatiles are produced through the lipoxygenase pathway.[133] In olive drupes, this biochemical pathway promotes the formation of C6 volatile compounds, from linoleic and linolenic hydroperoxides, rather than C9 compounds. Table 3 lists the main volatiles in VOO and their linear retention indices as provided by Youssef et al. using head space and solid phase microextraction (HS–SPME) for sample preparation and gas chromatography–flame ionization detection (GC–FID) and GC–mass spectrometry (CG–MS) for characterization and quantitation. In this way, more than 300 components of this fraction have been identified.[134]

The characteristic aroma of VOOs is influenced by several production and processing parameters,[135] such as agronomic features, olive cultivar, ripening degree and sanitary quality of olives, harvest system, postharvest storage of fruits, processing technology and oil bottling and storage. Among these parameters, processing technology seems to greatly determine the final quality of the oil. It is well known that the organoleptic properties of VOO are significantly affected by the crushing method, the kneading process of the olive paste, and the separation systems of crude oil or oil–water mixture from the olive paste.

The sensory characteristics of VOO change as a consequence of lipolysis and oxidation processes leading to deterioration of its pleasant flavor. Lipolysis usually starts when the oil is still in the fruit, while oxidation begins after the oil is obtained and proceeds mainly during storage. Owing to these processes, the sensory characteristics of the oil change from pleasant to unpleasant. Though VOO is considered to be a stable oil thanks to the presence of α-tocopherol and phenols such as hydroxytyrosol, tyrosol, caffeic acid, and other minor components,[136] it is susceptible to oxidation, like other vegetable oils, and some off flavors due to volatile compounds deterioration

can be detected when oxidation processes start. Consequently, the initially pleasant sensory characteristics of the oil eventually give way to unpleasant sensory attributes. While oxidation in refined vegetable oils is detected by the increase of total volatiles and the appearance of some specific volatile compounds such as hexanal,[137] VOO is initially endowed with a great amount of volatiles, some of which (as hexanal) are also present in the original flavor. Studies on oxidation markers in VOO have led to propose the hexanal–nonanal ratio to detect the starting point of oxidation and subsequent evolution.[138] Volatile compounds in oxidized VOO and threshold values, sensory properties and main hydroperoxide isomer precursors of off flavor components in VOO have also been reported.[138]

As far as the authors know, there is not information in the literature on the antioxidant character of volatiles in VOO, possibly because this property is very marked in most of the components of the minor fraction.

Phenols

These diverse compounds make up one of the major groups of secondary metabolites in plants. Phenols can be broadly divided into non-soluble compounds such as condensed tannins, lignins, and cell wall bound hydroxycinamic acids; soluble phenols such as phenolic acids, phenolic alcohols, and flavonoids; and secoiridoids. Figure 8 shows the structure of the main classes of soluble (or hydrophilic phenols), which can also be divided into simple and sterified or *seco* forms (secoiridoids); all them constituting an excellent natural defence against pathogens attack and playing a role in structuring the cell wall skeleton as cross-linkers between different polysaccharide moities. The total concentration of phenols in VOO, which widely varies but with a usual range between 500 and 1000 mg kg^{-1} oil, strongly depends on different factors such as maturation index, genetics, geographical origin, pedoclimatic conditions, agronomical and technological

factors. The refination process lowers phenols concentration in refined olive oils to 60–70 mg kg^{-1} oil.[139–142]

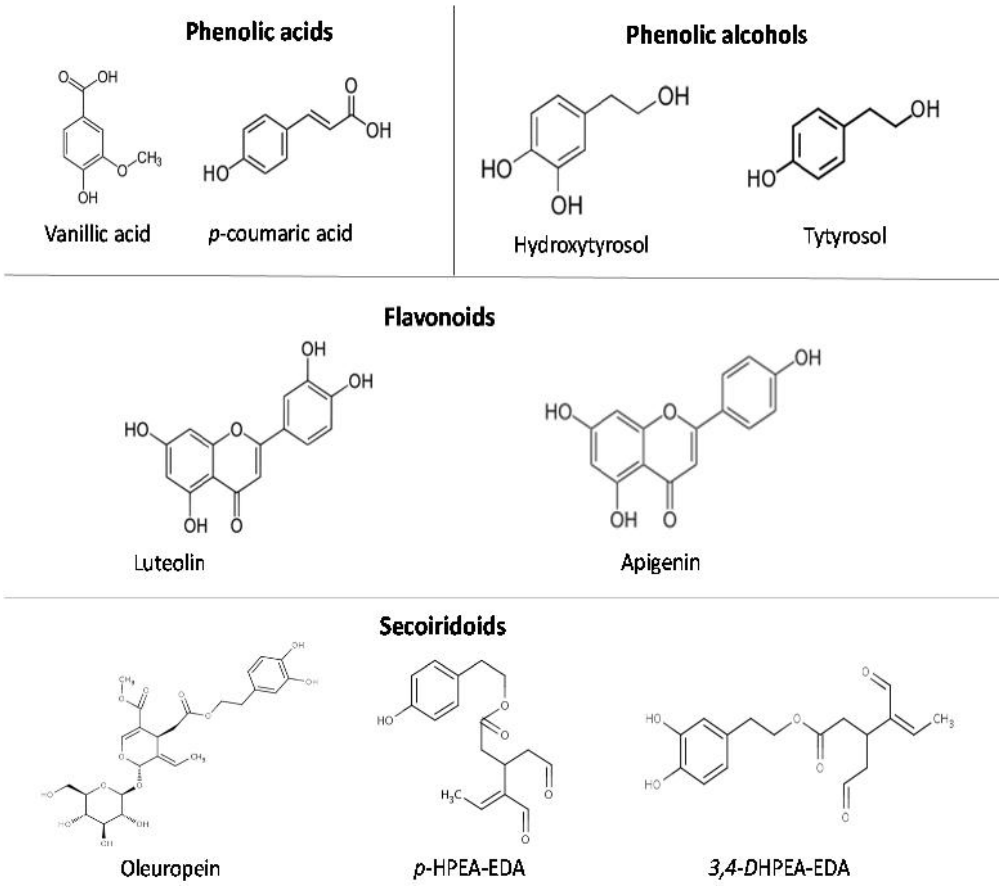

Figure 8. Chemical structures of the most representative hydrophilic phenols.

Phenols soluble in VOO are especially important to establish olive oil quality due to their antioxidant activity and effect on the organoleptic characteristics of VOO. Secoiridoids and lignans are the most abundant among these soluble secondary metabolites (despite the former could be classified as an especial type of saponifiable compounds because of their structure —see Figure 8). Within these selected classes are phenolic acids that include caffeic, vanillic, *p*-coumaric, *o*-coumaric, protocatechuic, sinapic,

p-hydroxybenzoic and gallic acids; and also ferulic and cinnamic acids, but in lower quantities (less than 1 mg kg^{-1} VOO). Key phenolic alcohols in VOO are 3,4-dihydroxyphenyl ethanol, also known as 3,4-DHPEA or hydroxytyrosol, and *p*-hydroxyphenyl ethanol *p*-HPEA or tyrosol.[143] These alcohols are at low concentration in fresh oils; concentration that increases during storage as a result of hydrolysis of olive oil secoiridoids containing 3,4-DHPEA and *p*-HPEA in their molecular structures.[144] Secoiridoids and lignans are the most concentrated secondary metabolites in VOO, and they are used as potential markers of geographical origin of olive cultivars. Most of these metabolites are also in a wide range of plants and influence the color and sensory properties of foods.[145]

Secoiridoids are both terpenic and hydroxy aromatic secondary metabolites, characterized by one or more hydroxyl substituents,[146] linked to the aromatic group that makes the basic phenol functionality. The structure of these compounds, some of them exclusive of *Olea Europaea* species, consists of a tyrosol or hydroxytyrosol molecule linked to elenolic acids or, in plants, to a glycosidic residue. They are erroneously referred to as polyphenols,[147–149] nomenclature borrowed from wine; nevertheless, secoiridoids are not polymers, but monomers of molecular weight up to 624, found in VOO in the range of 27–32 mg kg^{-1} VOO.[150] The main secoiridoids present in VOO are oleuropein and ligtroside; while the most important lignans in VOO are pinoresinol and acetoxipinoresinol, the concentrations of which are within the ranges 2–95 and 20–25 mg kg^{-1} VOO, respectively.[151]

Flavonoids are planar molecules divided into flavones, flavonols, flavanones, and flavanols classes. Luteolin and apigenin are the flavonoids present in VOO.

Concerning the evolution of VOO phenols after intake, Vissers et al. showed that oleuropein can be absorbed in the small intestine and hydrolyzed to hydroxytyrosol as it appears in urine.[152] Tyrosol, hydroxy-

tyrosol and their derivatives are absorbed by humans in a dose-dependent manner forming part of the phenolic content of the ingested VOO;[153] therefore, urinary tyrosol and hydroxytyrosol can be considered as biomarkers of phenolic compounds from VOO consumption, and a useful tool for monitoring compliance in clinical intervention studies.

The interest on phenols has increased in the last decades, becoming the most important components of the nonsaponificable fraction and stimulating multidisciplinary research on their composition, histological distribution and histochemical enzymatic localization to determine their biomolecular functions.[154] Nevertheless, the main reason of the growing research in this field lies in the healthy properties attributed to these compounds.

The excellent properties of phenols are a consequence of their function in the olive tree (namely, reactivity against pathogens attack and response to insect injury).[154] Olive phenols include a major group of secondary metabolites that display a wealth of both structural variety and key activities. The healthy effects of VOO —mainly due to the presence of these particular phenols— have been widely studied in the last decades.[145,155] Oleuropein, the most abundant phenol in olive leaves and also at high concentrations in pomace, has been used in a number of medical treatments since its first reference in the literature; thus, this phenol has shown to prevent cardiovascular diseases by protecting membrane lipid oxidation[156] acting on coronary dilation and antiarythmic action,[157] improves the lipid metabolism to mitigate obesity problems,[158] protects both enzymes and against hypertensive cell death in cancer patients,[159] and presents antiviral properties.[160] Hydroxytyrosol also improves cardiac and tumoral diseases with effects similar to those of oleuropein; in addition, it protects against atherosclerosis[161] and prevents diabetic neuropathies.[162] Recently, the EFSA (European Food Safety Authority) has considered that this phenolic alcohol is sufficiently characterized in relation to its claimed effect on protection of

LDL particles from oxitadive damage.[143] Also, the nutraceutical utility of other phenols such as verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside, present at high amounts in olive leaves and oil pomace, has been studied. Thus, verbascoside seems to exert repairing effects on brain's oxidative damage caused by heroin consumption, apigenin-7-glucoside to fight against Alzheimer's disease or liver diseases; luteolin-7-glucoside seems to avoid the abnormal proliferation of aortic vascular smooth muscle cells that is a common cause of pathogenesis such as atherosclerosis and restenosis.[162] However, among these olive phenols, only oleuropein is present in VOO at high amounts (between 2.3 and 9.0 mg kg^{-1}); while verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside are generally at low concentrations (lower than 1 mg kg^{-1}).[163,164] The continuous research on VOO phenolic compounds has led to the isolation of compounds such as oleocanthal, whose interesting ibuprofen like activity,[165] and recent evidence about its action on Alzheimer[166] have been reported.

Wider information on the levels and chemical structure of these compounds, the role on prevention/improvement of diseases, the ability to modulate the activity of enzymes and to bind to proteins, the stabilization of edible oils and preparation of food supplements can be found in the monographical review from Bendini et al.[145]

Olive phenols have promoted active research on raw materials to obtain them. The two main sources have been olive leaves and the pomace or waste from VOO production. In the case of the two phase oil production system (presently the most frequently used in this industry) the pomace is a polluting semisolid residue. It is a cheap source of natural antioxidants in concentrations up to 100 times higher than in VOO,[167] which results from the polar nature of both pomace and olive phenols and the low polar nature of oil; therefore olive leaves have the highest antioxidant and scavenging power between the different parts of the olive tree (e.g. taking oleuropein as an olive phenol model, its content in olive oil ranges between 0.005 and

0.12%; in pomace can reach up to 0.87% and in olive leaves ranges between 1 and 14%).[168]

CONCLUSIONS

Research on the characteristics and healthy properties of classes belonging to the nonsaponifiable fraction of VOO is in growing development, as shown by the number of both publications on this subject and projects supporting research on VOO.[169]

Assessment of the goodness of some of the compounds in this fraction by the competent authority has been reached,[61] but this is a pending goal for most of them; therefore, more research, particularly intervention studies, must be developed to support the results so far obtained.

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CAPÍTULO 2:

Nonsaponifiable compounds in virgin olive oil.

Part 2. Analytical methods

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Nonsaponifiable compounds in virgin olive oil. Part 2. Analytical methods

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Nonsaponifiable compounds in virgin olive oil. Part 2. Analytical methods

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Abstract

BACKGROUND: The traditional key role attributed to the minor fraction in virgin olive oil (VOO) on the beneficial effect of this oil on human health has promoted the study of the components of this fraction.

RESULTS: The wide number of methods for analysis of this fraction results from the variety of compounds that constitute this minor fraction. The very different chemical characteristics of the classes of compounds in this VOO fraction makes mandatory both different sample preparation and analysis methods; while the similitude among components of a given class or subclass calls for high-resolution analytical equipment when individual identification and/or quantitation is required. Both traditional and recent methods for either overall determination of a class or subclass, and present methods that have allowed identification of unkown compounds as well as determination of them at very low concentrations, are discussed in this review.

CONCLUSION: The critical point of view of analytical chemists on this subject may be of interest for scientists from other areas.

INTRODUCTION

The chemical characteristics and healthy effects of minor components in virgin olive oil (VOO) have been widely expossed and discussed in Part 1 of this review, while the present Part 2 is devoted to the methods required for analysis of the wide variety of compounds that constitute this minor fraction. The very different behavior of the classes of compounds integrated in this VOO fraction makes mandatory both different sample preparation and analysis methods. On the other hand, the similitude among components of a given class or subclass calls for high-resolution analytical equipment when individual identification and/or quantitation is required. Overall determination of a class or subclass requires simpler analytical equipment and provides sometimes enough information for given purposes. The state-of-the-art of both sample preparation and analysis of the components of this fraction are discussed in this review.

SAMPLE PREPARATION

Sample preparation is a key step of the analytical process that, in the case of the nonsaponifiable fraction can be very different depending on the given subfraction; therefore, distinction must be made among sample preparation for subsequent analysis of the lipophilic, hydrophilic, pigments, and volatile subfractions. Therefore, separate discussion of each is made starting from the official or traditionally used method and the improvements existing in the literature, the aims of them and the advantages they provide.

Lipophilic subfraction

The classes constituting this subfraction have traditionally required the most complex and time consuming sample preparation. Thus, the steps required to obtain an analytical sample able for being introduced into high resolution equipment —either gas chromatography (GC) or liquid chromato-

graphy (LC), and, sporadically, capillary electrophoresis (CE)—, as established by the IOC (International Olive Council) can be condensated as follows: (i) saponification, consisting of subjecting sample oil + 2 M KOH ethanolic solution to reflux until the reaction takes place, then continuing for a further 20 min period. (ii) Cooling, washing several times with distilled water, phase separation and washing of the aqueous phase three times with diethyl ether. (iii) Joint collection of the diethyl ether extracts, washing with distilled water until neutral pH of the water from washing and removal of water traces from the ether extract (usually by anhydrous sodium sulfate), filtration of the extract and evaporation to dryness. (iv) Reconstitution into the most appropriate solvent (usually chloroform) and fractionation of the different classes by thin layer chromatography (TLC) with subsequent scrape off of the different areas and reconstitution into the appropriate solvent. (v) Derivatization to form trimethylsilyl (TMS) derivatives for subsequent individual separation by nonpolar GC columns. It is worth emphasizing the high volumes of organic solvents disposed off when applying this sample preparation procedure, far for being green.[1–3]

Changes of the official method have been focused on improvement of either one or several of the steps or to an overally different sample preparation.

Shortening of the time required by the saponification step has been supported on the use of auxiliary energies such as microwaves (MW)[4] or ultrasound (US)[5,6] that reduced the required time since 1 h (official method) to not more than 10 min. Also the derivatization step has been drastically shortened by US assistance by applying the same probe to both the saponification and derivatization steps.[5,6] A recent comparison of saponification methods (viz. hot and cold saponification, MW and US assisted saponification) applied to the lipophilic subfraction of VOO —then using LC–MS and data treatment by multivariate statistical tools— has shown that: (i) hot saponification provided the highest efficiency in qualitative and quanti-

tative terms; (ii) cold and MW assisted saponification yielded similar composition (thus demonstrating that MW does not promote chemical alterations in this subfraction); (iii) US led to a more different composition of the extracts.[7] The time required by these methods ranges from 17 h of hot saponification to 10 min when assisted by US or MW. An in depth study of the degradation products formed under US energy could be of great interest to know the effect of cavitation on the target metabolites.

Following saponification, substitution of liquid–liquid extraction (LLE) by solid phase extraction (SPE) was one of the first proposed alternatives, based on both normal phase[8] and reversed phase sorbents,[9] with excellent results, as claimed by the authors. Later, the use of normal phase sorbents has demonstrated to be the most suitable to circumvent the higher complexity, development time and organic solvents consumption of LLE.[10] Recently, an overall sample preparation method based on only SPE has been proposed prior to determination of sterol, uvaol and erythodiol to meet IOC certification criteria for extra virgin olive oil (EVOO).[11] The nonsaponifiable fraction was separated by diatomaceous earth; then, sterol and triterpenic dialcohols were isolated by a novel activated silica SPE cartridge that, when compared to the IOC sample preparation method, provided higher concentrations of stigmasterol and β-sitosterol, and required only a third of the time.

Despite the well known advantages of SPE for sample preparation (short preparation time, simultaneous handling of several samples and drastic decrease of organic solvents consumption) improved by the appearance of new sorbents with better characteristics,[12] conventional time-consuming sample preparation is applied at present, particularly when the innovative step in the proposed method is the identification and/or quantitation step using mass detectors,[13–15] or a simplified analysis.[16]

TLC fractionation involves shortcomings in isolation of aliphatic alcohols of 22 to 28 carbon atoms because they do not move to a different band, but a "spray out" effect on the plate owing to the different number of carbon atoms in the aliphatic chain.[17] To avoid problems originated by this behavior and also to simplify sample preparation, the determination of aliphatic alcohols based on high resolution gas chromatography (HRGC) capillary columns without prior TLC isolation from other classes of nonsaponifiable compounds was reported by Frega in 1993.[18] In addition to avoid the tedious TLC step, the method based on HRGC allowed obtaining: (i) a fingerprint of the nonsaponifiable matter that permitted to differentiate the aliphatic alcohol classes in this fraction; (ii) assessment of the quantity and quality of the aliphatic alcohols, thus avoiding error owing to sample handling prior to the chromatographic individual separation; (iii) feedback on the method for oil extraction based on the presence of markers (erithrodiol, uvaol, oleanolic acid); (iv) evidence of oxidation products from squalene, indicative of the quality of the raw material.[18]

Despite aliphatic alcohols, sterols and tocophenols in vegetable oils are usually determined separately, also a method for their joint determination has been reported in which the TLC fractionation step in sample preparation was avoided.[19] In this case, after saponification and extraction with hexane, evaporation of this phase, reconstitution into chloroform and silylation, the analytical sample was injected into a GC column for individual separation and subsequent determination by flame ionization detection (FID).

Also the TLC step has been avoided by using LC–mass spectrometry (LC–MS) approaches. Thus, Segura-Carretero et al. evaporated diethyl ether after extraction, reconstituted the residue with methanol and injected this analytical sample into the LC–MS using ambient pressure chemical ionization (APCI) in the positive mode. In this way, they separated and identified 15 sterols and 2 dihydroxy triterpenes in soybean oil, palm oil, seed oil, sun-flower oil, olive pomace oil and VOO, and quantify some of them, with

results in agreement with those of the official method for the target compounds.[20]

Overall sample preparation of the lipophilic nonsaponifiable subfraction as an alternative to the IOC procedure has been mainly based on coupling LC (as a tool for both separation of the fatty acid —FA— major fraction and fractionation of the classes constituting the minor fraction) and GC for individual separation. In this way, almost all manual work is eliminated, and run of large number of samples in a short time is achieved.[21–24]

Grob et al. were pionners in proposing an online LC–GC method for the analysis of the unsaponifiable fraction of edible oils using normal phase columns in the LC preseparation step and previous derivatization of the compounds to be analyzed.[25–28] The use of normal phase in the LC step requires backflushing of the column after each analysis to eliminate any retained lipids which can affect the performance of the column (e.g. by deactivation of the silica gel by triglycerides). The use of a reversed phase circumvented this problem; however, transfer of polar solvents was difficult because of the very large volumes of produced vapor.[29] Further research on RPLC–GC was focused on development of interfaces suited to the transfer of aqueous eluents to prevent problems of this transfer. In this respect, the use of a programmed temperature vaporizer (PTV) as an interface was proven to be useful for the analysis of minor components as free sterols in edible oils.[29–31] The extremely large volume of vapor resulting from the aqueous eluent during LC–GC transfer was eliminated by removing the GC column end from the injector body. However, this approach did not allow automation of the system. Cortés et al. achieved automation of the LC–GC approach by an interface they called "through oven transfer adsorption desorption (TOTAD)", which allowed solvent venting and the introduction of the analytes into the GC column. In this way, automated and simultaneous analysis of different groups of minor components in edible oils could be

performed in only one run, and the whole procedure (LC preseparation, LC–GC transfer, and GC analysis) took about 60 min. In addition, analysis of only one group or class of compounds was possible by selecting the target compounds in the LC fraction. Filtered oil, diluted or not, was directly injected, thus avoiding saponification, LLE, fractionation and derivatization. The authors applied the approach to the determination of free sterols, tocopherols, squalene, and erythrodiol and uvaol in edible oils.[32] As the method did not permit quantitation of both free and esterified sterols, the authors later developed determination of the free fraction by the proposed method, and used an aliquot subjected to saponification and extraction with diethyl ether and injection of the extract into the LC–GC approach to determine esterified sterols.[33] In this case, only the TLC fractionation step was avoided.

Hydrophilic subfraction

The hydrophilic subfraction is mainly constituted by phenols, and the methods for isolation of the components of this subfraction from VOO are simple, fast and well established. The official IOC method only requires 15 min to transfer these minor components to an 80:20 (v/v) methanol–water mixture under US assistance for this time.[34] Subsequent LC separation makes direct use of the extract. This is not the case for previous methods in which extraction was carried out by an Ultraturra device and methanol, with subsequent evaporation of the extractant, reconstitution with acetonitrile and washing 3 times with acetone; then, evaporation of acetonitrile and reconstitution into acetone, followed by derivatization with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) for 1 h after injection into GC.[35] The difference between the two methods (this and the official from IOC) clearly reflects the analytical improvements achieved within the period 1996–2009.

Pigments

Most of the methods for determination of pigments in VOO are designed to be applied in attendance to legal aspects related to color. Therefore, the required determination is the overall response of all components contributing to this attribute of VOO. Sample preparation for this measurement is very simple as only homogeneization of the sample and, if turbid, filtration immediately before analysis using a medium pore size filter paper are necessary.

A method for individual determination of chlorophylls and carotenoids in olive oil requires appropriate sample preparation prior to chromatographic separation and detection, as that proposed by Mateos and García-Mesa. Sample preparation to obtain the pigment extract was based on either LLE or SPE.[36] The former used selective separation of the pigments by N,N'-dimethylformamide (DMF) and hexane. Lipids and the carotene fraction appeared in the hexane phase, while the DMF phase retained chlorophylls and xanthophylls.[37] LLE of the oil in DMF 6 times with 50 mL portions of hexane makes sample preparation very laborious and far for green. On the other hand, SPE sample preparation used C18 or diol cartridges. In the first case, the sorbent retained other components of the oil–hexane mixture circulated through them, while the hexane phase contained the β-carotene fraction and some amount of pheophytin a, which were measured directly in this solution using their respective extinction coefficients in hexane.[37] The retained pigments were eluted by acetone and injected into HPLC. The same procedure was applied using diol bonded phase cartridges. When compared the two sample preparation techniques, SPE required lower volumes of organic solvents and shorter times than LLE; between the two SPE procedures, the diol cartridge provided the best results.

Volatiles

The most usual methods for evaluation of volatile compounds involve sample preparation consisting of a preconcentration step because of the very low concentration of most of these compounds in VOO, despite their relative high concentration as compared to other oils. The simplest sample preparation methodologies for volatiles such as direct injection or static head space (SHS) provide insufficient preconcentration, so that detection is often not allowed (at least special SHS devices are used[38]). Therefore, sample preparation based on an enrichment step (dynamic head space, HS) involving stripping of the volatile compounds, trapping on a suitable adsorbent, subsequent thermal desorption or elution with a solvent is the most commonly used approach. The target method requires careful definition of all variables affecting the analysis to obtain comparable data. The influence of temperature, sample size, absolute amount of gas used for stripping, geometry of the trap, chemical–physical characteristics of compounds to be stripped on the amount of volatile compounds has been the subject of several studies.[39–41]

The most used sorbents to retain volatiles from VOO are Tenax and charcoal.[42] The former is usually adopted in purge and trap (P&T) methods because of its good adsorption capacity for medium and high boiling point compounds, high thermal stability, poor affinity against water and, the easiness of cleaning procedures.[43] The sampled compounds are thermally desorbed by heating the adsorbent at 280 ºC under the carrier gas flow and sent directly to the GC injector.[38] Charcoal, despite the negative aspect of good affinity against water, has been successfully adopted for preconcentration of volatiles from VOO since the matrix contains low amounts of water and, differently from Tenax, it shows a very strong adsorbent capacity towards all classes of chemical compounds, including those with low molecular weight.[43] In this way, information on oil quality and conditions of technological operations adopted during oil extraction is

obtained. Elution of volatiles from charcoal is generally performed at room temperature using an eluant such as diethyl ether; therefore, alteration of unsaturated thermolabile compounds is avoided.

Among new sample preparation methods for VOO volatiles, HS-solid phase microextraction (HS-SPME) is gaining importance because its efficiency and affordable price. The present variety of coated stationary phases for fibres makes the analysis of compounds within a wide range of polarity possible; nevertheless, the best efficiency in sampling VOO volatiles has been achieved using DVB/CAR/PDMS fibers.[44] Despite the number of compounds sampled by HS-SPME is sligthly lower than by dynamic HS, the former allows to analyze more than 130 chemical compounds (most of them belonging to the chemical classes aldehydes, alcohols, esters, hydrocarbons and ketones) which are enough to evaluate most of the volatile compounds related to the flavor and off flavor of the VOO.[42]

OVERALL DETERMINATION OR INDIVIDUAL SEPARATION AND IDENTIFICATION/QUANTITATION OF THE TARGET METABOLITES

Also distinction must be made in the analysis step between lipophilic, hydrophilic, pigments, and volatile subfractions.

Lipophilic subfraction

The official methods for the classes involved in this subfraction are based on individual separation by GC and FID detection using the retention times and commercial standards for identification and quantitation.[1–3]

Consistent with the progress in column technologies, GC columns for separation of lipophilic components have evolved from packed columns (3–8 mm inner diameter, ID) to capillary columns (0.1–0.3 mm ID) and fused silica capillary columns that contain coated and chemically bonded

stationary phases of variable polarity. They offer shorter analysis times and lesser peak interference, improvement in component resolution, and high thermal stability as compared with packed columns.[45] Individual separation of components with high boiling points requires high temperatures to be resolved; therefore, thermostable polar GC columns with low column bleeding, such as TAP Chrompack columns have been used with this aim.[46] In any case, FID continues being used to monitor the column effluents in routine analysis of the different classes of the lipophilic subfraction.[47] As FID lacks of selectivity, GC–FID analyses are often preceded by LC and/or TLC. The growing use of MS for structural identification and quantitation is mainly developed by single ion monitoring (SIM) or multiple ion monitoring (MIM).[45]

Derivatization of the components of the class under study prior to injection into the GC (to increase volatility and/or thermal stability to the detriment of analysis time) is an option that depends on the target class and if routine or sporadic analysis is being carried out. Simplification of this step is achieved by simultaneous derivatization and extraction using SPME with the fiber coated with the derivatization reagent (usually BSTFA).[46]

Use of an internal standard (IS) to eliminate analytical errors owing to fluctuations in instrument operational conditions and other experimental variables is a common practice in this area. GC peaks of the target compounds are represented by their retention times relative to a reference standard or an IS. A growing and more expensive way to correct losses of analytes during isolation and separation for final quantitation, mainly when obtained by MS, is the use of stable isotopes (mainly deuterated isotopes).[46,48]

Bidimensional (2D) GC has also recently been exploited for comprehensive analysis of the lipophilic subfraction with a long delay as compared with its application to the major fraction of vegetable oils in 2001.[49] The complexity of the fingerprint generated by the unsaponifiable

fraction fully justified to use 2D GC technology. The column set consisted of a low polarity first dimension, and a medium polarity secondary dimension, both characterized by a high thermal stability. Dual detection by MS and FI was applied by division of the effluent from the first column in such as way that roughly half of this was directed to the MS for identification purposes, while the remaining fraction reached the second column and then the FID for quantitation.[50] The main advantages of the proposed method over those based on common one dimension GC methods are: (i) creation of a two dimensional "fingerprint" of the entire unsaponifiable fraction; (ii) increased sensitivity (detection of minor constituents); (iii) greater peak capacity (separation of many more compounds); (iv) enhanced potential identification due to the formation of highly organized analyte patterns.

LC, as compared with GC, offers the advantage of operating under both milder column temperatures, and non destructive detection conditions. Therefore, it seems suitable for the analysis of thermally unstable lipophilic compounds. However, the high lipophilicity of some of these classes makes sample processing and chromatography difficult. Potential contamination of the column or the autoinjector has been minimized by using a high content of organic solvent.[46] Normal phase (NP) and reversed phase (RP) LC modes have been widely used for the analysis of classes of the lipophilic subfraction. RPLC uses less volatile polar organic solvents in water, and offers ready equilibration with the mobile phase solvents in a bonded silica stationary phase as compared with NPLC, thus justifying its more frequent use in this field.[51] Detection in dealing with LC has been carried out by UV absorption with conventional or photodiode array detector (DAD), refractive index (RI) detection, evaporative light scattering detection (ELSD), nuclear magnetic resonance (NMR) detection and mass spectrometry. A favorable characteristic of LC–MS analysis is that baseline separation of peaks is not always necessary, thus reducing the analysis time. Concerning ionization, highly lipophilic compounds with few functional groups are difficult to

ionize through conventional electrospray ionization (ESI) methods;[52] on the contrary, APCI was found to be suitable for these types of compounds, as is the case with sterols.[53] The use of this ionization source has recently been ratified by Segura-Carretero et al.,15 despite other ionization alternatives such as electron impact (EI), and atmospheric pressure photoionization (APPI) technique, have also demonstrated to be effective.[54]

The recent method reported by Segura-Carretero et al. based on LC–APCI–IT/MS for quantitative multicomponent analysis of sterols, tocopherols and triterpenic dialcohols in vegetable oils with a total of 15 quantitated compounds and 23 identified compounds is an example of the advantage of APCI over other ionization modes. Despite the authors refers to as rapid method, the adjective only applies to the analysis step, as the tedious IOC sample preparation procedure was used.[14]

Also the direct infusion approach in MS has been used to classify vegetable oils according to their botanical origin, based on direct injection of extracts into an MS with either ESI or APPI source.[55]

Supercritical fluid chromatography (SFC) has also been used for individual separation of components of the lipophilic subfraction, either after conventional sample preparation[56] or coupled to supercritical fluid extraction (SFE) for preconcentration, preparative fractionation and chromatographic quantitation in a single operation.[57,58] The promising results of the methods developed with the available SF chromatographs existing at that time could be widely surpassed with present equipment, in which the back pressure regulator has been greatly improved.

According to their capacities, the currently available separation techniques for this subfraction can be classified in the order GC > HPLC > SFC, the sensitivity being a function of the type of detector coupled to the given chromatograph.

Hydrophilic subfraction

Methods for determination of this subfraction are very different from those of the previous subfraction mainly due to the polar character of all its components. This aspect and their well known antioxidant properties have given place to two types of methods: (i) overall methods to determine a common property of all components; (ii) methods for the individual determination of either given phenols classes or given compounds.

Overall determination of phenols in VOO

Total determination of phenols after separation from the lipidic fraction involves selection of a single standard for relative quantitation, the most used being caffeic acid,[59,60] gallic acid,[61,62] or, less frequently, oleuropein.[63] The use of a single standard for determination of different phenols involves that predominance of compounds with molecular weight lower than that of the standard yields an overall phenols concentration higher than when the predominance corresponds to phenols with molecular weight higher than the standard. The two most popular overall methods are based on photometry: the Folin–Ciocalteu (F–C) method, and the total phenols index (TPI) method. The former is based on running a calibration curve with the selected standard after forming a colored compound with the F–C reagent, LLE of the phenols from the oil and interpolation of the absorbance at 700–765 nm of the extract within the calibration curve after formation of the colored compounds.[64] The TPI provides less selective information than the F–C method as it consists of measurement of absorption at 280 nm, where a number of interferents can contribute to the measured absorbance.[65]

Less common methods for overall determination of phenols are based on near infrared (NIR) spectroscopy,[66] or on fluorescence spectrometry,[67] in both cases with good correlation with the F–C method. Also NMR spectroscopy has been used as complementary technique for structural

assignment of VOO phenols.[68] However, the NMR spectra of phenols are complex and identification of the isolated compounds in the absence of appropriate standards is complicated. Also, the limited sensitivity that leads to the need for quantities of samples higher than those required by other detection techniques constitute a great limitation of NMR.

Individual determination of phenols in VOO

It could be said that, apart from FA determination, phenols are the compounds in VOO more frequently determined in the last decades.

The official IOC method for the hydrophilic components of the VOO nonsaponifiable fraction is very simple as compared with those for classes of the lipophilic subfraction. It is based on direct extraction of these polar compounds by methanol and subsequent quantitation, after individual separation by HPLC (C_{18} column), by UV molecular absorption detection at 280 nm. Syringic acid is used as the internal standard. The method could be considered as semiquantitative as the content of the natural and oxidised oleuropein and ligstroside derivatives, lignans, flavonoids and phenolic acids is expressed as mg kg^{-1} tyrosol.

GC based methods for individual determination of VOO phenols always require a derivatization step to obtain products both less polar and more volatile. One of the first methods in this area was proposed that by Solinas that required, after LLE, cleanup of the extract followed by azeotropic distillation to remove the extractant, low pressure column chromatography for further cleanup of the preconcentrate, derivatization by TMS esterification before injection into the GC. Despite the complexity of the method, only characterization of the simplest compounds was able as conjugated phenols were not identified because the FID used required the corresponding but non available standards.[69] MS detectors overcome this limitation and the quality of the results was improving from the use of chemical ionization (CI),[35] to ion trap (IT) and the subsequent MS/MS.[70]

Nevertheless, the necessary derivatization of phenols prior to GC separation that complicates sample preparation, considerably increases the molecular masses of the products reaching the detector, which may be displaced beyond the range of the detector; in addition to yield mixtures of partially derivatized phenols which decrease sensitivity and involve errors.[35]

LC based methods are nowadays the most popular for VOO analysis because the combination of resolution (approaching at levels similar to those provided by GC), efficiency, versatility and speed of analysis, and simple sample preparation as a technique suited for nonvolatile compounds.

RPLC is the preferred option for separation of VOO phenols,[71] using as stationary phase nonpolar octadecylsilane (C_{18}) bonded phase. Gradient or isocratic modes are used for LC separation. The former has demonstrated to provide better resolution thanks to the selectivity effect of one or more components of the mobile phase, but the use of gradient is most times mandatory owing to the complexity of the phenols profile of the majority of VOO samples.[72] The most common detectors in LC analysis of VOO phenols are based on UV–Vis molecular absorption, preferable DAD detectors for a higher versatility in the wavelengths for monitoring.[73,74] Despite other detectors such as fluorimetric[75] or electrochemical detectors[76] have been reported, MS detectors are massively used for research in this field, while DAD remains as the best for routine analysis because of its simplicity.[77] Therefore, while API-MS/MS,[78] IT and triple cuadrupole (QqQ) analyzers[79] have been coupled to LC for quantitation of hydrophilic components of the nonvolatile fraction of VOO, time of flight (TOF) with MS or MS/MS detectors have been mainly used to obtain overall profiles of these compounds and/or for identification of most of them.[80,81]

Despite MS has been the preferred detection technique to be coupled to LC in the last decade, and it is also that of the present decade, the LC–NMR arrangement has demonstrated its well recognized capacity in olive oil

analysis.[82,83] An excellent example was developed using the complex LC–SPE–NMR approach for identification and structure elucidation of simple phenols, lignans, flavonoids and a large number of secoiridoid derivatives, and also for identification of several new phenolic components which had not been reported previously as constituents of the polar fraction of olive oil.[84] The overall analytical process required sample preparation as described by Montedoro[85] (viz. repeated extraction by methanol–water, evaporation of the extractant and reconstitution into deuterated methanol) prior to injection in the LC system; collection of the eluted fraction and transference to an automated SPE device with subsequent transference of the SPE eluate to the NMR instrument by deuterated acetonitrile. Therefore, the method is clearly expensive (both in analytical equipment and application, in addition to the overall long time for development) but provides wide, unique information.

CE, characterized by fast analysis, high resolution and small sample and background electrolyte consumption, seemed to be a suitable approach for routine analysis of phenols in VOO. To date, the most widely used detectors are based on absorptiometry, mainly DAD.[86,87] Such is the case with the separation of phenols in VOO after enrichment of the oil with these compounds from different herbs such as rosemary, thyme or oregano.[88] The enrichment step, developed for 11 days under room temperature stirring, was monitored by capillary zone electrophoresis (CZE) after SPE preconcentration. The use of mass analyzers based on QqQ, IT or TOF has revalorized CE in this field.[89]

Pigments

Despite the importance of color as a quality attribute in olive oils that is the first perceived by the consumers, not much attention is paid to it when legal aspects related with this attribute of VOO are established. This behavior contrasts with the importance given to the color of other products for human consumption such as wines. Nevertheless, the need for having

appropriate methods to define olive oil color is unquestionable; hence, this parameter has for decades been assessed in different ways. The approaches to this end can be readily grouped into two main categories, visual and instrumental methods. The former are a part of sensory analysis of the product and can be carried out rather easily without the need for any instrumentation. Although a certain vocabulary has been established to define the colors thus studied, the description is largely subjective. In contrast, instrumental measurements allow an objective definition of color once the reference conditions necessary to carry out the readings are set.

Visual analysis of olive oil is commonly carried out by large panels of untrained panelists to determine preferences, whereas trained panelists are used for descriptive assessments. Trained judges perform quantitative descriptive analysis which can be correlated with instrumental or other type of data at a later stage.

The simplest way to assess visually the color of an olive oil is to compare it with color scales: a series of colored solutions, which are rather stable and can be made from easily available colorants, being bromothymol blue the most widely used.[90] Also colored glasses can be used as reference standards as an alternative to colored solutions.

Instrumental methods can be divided into total methods (either for joint or separated determination of carotenoids and chlorophylls) and individual methods for each target pigment.

The use of overall spectrometric methods started in the forties of the past century by using visual colorimeters for comparison of the colored glasses with the given olive oils. Correlation equations were established and accepted by the American Oils Chemists' Society (AOCS) upon recommendation of the Color Committee of Oils in the fifties of the last century. Spectrophotometric measurements were proposed to establish a method to control olive oil decoloration based on absorption maxima for given

pigments and the corresponding extinction coefficients, and on seven regions of maximum absorption influenced by variables such as acidity, temperature, and refractive index; and by transformations of the original pigments into derivatives with other chromatic characteristics.[91] Present routine overall methods for oils pigments are based on photometric measurements at 670 nm for the overall chlorophyll fraction and at 470 nm for that of carotenoids;[36] the results overally expressed for the content of chlorophyll pigments as mg of pheophytin a in 1 kg of oil and that of carotenes as β-carotene. This was the method used for Mateos and Mesa-García for comparison of sample preparation methods.[36]

Tristimulus colorimetry (based on the amount of radiant energy emitted by the source at each wavelength —emission spectrum of the source—, the way in which this energy is transmitted by the sample observed —emission spectrum—, and the response of the observer eye to the radiations of different frequencies —curve of eye sensitivity)[92–94] is applied to routine analysis of pigments in olive oils.[95,96] Concerning research involving NMR and pigments, VOO fingerprinting is the only approach used, which shows small or nil contribution of pigments, depending on their concentration.[97,98]

Research on individual determination of carotenoids in VOO has been mainly developed by LC–DAD which allows monitoring of each compound at the wavelength of maximum absorption.[36,99]

The controversy about the effects of carotenoids on the oxidation of triacylglycerides (TAGs) has been solved by LC–ESI/MS as suitable equipment to identify/quantify potential oxidation products. In this way, TAGs were found to be oxidized much faster in the presence of β-carotene than in its absence and the prooxidant effect seems to be dependent on the target TAG and promotes degradation of half of β-carotene after 3 h of thermal Rancimat treatment.[100] Conversely, astaxanthin seems to act as an

antioxidant as oxidation of TAGs is delayed up to 10 h of thermal Rancimat treatment;[101] therefore, this carotenoid could be proposed both for enrichement of olive oils and as a food colorant. Both carotenoids were found to significantly increase peroxide values of oils at exposure times longer than 10 h of thermal treatment.[100,101]

Volatiles

The key role of the volatile fraction in VOO is shown by the existence of international methods to assess its organoleptic characteristics and allow its classification. Thus, specific vocabulary exists on the possitive and negative organoleptic characteristics of VOO and intensity of them;[102] therefore, VOO is defined on the basis of its sensory characteristics which are mainly established by the volatile fraction.

Individual separation of the components of the volatile fraction of VOO is performed by capillary GC that allows separation of the compounds present in a wide range of concentrations and can be used as a routine basis for qualitative and quantitative analysis,[103] and also for enantioselective separations by using the appropriate chiral column.[104]

The most common detector in this field is FID the response and wide linear dynamic range of which meet the analytical needs of this fraction, but requires the corresponding standards for quantitation. Tentative identification can be achieved when GC is coupled to Fourier transform infrared (FTIR) spectroscopy or, more frequently, to MS detectors. The most widely used ionization technique in GC–MS for VOO volatiles research has for years been EI,[105] the main limitation of which is that, too often, the molecular ions do not survive fragmentation and, consequently, are not "seen" by the detector. Softer ionization sources such as CI overcomes this problem.[106] The mass spectra obtained by CI are simpler than those from EI, though most of the interpretable structural information is missing. Other soft ionization sources such as field ionization (FI) and field desorption (FD),

producing abundant molecular ions with minimal fragmentation, have also been applied to volatile compounds.[107,108] Concerning analyzers, diverse approaches such as QqQ, IT, QqTOF, etc., have been used for volatiles analysis, the last allowing the simultaneous acquisition of complete spectra with a constant mass spectral m/z profile for the whole chromatographic peak, while in qMS instruments the skewing effect is unavoidable. This fact enables application of spectral deconvolution,[109] and, potentially, a more accurate use of reference libraries for identification and confirmation of analytes may be possible. Nevertheless, development of TOF/MS dedicated mass spectral libraries for routine laboratory should be considered to complement the libraries presently generated by using qMS. Spectral matching is usually better when qMS data are compared.[110,111]

A recent research by Gomes da Silva et al. based on HS-SPME–GC–TOF/MS using different fibres for sample preparation and polar and unpolar chromatographic columns to detect coelutions, occurring with polar columns, has helped in the identification of a large number of volatiles.[106]

The development of new analytical methods that maximize volatiles separation has been a current trend of the last twenty years, of which comprehensive 2D GC is an example.[112,113] This approach has allowed identification of VOO key flavor compounds present at very low concentrations,[114] and has also demonstrated to be endowed with flexibility for the screening of flavors and other classes of semipolar compounds by using the conventional orthogonal approach and the reverse nonorthogonal approach to obtain ordered structures that simplify identification.[115] In addition, proper selection of the detectors in 2D GC has allowed comprehensive fingerprint of several olive oil matrices in a direct way or using image processing statistics.[116] Presently, most research on oil volatiles is focused on the establishment of the differences of these components among oils from different growing areas,[117,118] cultivars,[119] planting densities,[120] or irrigation regimes.[121] The most varied analytical couplings have been used to

obtain the required information: thus, an electronic nose,[120] GC–FID and GC–MS equipment[117,119,120] have been the preferred choices that provided the raw data. Treatment of raw data as required to clearly discriminate the differences among the variable under study (cultivar, growing area, irrigation regime or planting density) was based on Duncan's tests,[120] principal components analysis (PCA),[121] etc.

CONCLUSIONS

Methods for analysis of the nonsaponifiable fraction of VOO have traditionally required long and tedious sample preparation steps, thus making sample preparation the most critical part of the overall analysis as the main responsible for losses. Shortening or avoidance of these steps is a present field of research which could be favored by both assistance of auxiliary energies and cutting-edge analytical equipment.

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CAPÍTULO 3:

Comparison of saponification methods for characterization of the non saponifiable fraction of virgin olive oil

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Comparison of saponification methods for characterization of the non saponifiable fraction of virgin olive oil

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Comparison of saponification methods for characterization of the non saponifiable fraction of virgin olive oil

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Abstract

Despite the interest of the non saponifiable fraction of olive oil (composed by sterols, tocopherols, fatty alcohols, hydrocarbons, phenols, pigments and volatile compounds) there are no studies in the literature dealing with comparison of methods to isolate this fraction. Four saponification methods (*viz.* hot and cold saponification, and microwave- and ultrasound-assisted saponification) have been compared by analysis of the resulting extracts using LC–MS in high-resolution mode and data treatment with multivariate statistical tools. Principal component analysis and Venn diagrams were employed to compare qualitatively and/or semiquantitatively the metabolite profiles provided by the different methods. Hot saponification was the approach which provided the highest efficiency of this step in qualitative and quantitative terms. On the other hand, cold and microwave-assisted saponification led to non saponifiable extracts with similar composition according to unsupervised analysis. These results revealed that microwave assistance does not promote chemical alteration of the non saponifiable fraction. Finally, ultrasound-assisted saponification provided the extract with a more different composition.

INTRODUCTION

Virgin olive oil (VOO) obtained from mechanical pressing of olive drupes is considered one of the most priced vegetable oils due to its organoleptical properties and benefits on human health [1, 2]. Its chemical composition is characterized by the presence of two main groups of compounds: major components (about 98%), and minor components, which is only about 2% [3]. The former fraction includes mainly triacylglycerides (TAGs) and small concentrations of diacylglycerides (DAGs), monoacylglycerides (MAGs), free fatty acids, waxes and phospholipids, and is characterized by a high concentration of monounsaturated fatty acids (MUFA), especially oleic acid. The minor fraction forms a chemically heterogeneous group as it includes more than 230 compounds, which can be classified into two fractions, polar and non polar compounds [4]. The non polar fraction, commonly termed non saponifiable fraction and constituted by secondary metabolites, is extracted with non polar solvents after oil saponification [5]. This fraction is mainly composed by hydrocarbons, sterols, tocopherols and fatty alcohols, whereas the polar fraction includes phenols, pigments and volatile compounds.

Despite the small concentration of minor components, they play a key role in the nutritional and health benefits of VOO. Most of the families pertaining to the non saponifiable fraction have been recognized to possess anti-inflammatory [6], anti-bacterial [7], anti-oxidant [8, 9], anti-fungal [10], gastroprotective [11], anti-hyperlipidemic [12], and anti-carcinogenic properties [13] by *in vitro* experiments. One of the most bioactive fractions is formed by phytosterols, the main constituents of the lipid unsaponificable fraction in VOO. Phytosterols reduce biliary cholesterol absorption in the intestine by increasing faecal excretion, thus resulting in significant reduction in the risk of cardiovascular diseases [14].

Triterpenes is other of the families which has demonstrated benefits in preventing hypertension in *in vivo* studies [15]. Additionally, oleanolic and

maslinic acids are powerful pro-apoptotic agents in human colon cancer cells [16, 17]. Recently, it has been suggested that erythrodiol derivatives can prevent the harmful effects of ultraviolet rays that lead to skin aging or skin cancer [18]. Tocopherols and carotenoids are recognized as potent non polar oxidation inhibitors [19], while specific hydrocarbons such as squalene are valued by their antioxidant and anticancer properties [20].

The non saponifiable fraction is not only relevant from the organoleptic point of view but also in terms of VOO quality. Compounds such as triterpenes and phytosterols have been used as purity parameter to detect olive oil frauds with pomace olive oil or hazelnut oil [21]. In addition, tocopherols are involved in the mechanisms of oil oxidation and preserve oil quality during storage [22], while high amount of certain triterpenes seems to deteriorate organoleptic oil quality [23].

The great diversity of minor components in the non saponifiable fraction makes their characterization a complex task. First, oil saponification is required to remove triglycerides by extraction of the non saponifiable fraction into an appropriate organic solvent [4]. There are different ways to develop this step, with temperature assistance or at ambient temperature; with assistance of auxiliary energies such as microwaves [24, 25] or ultrasound [26]. Then, the non saponifiable matter can be fractionated into several classes of compounds by preparative chromatography —*e.g.* thin-layer chromatography (TLC) [27]— or solid-phase extraction (SPE) [28] for subsequent analysis. Other approach is based on direct analysis of the non saponifiable fraction by a high resolution separation technique such as gas chromatography (GC) coupled to either flame ionization detection (FID) or mass spectrometry (MS) [29]. A major drawback of GC for this task is the requirement of chemical derivatization —usually by trimethylsilylation (TMS)— prior to analyses [30]. In the last years, methods based on either liquid chromatography (LC) or nanoLC with MS detection have been developed [31–33].

Most methods in the literature are aimed at characterization of a specific family of compounds pertaining to the non saponifiable fraction [32–41] Thus, Zarrouk *et al.* [41] and Martínez-Vidal *et al.* [32] have reported methods based on LC–MS with atmospheric pressure chemical ionization combined with ion trap mass spectrometry (APCI–IT) for quantitative multicomponent analysis of sterols, tocopherols and triterpenic dialcohols. These methods allowed detecting and quantifying a great number of compounds belonging to these three families [41]. Nevertheless, there are no studies in the literature dealing with comparison of saponification methods for characterization of the non saponifiable fraction of VOO. For this reason, it would be desirable to compare different saponification alternatives to know the capability of them as sample preparation for the analysis of this scarcely studied fraction. With these premises, the aim of this research was to compare four saponification methods: hot and cold saponification, and microwave- and ultrasound-assisted saponification, for isolation of the non saponifiable fraction from VOO. For this purpose, the resulting extracts were characterized by liquid chromatography/time-of-flight mass spectrometry analysis in high resolution mode (LC–TOF/MS).

MATERIALS AND METHODS

Samples

Five samples of Picual and Hojiblanca VOOs from cultivars pertaining to different geographical areas in Andalusia (*viz.* Sevilla, Jaén, Málaga) were used in this study.

Reagents

The reagents used for characterization of the non saponifiable fraction of VOOs were: LC–MS grade acetonitrile (ACN) and formic acid for preparation of chromatographic mobile phases and LC grade ethanol for sample preparation from Scharlab (Barcelona, Spain). Potassium hydroxide and diethyl ether for saponification were from Panreac (Barcelona, Spain), and deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the mobile chromatographic phases.

Apparatus and Instruments

Ultrasound was applied by means of a Branson 450 digital sonifier (20 kHz, 450 W) equipped with a cylindrical titanium–alloy probe (12.70 mm diameter) immersed into a lab-made stainless-steel container with eight compartments to place test tubes. The microwave irradiation experiments were carried out with an MIC-II focused-microwave extraction system of 400 W maximum power (Puebla, Mexico) furnished with a manual power control unit. A Selecta Vibromatic electrical stirrer (Barcelona, Spain) was used in the cold saponification method. An MS2 minishaker from Ika (Wilmington, USA) was used for liquid–liquid extraction and to favor isolation of the non saponifiable fraction. A Büchi R-200 rotary evaporator (Postfach, Switzerland) furnished with a B-490 heating bath was used to concentrate the extracts.

All samples were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6540 LC–TOF/MS hybrid mass spectrometer with a dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected m/z ratios.

Saponification–Extraction Methods for Isolation of the Non Saponifiable Matter from VOO

The saponification methods available in the literature were hot [27, 42, 43] and cold [44] saponification, and microwave- and ultrasound-assisted saponification [45, 26]; therefore, they were used as proposed to evaluate their efficiency for extraction of the non saponifiable fraction from VOO.

Hot Saponification

The hot saponification method was a modification of the official method [27] proposed by Carretero *et al.* [31]. 5 g of VOO was saponified by refluxing for 30 min at 70 ºC with 50 mL of a 2 M KOH ethanolic solution. The solution was cooled in water, and 50 mL of deionized water plus 50 mL of diethyl ether were added. The non saponifiable matter was extracted twice and the combined diethyl ether fractions were mixed with 25 mL of 0.5 M KOH in ethanol and washed 2–3 times with deionized water until neutral pH of the washing solution. The diethyl ether phase was led to dryness in a rotary evaporator. The residue was dissolved in 5 mL of ACN.

Cold Saponification

5 g of VOO was weighed and 50 mL of 2 M KOH in ethanol was added, and the mixture shaken in the dark by an electrical stirrer for 17 h. Then, the resting steps were as in the hot saponification method for extraction, concentration and reconstitution of the non saponifiable fraction [44].

Microwave-Assisted Saponification

5 g of VOO was weighed into a flask and 50 mL of 2 M ethanolic KOH solution was added. The flask was subject to 400 W microwave radiation for 10 min, after that the resting steps were developed as in the hot saponification method [45].

Ultrasound-Assisted Saponification

1 g of each VOO was placed in a test tube with 10 mL 2 M KOH ethanol solution. The tubes were placed in an eight-position stainless-steel container which was immersed into a water-bath at 20 ºC, the ultrasonic probe located at an equidistant position of all tubes and 3 cm from the bath bottom (output amplitude 45% of the converter, duty cycle 50%, power 200 W applied for 10 min). Then, 10 mL of deionized water and 10 mL of diethyl ether were added. The non saponifiable matter was extracted twice and the combined diethyl ether fractions were mixed with 10 mL 0.5 M KOH in ethanol and washed 2–3 times with deionized water until neutral pH of the washing solution. The diethyl ether phase, separated by centrifugation, was led to dryness in a rotary evaporator, then dissolved in 1 mL of ACN [26].

LC–TOF/MS Analysis of Non Saponifiable Extracts

Analysis of the non saponifiable fraction of the target oils was conducted by LC–TOF/MS in high resolution mode using positive ionization mode. The analytical column was a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The mobile phases were: A (0.1% formic acid in water) and B (0.1% formic acid in ACN). The gradient program, at 0.5 mL/min constant flow rate, was as follows: initially 30% A and 70% B; 0–15 min, 30–0% A and 70–100% B; 15–35 min, 0% A and 100% B. After analysis, the column was equilibrated for 10 min. The volume of extract injected without any additional pretreatment was 5 μL. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V in positive ionization mode; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. The data were acquired in centroid mode in the extended dynamic range (2 GHz). The mass range and detection window were set at m/z 100–1100. Before the experiments, the instrument reported mass detection resolution of 12500 FWHM (Full Width at Half Maximum) at m/z 121.0509 and 40000 FWHM

at m/z 922.0098. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at m/z 121.0509 (protonated purine) and m/z 922.0098 (formate adduct of hexakis(1H,1H,3H-tetrafluoropropoxy)-phosphazine).

Data Pretreatment

Mass Hunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing the raw LC−TOF/MS data. Molecular features were extracted using the molecular feature extraction algorithm (MFE) from the raw data files prior to formula generation. Ions with identical elution profiles and related m/z values (representing different adducts, ions generated after specific neutral losses, or isotopic forms from the monoisotopic ions) were extracted as molecular features (MFs) in a matrix characterized by retention time (t_R), accurate mass and signal intensity in apex of chromatographic peaks as data. The isotope model corresponded to common organic molecules with peak spacing tolerance of m/z 0.0025±7.0 ppm. The MFE algorithm limited extraction to ions exceeding 5000 counts with charge state limited to a maximum of two. The allowed positive ions were protonated species and formate adducts. Dehydratation neutral losses were also allowed. The generated raw data files in compound exchange format (.cef files) were created for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) to create the data matrix for further processing. In the next step, alignment of t_R and m/z values was carried out across the sample set using a tolerance window of 0.5 min and 10 ppm, respectively. Protocols for reduction of MFs number based on frequency filters were performed. Principal component analysis was used to find clustering of samples attending to the saponification method used.

RESULTS AND DISCUSSION

Optimization of the LC–TOF/MS Analysis of the Non Saponifiable Fraction

Different chromatographic methods have been reported in the literature for the analysis of the non saponifiable fraction of VOO using LC–MS, for targeted analysis of characteristic compounds in this fraction [31–33]. These methods were used as starting point to achieve high resolution chromatographic profiles from a model non saponifiable extract (that obtained by the hot saponification method). Two gradient programs were tested with this purpose, both at a constant flow rate of 0.5 mL/min. The first one (gradient 1), based on the existing literature, was as follows: initial mobile phase 30% A and 70% B; then, 0–20 min, 30–0% A and 70–100% B; 20–40 min, 0% A and 100% B. The second gradient (gradient 2) was initiated at lower concentration of organic solvent 70% A and 30% B; then, 0–30 min, 70–0% A and 30–100% B; 30–50 min, 0% A and 100% B.

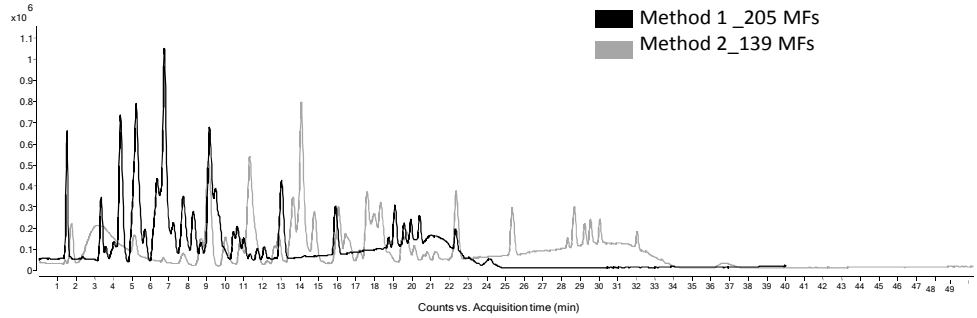

Figure 1. BPCs obtained by LC–TOF/MS analysis of extracts of the non saponifiable fraction corresponding to the chromatographic methods (A) and (B) (see text for details).

Figure 1 includes base peak chromatograms (BPCs) obtained by methods 1 and 2. As can be seen, chromatographic resolution was quite good by both gradients. However, a higher number of entities was provided by the gradient method 1 (205 MFs) than by the gradient method 2 (139 MFs), which started at a lower concentration of water in the mobile phase. For this

reason, this method was tested with variable gradient rates (27, 30, 35 min) from the initial 70% B to 100% B to compare the chromatographic resolution each gradient provided. **Supplementary Figure 1** shows the BPCs obtained by the analysis of the extract obtained after hot saponification using the three gradient methods. Visually, chromatographic resolution was better by the gradient method run for 35 min, which also allowed detecting higher number of molecular entities.

Alignment of Molecular Features Extracted After LC–TOF/MS Analysis

After extraction of the MFs, alignment of entities detected in the different samples was executed. The tolerance window for alignment of molecular features among different samples was set at 0.5 min and 10 ppm for elution time and mass accuracy, respectively. **Supplementary Figure 2** shows a frequency plot that allowed deducing that no all compounds were detected in all samples. This situation could be ascribed to the high variability among the different oils but also to variability promoted by the saponification method. To obtain a more representative data set, a frequency filter was applied to include those MFs present in at least 75% of all samples pertaining to one of the considered classes (one class per saponification method). This filter allowed reducing the data set from 463 to 362 molecular entities.

Figure 2 illustrates the BPCs obtained by LC−TOF/MS analysis of the extracts containing the non saponifiable fraction from one of the VOO samples. The chromatograms show critical differences in the profiles provided by the different saponification methods. Hot saponification provides the chromatographic profile with more qualitative and quantitative information (number and intensity of peaks). Lower number of peaks provided the extracts in which saponification was assisted by either micro-waves or ultrasound and, particularly, by cold saponification, which could be

attributed to a lower efficiency of saponification by insufficient energy for saponification.

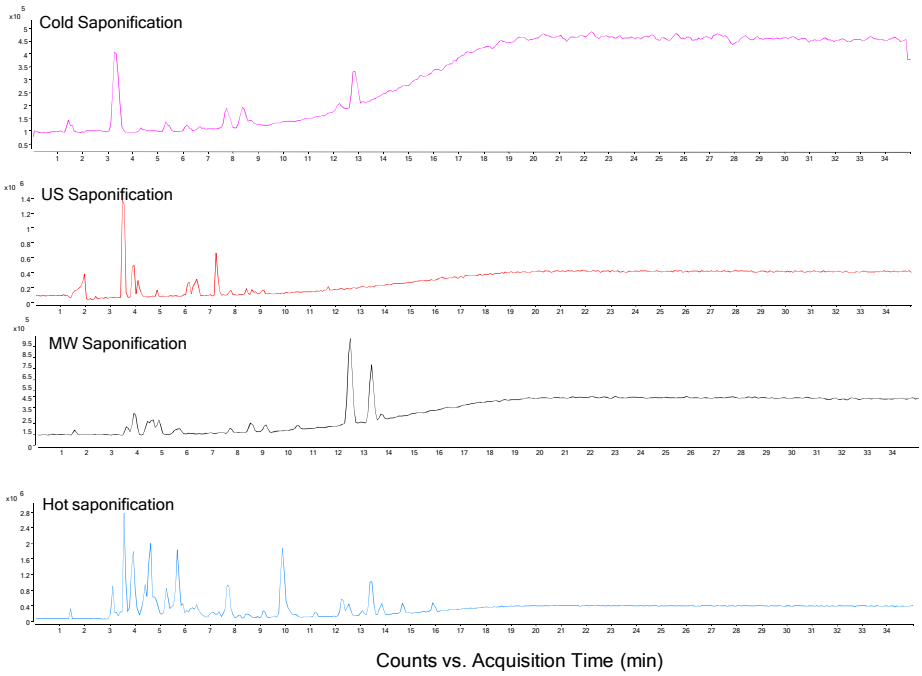

Figure 2. Base peak chromatograms from the extracts using the four saponification methods.

Qualitative and Relative Concentration Comparison of the Non Saponifiable Fraction Obtained by the Four Extraction Methods

The qualitative composition of the extracts isolated by the four saponification methods was compared using the profile of MFs obtained after subjecting the extracts to LC–TOF/MS analysis. **Figure 3** illustrates the Venn diagram generated by qualitative comparison of MFs profiles obtained after application of the filter by frequency for each type of extraction method. As can be seen, hot saponification reported the highest number of detected MFs (205), while cold saponification reported the lowest number

(175), the latter justified by the lack of applied energy. It is worth mentioning that 64 MFs were common to the four saponification methods.

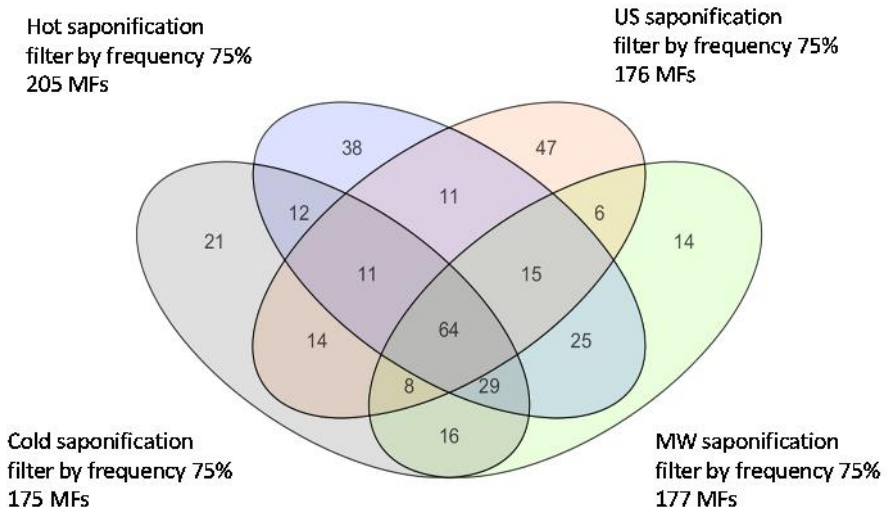

Figure 3. Venn diagram generated by qualitative comparison of MFs profiles obtained after application of the filter to each type of saponification method.

Supplementary Figure 3 shows the correlation map that, in contrast to Venn diagrams, takes into account qualitative information and relative concentrations information to find similarity patterns among the four saponification methods using the pretreated data set. As can be seen in the figure, similarity was found in the extracts from cold and microwave-assisted saponification. This result supports that microwave assistance under the operating conditions did not promote a significant alteration in the non saponifiable fraction. The extracts obtained after hot saponification were characterized by a low variability among them, although they were not highly-correlated with the rest. Neither the extract from ultrasound-assisted saponification showed similarity with those obtained from cold, hot or microwave-assisted saponification.

Venn diagrams comparing qualitative profiles of pair of saponification methods are illustrated in **Supplementary Figure 4**, which shows

maximum similarity coverage for the pair hot saponification/microwave-assisted saponification (34.81%), and the lowest similarity for hot saponification/ultrasound-assisted saponification (26.34%). Attending to the profiles of MFs obtained by the different methods, hot saponification seems to be the most suited approach to extract qualitatively and quantitatively non saponifiable compounds and remove the saponifiable fraction.

Multivariate Unsupervised Analysis

After application of the filter that reduced the data matrix from 463 entities to 362, a principal component analysis (PCA) was carried out to find similarity/dissimilarity patterns among the saponification methods. As can be seen in **Figure 4**, the main source of variability was associated to the saponification method but not to the variability among VOO samples. Thus, the PCA scores plot reveals separation among samples processed by the four saponification methods supported on the non saponifiable fraction. Discrimination along PC1 was observed for those samples processed under ultrasonic assistance. This fact could be supported on the Venn diagram comparing the qualitative profile associated to the four saponification methods since a high number of entities (47 MFs) were exclusively detected in the extract obtained after ultrasonic saponification, which could be explained by degradation. **Figure 4** shows that cold saponification and microwave-assisted saponification were the two approaches providing the most similar profiles of non saponifiable fraction, which agrees with the correlation map in **Supplementary Figure 3**. Apart from that, the microwave-assisted method was that providing the extract more similar to that obtained by hot saponification according to PCA. Therefore, a high temperature could be crucial to attain high-efficient saponification and, for this reason, cold and ultrasound-assisted saponifications were less efficient.

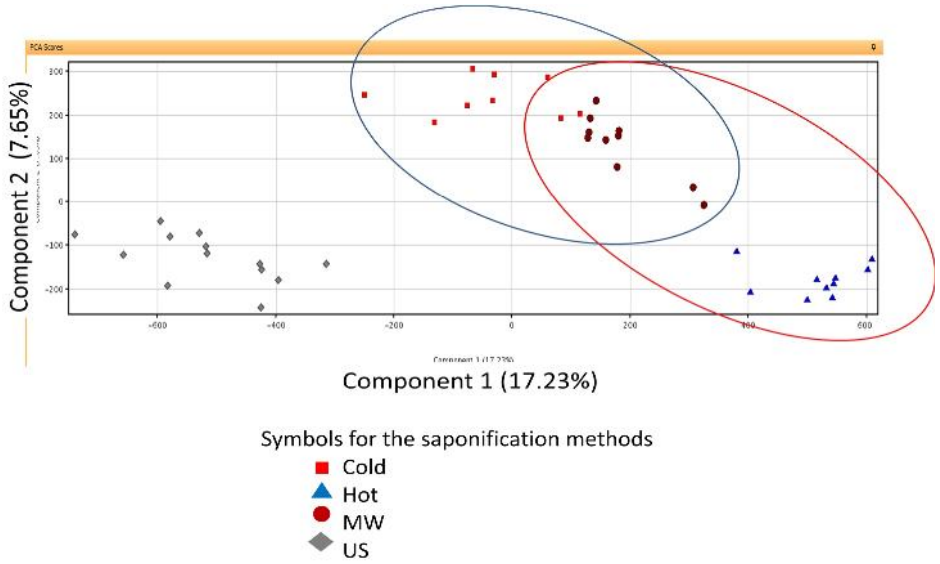

Figure 4. Score plots for PCA corresponding to the different saponification methods.

Representative compounds of different families of the non saponifiable fraction were monitored in the extracts isolated after application of the four saponification methods. The target compounds were Δ-5 avenasterol and campesterol representing phytosterols, tetradecadienal as fatty aldehyde, maslinic acid for triterpene compounds; α-tocopherol for tocopherols, lutein and β-carotene as carotenoids, and also two fatty acid derivatives (oleamide and *N*-palmitoyl valine). Critical differences were found for some of these compounds depending on the saponification method. Among them, Δ-5 avenasterol, campesterol and tetradecadienal were exclusively detected in the non saponifiable fraction of oils subjected to hot saponification and microwave-assisted saponification methods. These compounds could undergo degradation under ultrasound-assisted saponification, while cold saponification could not extract these compounds owing to insufficient energy. **Figure 5.A, B and C** shows stacked extracted ion chromatograms (EICs) for the three representative compounds of phytosterols and fatty aldehydes.

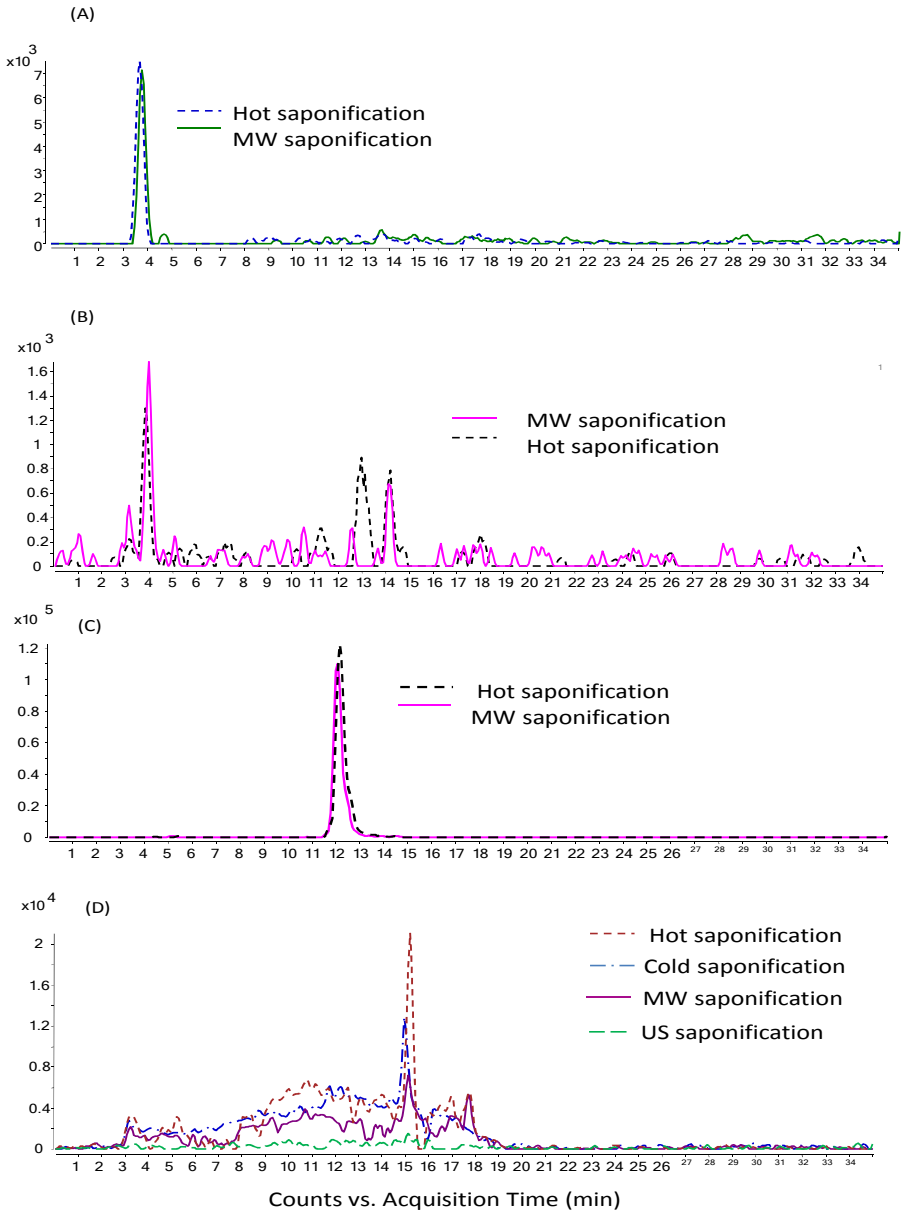

Figure 5. Stacked extracted ion chromatograms (EICs) provided by the different saponification methods for (A) Δ-5 avenasterol, (B) campesterol, (C) tetradecadienal, (D) maslinic acid.

Concerning maslinic acid, this triterpene was also detected at higher amounts in the non saponifiable fraction obtained by hot saponification, while cold saponification and microwave-assisted saponification produced lower content of this compound (**Figure 5.D)**. In contrast, this valuable triterpene was not detected by ultrasound-assisted saponification, possibly owing to degradation. α-Tocopherol, lutein and β-carotene are well-known because of their lipophilic antioxidant properties [19]. These three compounds were detected in the non saponifiable fraction of analyzed VOOs, although different patterns were observed, as shows **Figure 6.A, B and C**. Thus, α-tocopherol was preferentially found in the non saponifiable fraction from the hot saponification method; low levels contained the extracts from the other three approaches, especially that from the microwave-assisted protocol. This effect was not found for lutein as hot saponification and cold saponification provided the maximum efficiency for its isolation. β-Carotene was mainly found in the non saponifiable fraction from hot protocol, microwave-assisted saponification and cold saponification, while that assisted by ultrasound contained low levels of this antioxidant, possibly owing to degradation.

Finally, the capability of the saponification methods was also compared by monitoring two fatty acid derivatives such as oleamide and *N*-palmitoyl valine. Each type of compounds was preferentially isolated by a different method (**Figure 6.D and E**). Thus, oleamide was favorably extracted by hot saponification, while cold saponification improved the extraction of N-palmitoyl valine.

Comparison of saponification efficiency for the four protocols tested in this research enabled to establish that the hot saponification method provides the extract more enriched in the monitored compounds; probably less degradation is produced by the hot protocol as compared with those based on auxiliary energies. Multivariate data analysis revealed microwave-assisted saponification and cold saponification as the two closest

approaches to the hot saponification method. Assistance by ultrasonic energy did not show a clear benefit to enhance saponification.

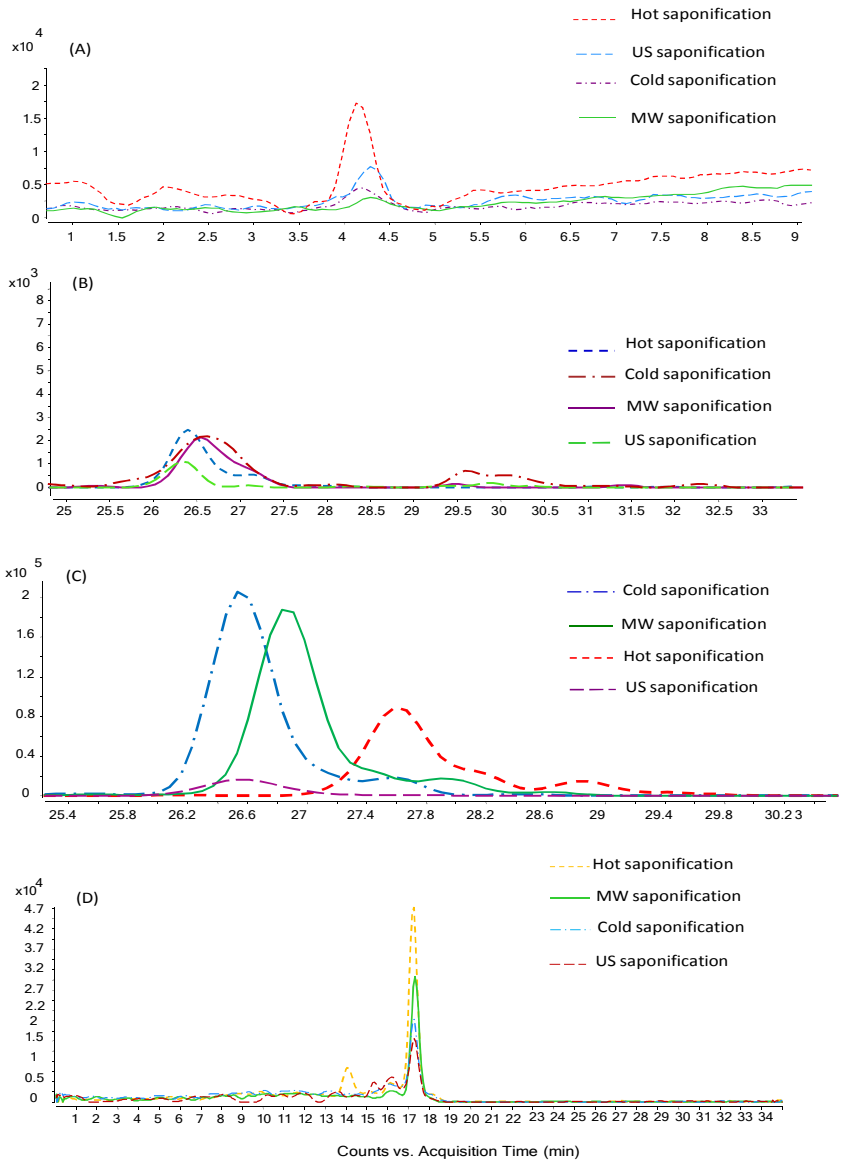

Figure 6. Stacked extracted ion chromatograms (EICs) provided by the different saponification methods for (A) a-tocopherol, (B) lutein, (C) tetradecadienal, (D) β-carotene, (E) N-palmitoyl valine.

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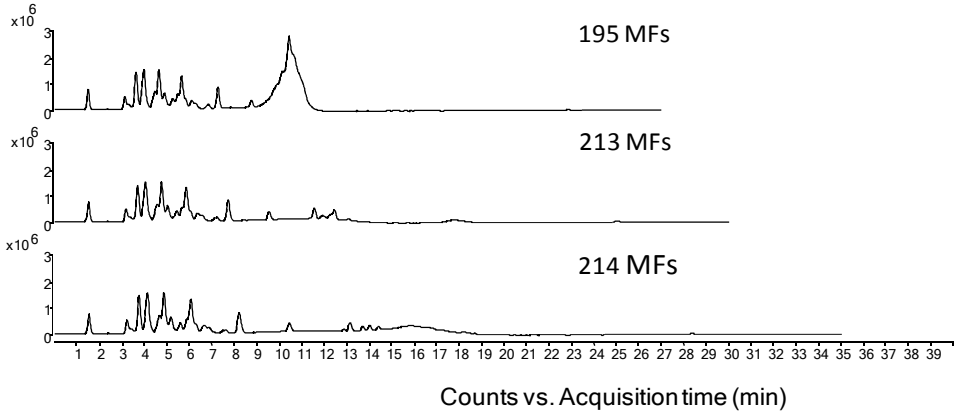
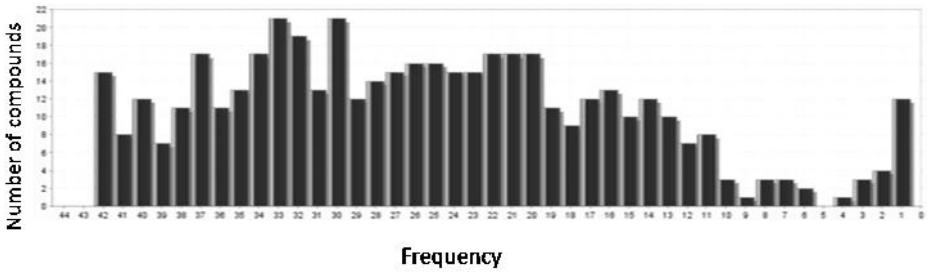
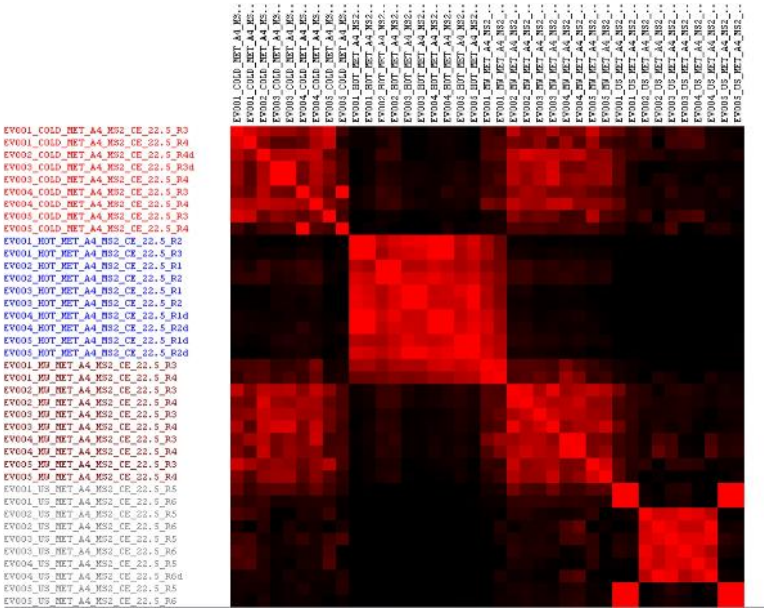
SUPPLEMENTARY MATERIAL



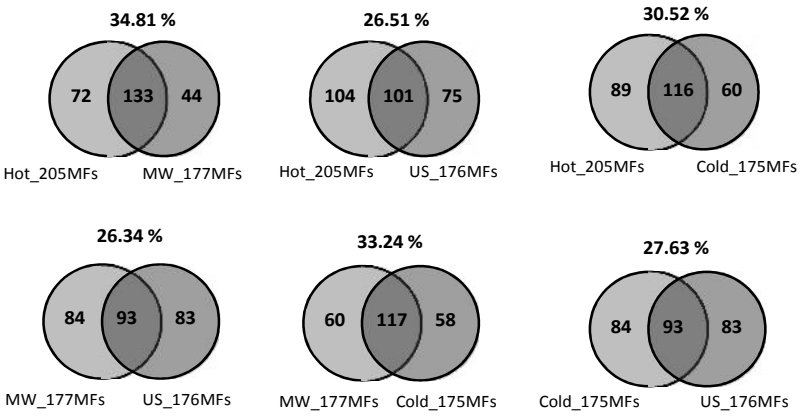
Supplementary Figure 1. BPCs obtained by LC−TOF/MS analysis of an extract of the non saponifiable fraction of VOO by the different saponification methods.



Supplementary Figure 2. Frequency plot of the different compounds in the extracts.



Supplementary Figure 3. Heat map based on correlation analysis according to the saponification method.



Supplementary Figure 4. Common and uncommon percentages of molecular features for the different extraction methods by Venn diagrams.

CAPÍTULO 4:

Comparison of the non-polar non-saponifiable fraction in virgin olive oil and refined vegetable oils by liquid chromatography–mass spectrometry in high resolution mode

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Comparison of the non-polar non-saponifiable fraction in virgin olive oil and refined vegetable oils by liquid chromatography–mass spectrometry in high resolution mode

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Comparison of the non-polar non-saponifiable fraction in virgin olive oil and refined vegetable oils by liquid chromatography–mass spectrometry in high resolution mode

Verónica Sánchez de Medina, Feliciano Priego-Capote*, María Dolores Luque de Castro*

Abstract

The non-saponifiable fraction from monovarietal virgin olive oils (VOOs) and refined vegetable oils —sunflower (RSO), soybean (RSoO), pomace (POO) and olive (ROO)— has been analyzed after subjected to a hot saponification method. The results from applying LC–MS in high-resolution mode and data treatment by multivariate statistical tools have been compared. Principal component analysis and Venn diagrams were employed to compare qualitatively and/or semiquantitatively the metabolite profiles from the different refined vegetable oils and those from VOOs. The results revealed that the non-saponifiable fraction from VOOs provides a more complete profile than that from refined oils. On the other hand, VOO and soybean refined oil reported the highest similarity, followed by POO and ROO. Representative non-saponifiable compounds from different families (e.g. Δ-5-avenasterol, stigmastatrianol, oleanolic and ursolic acids, 11-cis-retinol, δ-tocopherol, diapo-zeta-caroten, lutein, 2,4-octadienal, octanal, N-acetylsphingosine and oleamide) were monitored, and the results showed that the refining process considerably reduces the content of non-saponifiable compounds in edible oils. The analysis of the non-saponifiable fraction can be useful from an overall point of view to identify refined oils with a non-saponifiable fraction similar to that in VOO.

INTRODUCTION

The non-saponifiable fraction of vegetable edible oils constitutes 0.5–1.5% of the total weight [1], and encompasses different families of compounds classified into polar and non-polar compounds [2]. The latter, commonly named non-saponifiable fraction as such, is described as the extract obtained with non-polar solvents after oil saponification. This fraction is mainly composed by hydrocarbons, sterols, tocopherols and fatty alcohols, whereas the polar fraction includes phenols, pigments and volatile compounds [3].

The non-saponifiable fraction is particularly representative of edible oils, but also is strongly influenced by the refining process. VOO is one of the few vegetable oils that can be consumed as crude oil [4]. A refining process must be applied to most edible oils to reduce/eliminate organoleptical defects such as high acidity, unpleasant taste and odor, unsuitable color, and instability [5]. The two most commonly used methods for oil refining at the industrial level are based on chemical or physical treatments [6].

Chemical refining involves a degumming step of the crude oil to remove mainly phospholipids and lecithins and reduce levels of proteins, waxes and peroxides. This initial step can be performed by heating at about 70 ºC under agitation, or by acidification with citric acid and heating at 60 ºC [7]. Subsequently, a deacidification step is carried out to remove free fatty acids (FFA) — to lower the content below 0.03% (w/w)— with sodium hydroxide at high temperature (80–100 ºC), which can promote losses of sterols and tocopherols that do not surpass 5% [7]. The third step, known as bleaching, has the purpose of eliminating excessive oil coloration owing to different undesirable pigments such as carotenes, chlorophylls and xanthophylls. Apart from that, bleaching prevents oxidation of tocopherol derivatives and improves oxidative flavor stability of the final product [8].

Finally, deodorization is applied at low pressure (2–6 mbar) and high temperature (180–220 °C) to remove volatile compounds, mainly aldehydes and ketones, sterols, carotenoids, tocopherols or sulfur derivatives, which cause undesired odor [9].

On the other hand, physical refining involves degumming, bleaching —common stages with chemical refining— and finally deacidification/-deodorization is the step that stablishes a key difference to chemical refining. This step is based on steam distillation at high temperature (240–250 ºC) under vacuum to lower FFA to a final content below 0.05–0.1% [10]. Physical deacidification has reported to reduce tocopherol and carotene contents in vegetable oils owing to the high temperature applied [11].

Apart from the described modifications in refined oils, the appearance of hydrocarbons such as n-alkanes, alkadienes, isomerization products from squalene, isoprenoidal polyolefins and steroidal hydrocarbons has been reported [12]. Furthermore, triglyceride oligopolymers increase during refining —4 fold greater in refined oils—, while oxidized triglycerides decrease by an average of 49% in refined oils [13].

The analysis of the non-saponifiable fraction requires, first, oil saponification to remove triglycerides with subsequent extraction of non-saponifiable compounds into an appropriate organic solvent, commonly diethyl ether [14]. Once the non-saponifiable fraction is isolated, fractionation can be carried out by thin-layer chromatography (TLC) [15], or solid-phase extraction (SPE) prior to analysis [16], which is mainly carried out by gas chromatography (GC) after derivatization, followed by mass detection (MS), or by liquid chromatography (LC) and MS, without the need for derivatization. The universal detector based on flame ionization (FID) can also be used after GC, although selectivity and information are clearly lower than by MS-based methods [17].

There are not many studies focused on characterization of the non-polar non-saponifiable fraction, probably due to the complexity of sample

preparation. On the other hand, polar compounds such as phenols and volatile compounds have been more extensively studied because they can be analyzed after liquid–liquid extraction, which considerably simplifies sample preparation. Only phytosterols and aliphatic alcohols, are frequently used for quality control as proposed by the International Olive Oil Council (IOC) and especially for detection of adulteration of VOO with hazelnut oil or olive-pomace oil [18, 19]. Despite the low concentration of non-saponifiable compounds in vegetable oils they play a key role in their quality and stability [20], and also in their nutraceutical properties [21].

The aim of the present research was to compare the non-saponifiable fraction from different VOO and refined oils isolated by using a hot saponification protocol. Qualitative and relative quantitation analysis allowed establishing differences among the target oils but also selecting those refined oils with the closest composition to that of non-refined oils. Special emphasis was paid to a panel of representative compounds from different families to compare relative levels of them in the different oils.

MATERIALS AND METHODS

Samples

Four types of refined vegetable edible oils, selected according to their consumption in Spain, were used in this research. Refined sunflower oil (RSO), refined soybean oil (RSoO), pomace olive oil (POO) and refined olive oil (ROO) were selected, and all of them were purchased in local supermarkets. Apart from them, five monovarietal VOOs from Picual and Hojiblanca cultivars were provided from different geographical areas of Andalusia (viz. Sevilla, Jaén, Granada and Córdoba).

Reagents

LC grade ethanol, LC–MS grade acetonitrile (ACN) and formic acid from Scharlab (Barcelona, Spain) were used for preparation of the chromatographic mobile phases. Deionized water (18 MΩ•cm) from a Millipore Milli-

Q water purification system (Bedford, MA, USA) was used to prepare the aqueous chromatographic phases. Potassium hydroxide and diethyl ether from Panreac (Barcelona, Spain) were used in the saponification step.

Apparatus and Instruments

A Selecta Mixtasel centrifuge (Barcelona, Spain) was used for separation of the phases formed after saponification. A Büchi R-200 rotary evaporator (Postfach, Switzerland) furnished with a B-490 heating bath was used to concentrate the isolated non-saponifiable fraction. An MS2 minishaker from Ika (Wilmington, USA) was used to favor liquid–liquid extraction.

All samples were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6540 LC–TOF/MS hybrid mass spectrometer with dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected m/z ratios in each scan.

Sample Preparation

An amount of 5 g of oil was saponified by refluxing for 30 min at 70 ºC with 50 mL of a 2 M KOH ethanolic solution, which was cooled in water, and 50 mL of deionized water was added. The non-saponifiable matter was extracted twice by 50 mL of diethyl ether and the combined fractions were mixed with 25 mL of 0.5 M KOH in ethanol and washed 2–3 times with deionized water up to neutral pH of the washing solution. The diethyl ether extract was led to dryness in a rotary evaporator and the resulting residue reconstituted in 5 mL of ACN.

LC–TOF/MS Analysis of Non-Saponifiable Extracts

Analysis of the non-saponifiable fraction from the target oils was conducted by LC–TOF/MS in high-resolution mode using positive ionization mode. The analytical column was a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The mobile phases were: A (0.1% formic acid in water) and B (0.1% formic acid in

ACN). The gradient program, at 0.5 mL/min constant flow-rate, was as follows: initial composition 30% phase A and 70% phase B; 0–15 min, 30–0% phase A and 70–100% phase B; 15–35 min, 0% phase A and 100% phase B. After analysis, the column was equilibrated for 10 min. The injected volume of extract, without any additional pretreatment, was 5 μL. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V in positive ionization mode; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. The data were acquired in centroid mode in high resolution (2 GHz). The mass range and detection window were set at m/z 100–1100. Before experiments, the instrument reported mass detection resolution of 12500 FWHM (Full Width at Half Maximum) at m/z 121.0509 and 40000 FWHM at m/z 922.0098. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at m/z 121.0509 (protonated purine) and m/z 922.0098 (formate adduct of hexakis(1H,1H,3H-tetrafluoropropoxy)-phosphazine).

Data Pretreatment

Mass Hunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing the raw LC−TOF/MS data. Molecular features (MFs) were extracted using the molecular feature extraction algorithm (MFE) from raw data files prior to formula generation. The MFE algorithm limited extraction to ions exceeding 5000 counts with charge state limited to a maximum of two. The allowed positive ions were protonated species and sodium adducts. Dehydratation neutral losses were also allowed. Thus, ions with identical elution profiles and related m/z values (representing different adducts, ions generated after specific neutral losses, or isotopic forms from the monoisotopic ions) were extracted as MFs in a matrix characterized by retention time (t_R), accurate

mass and intensity in apex of chromatographic peaks. The isotope model corresponded to common organic molecules with peak spacing tolerance of m/z 0.0025 ± 7.0 ppm. The generated raw data files in compound exchange format (.cef files) were created for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) to create the data matrix for further processing.

RESULTS AND DISCUSSION

Alignment of Molecular Features Extracted after LC–TOF/MS Analysis

In this research, the non-saponifiable fraction from different refined oils was analyzed and compared with that obtained from non-refined VOOs. For this purpose, a liquid–liquid extraction process with a non-polar solvent such as diethyl ether was used for isolation of the non-saponifiable fraction after saponification [24, 25]. **Figure 1** shows the base peak chromatograms (BPCs) obtained by LC–TOF/MS analysis of extracts of the non-saponifiable fraction from one of the studied VOOs and also from refined oils. As can be seen in the chromatograms, the non-saponifiable fraction from VOOs provided a more complete profile than those from refined oils. The extract from RSO revealed almost non chromatographic signals to be assigned to non-saponifiable compounds. Apart from that, the extracts from POO and ROO were characterized by a similar profile along the chromatographic run, whereas the extract from RSoO reported the chromatogram with the highest presence of peaks among refined oils.

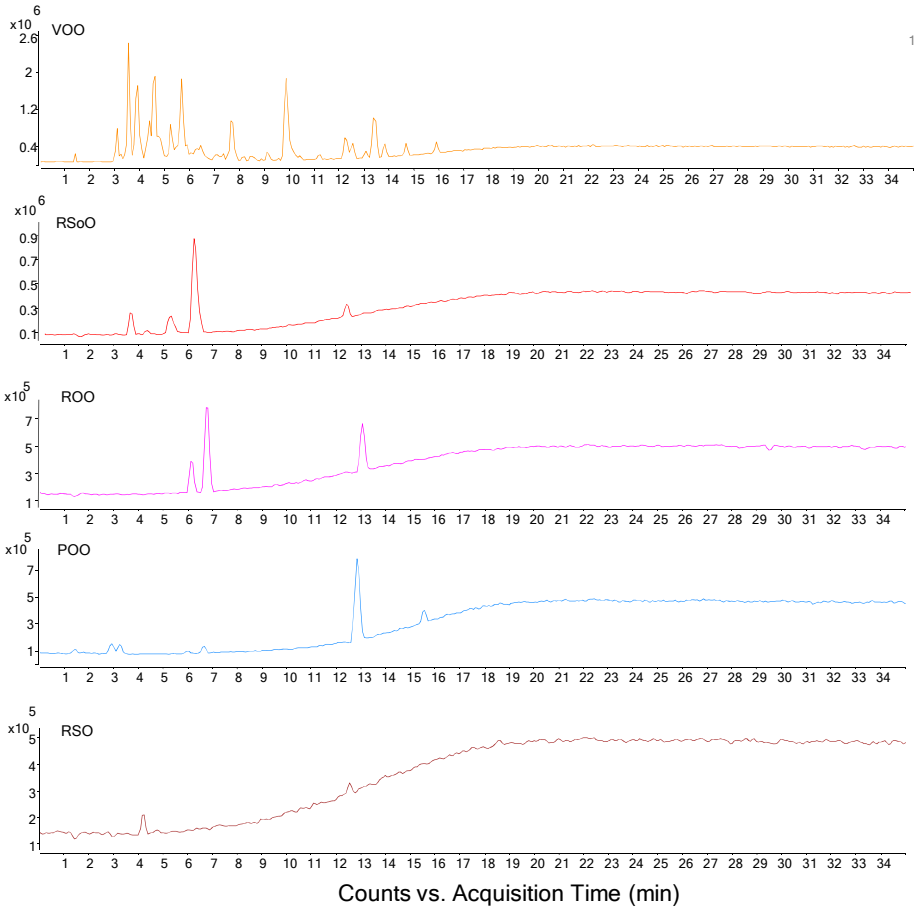

Figure 1. Base peak chromatograms (BPCs) obtained by LC−TOF/MS analysis of the extracts containing the non-saponifiable fraction from VOO and refined oils.

Data treatment was required prior to statistical analysis intended to compare the non-saponifiable chromatographic profiles from different extracts. For this purpose, MFs were extracted as described under "Data Pretreatment". The initial data set was built after alignment of potential MFs according to retention time and mass accuracy using a tolerance window of 0.5 min and 20 ppm. The alignment process resulted in a data set composed of 311 variables associated to different MFs in all samples. The frequency plot in **Supplementary Figure 1** shows the fraction of MFs detected in a

particular number of samples. As can be seen, 63.4% of MFs were present in 2/3 of the total number of samples (18 extracts). A filter was applied to the data matrix to retain the MFs present in at least 75% of the samples. This filtration algorithm reduced the molecular entities to 208.

Qualitative Comparison of the Non-Saponifiable Fraction Profile in Edible Oils

After data preparation, the first study was the qualitative comparison of the non-saponifiable fraction isolated from VOOs and refined oils. As can be seen in **Supplementary Figure 2** the non-saponifiable extracts from VOOs reported a number of MFs higher than refined oils (179 *vs* 121, 141, 157, and 161 for RSO, ROO, POO and RSoO, respectively). The decreased number of peaks in the profile of non-saponifiable fraction from refined oils can be ascribed to the treatment applied to obtain these oils. It is well-known that the stages of deacidification and deodorization cause a decrease in the concentration of non-saponifiable fraction of 5–10% [22], owing to the high temperature (80–100ºC and 180–220 ºC for the deacidification and deodorization steps, respectively) applied in these processes. Apart from that, deacidification is carried out at pressures ranging from 2 to 6 mbar; conditions that are responsible for partial degradation of the non-saponifiable fraction and total degradation of polar compounds. Qualitative comparison of non-saponifiable fraction profiles from the studied oils was carried out by Venn diagrams for each VOOs/refined oils pair. As shown in **Figure 2,** RSoO reported the highest similarity (53.15%) to VOOs, followed by POO and ROO (51.35 and 45.45%, respectively). The lowest similarity to VOOs corresponded to RSO (42.18%).

This study was complemented with a correlation analysis which, in contrast to Venn diagrams, took into account qualitative and semiquantitative information. Thus, different correlation levels can be set among the different oils according to the non-saponifiable fraction. **Figure 3** shows the heat map, in which, as expected, a high correlation among the five VOOs selected

for this work was found, although with a high variability, as these oils were obtained from different geographical areas [26]. On the other hand, no correlation exists between the non-saponifiable fraction of VOOs and that of ROO or POO, behavior attributed to the refining process. Nevertheless, a certain correlation between VOO and RSoO could be justified by a higher stability of this refined oil as compared to POO or ROO.

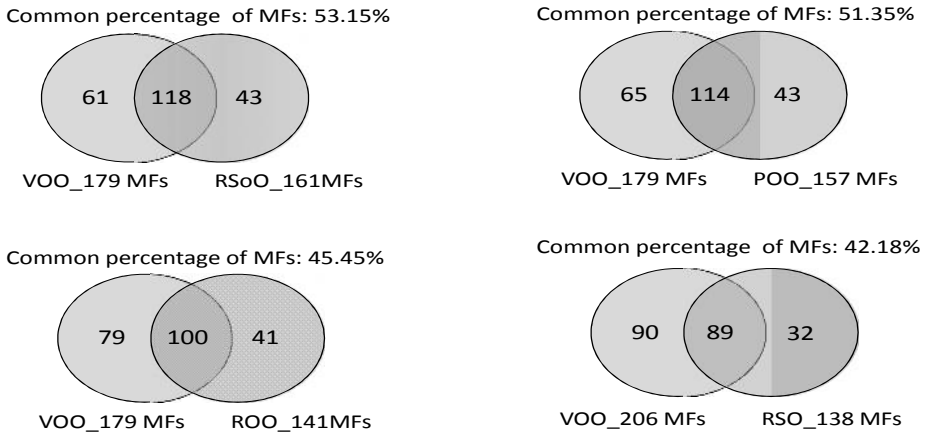

Figure 2. Venn diagrams generated by qualitative comparison of each VOOs–refined oil pair.

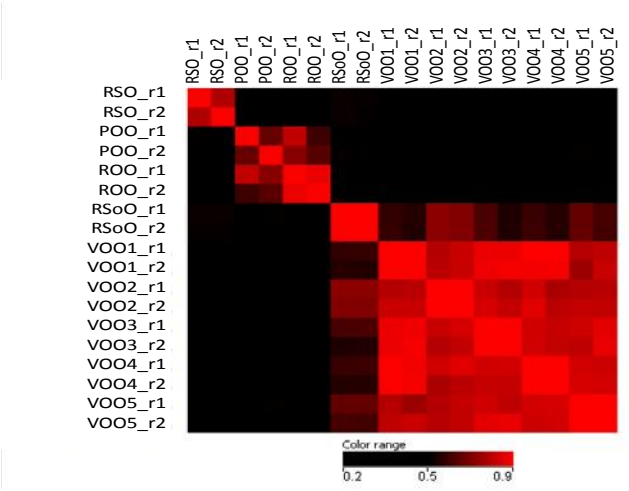

Figure 3. Heat map based on correlation analysis among non-saponifiable fraction from VOO and refined oils.

Multivariate Unsupervised Analysis

Unsupervised analysis based on PCA was developed to detect clusters as a function of the non-saponifiable fraction. As can be seen in **Figure 4**, the two-dimensional PCA scores plot allowed explaining 40.75% of variability with detection of some clusters. Total separation among the non-saponifiable fraction from the five monovarietal VOOs and that of refined oils reveals a high variability; while a certain similarity is found between RSoO and VOOs. In addition, a clustering between POO and ROO was found as a consequence of sharing the raw material (olive fruit). The highest separation was found between RSO and VOOs.

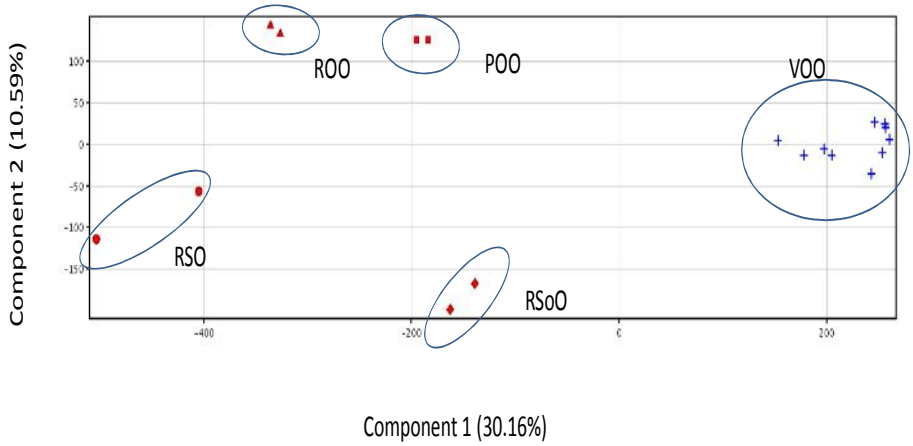

Figure 4. Score plots for PCA-2D corresponding to the non-saponifiable fraction from VOO and refined oils.

Representative compounds pertain to different families in the non-saponifiable fraction were selected to compare their relative concentration in different oils. The target compounds were Δ-5-avenasterol, representing sterols; stigmastatrianol as ergosterol; oleanolic and ursolic acids as representative triterpenes; 11-*cis*-retinol as diterpene; δ-tocopherol for the group of isomers with the same name; diapo-zeta-carotene (β-carotene derivative) and lutein for carotenoids; 2,4-octadienal and octanal as fatty aldehydes, and N-acetylsphingosine and oleamide for ceramides and fatty acid derivatives, respectively.

Significant differences caused by the presence/absence of these compounds in the oils under study were found. Thus, lutein, one of the main lipophilic oxidation inhibitors, was only detected in VOOs, which could be ascribed to absence in other edible oils or to degradation during the refining process [27].

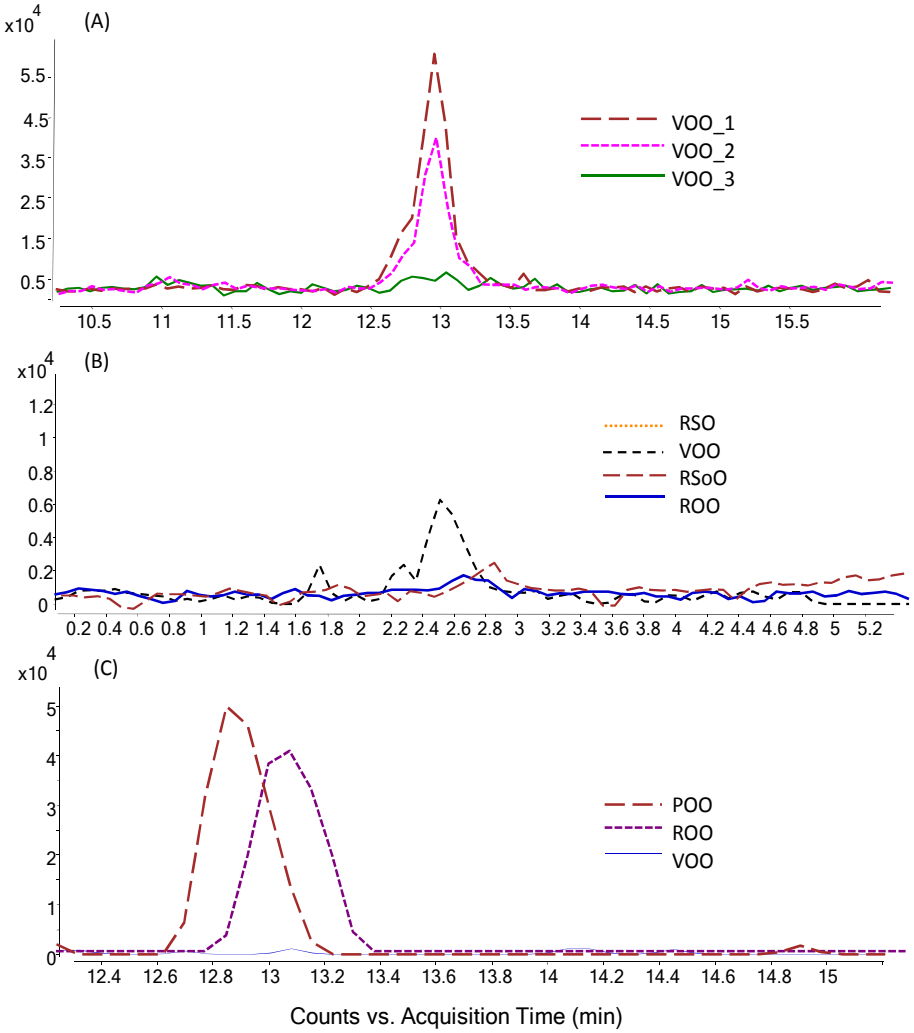

Figure 5. Overlaid extracted ion chromatograms for lutein (A), δ-tocopherol (B), and stigmastatrianol (C).

Figure 5.A shows the overlaid extracted ion chromatogram (EIC) for this compound in three different VOOs. δ-Tocopherol, a natural antioxidant which gives stability to vegetable oils, presented the highest concentration in RSO, in agreement with the results in the literature [1]; followed by VOOs, RSoO and ROO, as can be seen in **Figure 5.B**. It is well known that refining protocols promote dehydration of sterols to yield ergosterols such as stigmastatrianol, which allows detection of VOO adulterations [28]. **Figure 5.C** illustrates EICs for this ergosterol, which was only detected in ROO and POO, but not in VOOs. This behavior can be explained by the original content of stigmastatrianol in these oils. According to the literature, oleanolic and ursolic acids are found in POO at significant concentration (0.1%), as compared to VOO, in which this triterpene is at trace levels [29]. The EICs for oleanolic and ursolic acids in POO and a representative VOO are in **Figure 6.A**, which shows clear differences between these two oils. These results are consistent with previous studies which reported higher concentrations of both triterpenes in refined olive oils as compared to VOO [30]. In fact, the concentration of these triterpenes can be used to detect adulteration of VOO with low-quality refined olive oils. A β-carotene derivative (diapo-zeta-carotene) was only detected in ROO and VOOs. The content of this compound was significantly higher in VOOs than in ROO, as shows **Figure 6.B**, which could be explained by the low stability of this oxidation inhibitor [31]. The isomeric character of this compound, explained by the configuration of double bonds, justifies the presence of numerous chromatographic peaks.

Similarity between RSoO and VOOs was supported on characteristic compounds such as octanal, 2,4-octadienal, and 11-*cis*-retinol. The concentrations of 2,4-octadienal and retinol were considerably higher in VOOs than in RSoO (**Figure 6.C and D**). On the contrary, octanal was more concentrated in RSoO than in VOOs (**Figure 6.E**). Other compounds such as Δ-5-avenasterol, N-acetylsphingosine and oleamide were detected in all analyzed

oils. Δ-5-avenasterol and N-acetylsphingosine concentrations were significant in POO, while low levels of these compounds were found in VOOs and RSO. Oleamide was more concentrated in VOOs, and a low level of this compound was found in ROO.

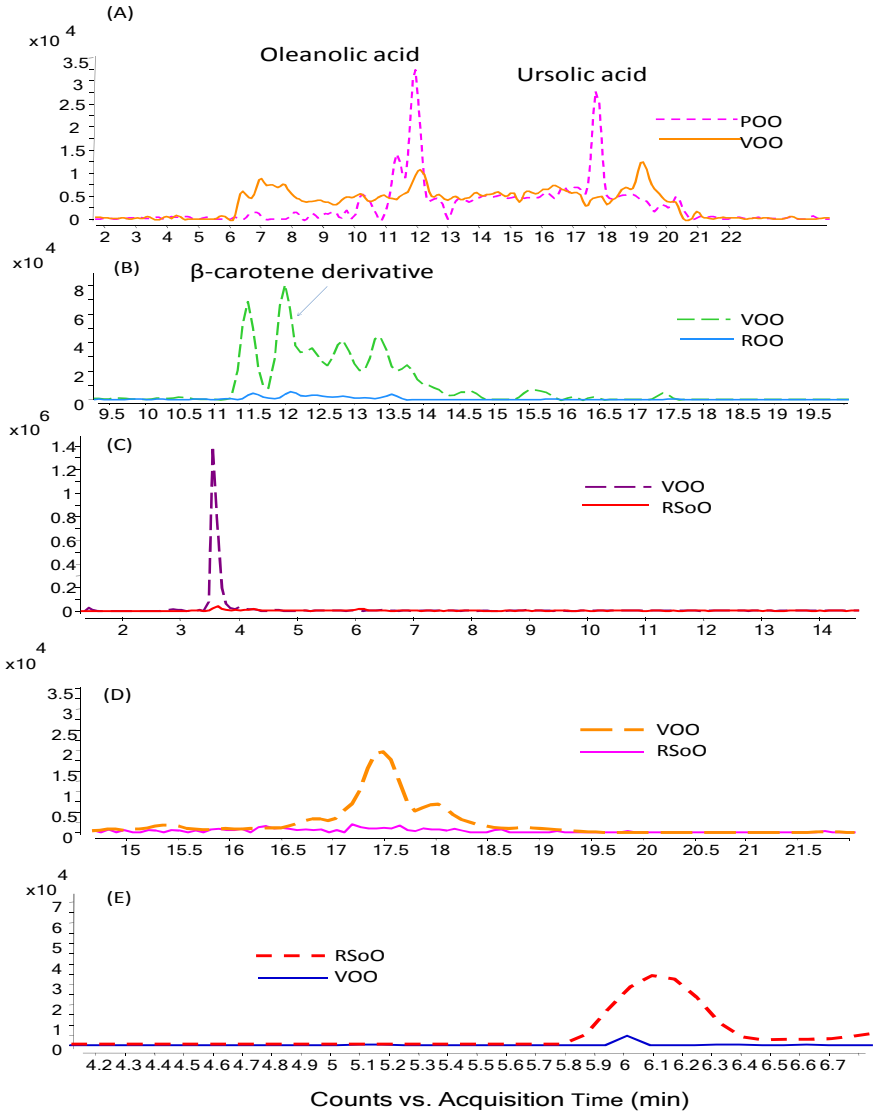

Figure 6. Overlaid extracted ion chromatograms for oleanolic and ursolic acids (A), β-carotene derivative (B), 2,4-octadienal (C), 11-cis-retinol (D) and octanal (E).

In summary, the refining process reduces the number of non-saponifiable compounds in general terms in edible oils. The observed differences in composition can be ascribed to the quality of the oils used in this study. Thus, POO and ROO are refined olive oils obtained from low-quality olive oils, which were obtained under different technological conditions to those used for VOO. The global analysis of the non-saponifiable fraction can be useful to compare refined oils and VOOs.

ACKNOWLEDGEMENTS

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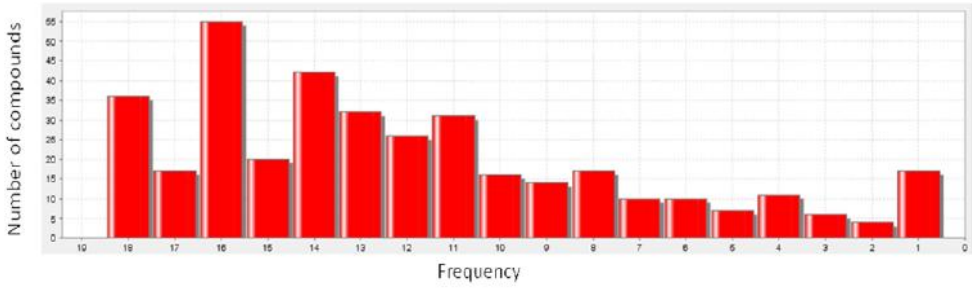
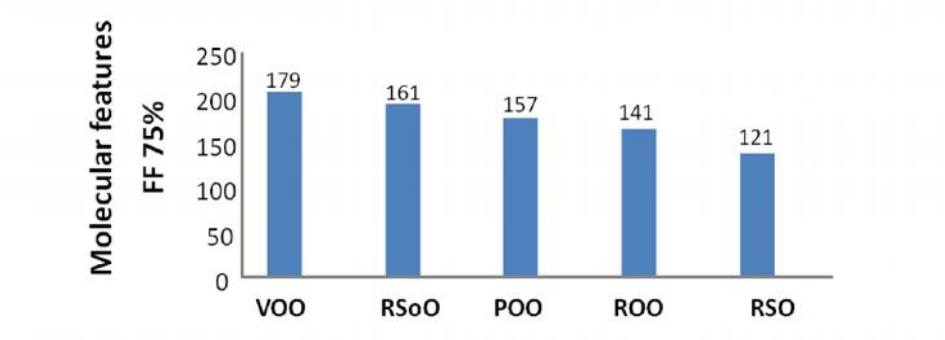
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Frequency plot of the molecular features detected in the non-saponifiable fraction of vegetable oils.



Supplementary Figure 2. Number of molecular features detected in the non-saponifiable fraction of vegetable oils after applying a filter by frequency (75%).

SECCIÓN B: CARACTERIZACIÓN DE LA FRACCIÓN FENÓLICA DE LOS VOOs POR ESPECTROMETRÍA DE MASAS. ESTUDIO DE LA INFLUENCIA DE LA MADURACIÓN Y EL GENOTIPO SOBRE ESTA FRACCIÓN

Las secciones de la B a la D tienen como denominador común el desarrollo y aplicación de herramientas analíticas y metabolómicas para su implementación en programas de mejora de olivo con el fin de demostrar su aplicabilidad. Las familias de compuestos estudiados fueron las de ácidos grasos y la fracción fenólica. La separación en diferentes secciones se justifica por la muy distinta naturaleza química de las familias de compuestos implicadas, que requiere una diferente instrumentación analítica y unos también diferentes procedimientos de preparación de la muestra.

Se ha dedicado esta Sección B a los compuestos fenólicos, ya que son los que imprimen un carácter diferencial más marcado a los VOOs respecto a otros aceites. De forma global, la investigación que recoge esta sección ha puesto de manifiesto la utilidad del análisis metabolómico orientado y global en los programas de mejora de olivo y, de forma particular, su aplicación para evaluar la influencia del genotipo y del grado de maduración del fruto en la composición de esta fracción.

CAPÍTULO 5:

Characterization of monovarietal virgin olive oils by phenols profiling

Enviado a Talanta

Characterization of monovarietal virgin olive oils by phenols profiling

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Characterization of monovarietal virgin olive oils by phenols profiling

Verónica Sánchez de Medina, Feliciano Priego-Capote*, María Dolores Luque de Castro

Abstract

The phenols profile of seven monovarietal virgin olive oils (VOOs) —*viz.* Arbequina, Arbosana, Cornicabra, FS-17, Hojiblanca, Picual and Sikitita— was characterized by using a quantitative strategy based on LC–MS/MS and the selected reaction monitoring (SRM) mode. Data dependent methods, based on precursor ion scanning, product ion scanning and neutral loss scanning, were developed for confirmatory analysis of secoiridoids. The observed phenols profiles were used to find correlation between pairs of phenols and trends similarity among the monovarietal VOOs. A Pearson analysis revealed several correlations among phenols with p-value < 0.01 and correlation coefficient (R) > 0.75, in the seven monovarietal VOOs. Cluster Analysis showed two main clusters between VOOs, formed by Arbequina/Hojiblanca/Cornicabra/Picual and Sikitita/Arbosana/FS-17. High correlations (R > 0.7) were observed for the following pairs of VOOs: Arbequina/Hojiblanca (R = 0.77), essentially supported on levels of hydroxytyrosol acetate (3,4-DHPEA-AC) and dialdehydic forms of secoiridoids; Cornicabra/FS-17 (R = 0.81) and Picual/FS-17 (R = 0.79), by correlation of flavonoids and secoiridoid derivatives in general. The highest correlation was observed for the pair Picual/Cornicabra (R = 0.99). This preliminary study allowed setting similarities and dissimilarities between monovarietal VOOs by analysis of the phenols profile. The observed connections between phenols for different varieties have been interpreted according to the main pathways for phenols biosynthesis.

INTRODUCTION

The minor fraction of virgin olive oil (VOO), formed by a chemically heterogenous group that includes more than 230 compounds, represents about 2% (w/w) of VOO. This fraction encompasses non-polar compounds such as phytosterols, waxes or hydrocarbons, and a polar or mid-polar sub-fraction[1] mostly constituted by volatile and phenolic compounds. Phenols present in VOO are also characterized by a chemical diversity of families such as phenolic alcohols and derivatives, phenolic acids, hydroxy-iso-chromans, flavonoids, lignans and secoiridoids. Among them, secoiridoids, the most concentrated phenols present in VOO, are specific compounds of *Oleaceae* plants.[2] In fact, secoiridoids differentiate VOO as unique among other vegetable oils.

Phenolic compounds have been widely studied because of their nutraceutical effects, relevant contribution to the sensory properties of VOO with special emphasis on bitterness and pungency, and their stabilizing role to ensure the long shelf-life of VOOs as compared to other vegetable oils.[3] The nutraceutical and stability properties of phenolic compounds have been linked to the capability of the phenolic structure for inhibiting oxidation processes. Numerous studies have evaluated the oxidation inhibition capability of phenols present in VOO, which allowed identifying hydroxy-tyrosol as the VOO phenol with the highest oxidation inhibition power by the DPPH test, followed by oleuropein aglycon (3,4-DHPEA-EA) and decarboxymethyloleuropein aglycon (3,4-DHPEA-EDA).[4] The EFSA (European Food Safety Authority) report has emphasized the inhibition activity of hydroxytyrosol and secoiridoids against the oxidation of phospholipids and LDL cholesterol.[5] In fact, the antioxidant capability of hydroxytyrosol is responsible for its prevention role against tumoral diseases, cardiovascular diseases such as atherosclerosis,[6] and diabetic neuropathies.[7] Secoiridoids also possess several health properties such as their implication in the inhibition of blood platelet aggregation, involvement in the synthesis of thromboxane in

human cells and protection of erythrocytes from oxidative damage.[8] Additionally, the anti-inflammatory activity of decarboxymethyl ligstroside aglycon (*p*-HPEA-EDA,[9] and recent evidences about its action on Alzheimer's disease[10] have been reported. One other particular family of phenols such as the flavonoids have also been associated to health benefits related with cancer and coronary heart diseases.[11–13] Concerning the organoleptical properties of VOOs, the bitter intensity has been correlated with the level of individual phenols,[14–16] mainly with hydroxytyrosol derivatives.[17–19] Two other secoiridoids such as 3,4-DHPEA-EDA and *p*-HPEA-EDA have also been associated to pungency character found in many VOOs.[20] Furthermore, a negative correlation has been found between the vanillin content of VOO and their bitter sensory attributes.[21]

Numerous studies have been published dealing with the quantitative and qualitative analysis of phenols in VOO. However, few of them have been focused on phenolic analysis of monovarietal VOOs for characterization of phenols profiles. Thus, Lorenzo-Sánchez et al. proposed a study on the influence of the phenolic composition determined in five monovarietal VOOs on the cytotoxic activity against human breast cancer cells.[22] The aim of this study was to characterize the phenolic composition of monovarietal VOOs by using a quantitative strategy based on LC–MS/MS and the selected reaction monitoring (SRM) mode. The observed phenols profiles were used to find correlation between pairs of phenols and trends of similarity among the monovarietal oils.

MATERIALS AND METHODS

Monovarietal Virgin Olive Oil Samples

The monovarietal VOO samples used in this work were obtained from olive fruits collected from 15th to 22nd November 2013 at intermediate ripening from different cultivars located in different areas of the south of Spain: Arbequina (Sevilla), Arbosana (Córdoba), Cornicabra (Toledo),

Hojiblanca (Málaga), Picual (Jaén), Sikitita (Córdoba) and FS-17 (Córdoba). Oils samples were stored in darkness at –20 ºC.

Reagents

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

Standards of target phenols for quantitative analysis of these metabolites in VOO were from Extrasynthese (Genay, France), consisting of hydroxytyrosol, tyrosol and verbascoside as simple phenols, and phenyletanoids such as oleuropein representing secoiridoids; luteolin, apigenin, apigenin-7-glucoside, luteolin-7-glucoside, diosmetin, quercetin and rutin as flavonoids; and vanillin, caffeic acid, *p*-coumaric acid, ferulic acid and vanillic acid as phenolic aldehydes and acids. Syringic acid, used as internal standard (IS), was from Extrasynthese (Genay, France). Individual stock standard solutions and multistandard solutions were prepared by dilution of each compound in methanol. The solutions were stored in a freezer at −20 ºC.

Apparatus and Instruments

An MS2 minishaker from Ika (Wilmington, USA) was used to enhance the transfer of phenols from oil to a methanol–water solution for individual quantification of the target compounds. Phenols extracts were analyzed by an Agilent 6410 triple quadrupole (QqQ) detector furnished with an electrospray ionization (ESI) source.

Extraction of Phenols from VOO

Phenolic compounds were extracted from VOO by shaking 1 g of oil with 1 mL of hexane and 1 mL of 60:40 methanol–water mixture for 1 min in the MS2 minishaker. The hydroalcoholic phase was directly injected into

the LC–QqQ. This approach has been widely validated in previous research and is accepted as sample preparation strategy for analysis of phenolic compounds in VOO.[23–25]

LC–MS/MS Analysis of Phenols Extracts

Analyses were performed by reversed-phase liquid chromatography followed by negative electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) detection.

10 μL of extract was injected in triplicate into the LC system for chromatographic separation of the target compounds using a Mediterranea C18 analytical column (150 mm×0.46 mm i.d., 3 μm particle size) from Teknokroma (Barcelona, Spain). The column compartment was kept at 30 C. The mobile phases were: phase A (0.1% formic acid in water) and phase B (0.1% formic acid in methanol). The gradient program, at 0.8 mL/min constant flow rate, was as follows: initially 96% phase A and 4% phase B kept for 2 min; from 2 to 17 min, the mobile phase A was from 96 to 34%; from 17 to 35 min, mobile phase A was from 34 to 0% A, while B ranged from 66 to 100% B. This composition was kept for 5 min. After each analysis, the column was equilibrated for 7 min up to the initial conditions.

The entire eluate was electrosprayed and ionized in negative mode and monitored by MS/MS in Selected Reaction Monitoring (SRM) mode by monitoring selective transitions from precursor to product ions. The flow rate and temperature of the drying gas (N_2) was 10 L/min and 300 C, respectively. The nebulizer pressure was 50 psi and the capillary voltage 3000 V. The dwell time was set at 200 μs. Identification and quantification of the phenols were performed using commercial standards, except for hydroxytyrosol acetate (3,4-DHPEA-AC) and secoiridoid derivatives, for which commercial standards were not available. For this reason, these compounds were relatively quantified as oleuropein equivalents.

Statistical Analysis

After LC–MS/MS analysis, a data set was built with the concentrations of phenols found in each analytical sample. Normalization by logarithmic transformation was used as pre-processing step. The definitive data set was exported to Statgraphic software (Centurion XV.I) for statistical analysis by Pearson correlation analysis. Additionally, the data set was also exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for Clustering Analysis, which allowed organizing samples into clusters based on similarity of their abundance profiles.

RESULTS AND DISCUSSION

Parameters of the Selected Reaction Monitoring Method

A panel of 16 phenols, which can be considered among the most important phenols in VOO with available commercial standards, was selected for determination purposes.[23,26,27] The monitored compounds were hydroxytyrosol and tyrosol, caffeic acid, *p*-coumaric acid, ferulic acid, vanillic acid, vanillin, verbascoside, apigenin, luteolin and their corresponding glucosides, diosmetin, quercetin, rutin and oleuropein.

The most sensitive transitions from precursor ions to product ions were used for quantitation of phenols, whereas secondary transitions were used as qualifiers for confirmatory analysis. The SRM transitions and the corresponding acquisition parameters such as isolation voltage of the first quadrupole and collision energy were optimized by using standard solutions resulting in the values listed in **Table 1**. SRM transitions with quantitative purposes agreed with those reported in methods previously published, which have also identified the chemical structure associated to product ions formed after MS/MS fragmentation.[28,29]

Table 1. Optimization of the MS/MS step for qualitative and quantitative determination of phenols.

Phenol	Fragmentor (V)	Precursor ion	Collision energy (eV)	Quantitative transition (*m/z*)	Product ion confirmation (*m/z*)
Hydroxytyrosol	110	153.1	10	153→123	108
Tyrosol	110	137	10	137→119	81
Caffeic acid	110	179	13	179→135	107
p-Coumaric acid	80	163	10	163→119	93
Ferulic acid	110	193	13	193→134	178
Oleuropein	170	539	23	539→307	275
Luteolin	170	285	35	285→133	175
Apigenin	170	269	35	269→117	151
Vanillic acid	80	167	11	167→151	108
Vanillin	80	151	14	151→136	108
Diosmetin	170	299	20	299→284	256
Quercetin	170	301	20	301→179	151
Apigenin-7-glucoside	220	431	35	431→268	225
Luteolin-7-glucoside	230	447	35	447→285	284
Verbascoside	80	623	35	623→161	179

Quantitation was carried out using the ratio between the peak area of each phenol and that of the IS. Calibration models were developed for each phenol using multistandard solutions. Nine concentration levels from 0.25 ng/mL to 5 μg/mL were analyzed in triplicate to obtain the calibration curves. The calibration equations, coefficients of regression (R^2) and limis of quantitation (LOQs) are listed in **Table 2**.

Other phenols for which no commercial standards are available were also included in this study due to their relevance in VOO in terms of concentration as well as nutraceutical properties and sensory contribution. They were 3,4-DHPEA-AC, *p*-HPEA-EDA and 3,4-DHPEA-EDA 3,4-DHPEA-EA and ligstroside aglycon (*p*-HPEA-EA). These phenols were quantified in relative terms by using the calibration curve of hydroxytyrosol in the case of 3,4-DHPEA-AC or that of oleuropein for the secoiridoids. Nevertheless, confirmatory analysis was required to prove their presence in monovarietal VOO samples included in this study due to the absence of standards. For this purpose, data-dependent methods were used to scan the

presence of these target metabolites by taking benefits from the versatility of the triple-quad mass analyzer, particularly, precursor ion scanning, product ion scanning and neutral loss scanning.

Table 2. Regression equations, correlation coefficients and limits of quantification for analysis of phenolic compounds.

Phenol	Calibration equations	Regression coeficient (R^2)	LOQ (μg/mL)
Hydroxytyrosol	y=15.938x-0.5866	0.993	0.25×10^{-3}
Tyrosol	y=0.0108x-0.0128	0.981	1
Caffeic acid	y=31.8938x-0.6562	0.995	0.25×10^{-3}
***p*-Coumaric acid**	y=20.2813x+0.6944	0.998	0.05
Ferulic acid	y=7.1723x-0.6368	0.991	0.01
Oleuropein	y=4.1947x-2.0536	0.995	0.25×10^{-3}
Luteolin	y=33.6232x-0.7341	0.998	0.25×10^{-3}
Apigenin	y=40.7716x-19.5034	0.993	0.25×10^{-3}
Vanillic acid	y=0.7035x-0.0135	0.994	0.01
Vanillin	y=2.2616x-0.0659	0.993	0.01
Diosmetin	y=21.9695x-1.1352	0.997	0.25×10^{-3}
Quercetin	y=0.4437x-0.0163	0.994	0.25×10^{-3}
Apigenin-7- glucoside	y=33.3276x-1.2567	0.992	0.25×10^{-3}
Luteolin-7-glucoside	y=11.2851x-0.2514	0.991	0.25×10^{-3}
Verbascoside	y=2.3377x-0.0526	0.992	0.25×10^{-3}

Confirmatory Analysis of Secoiridoids by Data-Dependent Methods

Oleuropein is the major secoiridoid found in the pulp of olives,[30] where its concentration reaches relatively high levels in immature fruits (around 2.7 mg/g).[31] However, its concentration is declined with progression of the maturation process of the fruit to form secoiridoid derivatives such as 3,4-DHPEA-EDA, *p*-HPEA-EDA, 3,4-DHPEA-EA and *p*-HPEA-EA as well as hydroxytyrosol, 3,4-DHPEA-AC, tyrosol and elenolic acid glucoside, which can be considered as indicators of the extent of olives maturation.[32–34] The formation of these secoiridoid derivatives as well as that of phenolic alcohols is significantly enhanced during the process for production of VOO by action of β-glucosidases, which are released during the pressing step. For this reason, these compounds are massively found in VOO instead of oleuropein.

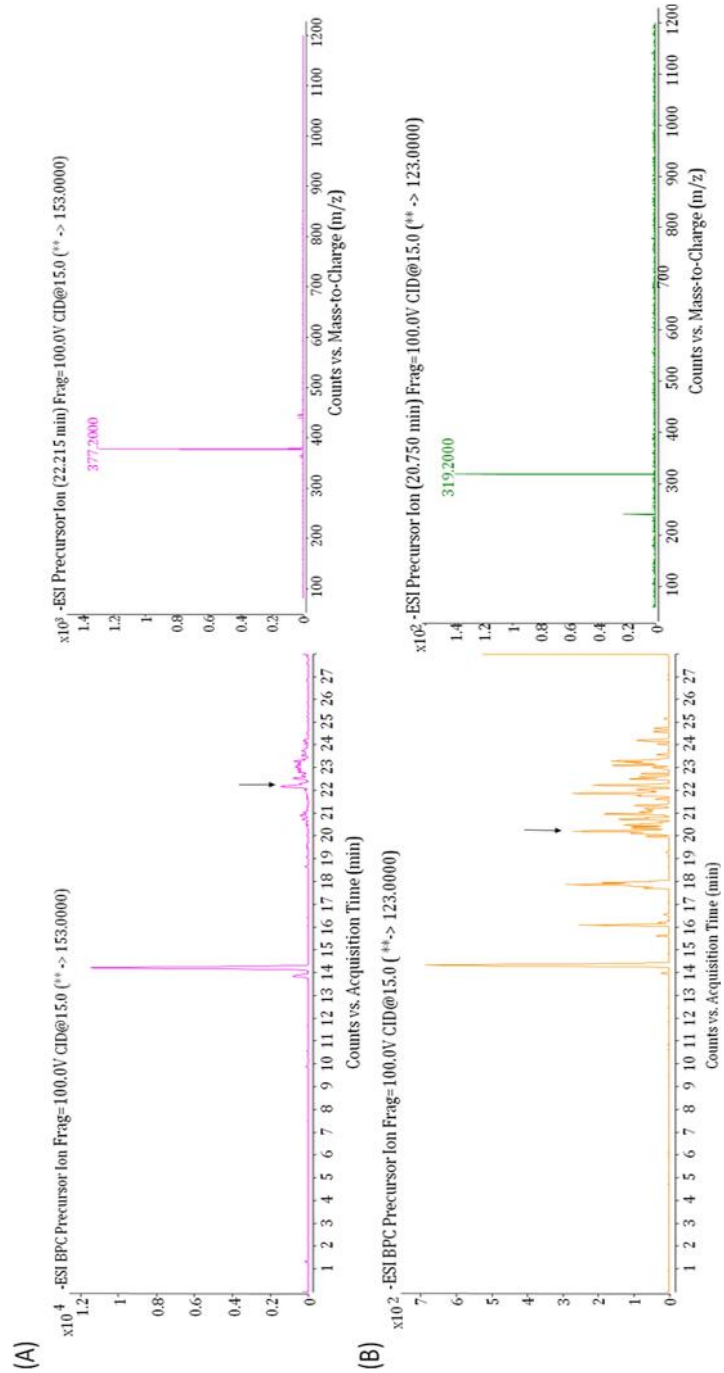

Figure 1. Precursor ion scanning for qualitative analysis of (A) 3,4-DHPEA-EA and (B) 3,4-DHPEA-EDA.

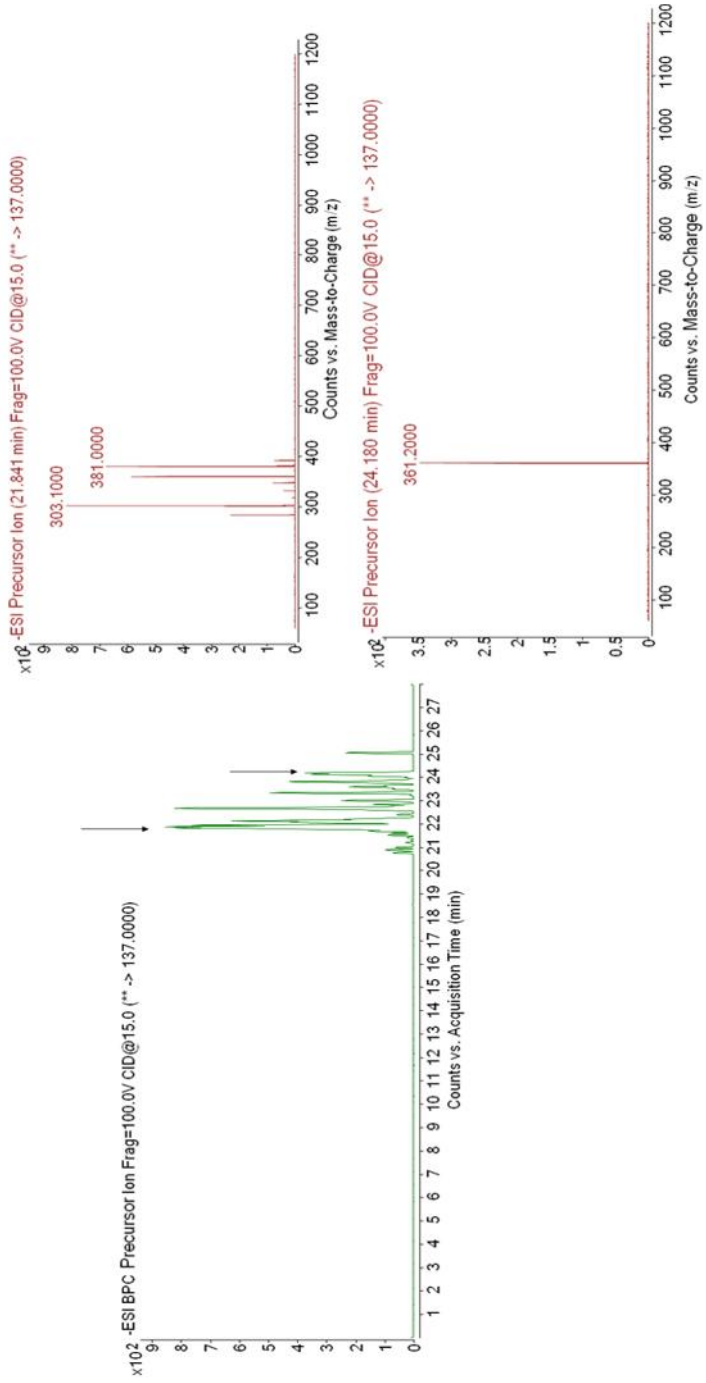

Figure 2. Precursor ion scanning for qualitative analysis of p-HPEA-EA and p-HPEA-EDA.

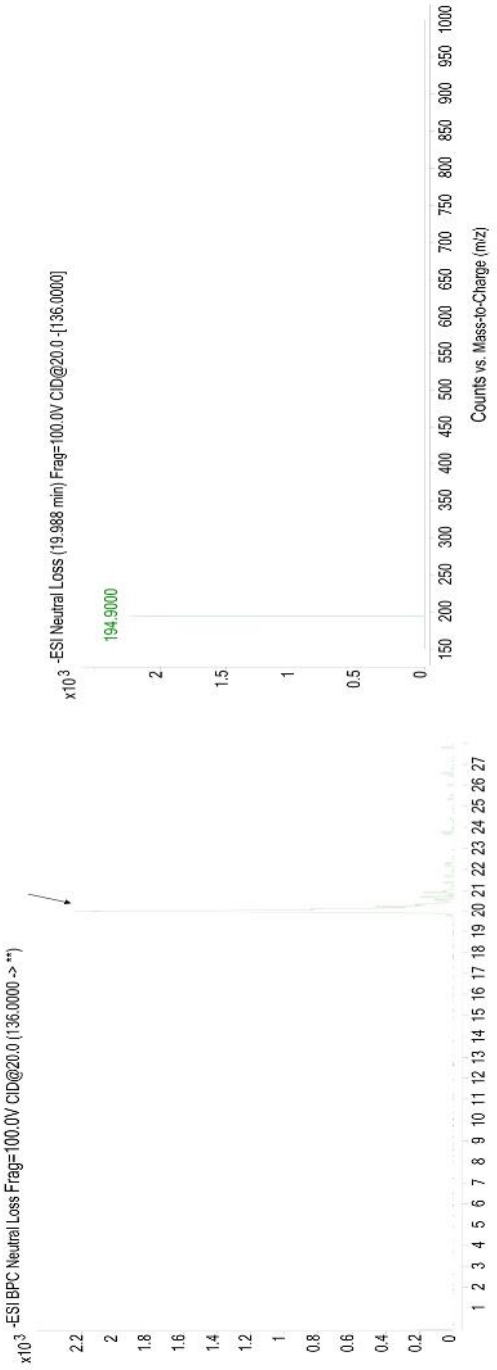

Figure 3. Neutral loss scanning for qualitative analysis of 3,4-DHPEA-AC.

Taking into account the structural similarity of these secoiridoid derivatives with hydroxytyrosol and tyrosol, data-dependent methods based on precursor ion scanning, product ion scanning and neutral loss scanning were developed. Thus, 3,4-DHPEA-EDA and 3,4-DHPEA-EA are esters of hydroxytyrosol, while *p*-HPEA-EDA and *p*-HPEA-EA are esters of tyrosol since the two sets of compounds are formed by hydrolysis of oleuropein and ligstroside, respectively. With these premises, a precursor ion scanning method by monitoring representative ions found for hydroxytyrosol and tyrosol (identified as molecular ions or stable fragments) at the third quadrupole (Q3) could scan compounds formed by condensation reaction of the two phenolic alcohols in the first quadrupole (Q1). Precursor ion scanning by monitoring the precursor ion for hydroxytyrosol m/z 153 at Q3 allowed detecting an ion with precursor ion m/z 377 at 22.2 min elution time as **Figure 1.A** shows, which fit to the $[M–H]^-$ ion of 3,4-DHPEA-EA. Similarly, one other compound with precursor ion m/z 319, which corresponded to 3,4-DHPEA-EDA, was detected by monitoring the m/z 123 at Q3 (20.75 min), which is the most stable product ion formed by fragmentation of hydroxytyrosol (**Figure 1.B**). Product ion scanning for m/z 377 and 319 revealed two selective transitions with quantitative purposes for 3,4-DHPEA-EA and 3,4-DHPEA-EDA by monitoring product ions at m/z 275 and 59, respectively, which have previously been detected.[28,29] Neutral loss scanning analysis by monitoring 102 and 260 mass units allowed confirming the presence of these two secoiridoid derivatives as **Supplementary Figure 1** shows.

The two derivatives obtained by hydrolysis of ligstroside, *p*-HPEA-EA and *p*-HPEA-EDA, were identified by the same strategy. The presence of the two derivatives was detected by precursor ion scanning by monitoring m/z 137 at Q3, which fits with the cleavage of tyrosol by the ester bound. Thus, two precursor ions with m/z 361 and 303 were detected at retention time 24.1 and 21.9 min as **Figure 2** shows, which were identified as *p*-

HPEA-EA and *p*-HPEA-EDA, respectively. Product ion scanning for both precursors enabled to find quantitative transitions from *m/z* 361 and 303 to product ions at *m/z* 291 and 59. Neutral losses observed for these transitions, 70 and 244 mass units, were scanned by tuning Q1 and Q3 to confirm the presence of both ligstroside derivatives (**Supplementary Figure 2**).

The presence of 3,4-DHPEA-AC was verified by precursor ion scanning by monitoring *m/z* 59 at Q3, which provided a chromatographic peak at 19.9 min with precursor *m/z* 195. The identity of this secoiridoid derivative was confirmed by neutral loss scanning of 136 Da, which fit the fragment formed by hydrolysis of the ester bound. These results are illustrated in **Figure 3**.

After verification and confirmatory analysis of oleuropein derivatives in monovarietal VOOs, optimization of the method for these compounds was carried out. **Table 3** summarized the main result obtained by data-dependent methods, as well as qualitative and quantitative MS/MS parameters.

Table 3. Optimization of the MS/MS step for qualitative and quantitative determination of oleuropein derivatives.

Phenol	Precursor ion	Neutral loss	Fragmentor (V)	Collision energy (eV)	Quantitative transition (*m/z*)	Product ion confirmation (*m/z*)
3,4-DHPEA-AC	195.1	136	110	12	195→59	135
3,4-DHPEA-EDA	319.1	260	110	12	319→59	139
***p*-HPEA-EDA**	303.1	244	110	12	303→59	137
3,4-DHPEA-EA	377	102	110	12	377→275	307
***p*-HPEA-EA**	361.1	70	110	12	361→291	101

Statistical Description of Phenols Profiles in Monovarietal VOOs

The averaged concentrations found for target phenolic compounds in each monovarietal VOO and standard deviation (SD) values are listed in **Table 4**. Hydroxytyrosol, the most representative phenol in VOO[35] was found at levels below 5 μg/g in all monovarietal VOO, except in FS-17, which provided very high levels of this phenol —around 25 μg/g— (**Figure 4.A**). This variety also reported the highest concentration of tyrosol (23.6 μg/g), followed by Arbosana with 18.7 μg/g, while the resting varieties gave levels below 6 μg/g (**Figure 4.B**).

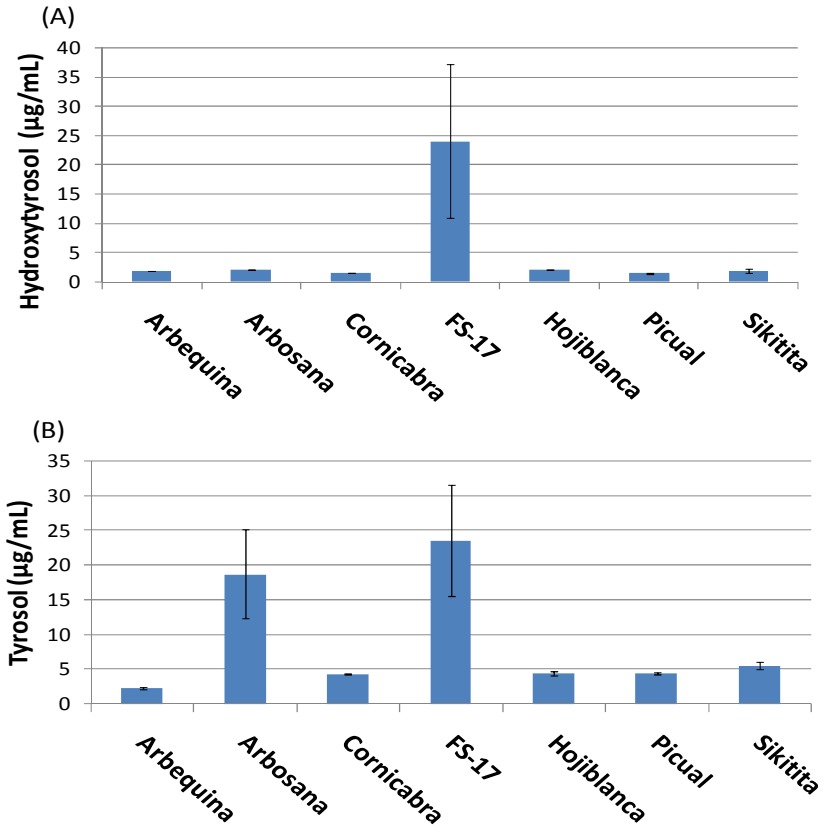

Figure 4. Concentration plots corresponding to: (A) hydroxytyrosol (B) tyrosol, in monovarietal VOOs.

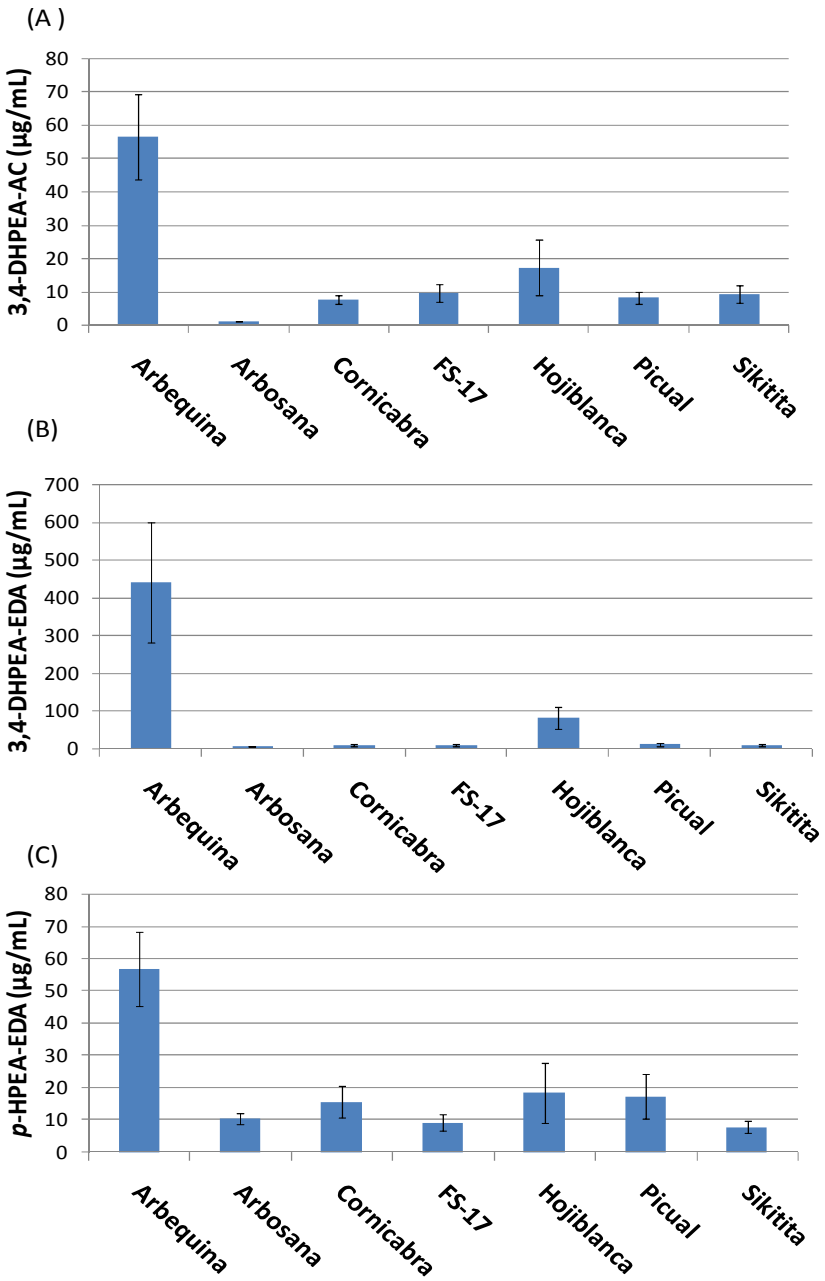

Figure 5. Concentration plots corresponding to: (A) 3,4-DHPEA-AC;(B) 3, 4-DHPEA-EDA; (C) p-HPEA-EDA, in monovarietal VOOs.

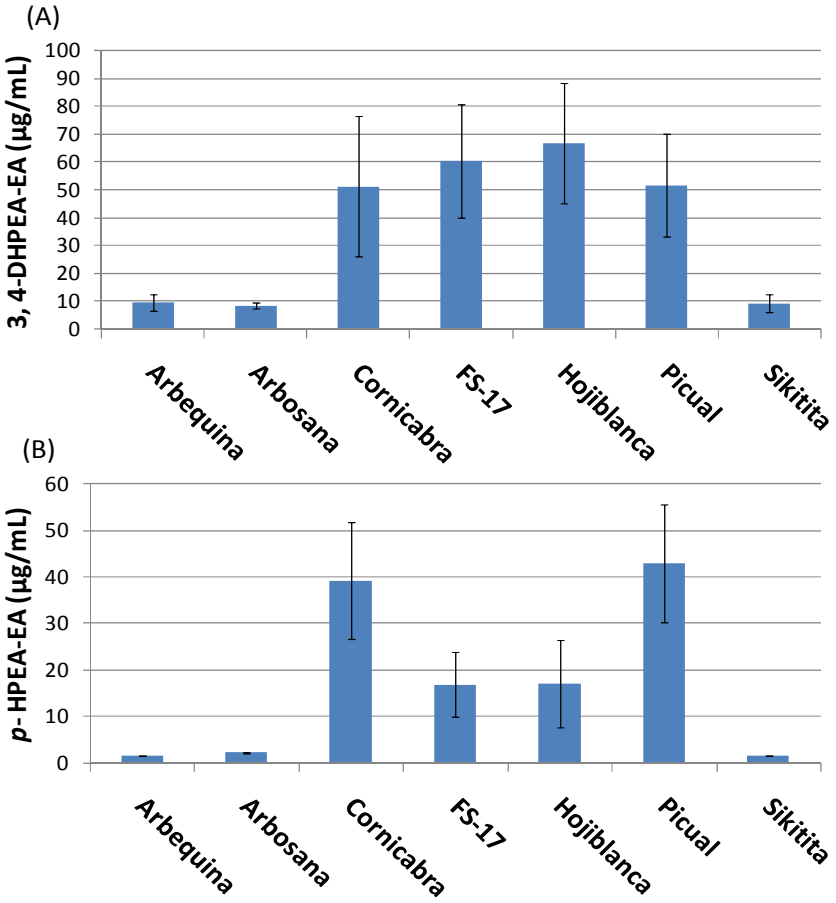

Figure 6. Concentration plots corresponding to: (A) 3, 4-DHPEA-EA (B) p-HPEA-EA, in monovarietal VOOs.

One other variety such as Arbequina was clearly outstanding in terms of 3,4-DHPEA-AC, 3,4-DHPEA-EDA and *p*-HPEA-EDA, which is clearly correlated to the bitter character of VOOs from Arbequina fruits (**Figure 5.A, B, and C**). Apart from Arbequina, Hojiblanca VOO was also characterized by extra levels of these secoiridoid derivatives as compared to the resting VOO varieties. On the other hand, four varieties reported VOOs with high levels of the aglycon forms of oleuropein and ligstroside, 3,4-DHPEA-EA and *p*-HPEA-EA, respectively. These varieties were Cornicabra, FS-17, Hojiblanca and Picual (**Figure 6. A and B**). Levels of these two

derivatives are strongly related to the pungent character of VOOs. Two phenolic compounds found at low concentrations were oleuropein, which did not exceed 0.03 μg/g in the different varieties, while verbascoside was only detected in Arbequina and Sikitita monovarietal VOOs.

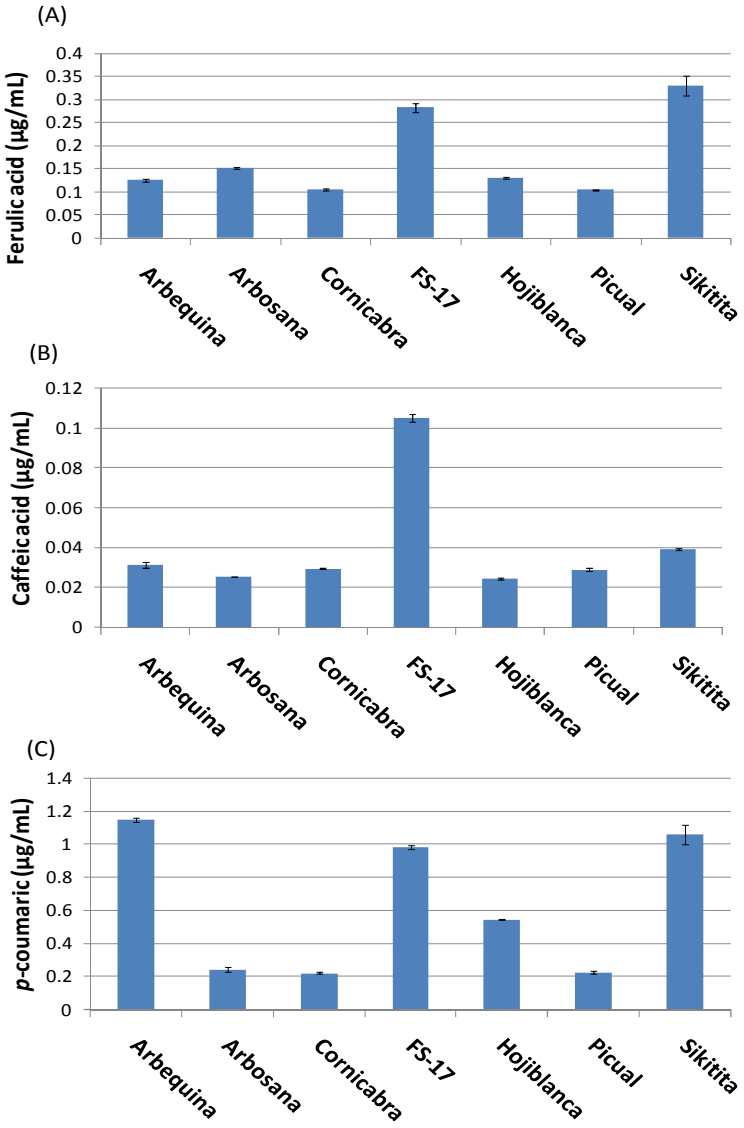

Figure 7. Concentration plots corresponding to: (A) ferulic;(B) caffeic; (C) p-coumaric acids, in monovarietal VOOs.

Ferulic, *p*-coumaric and caffeic acids are synthesized through the phenylpropanoid pathway, by which *p*-coumaric acid is hydroxylated to caffeic acid, and that methoxylated to ferulic acid. As **Figure 7.A, B** and **C** shows, these phenolic acids were more concentrated in FS-17, Arbequina and Sikitita VOOs, which could be attributed to a higher activity of the enzymes involved in this pathway. Vanillin and its carboxylic acid vanillin presented an opposite behavior. Thus, Cornicabra and Picual VOOs reported the lowest concentration of vanillic acid, whereas vanillin was high-concentrated in these VOOs as compared to the resting varieties (**Figure 8.A** and **B**). The activity of the oxidation enzyme to convert vanillin into vanillic acid could be responsible for this result.

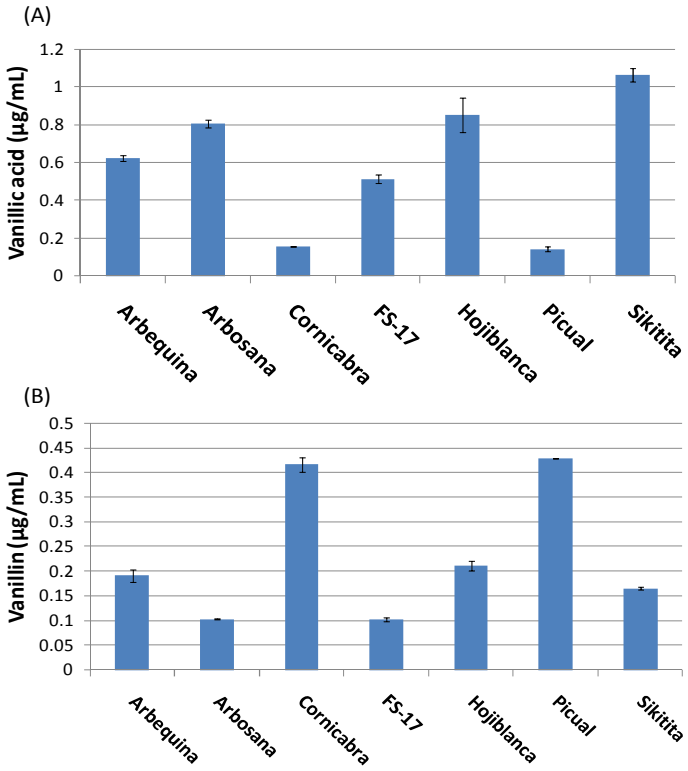

Figure 8. Concentration plots corresponding to: (A) vanillic acid; (B) vanillin, in monovarietal VOOs.

Concerning flavonoids, Arbequina, Arbosana, Hojiblanca and Sikitita VOOs provided higher levels of luteolin, apigenin and diosmetin than the other varieties (**Figure 9.A, B** and **C**). Levels of quercetin were slightly higher in Arbequina and Sikitita monovarietal oils by comparison to the resting varieties. Finally, no critical differences were observed between levels of glucoside flavonoids such as luteolin-7-glucoside, apigenin-7-glucoside and rutin.

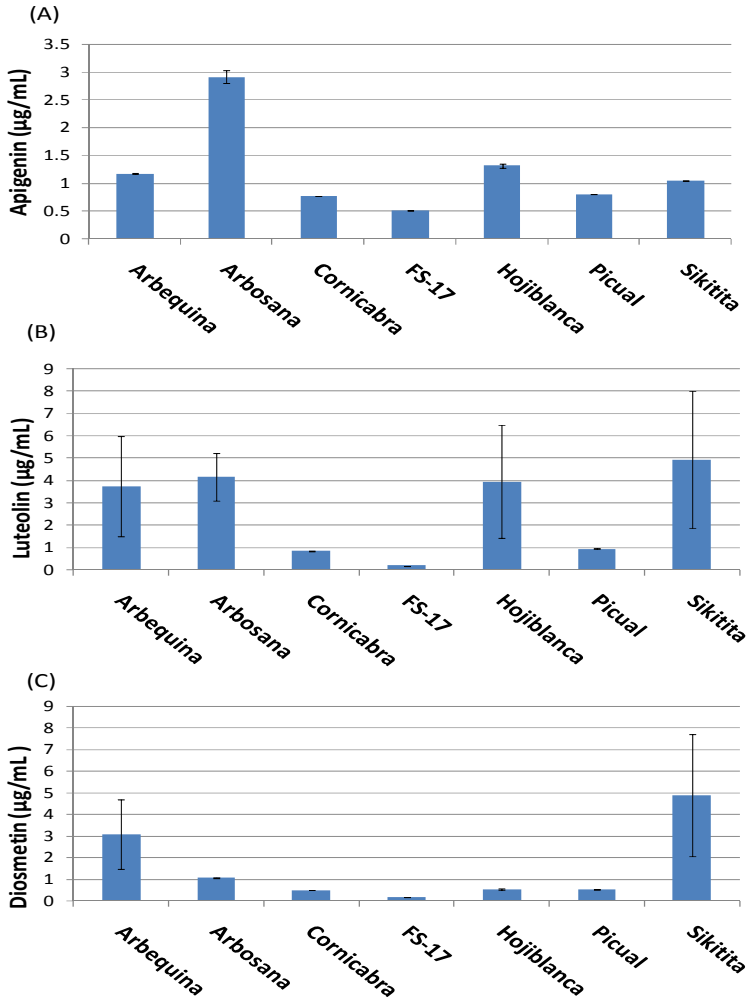

Figure 9. Concentration plots corresponding to: (A) apigenin; (B) luteolin; (C), diosmetin, in monovarietal VOOs.

Statistical Correlation between Phenolic Compounds

Considering as data set that formed by concentrations of phenols in the seven monovarietal VOO, a Pearson correlational analysis was carried out to find correlations between phenolic compounds. Several correlations were found between several pairs of phenols with *p*-value < 0.01 and correlation coefficient (*R*) > 0.75, which are indicative at least of a moderate relationship between them (**Supplementary Table 1**). A strong positive correlation was found between *p*-HPEA-EA and 3,4-DHPEA-EA (*R* = 0.93, *p*-value = 0.002) as well as between *p*-HPEA-EDA and 3,4-DHPEA-EDA (*R* = 0.89, *p*-value = 0.007). In the pathway for oleosides biosynthesis ligstroside is converted into oleuropein by hydroxylation.[36] This process is quite active at the early stages of maturation through which fruits are highly-concentrated in oleuropein. However, it can be assumed that ripening as well as crushing and malaxation of olive fruits during oil production releases β-glucosidases, which produce the aglycon forms of ligstroside and oleuropein (*p*-HPEA-EA and 3,4-DHPEA-EA),[30] which would support the positive correlation observed. Additionally, the aglycon forms are hydrolyzed and turned into the dialdehydic form of secoiridoids (*p*-HPEA-EDA and 3,4-DHPEA-EDA) by action of a decarboxymethylase, which also justifies the positive correlation found for the two dialdehydic secoiridoid forms. **Supplementary Figure 3** shows the biosynthetic pathways of secoiridoids.

Consistent positive correlations were also found within the flavonoids class. The highest correlation was detected for apigenin and luteolin glucosides (*R* = 0.91, *p*-value = 0.003). Both compounds are formed through the flavone and flavonols biosynthesis from their respective aglycone by glycosyltransferase. This common pathway explains the strong positive correlation. A significant negative correlation was observed between quercetin and vanillic acid (*R* = –0.91, *p*-value = 0.004). Vanillic acid is synthesized through the phenylalanine pathway, whereas quercetin is obtained in the biosynthesis of flavonoids. There is a connection point

between both pathways: the *p*-caffeoyl-CoA; therefore, depending on the activity of enzymes using p-caffeoy-CoA as substrate the prevalence of vanillic acid or quercetin would be justified. High activity of methyl-transferase enzyme would imply the formation of vanillic acid, while the synthesis of quercetin would be supported on the activity of chalcone synthase (**Supplementary Figure 4**).

Significant correlations with *p*-value between 0.01 and 0.05 were also found for some pairs of phenols. Thus, 3,4-DHPEA-EDA and 3,4-DHPEA-AC exhibited positive correlation (R = 0.89, *p*-value = 0.03) that could be explained because 3,4-DHPEA-AC is obtained by cleavage of 3,4-DHPEA-EDA.[8] Luteolin was also positively correlated to other two aglycon flavonoids such as diosmetin and apigenin (R = 0.81, *p*-value = 0.03 and R = 0.79, *p*-value = 0.03, respectively). Apigenin is the substrate for the synthesis of luteolin in the flavonoids pathway by action of a hydroxylase, which allows explaining this positive correlation. As previously mentioned, diosmetin is a methoxyderivative of luteolin and, for this reason, their concentrations in VOO could be correlated. Apigenin was also positively correlated with apigenin-7-glucoside (R = 0.82, *p*-value = 0.02), which could be ascribed to the glycosylation step that favors storage of certain phenols in VOOs.

Other remarkable correlations were those between vanillin–vanillic acid and luteolin–caffeic acid (R = –0.79, *p*-value = 0.03 and R = –0.76, *p*-value = 0.05, respectively). The former correlation can be explained because vanillic acid is obtained by oxidation of vanillin. Luteolin and caffeic are produced in two different pathways: the flavonoids and phenylpropanoid pathways, respectively. However, there is a nexus between them through *p*-coumaric acid. Consequently, the degree of concentration of each compound depends on the enzymatic activity. In this manner, higher concentration of caffeic acid implies a high hydroxylase activity. On the other hand, high

levels of luteolin assume an increased activity of the flavonoids biosynthesis pathway (**Supplementary Figure 5**).

Differences in the Phenolic Profiles of Monovarietal VOOs

The next step was to find similarities and differences between the phenols profiles of the analyzed monovarietal VOOs by Clustering Analysis. This analysis was applied to the complete data set. **Supplementary Figure 6** shows the cluster graph obtained by comparing phenolic profiles of monovarietal VOOs. The dendrogram reveals the relationships between phenols in one dimension and between VOO samples in the other dimension. Two main clusters can be observed in the graph: Arbequina, Hojiblanca, Cornicabra and Picual were classified within the same cluster, whereas the second one was formed by Sikitita, Arbosana and FS-17. Each cluster was divided into subclusters, which were constituted by VOO pairs, except for Sikitita VOO. The detected subclusters were Arbequina/Hojiblanca, Cornicabra/Picual and Arbosana/FS-17. Among them, the higher similarity was observed between Cornicabra and Picual VOOs since a parallelism was found in the levels of most phenols both with positive and negative correlation.

The main differences between VOO monovarietal samples were observed in the levels of certain groups of phenols. Thus, it was clear that levels of secoiridoids and derivatives are critical to establish differences between VOOs. In particular, levels of 3,4-DHPEA-AC, 3,4-DHPEA-EDA and *p*-HPEA-EDA followed an opposite trend to those observed for 3,4-DHPEA-EA and *p*-HPEA-EA. This was clearly detected in Arbequina and Hojiblanca VOOs, which provided high levels of EDA phenols and 3,4-DHPEA-AC (particularly Arbequina VOO) as compared to the other varieties. The contrary situation was observed for Sikitita and Arbosana VOOs, which were characterized by low levels of the EA secoiridoid derivatives, and for Cornicabra and Picual, which provided relatively high levels for these compounds. One other aspect to be emphasized was the high concentrations

of phenolic alcohols, hydroxytyrosol and tyrosol, and phenolic acids observed in FS-17. **Table 5** shows the correlation between the pairs of VOO samples according the measured concentration of phenolic compounds. As can be seen, high correlations ($R > 0.7$) were observed for the following pairs of VOO: Arbequina/Hojiblanca ($R = 0.77$), essentially supported on levels of 3,4-DHPEA-AC and EDA derivatives; Cornicabra/FS-17 ($R = 0.81$), Picual/FS-17 ($R = 0.79$) and Picual/FS-17 ($R = 0.74$), by correlation of flavonoids and secoiridoid derivatives in general. The highest correlation was observed for the pair Picual/Cornicabra ($R = 0.99$), which agrees with the parallelism between phenolic levels previously described.

This preliminary study allowed setting similarities and dissimilarities between monovarietal VOOs by analysis of the phenolic profile. The observed connections between phenolic compounds for different varieties have been interpreted according to the main pathways for biosynthesis of phenols.

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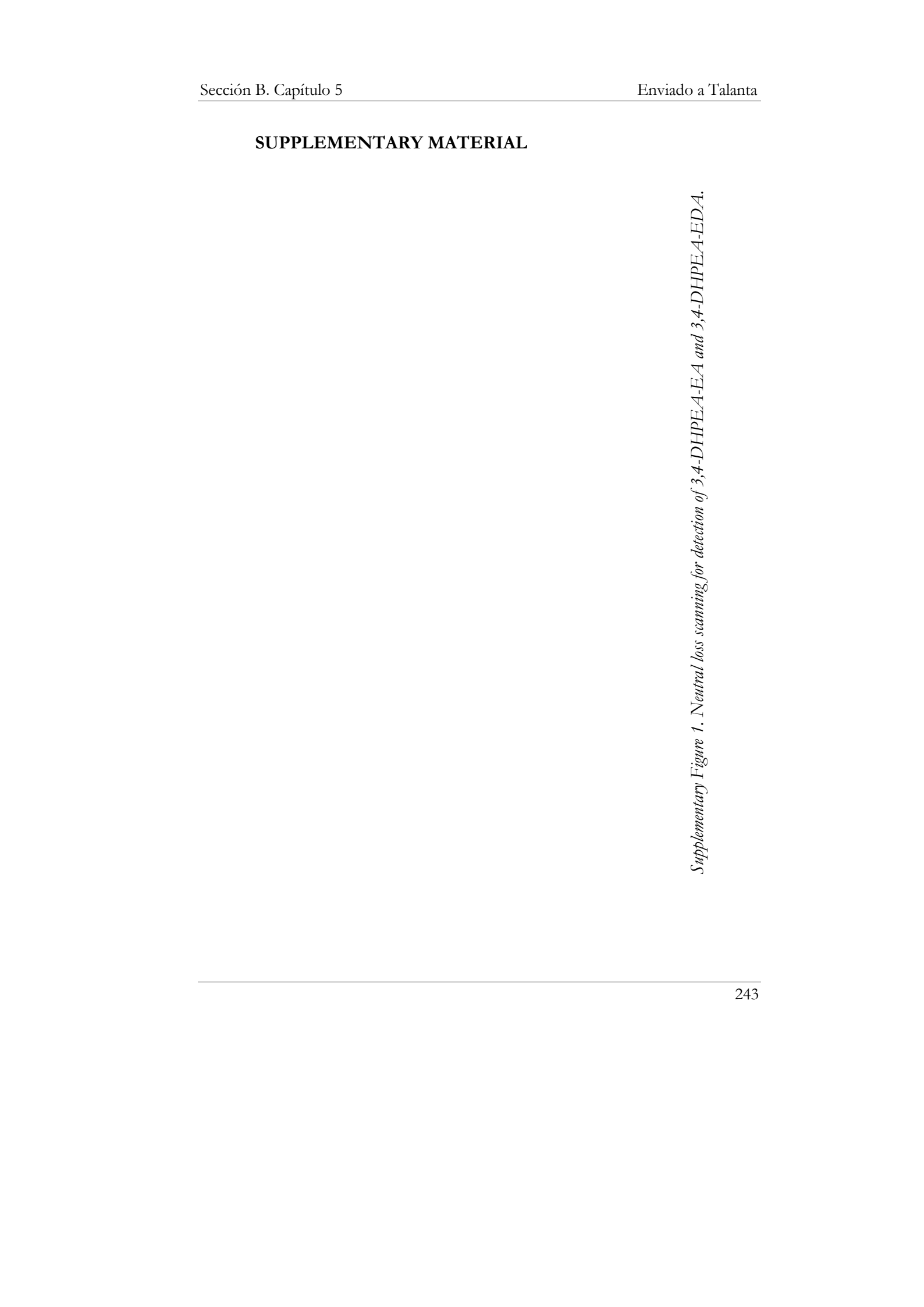
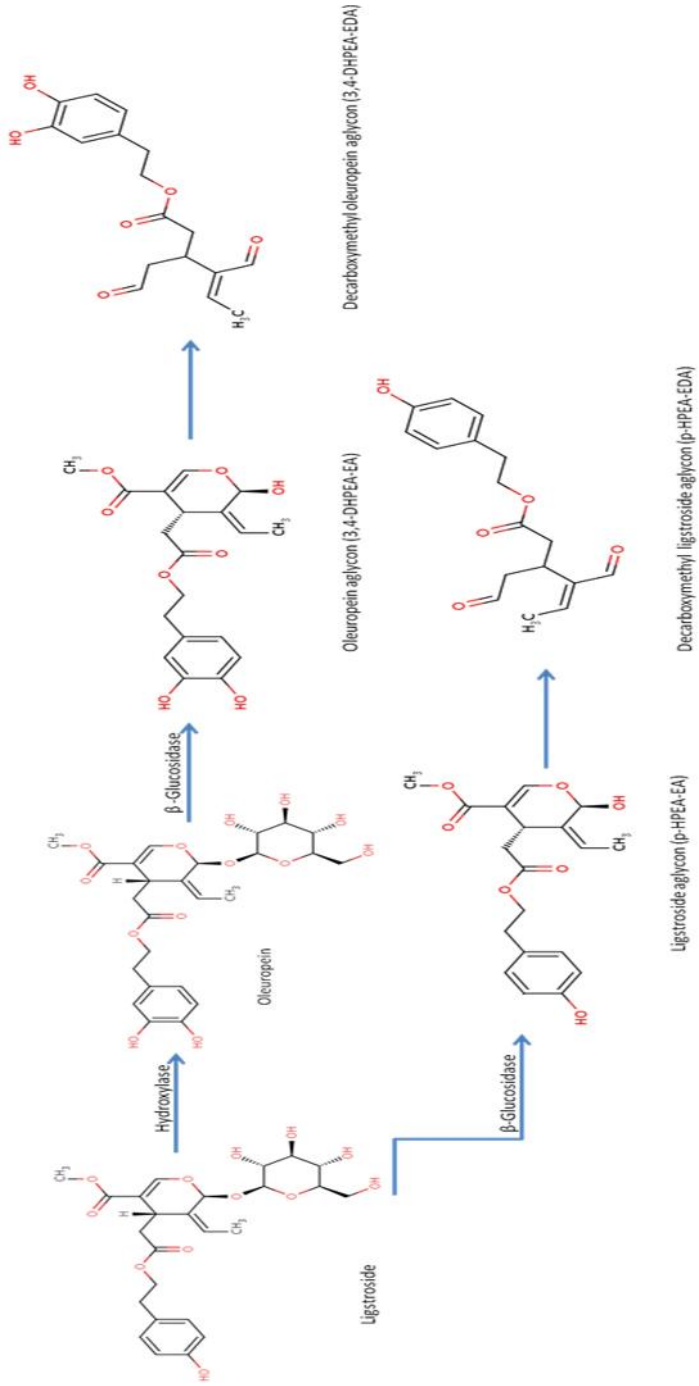
SUPPLEMENTARY MATERIAL

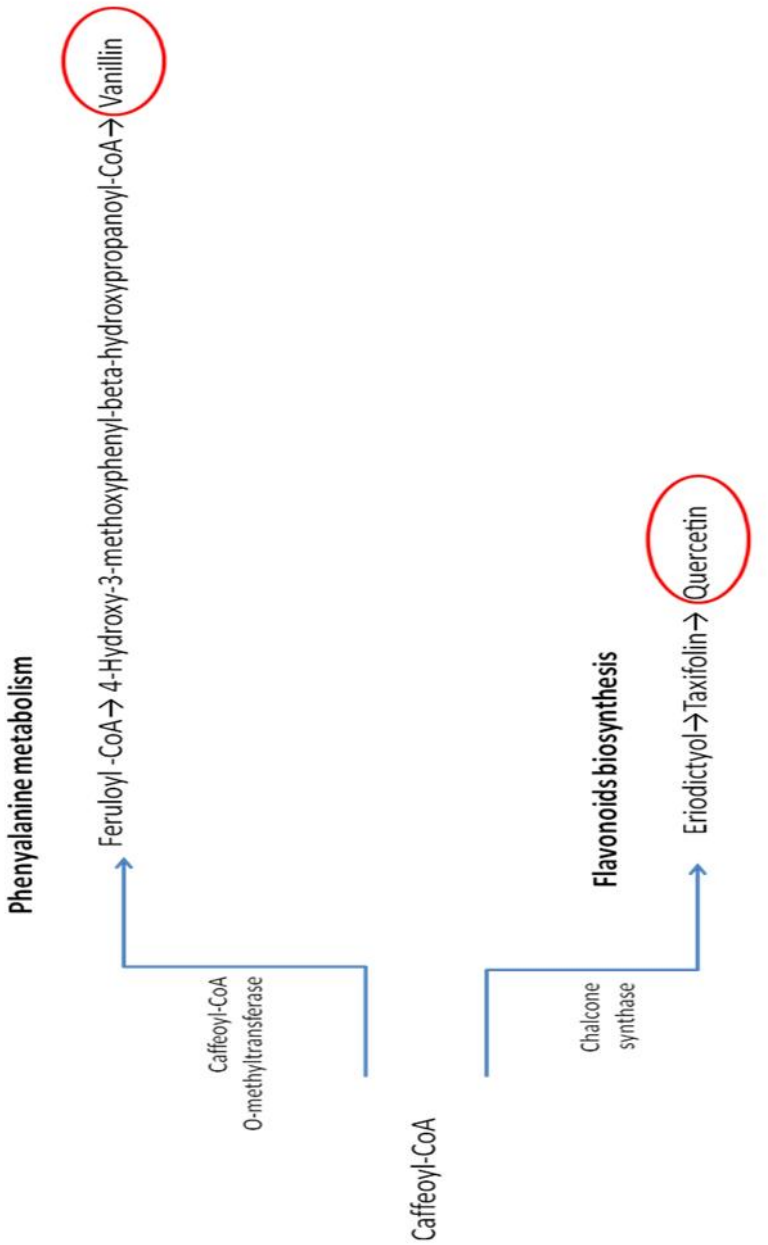


Supplementary Figure 1. Neutral loss scanning for detection of 3,4-DHPEA-EA and 3,4-DHPEA-EDA.

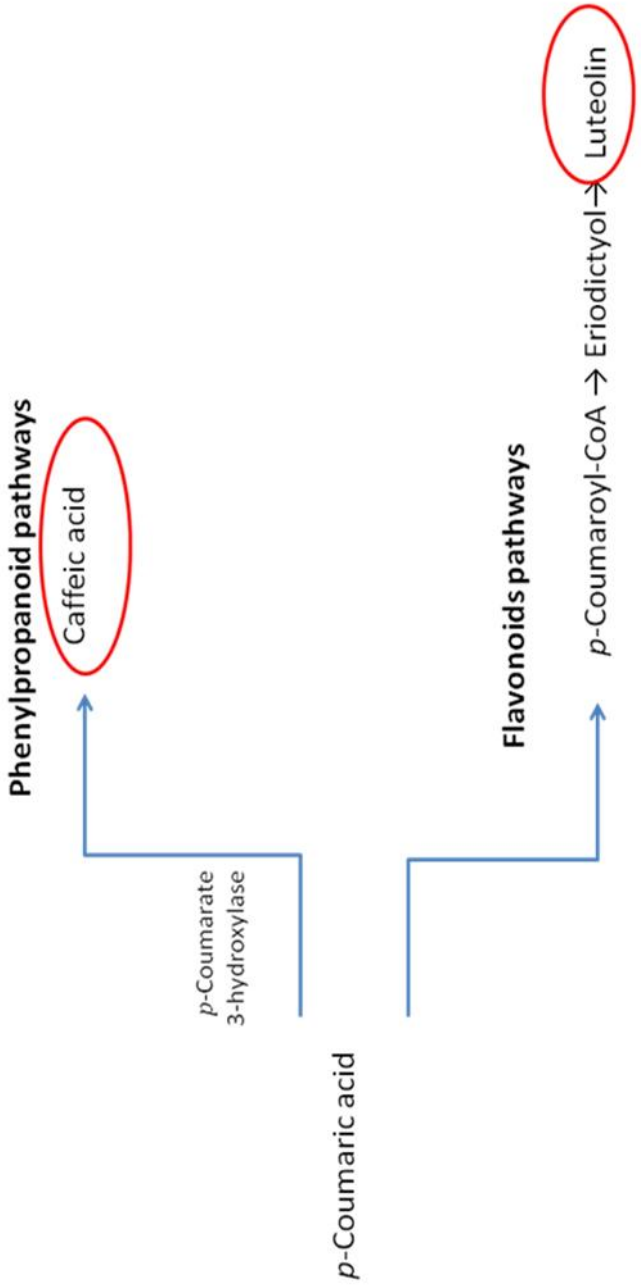
Supplementary Figure 2. Neutral loss scanning for detection of p-DHPEA-EA and p-DHPEA-EDA.



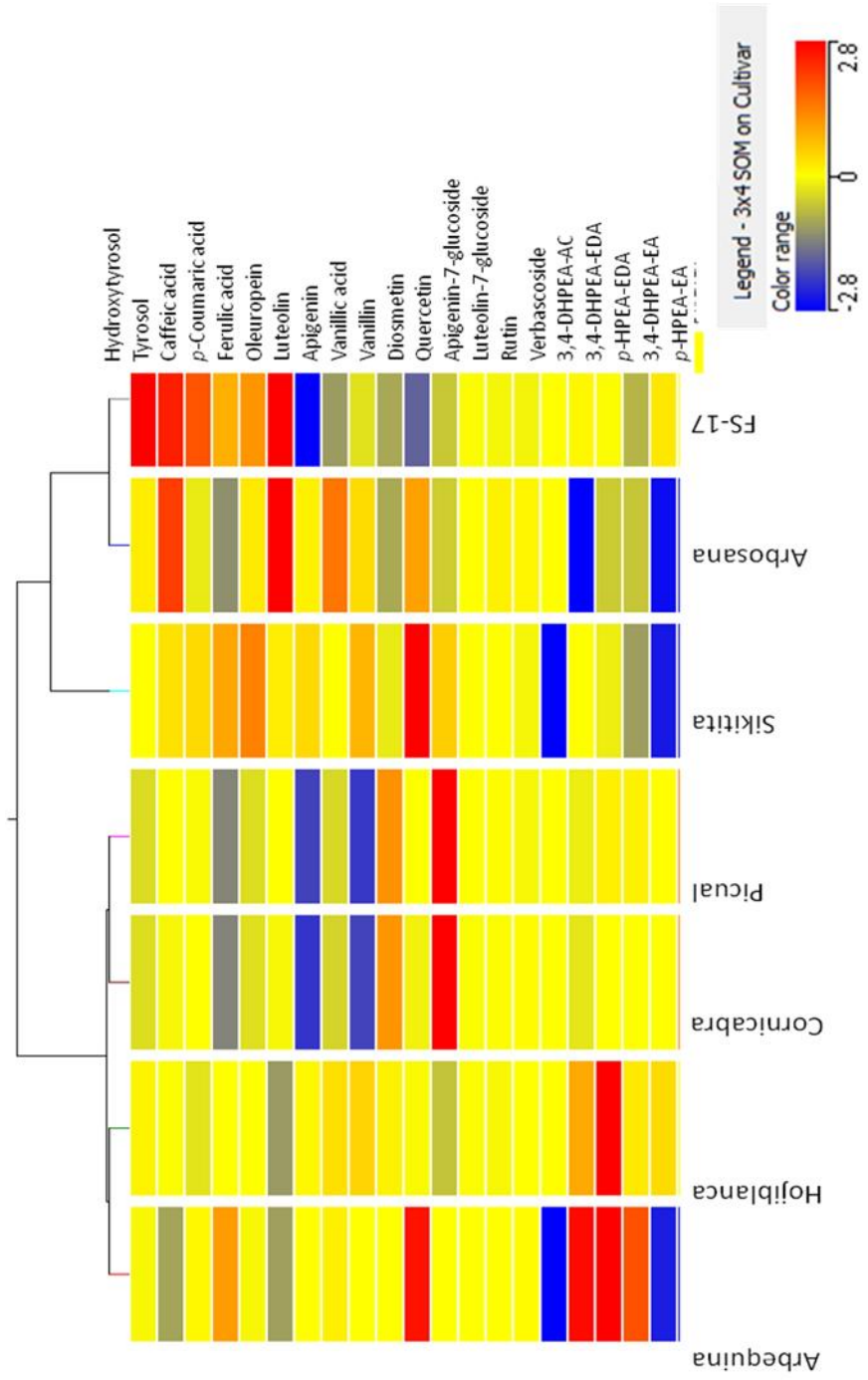
Supplementary Figure 3 Biosynthetic pathways of secoiridoids.



Supplementary Figure 4. Biosynthetic pathways of vanillin and quercetin.



Supplementary Figure 5. Biosynthetic pathways of caffeic acid and luteolin.



Supplementary Figure 6. Cluster diagrams for the seven monovarietal VOOs.

Supplementary Table 1. p-Values and correlation coefficients (R) obtained by Pearson correlational analysis of monovarietal VOO for each phenol pair.

FALTA ALGO	3,4-DHPEA-AC	3,4-DHPEA-EA	3,4-DHPEA-EDA	Apigenin
3,4-DHPEA-AC		0.1334	0.8093	-0.4628
		0.7755	0.0274	0.2957
3,4-DHPEA-EA	0.1334		-0.132	-0.6236
	0.7755		0.7779	0.1346
3,4-DHPEA-EDA	0.8093	-0.132		0.0926
	0.0274	0.7779		0.8435
Apigenin	-0.4628	-0.6236	0.0926	
	0.2957	0.1346	0.8435	
Apigenin-7-glucoside	-0.7773	-0.5497	-0.3054	0.8278
	0.0397	0.2012	0.5053	0.0215
Caffeic acid	0.0994	0.2343	-0.2459	-0.6651
	0.8321	0.613	0.5951	0.103
Diosmetin	0.1921	-0.8545	0.3118	0.4596
	0.6799	0.0143	0.496	0.2995
Ferulic acid	-0.0958	-0.2655	-0.3253	-0.1866
	0.8382	0.565	0.4765	0.6887
Hydroxytyrosol	-0.0186	0.3092	-0.1957	-0.4751
	0.9684	0.4998	0.6741	0.2813
Luteolin	-0.0097	-0.6689	0.3507	0.7974
	0.9836	0.1004	0.4406	0.0317
Luteolin-7-glucoside	-0.6403	-0.6529	-0.2382	0.7629
	0.1213	0.1118	0.6069	0.0461
Oleuropein	-0.285	-0.1209	0.0436	0.3086
	0.5356	0.7962	0.926	0.5007
***p*-Coumaric**	0.6212	-0.2718	0.4743	-0.252
	0.1365	0.5554	0.2822	0.5856
***p*-HPEA-EA**	-0.0265	0.9309	-0.2902	-0.5739
	0.955	0.0023	0.5279	0.1779

	3,4-DHPEA-AC	3,4-DHPEA-EA	3,4-DHPEA-EDA	Apigenin
***p*-HPEA-EDA**	0.7003	-0.1083	0.89	0.059
	0.0797	0.8172	0.0073	0.9
Quercetin	-0.0326	0.3432	-0.3109	-0.3838
	0.9447	0.4511	0.4973	0.3954
Rutin	-0.3327	-0.3911	0.208	0.7979
	0.4659	0.3856	0.6544	0.0315
Tyrosol	-0.7028	0.0519	-0.6481	0.0643
	0.0782	0.9121	0.1154	0.8911
Vanillic acid	0.0062	-0.5406	0.2453	0.493
	0.9895	0.2103	0.596	0.2609
Vanillin	0.2691	0.4189	0.0533	-0.325
	0.5596	0.3496	0.9096	0.4769
Verbascoside	-0.5149	0.7073	-0.4407	-0.056
	0.237	0.0755	0.3223	0.9051
	Apigenin-7-glucoside	**Caffeic acid**	**Diosmetin**	**Ferulic acid**
3,4-DHPEA-AC	-0.7773	0.0994	0.1921	-0.0958
	0.0397	0.8321	0.6799	0.8382
3,4-DHPEA-EA	-0.5497	0.2343	-0.8545	-0.2655
	0.2012	0.613	0.0143	0.565
3,4-DHPEA-EDA	-0.3054	-0.2459	0.3118	-0.3253
	0.5053	0.5951	0.496	0.4765
Apigenin	0.8278	-0.6651	0.4596	-0.1866
	0.0215	0.103	0.2995	0.6887
Apigenin-7-glucoside		-0.3442	0.169	-0.088
		0.4496	0.7171	0.8511
Caffeic acid	-0.3442		-0.422	0.6889
	0.4496		0.3456	0.0869
Diosmetin	0.169	-0.422		0.162
	0.7171	0.3456		0.7285

	Apigenin-7-glucoside	Caffeic acid	Diosmetin	Ferulic acid
Ferulic acid	-0.088	0.6889	0.162	
	0.8511	0.0869	0.7285	
Hydroxytyrosol	-0.1735	0.9315	-0.5823	0.5936
	0.7098	0.0023	0.1701	0.16
Luteolin	0.3835	-0.7617	0.8103	-0.1223
	0.3958	0.0466	0.0271	0.7939
Luteolin-7-glucoside	0.9169	-0.4968	0.3738	-0.2467
	0.0037	0.2568	0.4089	0.5939
Oleuropein	0.4775	0.3923	-0.3168	0.2768
	0.2786	0.3841	0.4888	0.5479
p-Coumaric	-0.4388	0.5235	0.3426	0.6637
	0.3246	0.2279	0.4519	0.104
p-HPEA-EA	-0.4078	0.08	-0.8053	-0.4402
	0.3638	0.8647	0.0289	0.3229
p-HPEA-EDA	-0.195	-0.3658	0.242	-0.6236
	0.6753	0.4197	0.6011	0.1345
Quercetin	-0.2343	-0.2663	-0.1432	-0.5301
	0.6131	0.5638	0.7593	0.221
Rutin	0.6927	-0.6514	0.1893	-0.5424
	0.0845	0.113	0.6843	0.2084
Tyrosol	0.4671	0.5681	-0.4846	0.5142
	0.2907	0.1834	0.2704	0.2377
Vanillic acid	0.2365	0.0738	0.4601	0.6078
	0.6096	0.8751	0.2988	0.1477
Vanillin	-0.4216	-0.472	-0.0554	-0.6841
	0.3461	0.2849	0.9061	0.0901
Verbascoside	0.1946	0.0102	-0.8743	-0.3801
	0.6758	0.9827	0.01	0.4003

	Hydroxytyrosol	Luteolin	Luteolin-7-glucoside	Oleuropein
3,4-DHPEA-AC	-0.0186	-0.0097	-0.6403	-0.285
	0.9684	0.9836	0.1213	0.5356
3,4-DHPEA-EA	0.3092	-0.6689	-0.6529	-0.1209
	0.4998	0.1004	0.1118	0.7962
3,4-DHPEA-EDA	-0.1957	0.3507	-0.2382	0.0436
	0.6741	0.4406	0.6069	0.926
Apigenin	-0.4751	0.7974	0.7629	0.3086
	0.2813	0.0317	0.0461	0.5007
Apigenin-7-glucoside	-0.1735	0.3835	0.9169	0.4775
	0.7098	0.3958	0.0037	0.2786
Caffeic acid	0.9315	-0.7617	-0.4968	0.3923
	0.0023	0.0466	0.2568	0.3841
Diosmetin	-0.5823	0.8103	0.3738	-0.3168
	0.1701	0.0271	0.4089	0.4888
Ferulic acid	0.5936	-0.1223	-0.2467	0.2768
	0.16	0.7939	0.5939	0.5479
Hydroxytyrosol		-0.728	-0.4389	0.6559
		0.0636	0.3246	0.1097
Luteolin	-0.728		0.4614	-0.161
	0.0636		0.2973	0.7302
Luteolin-7-glucoside	-0.4389	0.4614		0.1585
	0.3246	0.2973		0.7343
Oleuropein	0.6559	-0.161	0.1585	
	0.1097	0.7302	0.7343	
p-Coumaric	0.4349	0.0439	-0.5013	0.2151
	0.3295	0.9255	0.2518	0.6432
p-HPEA-EA	0.1053	-0.656	-0.4102	-0.2933
	0.8223	0.1096	0.3607	0.5232
p-HPEA-EDA	-0.3561	0.2346	-0.0109	-0.1026

	Hydroxytyrosol	**Luteolin**	**Luteolin-7-glucoside**	**Oleuropein**
	0.433	0.6126	0.9815	0.8267
Quercetin	-0.4571	-0.2597	0.0561	-0.7729
	0.3025	0.5738	0.905	0.0416
Rutin	-0.4547	0.5152	0.6745	0.283
	0.3053	0.2367	0.0965	0.5385
Tyrosol	0.7155	-0.4323	0.1698	0.7208
	0.0706	0.3327	0.7159	0.0676
Vanillic acid	0.1805	0.5355	0.0252	0.4948
	0.6985	0.2154	0.9572	0.2589
Vanillin	-0.6184	-0.0467	-0.1336	-0.8528
	0.1388	0.9208	0.7752	0.0147
Verbascoside	0.2327	-0.4957	0.0053	0.2599
	0.6156	0.2579	0.991	0.5735
	***p*-Coumaric**	***p*-HPEA-EA**	***p*-HPEA-EDA**	**Quercetin**
3,4-DHPEA-AC	0.6212	-0.0265	0.7003	-0.0326
	0.1365	0.955	0.0797	0.9447
3,4-DHPEA-EA	-0.2718	0.9309	-0.1083	0.3432
	0.5554	0.0023	0.8172	0.4511
3,4-DHPEA-EDA	0.4743	-0.2902	0.89	-0.3109
	0.2822	0.5279	0.0073	0.4973
Apigenin	-0.252	-0.5739	0.059	-0.3838
	0.5856	0.1779	0.9	0.3954
Apigenin-7-glucoside	-0.4388	-0.4078	-0.195	-0.2343
	0.3246	0.3638	0.6753	0.6131
Caffeic acid	0.5235	0.08	-0.3658	-0.2663
	0.2279	0.8647	0.4197	0.5638
Diosmetin	0.3426	-0.8053	0.242	-0.1432
	0.4519	0.0289	0.6011	0.7593
Ferulic acid	0.6637	-0.4402	-0.6236	-0.5301
	0.104	0.3229	0.1345	0.221
	***p*-Coumaric**	***p*-HPEA-EA**	***p*-HPEA-EDA**	**Quercetin**

Hydroxytyrosol	0.4349	0.1053	-0.3561	-0.4571
	0.3295	0.8223	0.433	0.3025
Luteolin	0.0439	-0.656	0.2346	-0.2597
	0.9255	0.1096	0.6126	0.5738
Luteolin-7-glucoside	-0.5013	-0.4102	-0.0109	0.0561
	0.2518	0.3607	0.9815	0.905
Oleuropein	0.2151	-0.2933	-0.1026	-0.7729
	0.6432	0.5232	0.8267	0.0416
p-Coumaric		-0.546	0.1425	-0.6337
		0.2048	0.7605	0.1265
p-HPEA-EA	-0.546		-0.1097	0.633
	0.2048		0.8148	0.127
p-HPEA-EDA	0.1425	-0.1097		0.0576
	0.7605	0.8148		0.9024
Quercetin	-0.6337	0.633	0.0576	
	0.1265	0.127	0.9024	
Rutin	-0.4416	-0.2805	0.3381	-0.1238
	0.3212	0.5424	0.4583	0.7914
Tyrosol	-0.0712	-0.0005	-0.7154	-0.394
	0.8794	0.9992	0.0707	0.3818
Vanillic acid	0.6409	-0.7875	-0.148	-0.9128
	0.1209	0.0355	0.7515	0.0041
Vanillin	-0.5067	0.6198	0.3232	0.8943
	0.2459	0.1377	0.4796	0.0066
Verbascoside	-0.7007	0.7563	-0.3051	0.2282
	0.0795	0.0492	0.5057	0.6226

	Rutin	**Tyrosol**	**Vanillic acid**	**Vanillin**	**Verbascoside**

3,4-DHPEA-AC	-0.3327	-0.7028	0.0062	0.2691	-0.5149
	0.4659	0.0782	0.9895	0.5596	0.237
3,4-DHPEA-EA	-0.3911	0.0519	-0.5406	0.4189	0.7073
	0.3856	0.9121	0.2103	0.3496	0.0755
3,4-DHPEA-EDA	0.208	-0.6481	0.2453	0.0533	-0.4407
	0.6544	0.1154	0.596	0.9096	0.3223
Apigenin	0.7979	0.0643	0.493	-0.325	-0.056
	0.0315	0.8911	0.2609	0.4769	0.9051
Apigenin-7- glucoside	0.6927	0.4671	0.2365	-0.4216	0.1946
	0.0845	0.2907	0.6096	0.3461	0.6758
Caffeic acid	-0.6514	0.5681	0.0738	-0.472	0.0102
	0.113	0.1834	0.8751	0.2849	0.9827
Diosmetin	0.1893	-0.4846	0.4601	-0.0554	-0.8743
	0.6843	0.2704	0.2988	0.9061	0.01
Ferulic acid	-0.5424	0.5142	0.6078	-0.6841	-0.3801
	0.2084	0.2377	0.1477	0.0901	0.4003
Hydroxytyrosol	-0.4547	0.7155	0.1805	-0.6184	0.2327
	0.3053	0.0706	0.6985	0.1388	0.6156
Luteolin	0.5152	-0.4323	0.5355	-0.0467	-0.4957
	0.2367	0.3327	0.2154	0.9208	0.2579
Luteolin-7-glucoside	0.6745	0.1698	0.0252	-0.1336	0.0053
	0.0965	0.7159	0.9572	0.7752	0.991
Oleuropein	0.283	0.7208	0.4948	-0.8528	0.2599
	0.5385	0.0676	0.2589	0.0147	0.5735
***p*-Coumaric**	-0.4416	-0.0712	0.6409	-0.5067	-0.7007
	0.3212	0.8794	0.1209	0.2459	0.0795
***p*-HPEA-EA**	-0.2805	-0.0005	-0.7875	0.6198	0.7563
	0.5424	0.9992	0.0355	0.1377	0.0492
***p*-HPEA-EDA**	0.3381	-0.7154	-0.148	0.3232	-0.3051
	0.4583	0.0707	0.7515	0.4796	0.5057
Quercetin	-0.1238	-0.394	-0.9128	0.8943	0.2282
	0.7914	0.3818	0.0041	0.0066	0.6226
Rutin		-0.0498	0.1061	-0.0778	0.1507
	Rutin	**Tyrosol**	**Vanillic acid**	**Vanillin**	**Verbascoside**

		0.9155	0.8208	0.8684	0.7471
Tyrosol	-0.0498		0.2209	-0.6996	0.4719
	0.9155		0.6341	0.0801	0.285
Vanillic acid	0.1061	0.2209		-0.79	-0.46
	0.8208	0.6341		0.0345	0.299
Vanillin	-0.0778	-0.6996	-0.79		0.1372
	0.8684	0.0801	0.0345		0.7693
Verbascoside	0.1507	0.4719	-0.46	0.1372	
	0.7471	0.285	0.299	0.7693	

CAPÍTULO 6:

Mass spectrometry to evaluate ripening effect on phenols of virgin olive oils

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Mass spectrometry to evaluate ripening effect on phenols of virgin olive oils

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Mass spectrometry to evaluate ripening effect on phenols of virgin olive oils

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Abstract

A global approach has been developed to study the influence of ripening of olive fruits on the phenolic fraction present in virgin olive oil (VOO) from different 12 different genotypes of olives. For this purpose, a non-targeted method based on the analysis of phenolic extracts by mass spectrometry in high resolution mode was applied. The phenolic profiles obtained by LC–QqTOF MS/MS were compared by statistical multivariate analysis tools such as principal component analysis and heat correlation maps. In overall terms, discrimination was only observed for specific crop dates and ripening index values, which should be ascribed to a strong influence of the genotype. The ripening process of each genotype was also studied to demonstrate the variation of phenolic profile in relation to the ripening index. This study was also extended to a panel of representative phenols appreciated by their health and nutritional properties. The application of omics technologies to plant breeding programs could be considered as one of the main pillars on which improvement of products quality can be supported.

Practical applications: Olive oil phenols have a great interest since they contribute to autoxidation stability, health properties and organoleptic characteristics of virgin olive oil. These phenols are considered a key to assess virgin olive oil quality. This study is focused on the effect of the ripening process on the phenolic fraction from a metabolomics point of view. This advanced omic technology (foodomics) applied to plant breeding programs can be one of the pillars to improve products quality.

INTRODUCTION

Olive oil, particularly virgin olive oil (VOO), is gaining consumers around the world after its beneficial nutritional properties have been widely proved [1]. The chemical composition and quality of VOO are influenced by a variety of factors such as genetic, pedo-climatic, geographical origin, agronomic (irrigation, fertilization and harvesting time), and technological factors (extraction process and post-harvest storage) [*2–5*].

Harvesting time influences fruit weight, pulp-to-stone ratio, color, oil content and chemical composition, the last being intimately related to the organoleptical and nutraceutical characteristics of VOO [6, 7]. Thus, oils extracted from fruits harvested in an early ripening state, especially during green-to-violet evolution, are characterized by higher quality as olive fruits (or drupes) [8], lower polyunsaturated fatty acid content and higher phenolic content, which define high-quality olive oil [9,10]. In addition, oils from early harvested fruits are more stable to oxidation than mature oils since they are richer in antioxidant compounds. On the other hand, particular families of phenols such as secoiridoids contribute also to bitterness and pungency, which are undesirable organoleptic properties when in excess and, consequently, early harvesting may influence negatively oil yield [10].

The effect of drupe ripeness on oil quality is supported on a great number of studies. For instance, Baccouri et al. [11] have investigated the effect of harvest date and crop year on the chemical composition and quality of VOOs extracted from fruits of seven selected *Olea oleaster*. The results showed that changes in olive oil quality were mainly due to fruit maturation, while the crop season had a minor importance. Analytical parameters such as peroxide index, photometric absorption at 270 nm, total phenol content, carotenoids and chlorophyll pigments decreased during ripening, whereas oil content, free acidity and linoleic acid level clearly increased. A wider study by Gutiérrez et al. [12] dealt with the effect of ripeness on the oxidative stability of VOO extracted from tree cultivars of Picual and Hojiblanca. In particular,

they determined acidity, peroxide index, oxidative stability, UV absorption at 232 and 270 nm, fatty acid composition, total phenols, orthodiphenolic pigments, tocopherols, sterols and also developed sensory analysis. These authors found a decrease in all parameters under study, except for linoleic acid, Δ-5-avenasterol and total oil content which increased during ripening. Other investigations have been focused on the study of specific families of compounds such as phenols [13], sesquiterpenes [14] or volatile compounds [15], and, of course, fatty acids [16]. Nevertheless, not so many studies have been devoted to ripeness and different genotypes. The few studies existing in the literature focused on this objective have been targeted to a representative panel of phenols of interest such as the study reported by El Riachy et al. [17], who monitored hydroxytyrosol, tyrosol, 3,4-DHPEA-EDA, vanillic acid, coumaric acids, luteolin and apigenin. This study allowed concluding that genetic influence on the concentration of the defined phenolic compounds was stronger than that of fruit ripening. Each genotype was characterized by a different ripening pattern which contributed to explain the variability of the monitored phenolic compounds. However, it is worth mentioning that the study on maturation focused on the global analysis of the phenolic fraction had not been afforded yet.

The growing demand for high-quality VOO increases the interest in olive breeding programs. The methods proposed to study specific families of compounds such as phenols and fatty acids have been partially integrated in olive breeding programs that primed the quality of olive oil [17–19]. The interest of VOO phenols is supported on their contribution to the auto-oxidation stability and organoleptic characteristics of the oil [20]. Apart from that, the nutraceutical properties of these compounds have revealed relevant health benefits such as the inhibition capability of *in vitro* LDL oxidation, a key step in the formation of atherosclerotic plaques [21].

The objective of the present work was to study the effect of the ripening process on the phenolic fraction from an overall point of view.

Previous studies have evaluated the influence of ripening on panel of interesting phenols from the point of view of VOO quality by application of targeted methods [17]. In this research, a global strategy was selected to obtain phenolic fingerprints and their variability associated to the ripening of olive fruits. For this purpose, the approach based on analysis of phenolic extracts by mass spectrometry in high resolution mode was applied. Taking into account the interconnection of biochemical pathways involved in the synthesis of olive phenols, the purpose of this research was addressed to metabolomics.

MATERIALS AND METHODS

Samples

Selections evaluated in this research come from crosses between 'Arbequina', 'Frantoio' and 'Picual' cultivars carried out in the olive cross-breeding program of Córdoba in 1991–1992 coordinated by the Department of Agronomy in the University of Córdoba and the Andalusian Institute of Agricultural Research and Training (IFAPA, Junta de Andalucía). Several genotypes were selected in the initial seedling population after three consecutive harvest seasons using as criteria their early bearing (short juvenile period) and high oil content. The selected genotypes were propagated by soft-wood cuttings and planted, in 2001, in a comparative field trial together with the three genitors as control at 6 × 5 m spacing. Trees were trained as single-trunk vase, with three to four main branches, and minimal pruning was carried out to allow early bearing. Standard cultural practices were followed, including irrigation supply by in-line drips to avoid water stress of plants. Results of their agronomic evaluation at the initial seedling stage [22] and comparative field trials [23, 24] have so far been reported.

Olive fruits were manually collected from 'Arbequina', 'Frantoio' and 'Picual' cultivars as well as from nine selections as follows: three from 'Arbequina' × 'Picual', three from 'Picual' × 'Arbequina' and three from

'Frantoio' × 'Picual' at five ripening stages from 1st October to 26th November 2009 (every two weeks) and two trees-per-genotype were sampled each date. No samples were available from 'Picual' at the first date (1st October); therefore, the cohort was formed by 118 samples —(12genotypes*2replications*5 dates)–2=118 samples.

Determination of the Ripening Index

Ripening index of fruit samples was recorded as described by El Riachy et al. [25]. Briefly, an aliquot of 100 randomly selected fruits was taken from each fruit sampling, and fruits were classified into the following categories of skin color: 0= deep or dark green; 1= yellow or yellowish–green; 2= yellowish with reddish spots; 3= reddish or light violet; 4= black.

The total number of olives in each category (n0, n1,…, n4) was recorded, and the following equation was applied to determine the ripening index (RI):

$$RI = ((0\times n0) + (1\times n1) + (2\times n2) + (3\times n3) + (4\times n4)) / 100.$$

Then, RI was categorized as follows: RI≤0.5 (RI0); RI=0.5–1.5 (RI1); RI=1.5–2.5 (RI2); RI=2.5–3.5 (RI3) and finally RI≥3.5 (RI4).

Olive Oil Extraction

Olive fruit samples were processed using an Abencor system (MC2 Ingenierías y Sistemas, Sevilla, Spain), which consists of three essential elements: the mill, the thermo-malaxer and the olive paste centrifuge. VOO was extracted according to the manufacturer protocol, which is schemed as: (i) olives were crushed with the hammer mill; (ii) the olive paste thus obtained was malaxated for 30 min with the water bath set at 28±1 ºC; (iii) olive oil was separated from the paste by centrifugation for 2 min; finally, (iv) the oil was separated from the wastewater by decantation and collected in dark brown glass bottles and stored at –20 ºC until analysis.

Reagents

The reagents used for characterization of vegetable oils were: LC grade methanol and hexane from Scharlab (Barcelona, Spain). LC–MS grade

acetonitrile and formic acid for preparation of chromatographic mobile phases were also from Scharlab. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the mobile chromatographic phases.

Apparatus and Instruments

An MS2 minishaker from Ika (Wilmington, USA) was used to favor phenols transfer from oil to methanol for individual quantification of the target compounds.

All samples were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6540 QqTOF hybrid mass spectrometer with a dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected m/z ratios.

Extraction of Phenolic Compounds from VOO

Phenolic extracts were obtained by shaking 1 g of each oil with 1 mL of hexane and 1 mL of 60:40 methanol–water mixture for 1 min in the MS2 minishaker. The hydroalcoholic phase was directly injected into the LC–TOF/MS [18].

LC–QqTOF MS/MS A of Phenolic Extracts

Olive phenols analysis was conducted by LC–TOF/MS in accurate mode. The analytical column was a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The mobile phases were: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient program, at 0.4 mL/min constant flow rate, was as follows: initially 96% A and 4% B; 0–8 min, 96–0% A and 4–100% B; 8–10 min, 0% A and 100% B. After analysis, the column was equilibrated for 5 min.

The injected volume of extract was 5 μL, without any additional pretreatment. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas

temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3500 V in negative ionization mode; skimmer, 65 V; octopole radiofrequency voltage, 750 V; fragmentor voltage, 175 V. Data were acquired in centroid mode in the extended dynamic range (2 GHz). Full scan was carried out at 1 spectrum/s within the m/z range 100–1700 with subsequent activation of the three most intense precursor ions per scan (only single or double charged ions were allowed) by MS/MS using a collision energy of 20 eV at 1 spectrum/s within the m/z range 100–1700. An active exclusion window was programmed after one MS/MS spectrum and released after 0.75 min to avoid repetitive fragmentation of the most intense precursor ions and, in this way, increase the detection coverage. Before the experiments, the instrument reported mass detection resolution of 25000 FWHM (Full Width at Half Maximum) at m/z 112.985587 and 45000 FWHM at m/z 966.000725. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at m/z 119.0362 (proton abstracted purine) and m/z 966.0007 (formate adduct of hexakis(1H,1H,3H-tetrafluoropropoxy)-phosphazine).

Data Pretreatment

After LC–QqTOF MS/MS analysis, raw data files were converted to mzData using Mass Hunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) and processed in R statistical language (version 2.15.0)[26] using the open-free XCMS software (version 1.24)[27] with the CAMERA workflow [28]. This software allows the peak finding, alignment and peak picking of mass spectral features resulting in a list of m/z precursor ions per retention time with MS/MS spectra obtained in each scanning cycle. Parameters for XCMS [27] were taken from the centWave peak finder method designed for high mass accuracy data [29]. The extraction of molecular features (MFs) was carried out using a prefilter to take at least two ions with intensity above 2000 in each cycle. Briefly, peaks were deconvoluted and aligned across samples

using a signal/noise threshold of 10, a maximum tolerated m/z deviation of 5 ppm and a peak width of 10–60 s. The aligned output consisted of accurate mass, retention time and intensity of each peak. The file obtained with this pre-processing was created for each sample in comma separated values files (.csv). Multiple peaks associated with adducts, dimers, neutral losses and isotopes were excluded for further statistical analyses. The definitive files were exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for statistical analysis, which was carried out by unsupervised analysis by Principal Component Analysis (PCA) to find clustering of samples attending to crop date or ripening index.

Identification of representative phenolic compounds was proceeded by generation of candidate formulae based on monoisotopic masses and isotopic distribution which includes isotope abundance and isotope spacing. Tolerance in mass accuracy was set at 10 ppm. The contribution to mass accuracy, isotope abundance and isotope spacing scores was 100.00, 60.00 and 50.00, respectively. Retention times, formulae, experimental and theoretical masses, and errors, in ppm, obtained by accurate mass measurements were considered in the identification step. Identification of two or three representative fragments for each phenolic compound in tandem mass spectrometry supported the identification step. Compound identification was supported on searching m/z values of each precursor ion and the most representative fragment ions in the METLIN Metabolite and MS/MS [30] and PlantCyc Database[31]. The allowed negative precursor ions for identification were formate adducts and deprotonated ions. Dehydratation neutral losses were also allowed.

RESULT AND DISCUSSION

Data Pretreatment

The initial data set was formed by 850 variables associated to different MFs. Taking into account that the aim of this study was focused on the phenolic fraction, it was studied prior to statistical analysis. **Supplementary Figure 1** shows base peak chromatograms (BPCs) provided by phenolic extracts corresponding to the three genitors at the different crop dates. As can be seen, the most significant changes in the chromatogram profiles are produced in the central part for the three genitors, which corresponds to the detection of phenolic compounds. According to this observation, the extraction of molecular features was delimited to this elution window (2.0–7.6 min), where the representative phenols in VOO elute. The chromatographic separation of phenolic extracts from VOO by C_{18} columns is characterized by early elution of simple phenols such as hydroxytyrosol, tyrosol and glucoside derivatives, then, elution of complex secoiridoids and, finally, elution of flavonoids, which are the less polar phenols present in VOO [32]. Thus, hydroxytyrosol, detected at m/z 153.0559, was eluted at 2.8 min, while apigenin, detected at m/z 269.0455, was eluted at 6.4 min. In this way, the data set is representative of the phenolic family. This filtering step led to reduce the data set to 705 molecular entities (118 samples × 705 MFs). An additional filtering step was included to simplify the data set by removal of redundant variables. For this purpose, a fold-change algorithm was applied independently of the data set as a function of the crop date or the ripening index obtaining two new data matrices. The fold change algorithm allowed retaining only those entities that experienced a change in relative concentration equal or higher than 2.0 between consecutive dates or ripening indexes. This cut-off value was set to keep in the data matrices only MFs that changed significantly their relative concentration between pairs of consecutives crop dates or ripening indexes. The new data sets contained 163 and 130 MFs for the studies involving crop date and ripening index, respectively. **Supplementary Figure 2** illustrates the Venn diagram generated by qualitative comparison of MF profiles obtained after application of the fold-

change algorithm. As can be seen, 63 MFs were in common in both data sets, whereas 100 and 67 were only present in data sets for the crop date and ripening index studies, respectively. This result reveals that the composition of phenolic extracts from VOO evolves differently according to both parameters.

Influence of Crop Date and Ripening Index on the Phenolic Composition of VOO

Due to variability between crop years, harvesting date is not a normalized parameter to assess the production and quality of VOO from agronomical and chemical perspectives. There are numerous variables that can affect the production and quality of VOO such as climate, soil properties, agronomical practices, genetic variability, production cycles and technological factors. Therefore, other parameters have been developed to standardize the maturation influence such as ripening index, fruit skin color, fruit firmness, and overall amount of chlorophylls and carotenoids in the oil [33]. Ripening index, one of the most frequently used standardization parameters, allows scaling from 0 to 4 as a function of fruit color as described in the Experimental section. Color variation is one of the most significant observable changes during fruit maturation. The initial green fruits undergo color changes and associated physiological modifications leading to purplish-black fruits, which is indicative of the end of morphological development. Color change is associated with the decline in chlorophyll and oleuropein levels and appearance of anthocyanins that color fruits with purplish-black tone [33]. With these premises, three main biosynthetic pathways are involved in color evolution and, therefore, in the ripening index: synthesis and degradation of chlorophylls, biosynthesis of complex secoiridoids (oleuropein and ligstroside as main forms) and degradation to aglycone secoiridoids and derivatives (3,4-DHPEA-EDA, 3,4-DHPEA-EA, p-HPEA-EDA and *p*-HPEA-EA) and the phenylpropanoid pathway which gives place to synthesis of anthocyanins through a narigenin chalcone

intermediate. As two of these pathways are involved in the synthesis of olive oil phenols, global analysis of phenolic extracts from VOO could be correlated with the ripening index. **Figure 1** shows the variation in relative concentration of MFs (potential phenolic compounds) according to the crop date (A) and ripening index (B). As can be seen, the evolution plots for crop date and ripening index have a common profile; there are MFs that show upward or downward trends, which could be connected to the previously described pathways.

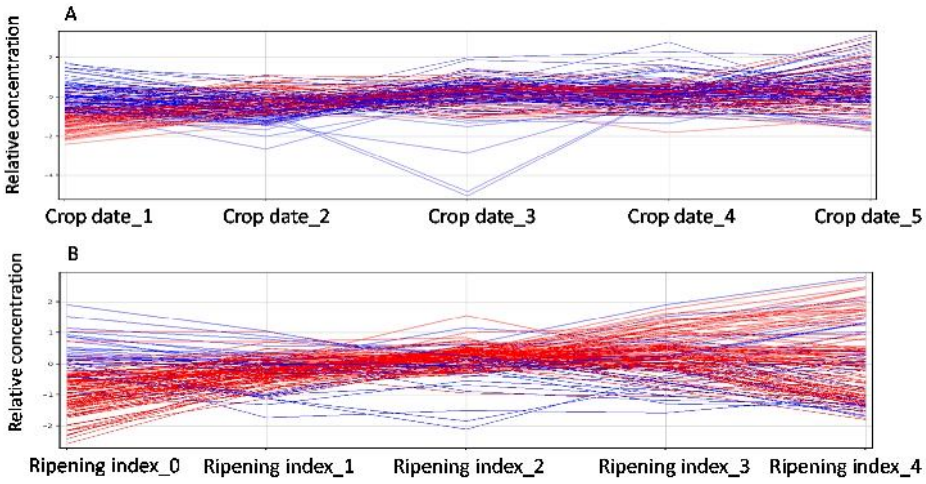

Figure 1. Relative concentration plots of MFs tentatively associated to the phenolic fraction according to the crop date (A) and ripening index (B).

Unsupervised Analyses of the Ripening Process

Once the data sets were filtered, the qualitative composition of phenolic extracts was studied to check the incidence of the crop date and ripening index. Statistical analysis was applied to find discrimination tools based on crop date and ripening index. For this purpose, PCA was tested for detection of grouping trends by application of an unsupervised approach. The aim was to compare the discrimination pattern of the global phenolic fraction according to the ripening index and crop date. **Figure 2** shows the scores plots obtained for both cases. As can be seen, discrimination was not

complete for either crop date (A) or ripening index (B). In the case of the crop date, only those samples collected in the fifth date were partially differentiated from the rest of the samples as a function of the phenolic profile. On the other hand, samples collected in the 1st and 2nd crop dates and 3rd and 4th crop dates were overlapped, respectively. A similar behavior was observed in the case of the ripening index since no complete discrimination was found although certain spatial distribution could be detected from early to advanced values of ripening index.

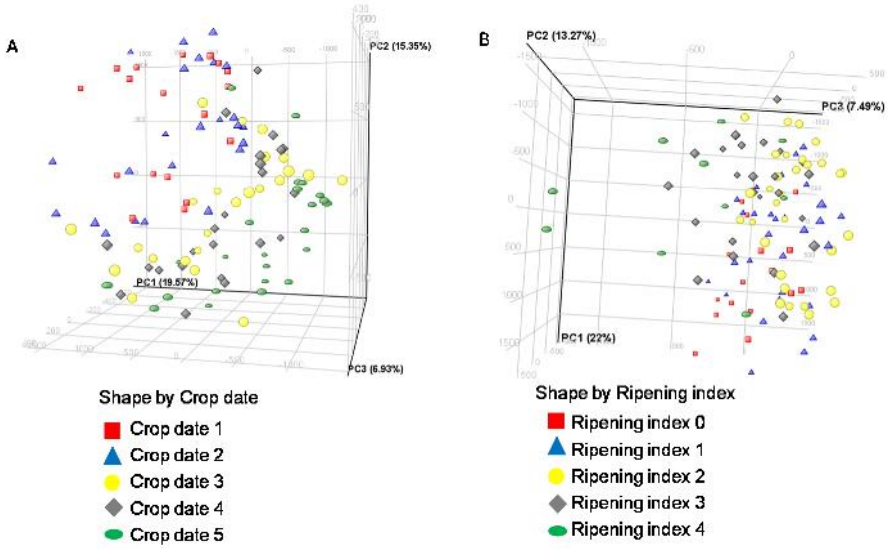

Figure 2. PCA scores plots associated to the phenolic fraction as a function of the crop date (A) and the ripening index (B).

The behavior of ripening index and crop date could be associated to the differences in the maturation process for different genotypes. El Riachy et al. [25] compared the influence of genotype and fruit ripening on the concentration of representative phenolic compounds present in VOO. A dual effect of fruit ripening and genotype on the phenolic profile was observed with more pronounced genetic influence. In fact, the highest degree of variability between genotypes was detected at early ripening stages. These results were of interest for breeding programs since this targeted approach

suggested a strategy based on early harvesting of fruits (at the first three ripening indexes) for better comparison and selection of genotypes in further crosses aimed at improving the quality of VOO. This variability is also found with this global approach since early ripening indexes were not well-differentiated in the PCA plots.

The strong influence of the genotype can be evaluated by comparison of the number of entities changing their relative concentration between pairs of consecutive ripening indexes, if present. If this comparison is globally carried out for the different genotypes (**Figure 3.A**), the main metabolic changes are produced at early and advanced stages of ripening, which means between pairs of ripening index 0–1 and 3–4.

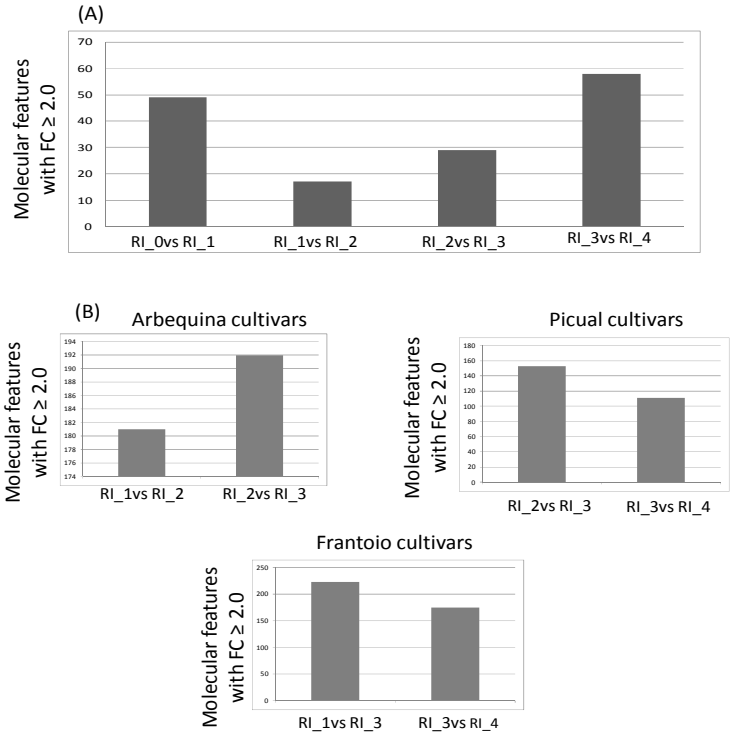

Figure 3. Metabolic changes represented by the number of MFs with fold change > 2.0 between consecutive ripening index values for all genotypes (A) and for each genotype (B).

If all genotypes were following this trend independently, the concentration of phenolic compounds in VOO could be modeled as a function of the ripening index. However, each genotype behaves differently, as shows **Figure 3.B**. Arbequina cultivars showed a trend different to that observed for Picual and Frantoio cultivars. This diversity was also found for crosses between cultivars. Thus, metabolic changes were more important at advanced ripening index for Picual × Arbequina crosses, while changes were more sensitive at early ripening stages for Frantoio × Picual crosses. On the other hand, metabolic changes were significant both at early and advanced stages for Arbequina × Picual crosses. These results were confirmed by independent PCA studies of the different genotypes, as shows **Figure 4**.

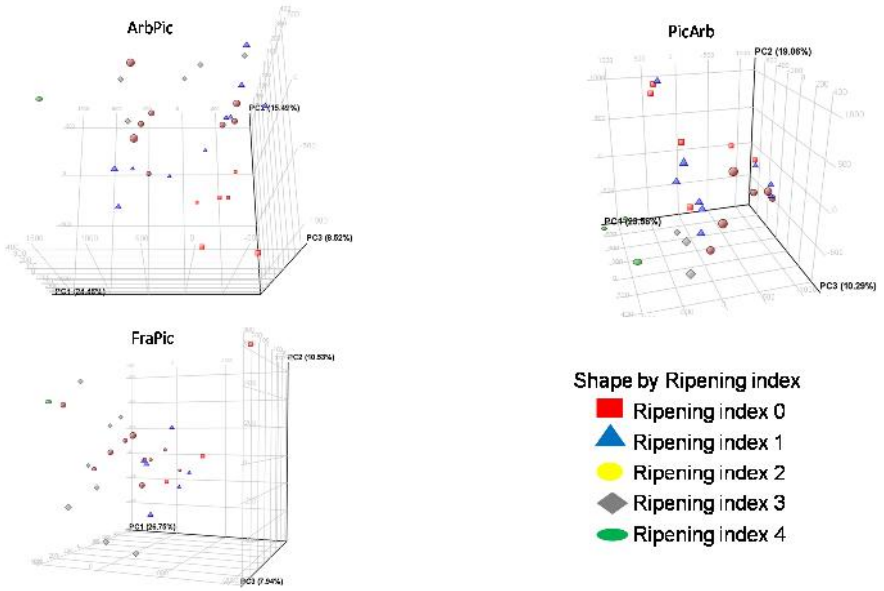

Figure 4. PCA scores plots associated to the phenolic fraction as a function of the ripening index for the three selections obtained by crosses.

In this case the phenolic profiles were differentiated according to the ripening index for all genotypes obtained by crosses of the three genitors. Thus, selections from the three crosses showed a discrimination pattern according to the ripening index, which can be considered as a standard

parameter. This analysis supports the incidence of the genotype as the main parameter regulating the variability in the ripening process. In fact, the correlation map for the ripening index (**Supplementary Figure 3.A**) revealed the absence of trends or patterns associated to the ripening index. As can be seen, samples were ordered as a function of the ripening index but no correlation was observed according to this parameter. On the other hand, high correlation was detected for genotypes (**Supplementary Figure 3.B**). Thus, the phenolic profiles of VOOs from Arbequina cultivars were highly-correlated with those from Arbequina descendants. It is quite interesting to observe that correlation was higher in Arbequina × Picual than in Picual × Arbequina. The same behavior was found for Picual cultivar and crosses.

Targeted Monitoring of Representative Phenols in VOO

The global approach described here also allows monitoring representative phenols present in VOO or any other metabolite which could be of interest. Thus, the evolution of target phenols according to crop date and ripening index can be compared. **Table 1** lists the identification parameters after LC–MS/MS analysis for confirmation of the presence of these compounds in VOO. Suppl. Fig. 4 shows the evolution plots observed for each representative phenol with crop date of olive fruits and ripening index. As can be seen, there are compounds which presented a similar trend for certain genotypes as hydroxytyrosol in Arbequina × Picual crosses or 3,4-DHPEA–EDA in Picual × Arbequina crosses. However, different trends were often observed in many genotypes. As an example, in Arbequina × Picual crosses a decreasing trend was detected for *p*-HPEA-EDA, while the concentration decrease was limited in ripening index from 0 to 1 stages. Other representative example is tyrosol in Picual × Arbequina crosses because a different trend was found for crop date and ripening index.

According to the results provided by this research, global metabolomics approaches implemented in plant breeding programs to improve quality are of great interest and can be one of the pillars on which

the attempts to improve products quality can be supported. This discipline has demonstrated to contribute widely to a better knowledge of food, thus giving place to a new subdiscipline known as foodomics, in which mass spectrometry in high resolution mode enables to develop a diverse range of studies to take benefits from its high sensitivity and selectivity.

Table 1. Representative phenols identified in extracts from VOO by LC–QqTOF MS/MS analysis.

Phenol	Formula	Experimental *m/z*	Theoretical *m/z*	Error (ppm)	t_r	Fragments
Hydroxytyrosol	$C_8H_{10}0_3$	153.0557	153.0557	0	2.8	123.0450 105.0339
Tyrosol	$C_8H_{10}O_2$	137.0615	137.0608	5.1	4.07	119.0505 111.0098
3,4-DHPEA-EDA	$C_{17}H_{20}O_6$	319.1188	319.1187	0.3	5.90	123.0446 139.0769
3,4-DHPEA-EA	$C_{19}H_{22}O_8$	377.1264	377.1242	5.8	6.60	123.0446 255.0869
***p*-HPEA-EDA**	$C_{17}H_{20}O_5$	303.1238	303.1238	0	6.50	59.1
***p*-HPEA-EA**	$C_{19}H_{22}0_7$	361.1299	361.1293	1.6	6.05	291.1
Luteolin	$C_{15}H_{10}O_6$	285.0405	285.0405	0	5.96	133
Apigenin	$C_{15}H_{10}O_5$	269.0463	269.0455	2.9	6.40	117

ACKNOWLEDGEMENTS

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contract (RYC-2009-03921). Luis Rallo (Agronomy Department, University of Córdoba) and Lorenzo León (IFAPA research center "Alameda del Obispo", Córdoba) are acknowledged for providing the oils.

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SUPPLEMENTARY MATERIAL

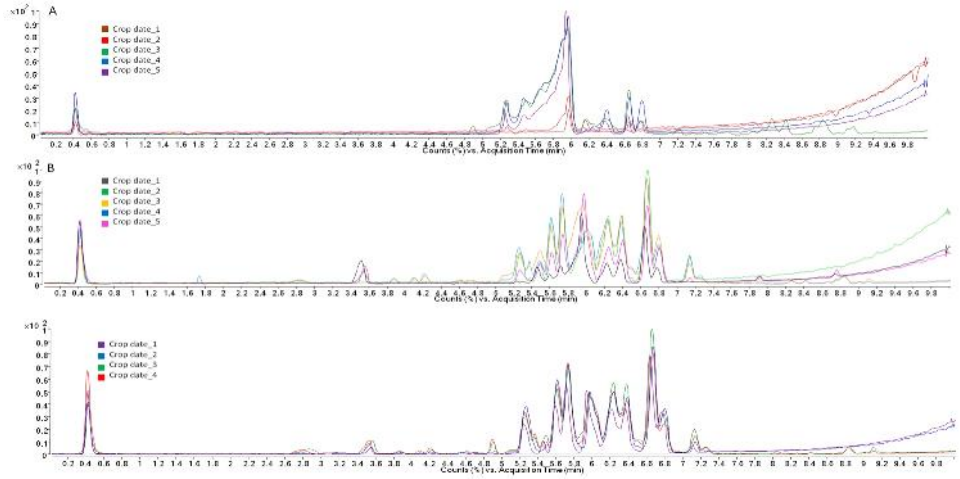


Figure 1. Base peak chromatograms (BPCs) obtained by LC–TOF/MS analysis of phenolic extracts from VOO of Arbequina (A), Frantoio (B) and Picual (C) genitors at the different crop dates.

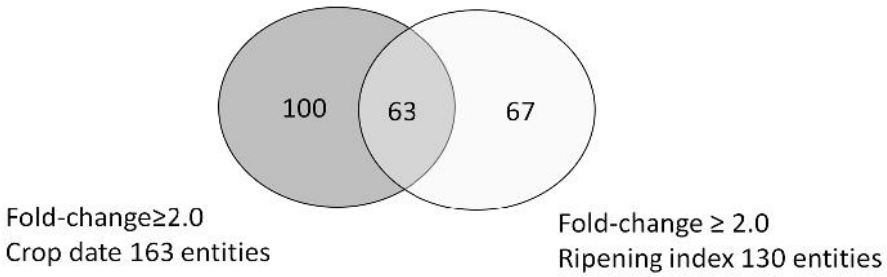


Figure 2. Venn diagram generated by qualitative comparison of MF profiles obtained after application of the fold-change algorithm.

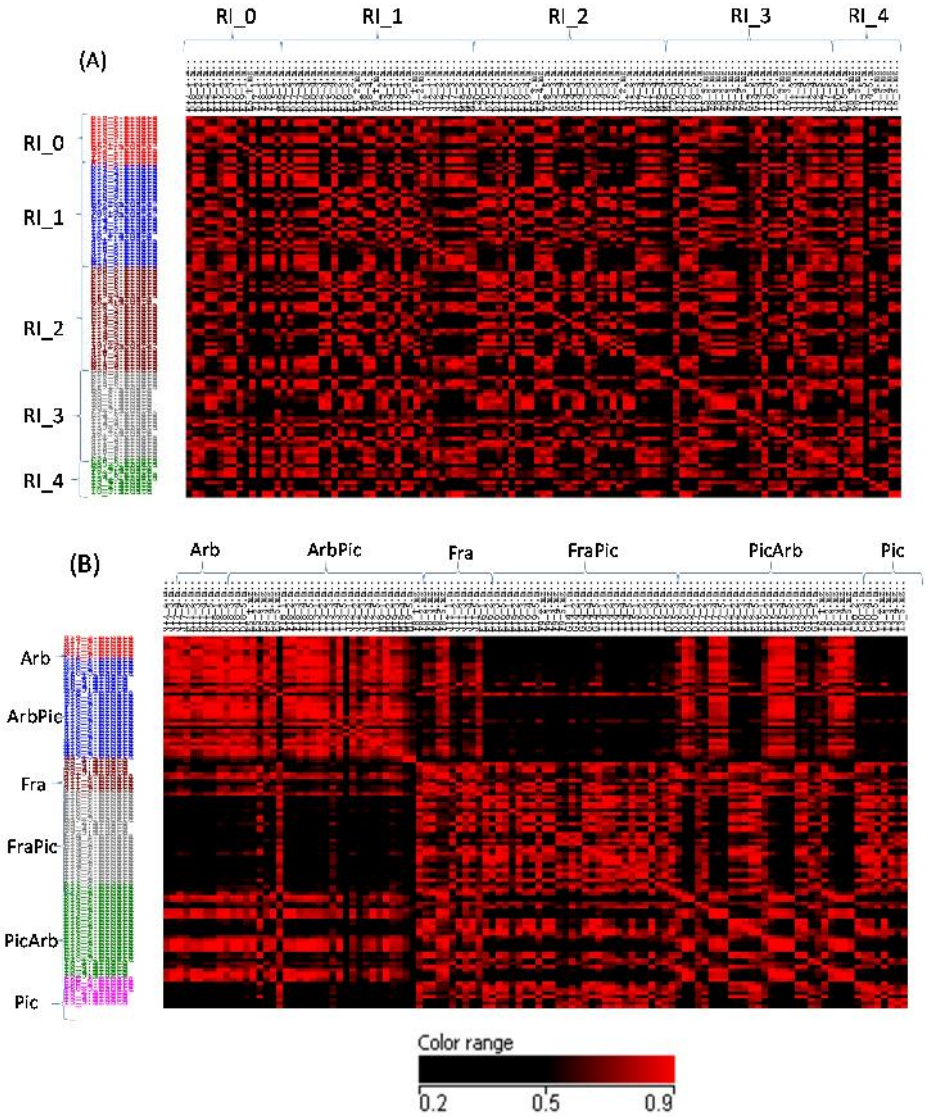
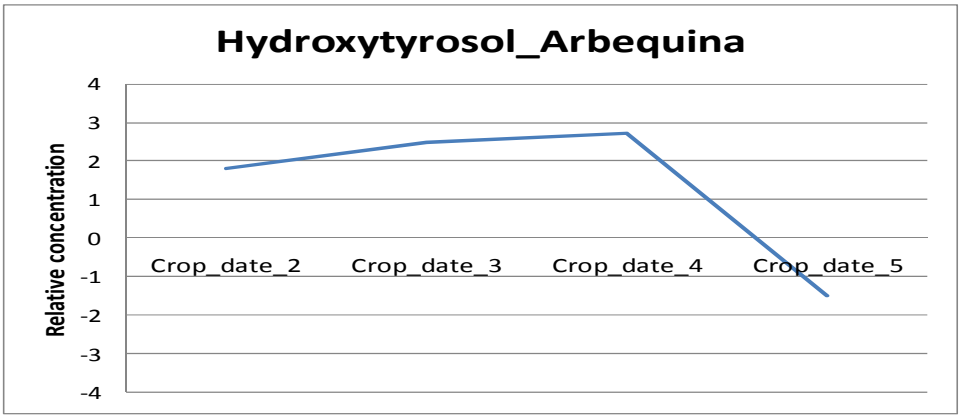


Figure 3. Heat maps based on correlation analysis according to the ripening index (A) and genotype (B).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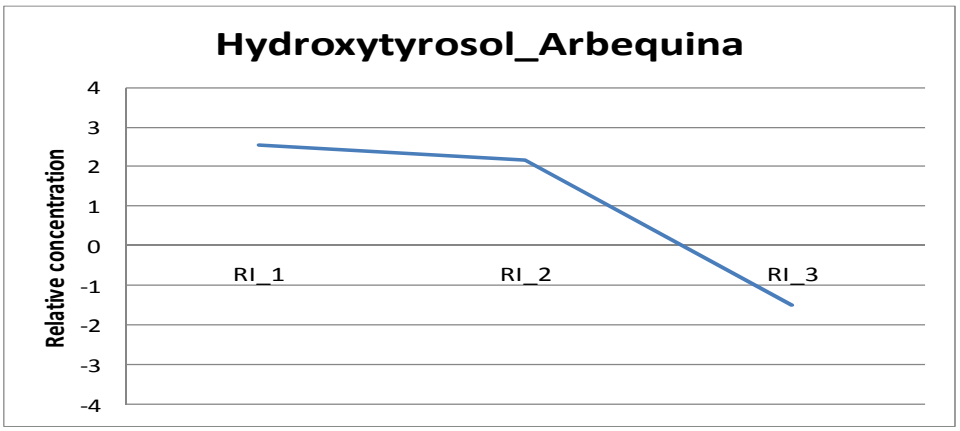
Crop_date_4
Crop_date_5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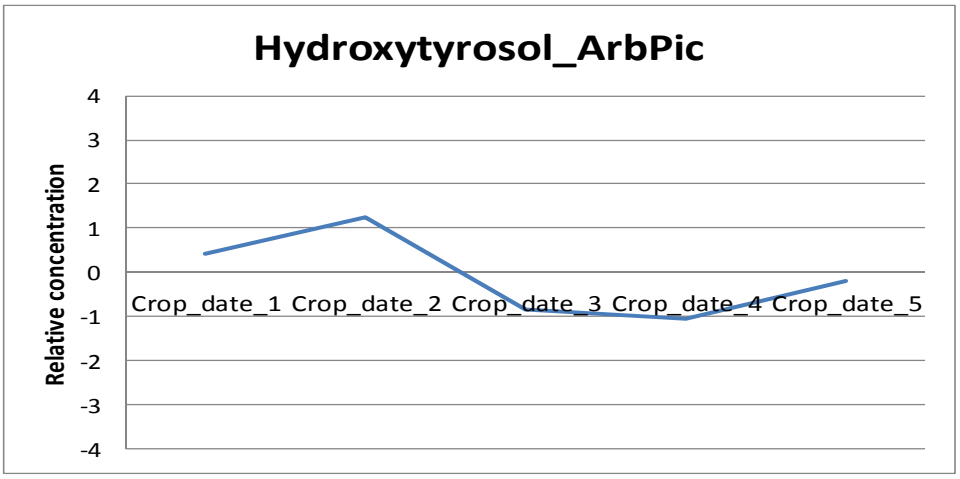
RI_1
RI_2
RI_3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Crop_date_3
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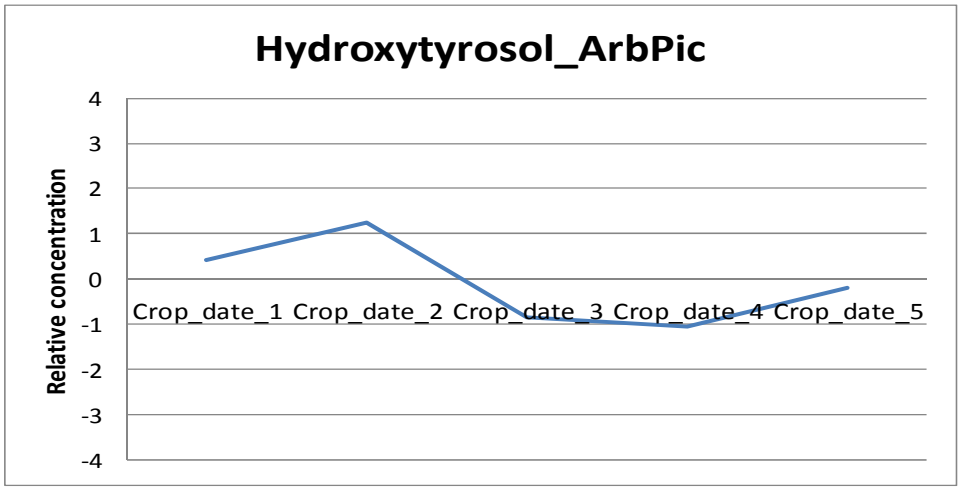
Crop_date_5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Relative concentration
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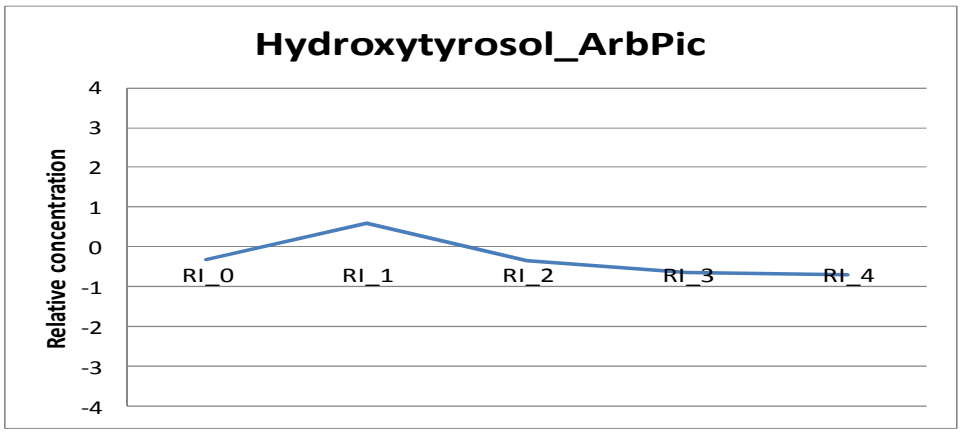
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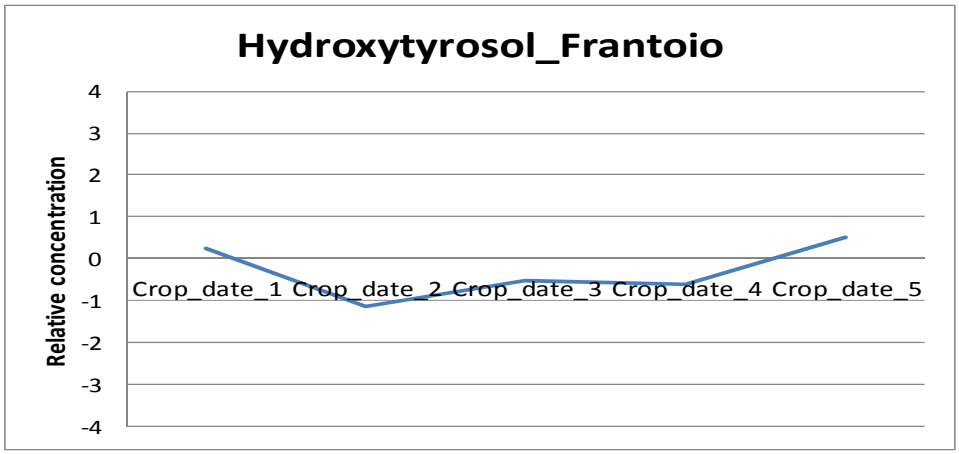
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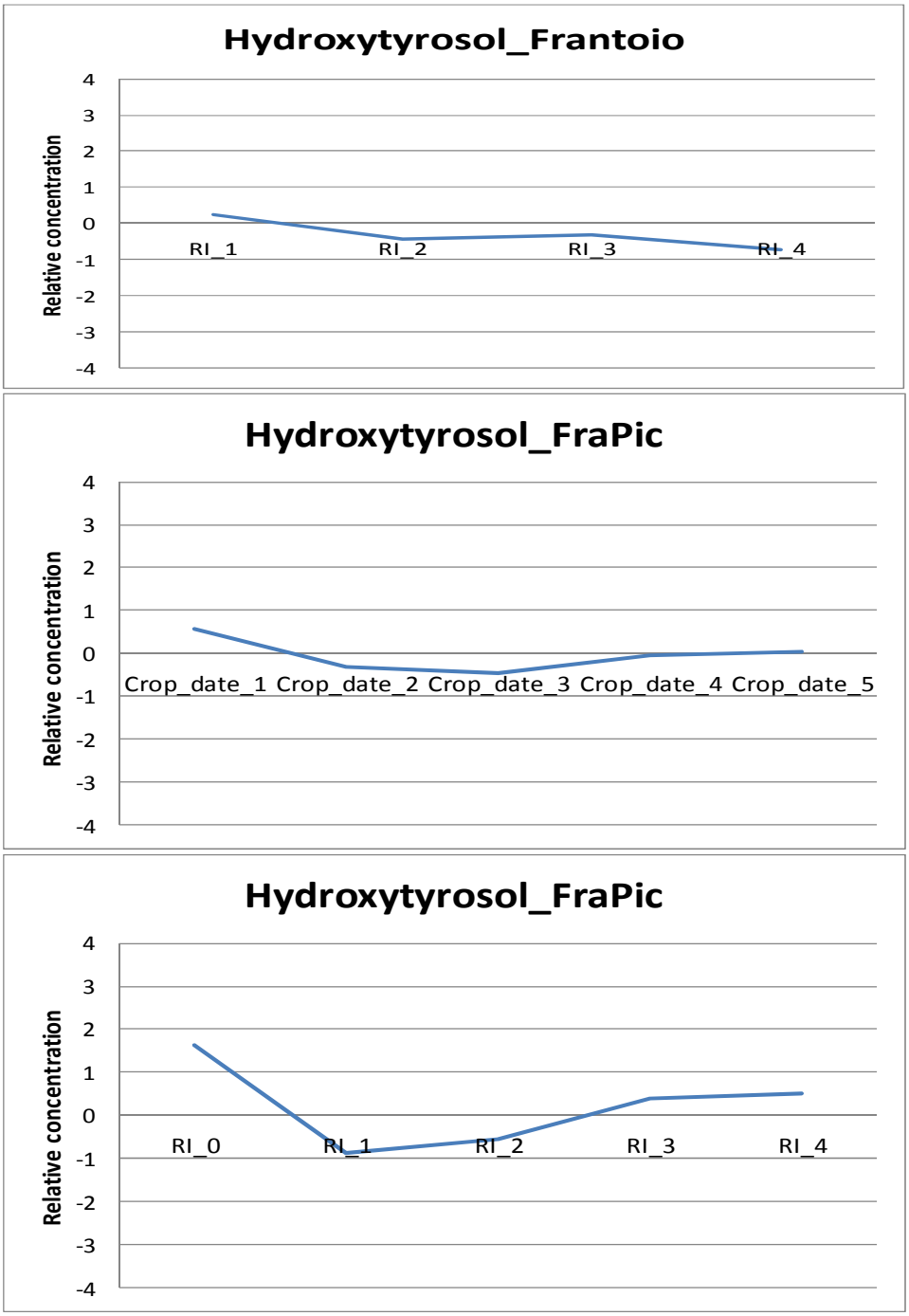
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Hydroxytyrosol_FraPic
Relative concentration
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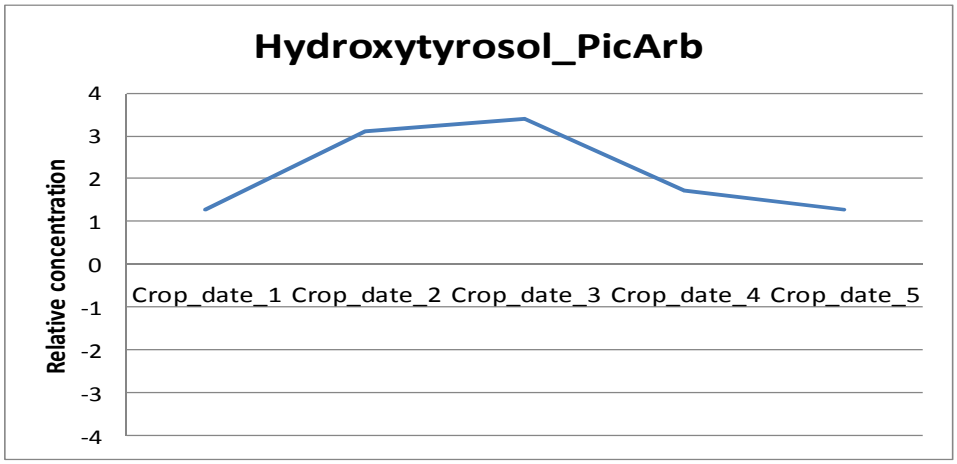
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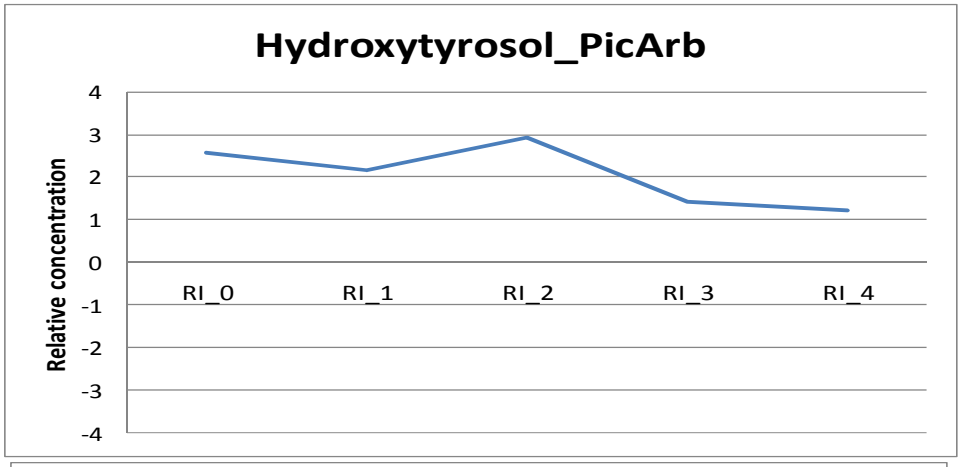
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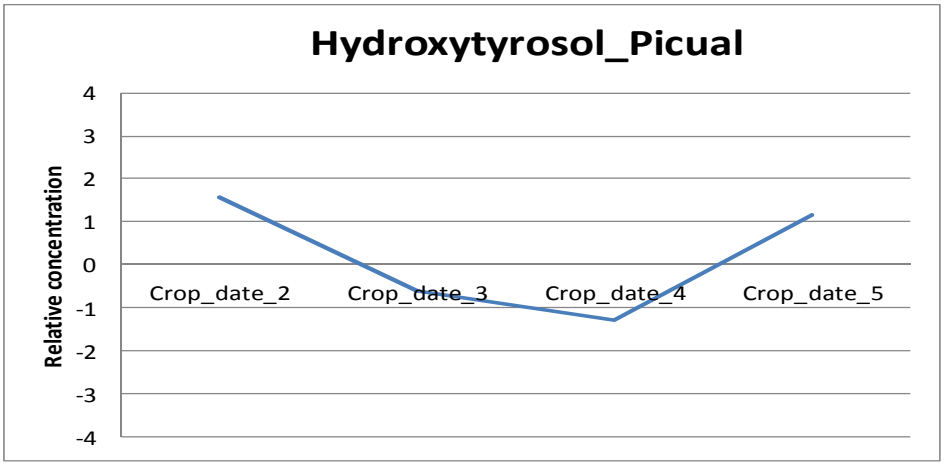
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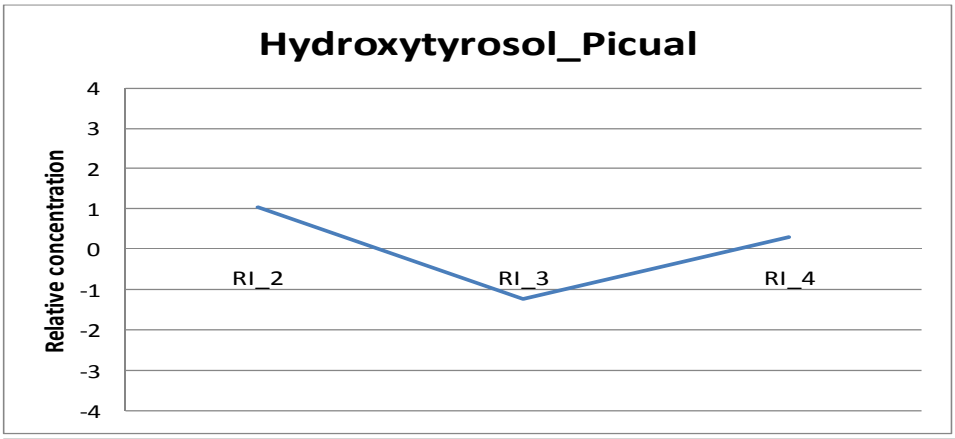
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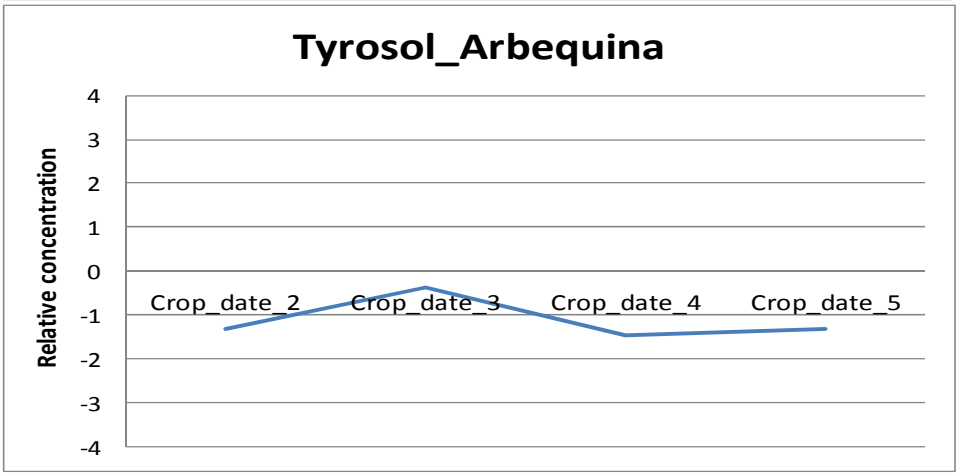
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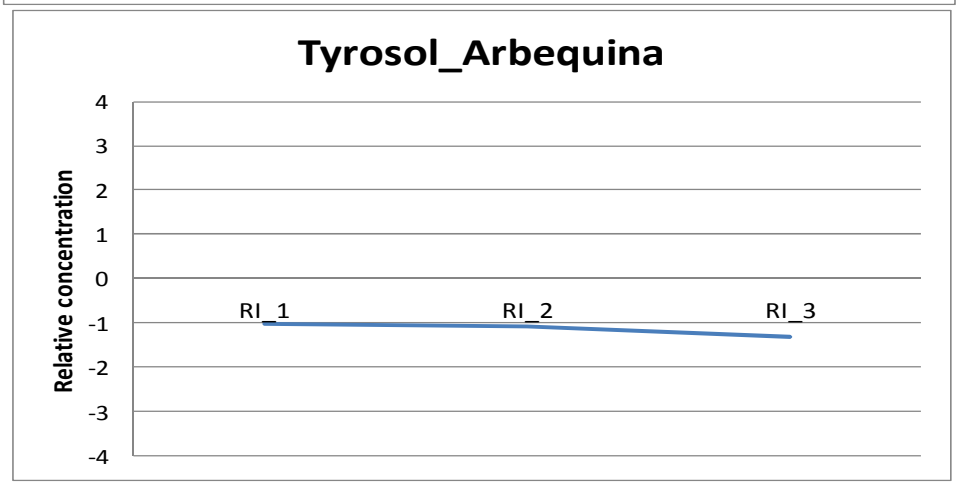
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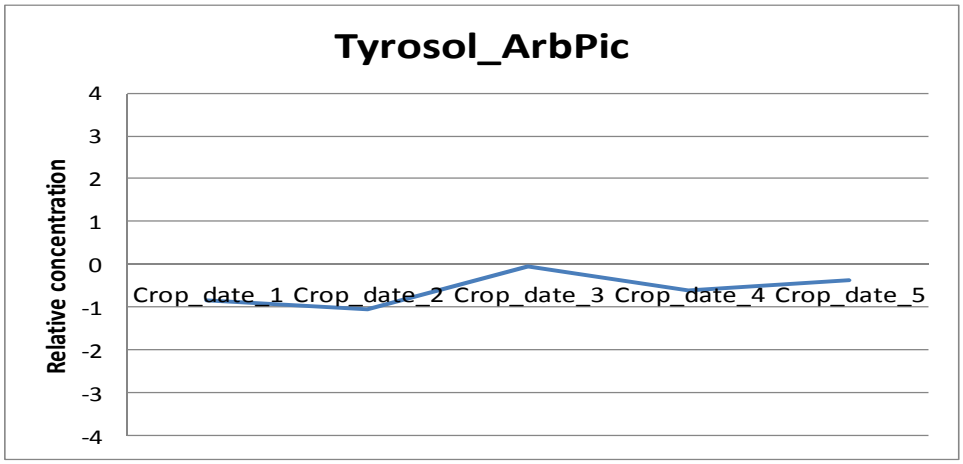
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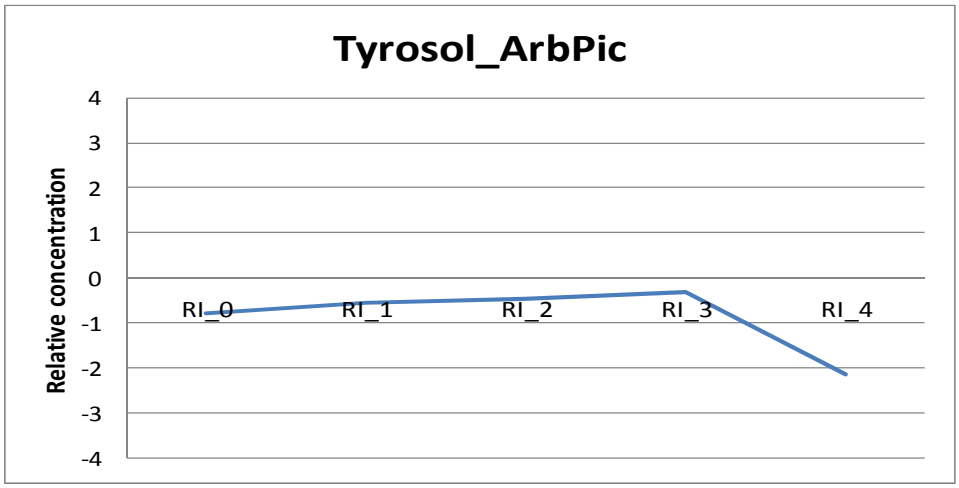
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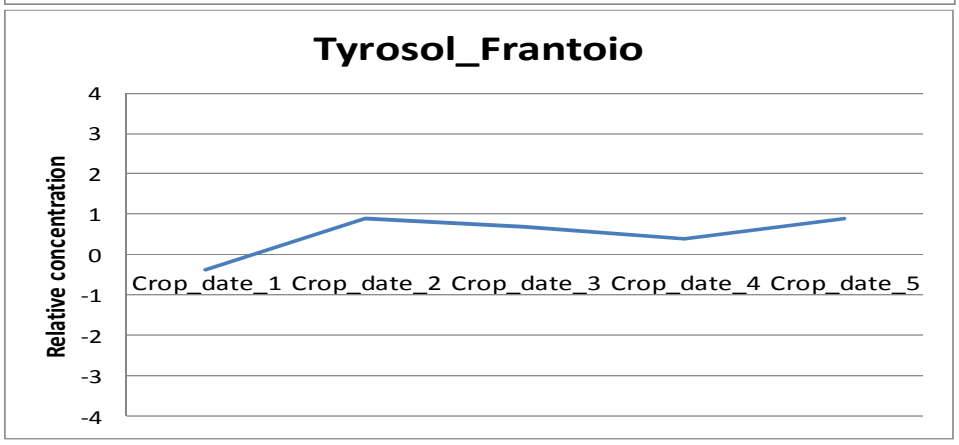
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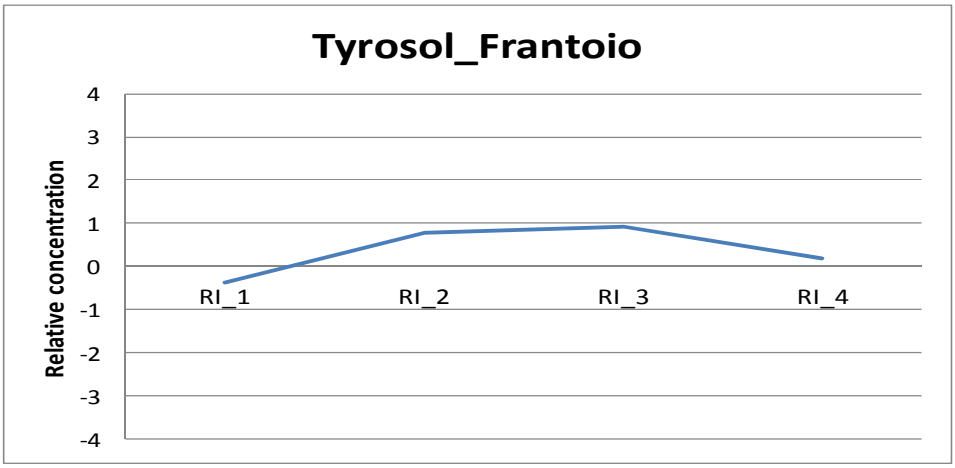
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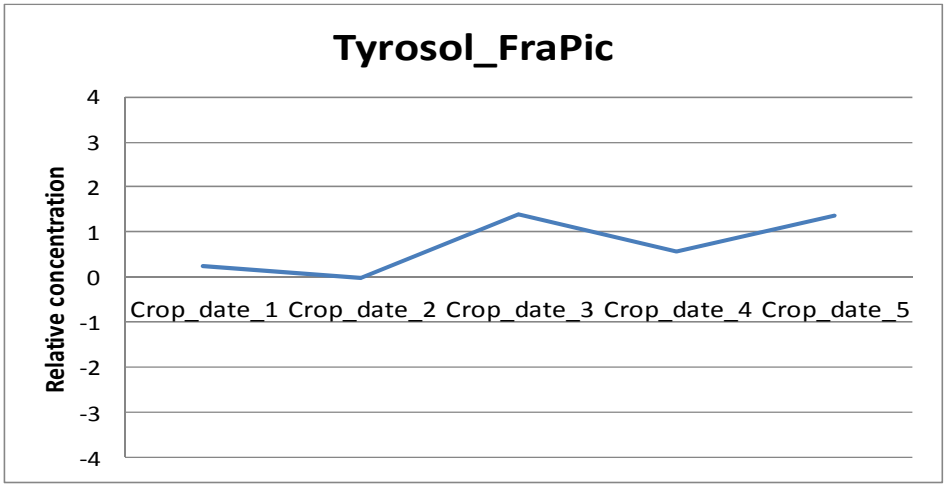
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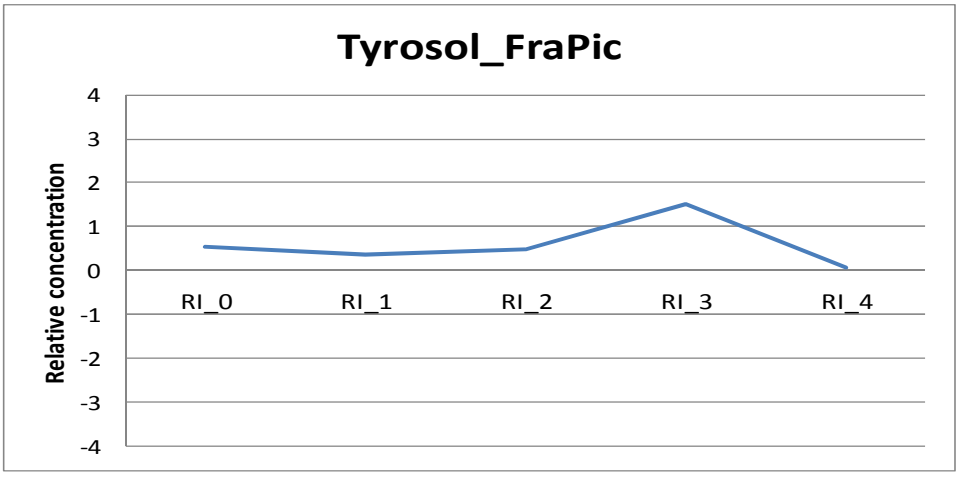
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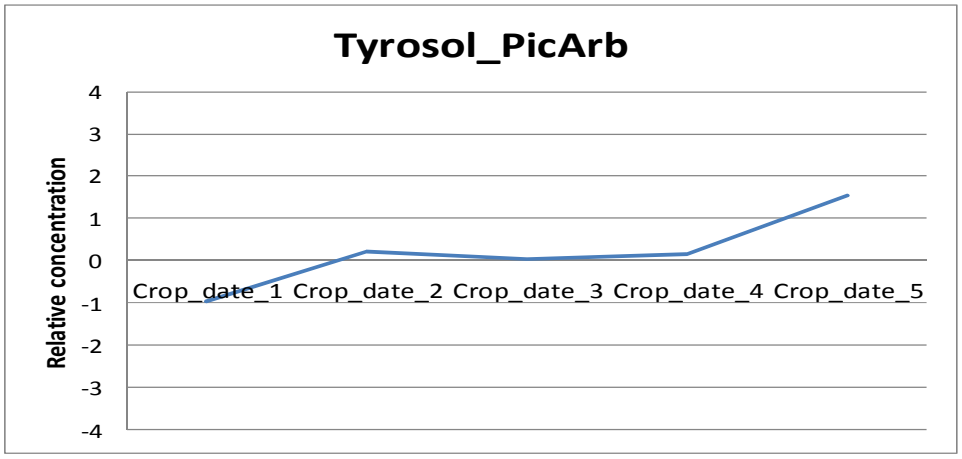
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Relative concentration
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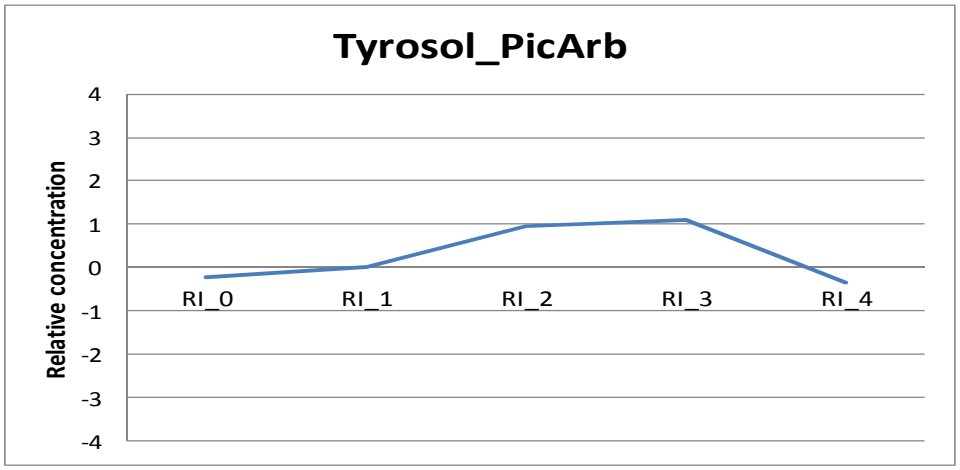
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Relative concentration
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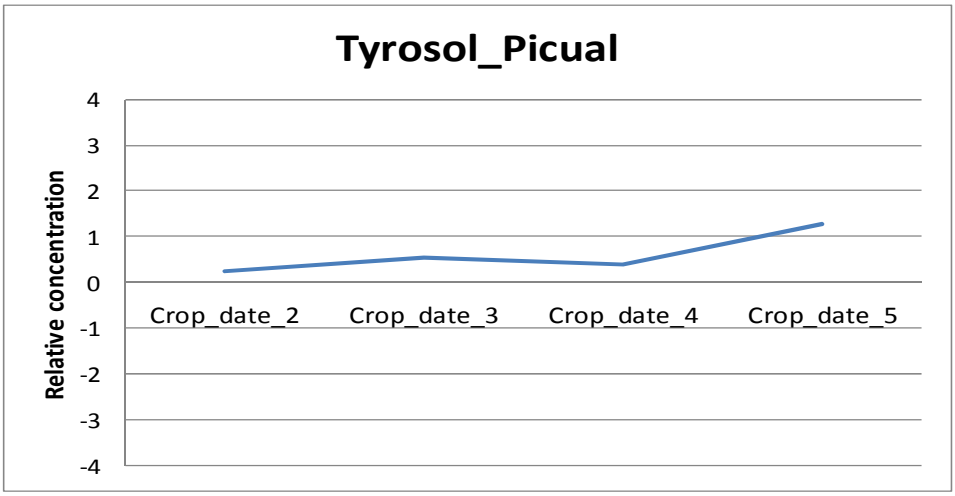
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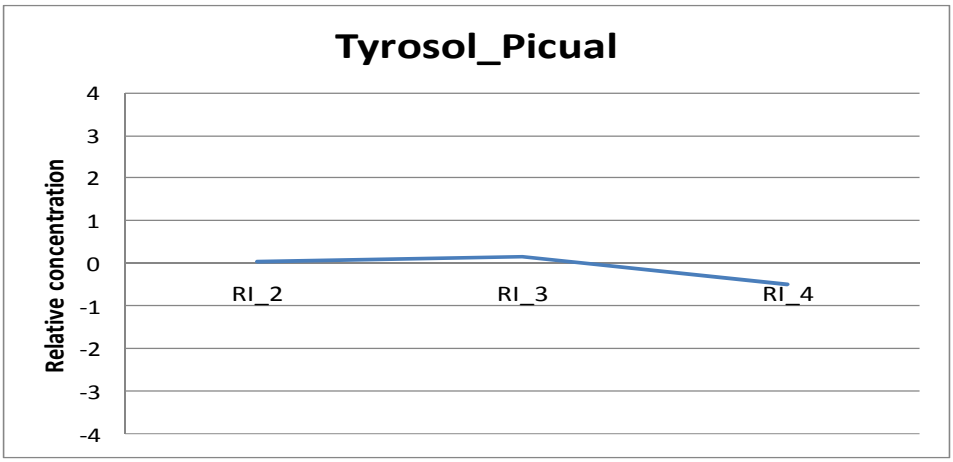
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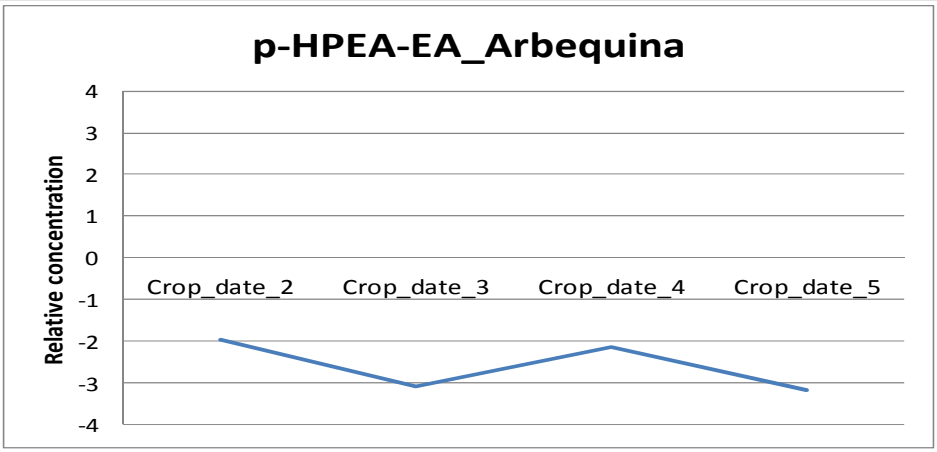
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Relative concentration
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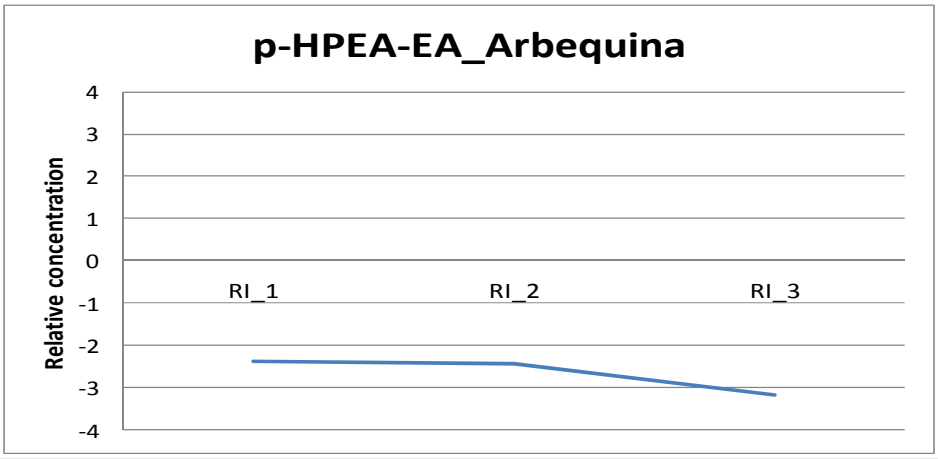
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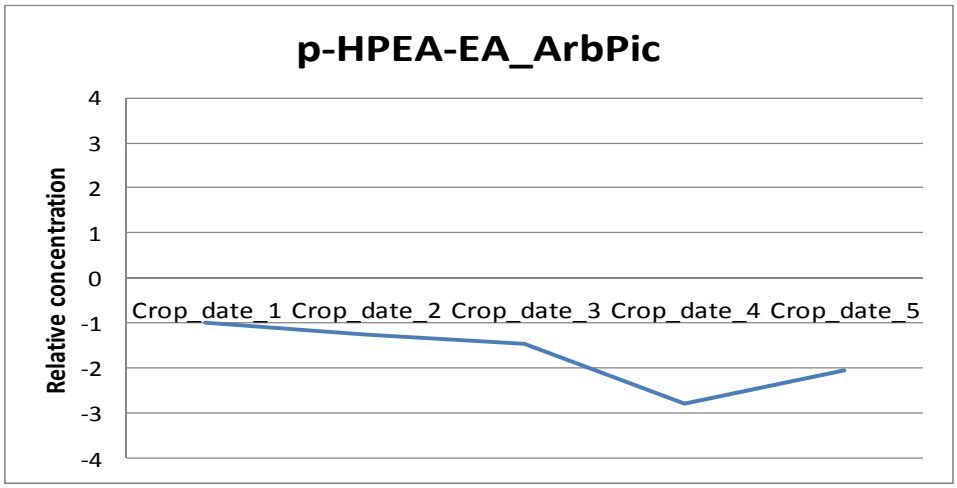
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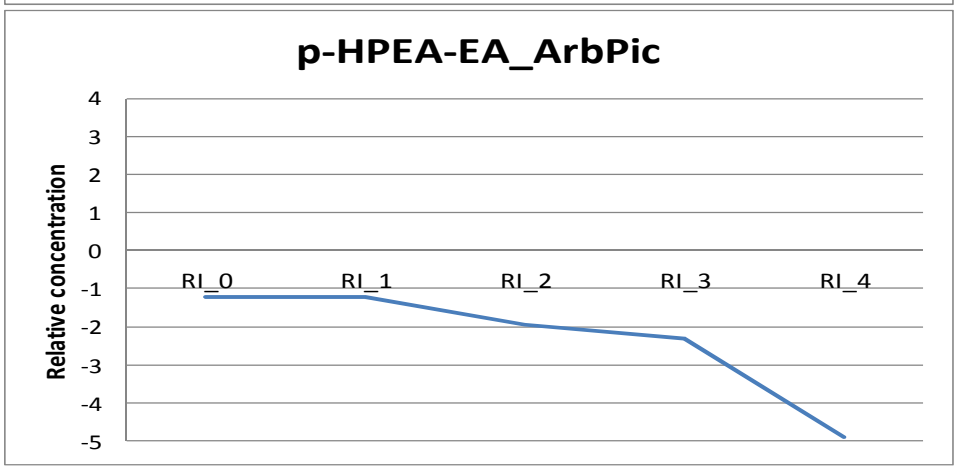
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Relative concentration
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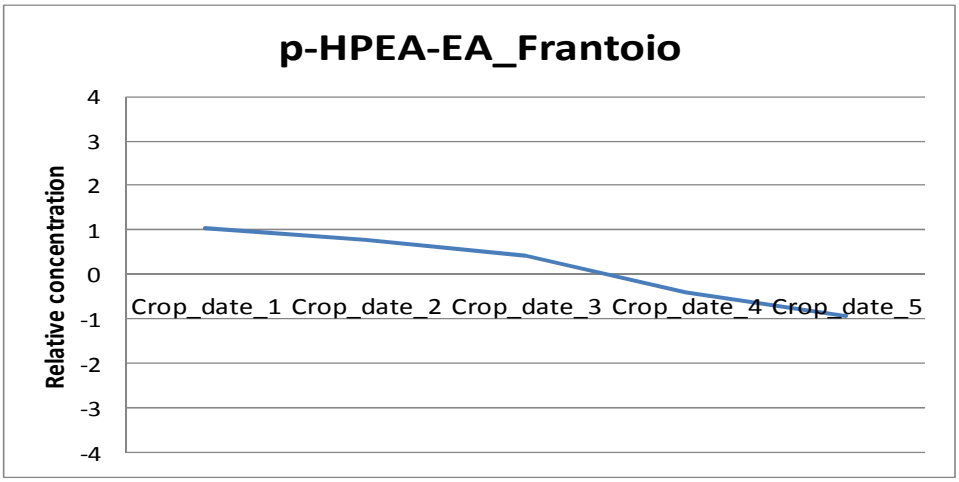
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Relative concentration
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-1
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-3
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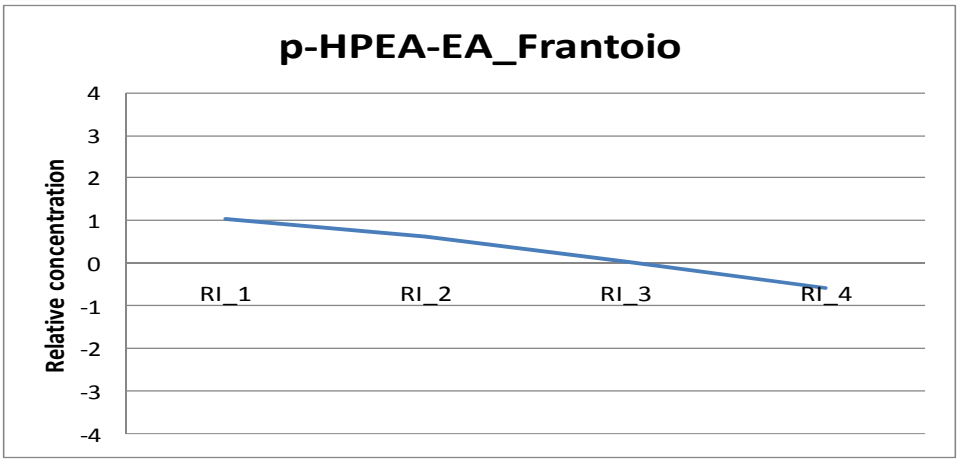
Crop_date_1 Crop_date_2 Crop_date_3 Crop_date_4 Crop_date_5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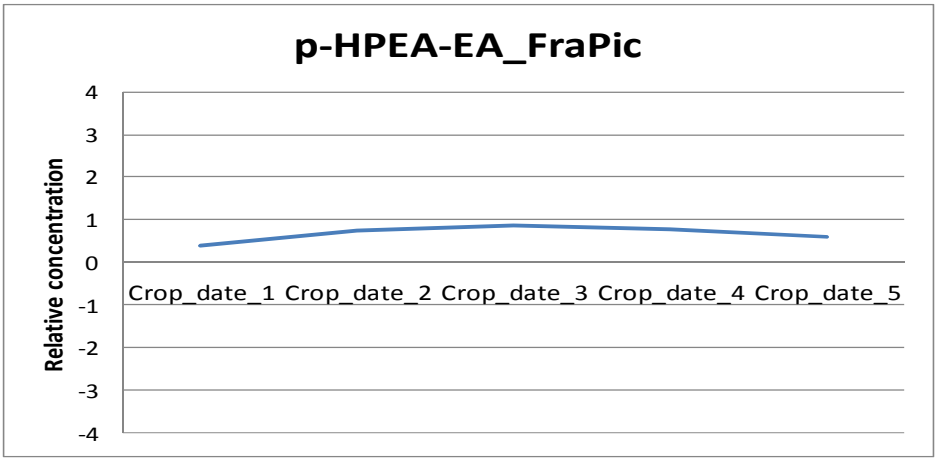
RI_3
RT_4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Relative concentration
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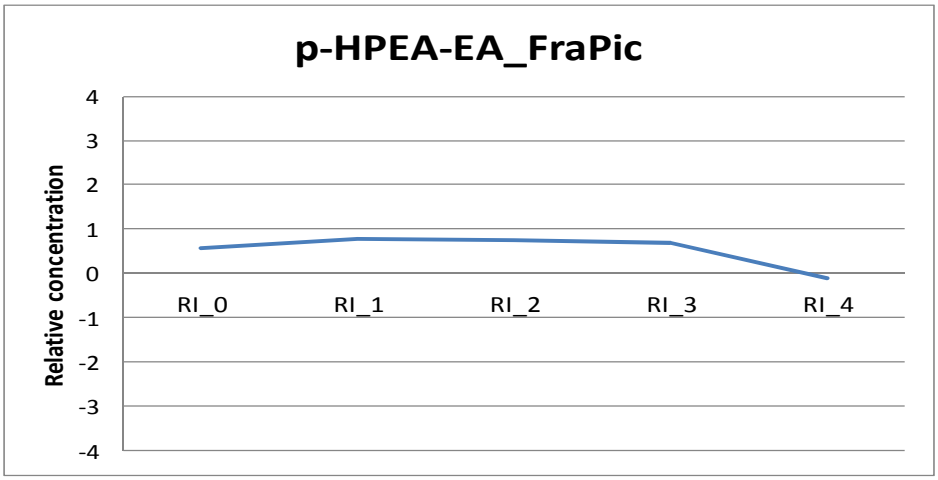
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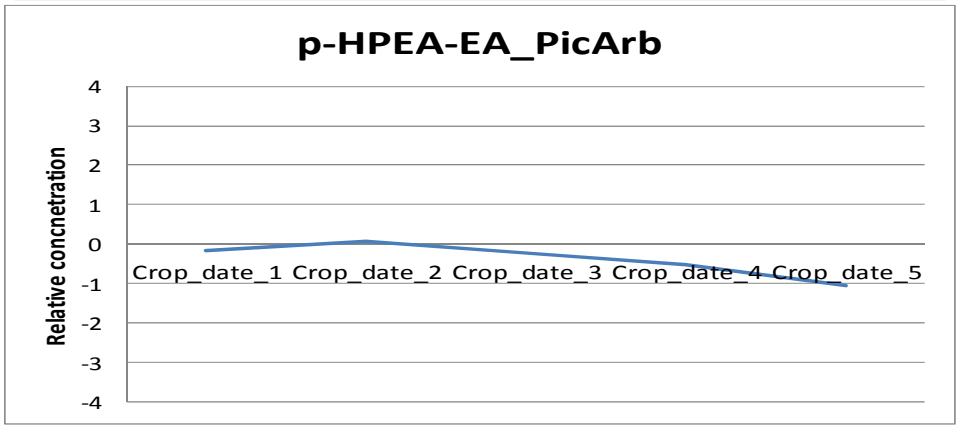
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Relative concnetration
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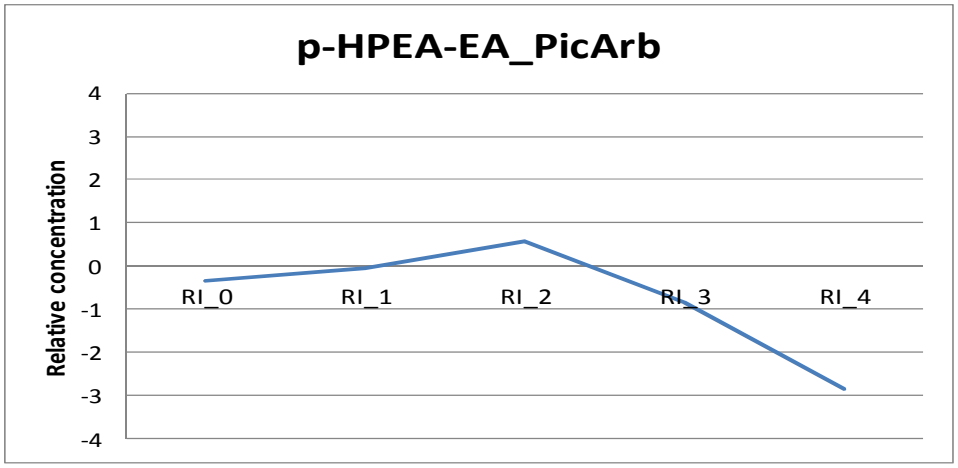
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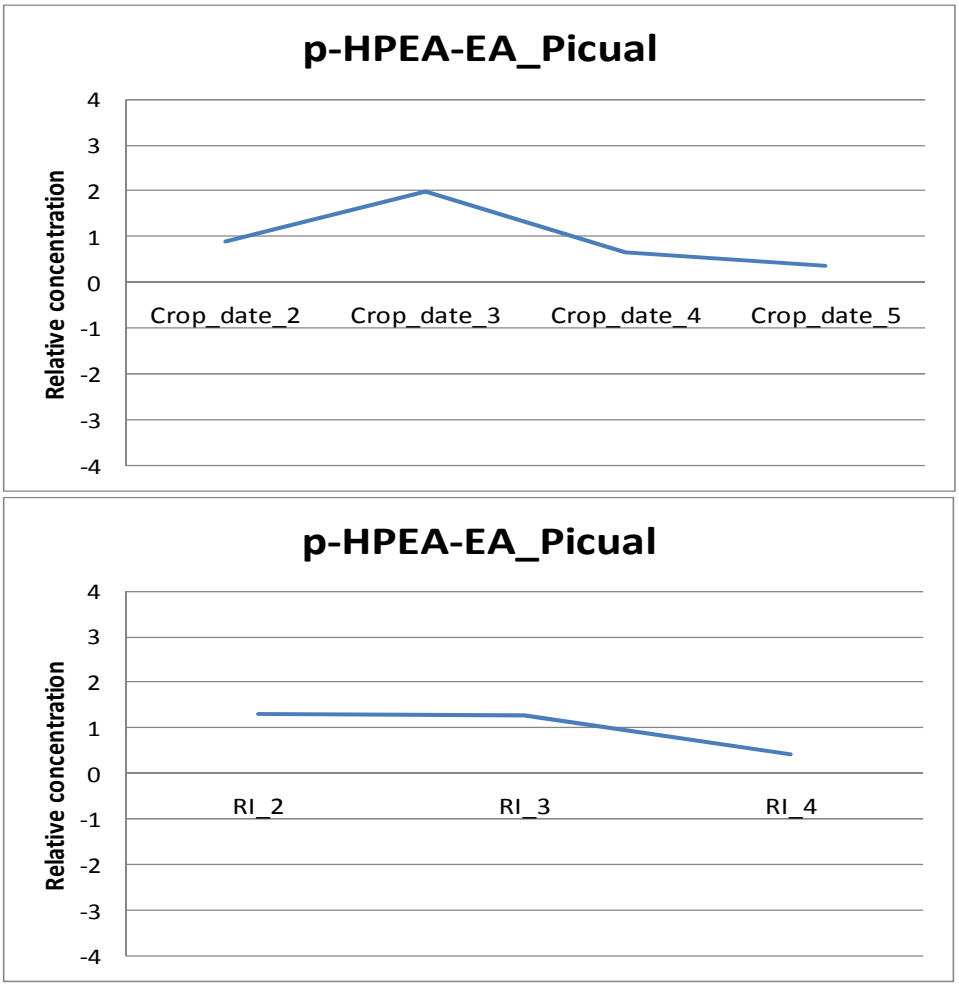

Figure 4. Evolution plots for representative phenols with crop date and ripening index.

CAPÍTULO 7:

High-resolution mass spectrometry to evaluate the influence of cross-breeding segregating populations on the phenolic profile of virgin olive oils

Aceptado en Journal of the
Science of Food and Agriculture

High-resolution mass spectrometry to evaluate the influence of cross-breeding segregating populations on the phenolic profile of virgin olive oils

Verónica Sánchez de Medina[1,2,3], Mónica Cálderón-Santiago[1,2,3], Milad El Riachy [4],Feliciano Priego-Capote[*,1,2,3], María Dolores Luque de Castro[*1,2,3]

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High-resolution mass spectrometry to evaluate the influence of cross-breeding segregating populations on the phenolic profile of virgin olive oils

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Abstract

BACKGROUND: The growing demand of high quality virgin olive oils (VOOs) has increased the interest in olive breeding programs. Cross-breeding is considered, within these programs, the best strategy to generate new cultivars as an attempt to improve the present cultivars. In this research, the phenolic profile of VOOs from target crosses (Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana) and their corresponding genitors (Arbequina, Arbosana, Koroneiki, Picual and Sikitita) has been evaluated using a targeted metabolomics approach.

RESULTS: The phenolic profiles were obtained by LC–QqTOF targeted analysis of thirty-seven phenols or compounds involved in the main pathways for their biosynthesis. Statistical multivariate analysis by Principal Component Analysis (PCA) was applied to study the influence of the genotype on phenols composition. Phenolic compounds with the highest contribution to explain the observed variability associated to genotype were identified through fold change algorithms (cut-off>2.0) and *t*-test analysis.

CONCLUSION: A total of nine phenols (*viz.* quercetin, ligstroside aglycon (*p*-HPEA-EA), demethyloleuropein aglycon, oleuropein aglycon (3,4-DHPEA-EA), hydroxypinoresinol, hydroxytyrosol, phenolic acids such as *p*-coumaric acid, ferulic acid and protocatechuic acid) contributed to explain the observed variability with 99% confidence ($p<0.01$).

INTRODUCTION

The present olive oil industry is developing breeding programs based on cross-breeding among the most outstanding cultivars by proper selection of genitors. Cross-breeding is considered the best strategy in olive breeding programs due to the high level of heterozygosity in *Olea europaea* species. Thus, new genotypes can be generated to take profit from the good qualities of the genitors to get improved new cultivars.[1] These programs are aimed at improving oil yield and quality, resistance to diseases, suitability to mechanical harvesting, early bearing, high productivity and oil content, and shortening of the juvenile period.[2,3] However, there are few studies focused on the improvement of virgin olive oil (VOO) quality as a result of breeding programs. Rjiba et al.[4], Serrilli et al.[5] and El Riachy et al.[6] have studied the effect of genotype on minor compounds of VOO from olive breeding programs. These works used a panel formed by a reduced number of representative phenols to assess the genetic influence on VOO quality due to the strong contribution of phenols to oil stability and organoleptic characteristics.[7,8] Apart from that, these compounds have revealed health benefits such as the inhibition capability of *in vitro* LDL oxidation, a key step in the formation of atherosclerotic plaques.[9]

There is at present a great interest on nutritional and, mainly, functional properties of VOO phenols as a result of an increased social concern on these topics.[10] In this context, metabolomics is aiding to improve VOO quality and confirm health benefits associated to phenols. From the point of view of VOO quality, metabolomics currently enables to: (i) differentiate among diverse quality levels of olive oils (*viz.* VOO, olive oil pomace, refined olive oil) according to the phenolic profile; (ii) reveal VOO adulterations;[11] (iii) predict the genetic variety used to obtain different VOOs;[12] (iv) build a phenols panel to be used as metabolites fingerprint for oil quality.[13] Few papers have been published on the relationship between

VOO quality and olive genotype from the metabolomics perspective.[14] Metabolomics seems to be specially suited to characterize VOO phenols as a function of olive genotypes.

Mass spectrometry in high resolution mode is one of the most relevant detection techniques for implementation of metabolomics methods. Measurement of accurate mass of precursor and product ions is crucial to confirm the presence of monitored compounds in target samples. In this research, LC–QqTOF in high resolution mode has been used to evaluate phenolic profiles (composed by thirty-seven representative phenols or compounds involved in the main pathways for biosynthesis of phenols present in VOO according to literature of VOOs from cross-breeding segregating populations obtained by a controlled planning. [6,15,16] For this purpose, phenols profiles VOOs extracted from target crosses (Arbequina×-Arbosana, Picual×Koroneiki and Sikitita×Arbosana) and corresponding genitors (Arbequina, Arbosana, Koroneiki, Picual and Sikitita) have been used to find discrimination patterns associated to the genotype. Significant differences in the phenols profile for each cross-breeding have been identified.

EXPERIMENTAL

Plant Material and Olive Oil Extraction

Healthy olive fruits were manually picked from two different trees of each cultivar: Arbequina, Arbosana, Koroneiki, Picual and Sikitita, and also from 25 trees from each of the following crosses: Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana. Crosses were made in Spring 2004-–2005 and seedlings were planted in 2006–2007 in field trials in Córdoba, coordinated by the Department of Agronomy in the University of Córdoba and the Andalusian Institute of Agricultural Research and Training (IFAPA, Junta de Andalucía). Seedlings were subjected to a forcing growth protocol from seed germination to greenhouse and field growth, as described by

Santos-Antunes et al.[17] Olive fruits were collected at a similar ripening index in the 2009/2010 harvest season (from 2 November to 10 December) to avoid the influence of this variable on the VOO components. In fact, 95% of the collected olive fruits samples were characterized by a ripening index 2–3 according to El Riachy et al.[18] It is worth mentioning that the same agronomical practices were applied to all cultivars.

An Abencor laboratory oil mill (MC2 Ingenierías y Sistemas, Sevilla, Spain) was used to extract the oil. This system reproduces the industrial process at a laboratory scale through three basic elements: a hammer mill, a thermobeater and a paste centrifuge. Approximately 1 kg of fruits was ground to a paste using the hammer mill; then, the paste was placed in the thermobeater and stirred for 30 min with the water bath set at 28±1 ºC in the presence of micronized talc and without water addition. Subsequently, a vertical centrifugation for 1 min permitted phases separation, and the oily phase was collected and left to decant for 24 h. Finally, the oil was separated and stored in glass vials without headspace at −20 ºC until analysis.[19]

Reagents

The reagents used for characterization of VOO were LC grade methanol and hexane from Scharlab (Barcelona, Spain). LC–MS grade acetonitrile and formic acid for preparation of chromatographic mobile phases were also from Scharlab. Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare the chromatographic phases.

Apparatus and Instruments

An MS2 minishaker from Ika (Wilmington, USA) was used to enhance the transfer of phenols from oil to a methanol–water solution for individual quantification of the target compounds.

All samples were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6540 QqTOF hybrid mass spectrometer with a dual electrospray ionization (ESI) source for

simultaneous spraying of a mass reference solution that enabled continuous calibration of detected m/z ratios.

Extraction of Phenolic Compounds from VOO

Phenols extracts were obtained by shaking 1 g of each oil with 1 mL of hexane and 1 mL of 60:40 methanol–water mixture for 1 min in the MS2 minishaker. The hydroalcoholic phase was directly injected into the LC–QqTOF. This approach has been widely validated in previous publications and is accepted as preparation strategy for analysis of phenolic compounds in VOO.[6,20,21]

LC–QqTOF Analysis of Phenolic Extracts

Olive phenols analysis was conducted by LC–QqTOF in accurate mode. The analytical column was a C18 Pursuit XRs Ultra (50×2.0 mm i.d., 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The mobile phases were: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient program, at 0.4 mL/min constant flow rate, was as follows: initially 96% A and 4% B; 0–8 min, 96–0% A and 4–100% B; 8–10 min, 0% A and 100% B. After analysis, the column was equilibrated for 5 min.

Five μL of extract was injected in the LC system without any additional pretreatment. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L min^{-1}; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L min^{-1}; capillary voltage, 3500 V in negative ionization mode; skimmer, 65 V; octopole radiofrequency voltage, 750 V; fragmentor voltage, 175 V. Data were acquired in centroid mode in high resolution (2 GHz). Full scan was carried out at 1 spectrum s^{-1} within the m/z range 100–1700 with subsequent activation of the three most intense precursor ions per scan (only single or double charged ions were allowed) by MS/MS using a collision energy of 20 eV. MS/MS scanning was carried out at 1 spectrum/s within the m/z range 100–1700. An active exclusion window was programmed after one MS/MS

spectrum and released after 0.75 min to avoid repetitive fragmentation of the most intense precursor ions and, in this way, increase the detection coverage. Before experiments, the instrument reported mass detection resolution of 25000 FWHM (Full Width at Half Maximum) at m/z 119.0362 (proton abstracted purine) and 45000 FWHM at m/z 966.0007 (formate adduct of hexakis (1H,1H,3H-tetrafluoropropoxy)-phosphazine). To assure the desired mass accuracy of recorded ions, internal calibration was performed during analyses with the dual ESI source by continuous infusion of a solution containing both standards.

Data Pretreatment

MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing the raw LC−QqTOF data files. Phenols were searched in the raw data files using a representative phenols database previously built.

Ions with identical elution profiles and related m/z values (representing different adducts, ions generated after specific neutral losses, or isotopic forms from the monoisotopic ions) were extracted in a matrix characterized by retention time (RT), accurate mass and containing intensity in apex of chromatographic peaks as data. The isotope model corresponded to common organic molecules with peak spacing tolerance of m/z 0.0025 ± 7.0 ppm. The search algorithm limited extraction to ions exceeding 1000 counts with charge state limited to a maximum of two. The allowed negative ions were deprotonated species and formate adducts. Dehydratation neutral losses were also allowed. The generated raw data files in compound exchange format (.cef files) were created for each phenolic extract and exported into the Mass Profiler Professional (MPP) software package (version 12.0, Agilent Technologies, Santa Clara, CA, USA) to create the data matrix for further processing. In the next step, alignment of RT and m/z values was carried out across the extract set using a tolerance window of 0.5 min and 10 ppm, respectively. Then, a statistical analysis by unsupervised PCA was performed

to find clustering of samples attending to cross-breeding (scores graphs). A fold change algorithm was applied with the aim of retaining only those phenols that experienced a change in relative concentration between cross-breeding classes equal to or higher than 2.0. Finally, a *t*-test analysis was performed to support statistically the differences between target crosses.

RESULTS AND DISCUSSION

Data File Preparation and Pretreatment

The study was focused on a panel of compounds which are the most common phenols present in VOO according to literature.[6,15,16] Moreover, other compounds implicated in the main pathways for biosynthesis of phenols were also included. The monitored compounds were hydroxytyrosol, tyrosol and derivatives, iridoid precursors, hydroxycinamic acid derivatives, phenolic acids and derivatives, phenolic alcohols, secoiridoids, lignans, and flavonoids. Monitored parameters for LC–QqTOF confirmatory analysis are listed in **Table 1**. After building the database, the target phenols were searched in the data file of each sample analyzed by LC–QqTOF. The requisite for inclusion of a given compound in the database was to be present in all extracts. Thus, the initial data set was formed by 37 variables associated to monitored phenols or compounds involved in the main pathways for biosynthesis of phenols. After extraction of each compound, alignment of entities in the different extracts was executed. The tolerance window for alignment was 0.5 min and 10 ppm for elution time and mass accuracy, respectively.

Instrumental and methodological variability was evaluated by a repetitive analyses of one of the samples by the LC–QqTOF method used in this research. The resulting data were evaluated by PCA to detect variability sources that could be associated to internal variability of the method. **Supplementary Figure 1** shows the overlapped chromatograms and PCA

scores plot, which does not show any trend related to methodological variations or sample modifications.

Table 1. Parameters for identification of target phenols by LC–QqTOF.

Compound	Theoretical *m/z*	Experimental *m/z*	Error (ppm)	Adduct	Retention time	Fragments
Hydroxytyrosol and tyrosol derivatives						
Hydroxytyrosol	153.0557	153.0559	-1.3067	$[M-H]^-$	2.60–3.00	123.0450 105.0339
Hydroxytyrosol glucoside	315.1085	315.1082	0.9520	$[M-H]^-$	3.08–3.48	123.0446 153.0570
Tyrosol	137.0604	137.0609	-3.6480	$[M-H]^-$	3.80–4.20	119.0505 111.0098
Hydroxytyrosol acetate	195.0663	195.0659	2.0505	$[M-H]^-$	5.70–6.10	123.0450 105.0339
Iridoids precursors						
Loganic acid	375.1297	375.1296	0.2666	$[M-H]^-$	3.38–3.78	151.0776 113.0248
Secologanol	389.1453	389.145	0.7709	$[M-H]^-$	3.42–3.82	151.0776 113.0248
Oleoside	389.1089	389.1089	0	$[M-H]^-$	4.00–4.40	121.0660 101.0243 209.0477 345.1253
Oleoside 11-methylester	403.1246	403.1243	0.7442	$[M-H]^-$	4.60–5.00	101.0244 119.0350 223.0626 179.0707
Hydroxycinamic acid derivatives						
Verbascoside	623.1981	623.1959	3.5302	$[M-H]^-$	4.77–5.17	461.1744 161.0254
Phenolic acid and derivatives						
Quinic acid	191.0561	191.0558	1.5702	$[M-H]^-$	0.26–0.66	-

Table 1. Parameters for identification of target phenols by LC–QqTOF (cont.).

Compound	Theoretical *m/z*	Experimental *m/z*	Error (ppm)	Adduct	Retention time	Fragments
Ferulic acid	193.0506	193.0513	3.6260	$[M-H]^-$	5.50–5.90	134.0377
p-Coumaric acid	163.0401	163.0388	7.9735	$[M-H]^-$	5.70–6.10	119.0503
Eleanolic acid	241.0718	241.0716	0.8296	$[M-H]^-$	5.80–6.20	-
Protocatechuic acid	153.0193	153.0181	7.8421	$[M-H]^-$	5.89–6.29	109.0295
Sinapic acid	223.0612	223.0598	6.2763	$[M-H]^-$	5.90–6.30	-
Vanillic acid	167.035	167.0334	9.500	$[M-H]^-$	5.98–6.38	-
Caffeic acid	179.035	179.0343	3.9098	$[M-H]^-$	6.40–6.80	135.0449
Phenolic alcohols						
3,4-DHPEA-EDA	319.1187	319.1185	0.6267	$[M-H]^-$	5.70–6.10	123.0446 139.0769
p-HPEA-EA	361.1293	361.1287	1.6614	$[M-H]^-$	5.90–6.30	-
p-HPEA-EDA	303.1238	303.1235	0.9897	$[M-H]^-$	6.30–6.70	-
3,4-DHPEA-EA	377.1242	377.1241	0.2652	$[M-H]^-$	6.50–6.90	123.0446 255.0869
Secoiridoids						
Demethyloleuropein	525.1614	525.1605	1.7137	$[M-H]^-$	4.50–4.90	-
Oleuropein	539.177	539.176	1.85467	$[M-H]^-$	5.20–5.60	377.1306 307.0874 275.0962
Ligstroside	523.1821	523.1812	1.7202	$[M-H]^-$	5.00–5.40	-
Demethyloleuropein aglycon	363.1085	363.1078	1.9278	$[M-H]^-$	5.82–6.22	-

Table 1. Parameters for identification of target phenols by LC–QqTOF (cont.).

Compound	Theoretical *m/z*	Experimental *m/z*	Error (ppm)	Adduct	Retention time	Fragments
Lignans						
Pinoresinol	357.1344	357.1335	2.5201	$[M-H]^-$	5.90–6.30	-
Hydroxypinoresinol	373.1293	373.1272	5.6281	$[M-H]^-$	6.70–7.10	-
Flavonoids						
Rutin	609.1461	609.1459	0.3283	$[M-H]^-$	4.79–4.19	301.0391
Luteolin-7 glucoside	447.0933	447.0928	1.1183	$[M-H]^-$	4.85–5.25	285.0423
Apigenin-7-glucoside	431.0984	431.1098	-3.5259	$[M-H]^-$	5.10–5.50	269.0450
Luteolin	285.0405	285.0414	-3.1574	$[M-H]^-$	5.80–6.20	-
Quercetin	301.0354	301.0351	0.9966	$[M-H]^-$	5.90–6.30	-
Apigenin	269.0455	269.0448	2.6018	$[M-H]^-$	6.20–6.60	-
α-Taxifolin	303.051	303.0511	-0.3300	$[M-H]^-$	6.00–6.40	-
Diosmetin	299.0561	299.0546	5.0158	$[M-H]^-$	6.30–6.70	-
Other phenols						
Vanillin	151.0401	151.0401	0	$[M-H]^-$	4.40–4.80	-
Hydroxy-isocromans						
1-3 methoxy 4-hydroxy-phenyl-6-7-dihydroxy-isochroman	287.0925	287.0909	5.5731	$[M-H]^-$	6.40–6.80	-

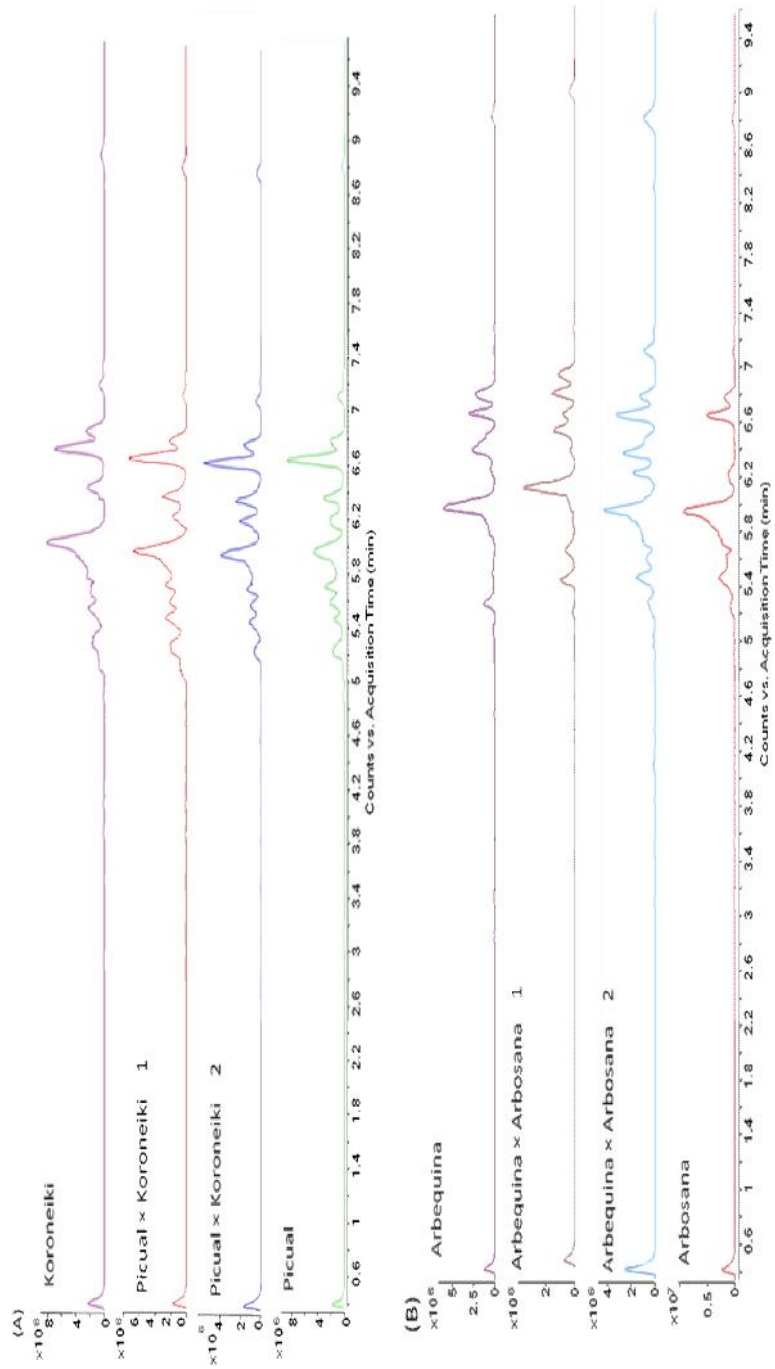

Figure 1. Total chromatograms compounds (TCCs) obtained by LC–QqTOF of phenolic extracts from the crosses Picual×Koroneiki (A), Arbequina×Arbosana (B) and their corresponding genitors.

Total chromatograms compounds (TCCs) obtained by LC–QqTOF of phenolic extracts corresponding to the crosses Picual×Koroneiki and Arbequina×Arbosana as well as their genitors are shown in **Figure 1.A** and **1.B**, respectively. These chromatograms illustrate fingerprints representative of the content of polar and mid-polar phenols in VOO extracts. **Figure 1.A** shows chromatographic profiles provided by the cross Picual×Koroneiki and those from the genitors. As shown, chromatographic profiles were similar for genitors and crosses. On the other hand, clear differences in the chromatographic profiles from Arbequina×Arbosana cross and their genitors can be observed. As **Figure 1.B** shows, the phenolic profile of Arbequina×Arbosana 1 is more similar to that obtained by analysis of Arbosana VOO, while the phenolic profile of Arbequina×Arbosana 2 was more similar to that of Arbequina VOO. This visual comparison was subsequently analyzed to assess the statistical significance of the genotype in the profile of phenolic compounds from cross-breeding.

Variability of Phenolic Composition in Cross-Breeding Segregating Populations

After data set preparation, the next step was to study the variability of the phenolic composition in the extracts from the VOOs obtained from the selected cross-breeding segregating populations. It is worth emphasizing that the influence of fruit ripening in the samples cohort was reduced since most of the samples were collected with ripening index 2–3. As previously reported, these ripening index stages had been identified as those with the lowest incidence on the phenolic composition of VOO.[22] Therefore, the variability in the phenolic composition of VOO should be ascribed to the genotype influence. Principal component analysis (PCA) was applied to find clusters of VOO samples attending to their phenolic content. **Figure 2** shows a partial discrimination observed according to the type of crosses in this scores plot. VOO extracts from the cross Picual×Koroneiki were separated along PC2 from Arbequina×Arbosana and Sikitita×Arbosana

crosses, which showed overlapping ascribed to the common genitor Arbosana. Therefore, cross-breedings could contribute to explain the variability associated to the composition of the main phenols present in VOO. The next step involved comparison of pairs of crosses to detect discrimination patterns according to phenols profiles between them.

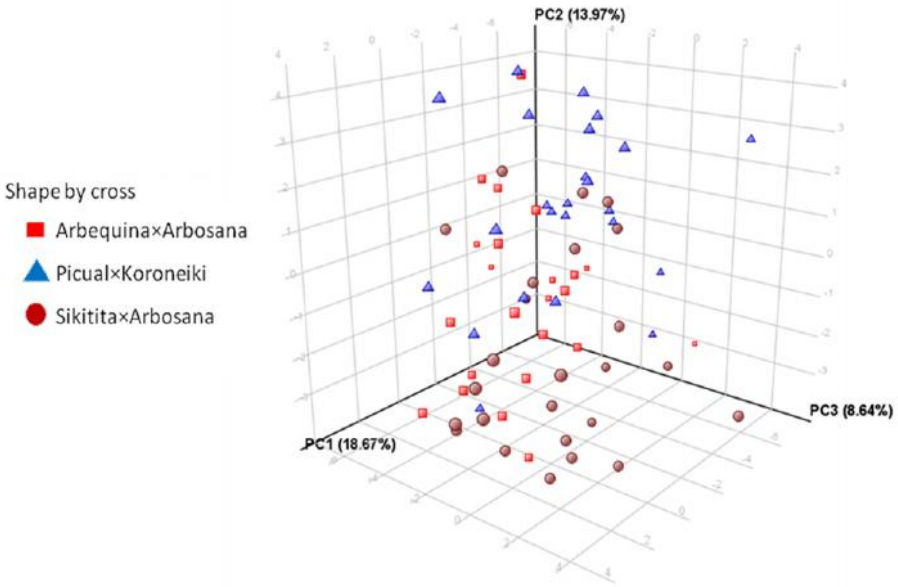

Figure 2. PCA scores plots from crosses: Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana.

Influence of the Genotype on the Phenolic Profile of Cross-Breeding Segregating Populations

The influence of the genotype on the total phenolic content of VOO from different crosses or within the same cross has been proved by different authors.[6,23,24] In this research, the phenolic profile of VOOs from cross-breeding segregating populations was compared to assess statistical differences among them. **Figure 3** illustrates PCA scores plots provided by pairs of cross-breeding populations: (A) Sikitita×Arbosana *vs* Picual×Koroneiki, (B) Sikitita×Arbosana *vs* Arbequina×Arbosana, (C) Picual×Koroneiki *vs* Arbequina×Arbosana.

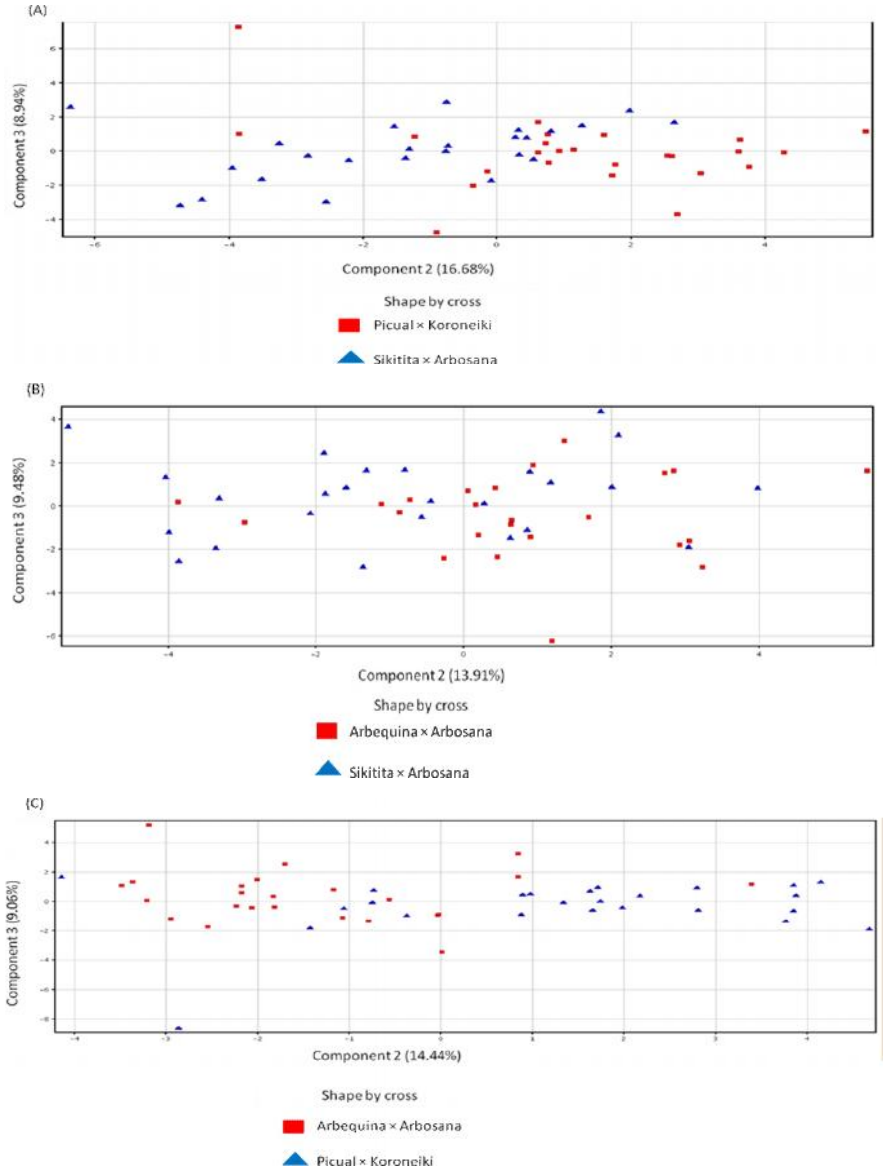

Figure 3. PCA scores plots from cross pairs (A) Sikitita×Arbosana vs Picual×Koroneiki, (B) Sikitita×Arbosana vs Arbequina×Arbosana, (C) Picual×Koroneiki vs Arbequina×Arbosana.

Separation was observed between the two cross-breeding populations compared in each case, which allows setting the influence of the genotype as

variability source to explain the concentration of phenols or those compounds involved in the pathways for biosynthesis of phenols. Segregation was higher for comparisons involving Picual×Koroneiki *versus* the other two crosses; while Sikitita×Arbosana and Arbequina×Arbosana showed partial overlapping.

Table 2. Results from application of the fold-change algorithm with cut-off set 2.0 for each pair of cross-breeding populations.

Compound	**Sikitita×Arbosana[1] *vs* Arbequina×Arbosana[2]**
Hydroxytyrosol	Up
o-Coumaric acid	Up
Caffeic acid	Down
Quercetin	Up
Pinoresinol	Up
Hydroxypinoresinol	Up
Oleoside	Down
Luteolin-7-glucoside	Down
Demethyloleuropein	Down
Verbascoside	Up
Compound	**Picual×Koroneiki[1] *vs* Arbequina×Arbosana[2]**
Protocatechuic acid	Up
o-Coumaric acid	Up
p-HPEA-EA	Up
Demethyloleuropein aglycon	Up
Demethyloleuropein	Down
Verbascoside	Up

Table 2. Results from application of the fold-change algorithm with cut-off set 2.0 for each pair of cross-breeding populations (cont.).

Compound	**Sikitita×Arbosana[1] *vs* Picual×Koroneiki[2]**
p-Coumaric	Down
Ferulic acid	Up
Apigenin	Up
1-3-methocy-4-hydroxy-phenyl-6,7-dihydroxyisochroman	Up
Diosmetin	Up
Quercetin	Up
Pinoresinol	Up
p-HPEA-EA	Down
Demethyloeuropein aglycon	Down
Hydroxypinoresinol	Up
3,4-DHPEA-EA	Down
Oleoside	Down
Secologanol	Up
Luteolin-7-glucoside	Down
Rutin	Up

Up: increased concentration in 1 as compared to 2.
Down: decreased concentration in 1 as compared to 2.

Apart from that, the PCA loadings plots can be used to detect phenols with the highest contribution to explain the observed variability (**Supplementary Figure 2. A**, **B**, **C**). The preferred presence of some phenols in certain cross-breeding populations could be used to define the organoleptical and nutraceutical characteristics of VOOs. Identification of the most significant phenols contributing to explain the variability observed in each case was carried out by fold change analysis with cut-off set at 2.0. **Table 2** lists the main phenols differentiating the studied populations according to the fold change algorithm. Most of the phenols (75%) were identified among those that contribute to explain the variability associated to

PC2 (value of the PC2 coordinate >0.1 or <–0.1), which allowed discriminating among cross-breedings olive oil. As can be seen, the panel of phenols contributing to discrimination between genotypes was different for each case under study, which emphasizes the relationship between genotype and phenols profile. The next step should be to identify the phenols present in VOO with the highest influence on genotype discrimination.

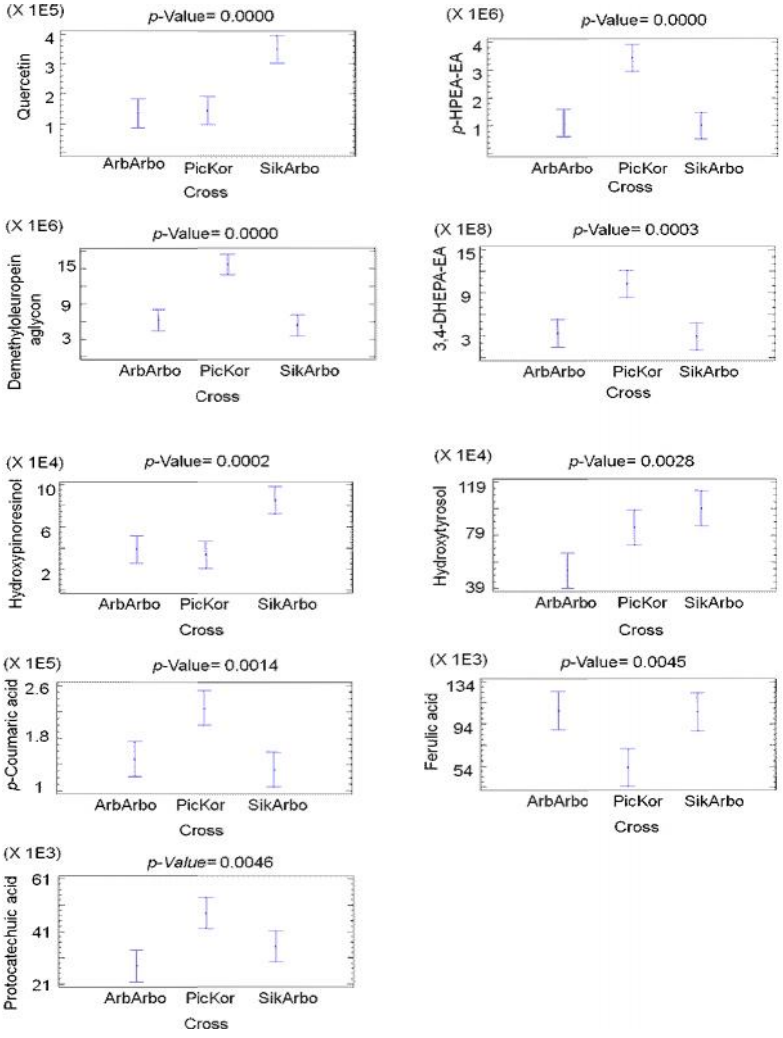

Figure 4. Average means plots for the main significant phenols in each cross. ArbArbo (Arbequina×Arbosana); PicKor (Picual×Koroneiki); SikArbo (Sikitita×Arbosana).

Differences in the Phenolic Profiles Observed for the Studied Populations

Significant differences in the profile of phenols were independently evaluated for the crosses under study. For this purpose, significance was focused on phenols that changed relatively their concentration according to the fold change algorithm. This evaluation was statistically supported on *t*-test analysis. Among the phenols that discriminated the target crosses, nine of them showed significance with 99% confidence ($p<0.01$). These were quercetin (*p*-value=0.0000), secoiridoids such as *p*-HPEA-EA (*p*-value= 0.0000), demethyloleuropein aglycon (*p*-value=0.0000) and 3,4-DHPEA-EA (*p*-value=0.003), hydroxypinoresinol (*p*-value=0.0002), hydroxytyrosol (*p*-value=0.0028) and phenolic acids such as *p*-coumaric acid (*p*-value=0.0014), ferulic acid (*p*-value=0.0045) and protocatechuic acid (*p*-value=0.0046). **Figure 4** shows the means average plots for concentration of these phenols in the three target crosses. Among phenols, it is worth emphasizing the group of secoiridoids since the same trend was observed for *p*-HPEA-EA, demethyloleuropein aglycon and 3,4-DHPEA-EA in VOOs from the three cross-breeding segregating populations. Thus, the levels found for these three secoiridoids were clearly higher in Picual×Koroneiki VOOs, while no distinction was observed between Sikitita×Arbosana and Arbequina×Arbosana. This is a key aspect taking into account the relevance of secoiridoids from the organoleptical and nutraceutical perspectives.[25] Levels of *p*-HPEA-EA and 3,4-DHPEA-EA in the crosses were similar to those in their genitors, as can be seen in **Supplementary Figure 3**, which shows the range of concentration found for significant phenols in varieties and genitors. However, demethyloleuropein aglycon levels in samples from the cross Picual×Koroneiki were below the levels observed in samples from Picual. Therefore, this cross was closer to the Koroneiki genitor in terms of this phenotypic character. One other significant phenol with a particular behavior was quercetin, which is less characteristic of VOO than apigenin or luteolin,

but allowed significant discrimination (p-value=0.0001) of Sikitita×Arbosana population, as **Supplementary Figure 4** shows. This cross was also distinguished by contribution of hydroxypinoresinol levels, which is one of the most important lignans present in VOO. Levels of this lignan in Sikitita×Arbosana VOO samples were similar to those in Sikitita samples while Arbosana genitors were characterized by lower levels of this phenol (**Supplementary Figure 4**).

Identification of hydroxytyrosol as one of the phenols with the highest separation contribution is a very interesting aspect from this research. This is based on the nutritional studies supporting the potential of this bioactive compound.[26,27] This phenol reported higher levels in Sikitita×Arbosana and Picual×Koroneiki VOOs as compared to Arbequina×Arbosana VOO. Phenolic acids are other characteristic group of compounds forming part of the phenolic family in VOO. Three of these compounds were found with discrimination capability (*p*-coumaric acid, ferulic acid and protocatechuic acid). *p*-Coumaric acid and ferulic acid are synthesized through the same pathway (the phenylpropanoid pathway), being *p*-coumaric acid the precursor of ferulic acid through caffeic acid. Both acids showed an opposite trend, as can be seen in their plots. Thus, *p*-coumaric acid was detected at higher concentration in Picual×Koroneiki VOO samples *versus* the other two crosses, while ferulic acid was lower concentrated in Picual×Koroneiki VOO samples. This behavior can be attributed to differences of activity corresponding to the enzymes involved in this pathway. It is also worth mentioning that levels of *p*-coumaric acid in Picual×Koroneiki VOO samples were intermediate between the high levels in Koroneiki VOOs and the low levels in Picual VOO samples (**Supplementary Figure 5**). On the other hand, protocatechuic acid, synthesized through the shikimate pathway, also allowed differentiating VOO samples from Picual×Koroneiki cross.

The dependence of the phenols profile on the genotype and the variability induced by obtaining cross-breeding segregating populations opens

the door to VOOs with a specific phenolic profile. The organoleptical and nutraceutical interest on phenolic compounds supports their inclusion in olive breeding programs and metabolomics analysis to find particular patterns associated to genotypes. The most important results from this research are: (i) the discrimination of controlled crosses according to the phenols profile of VOOs; (ii) the identification of the most significant phenols contributing to the variability of the VOOs from different crosses; and (iii) the suitability of mass spectrometry as a decision tool to be used in olive breeding programs to improve oil quality.

ACKNOWLEDGEMENTS

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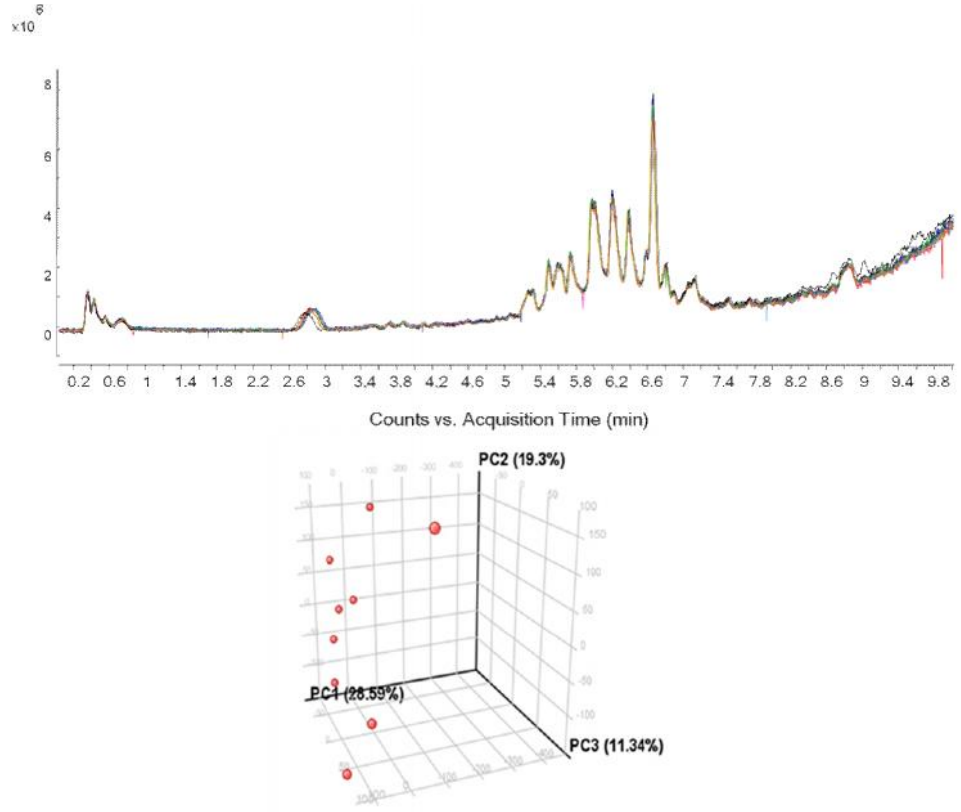
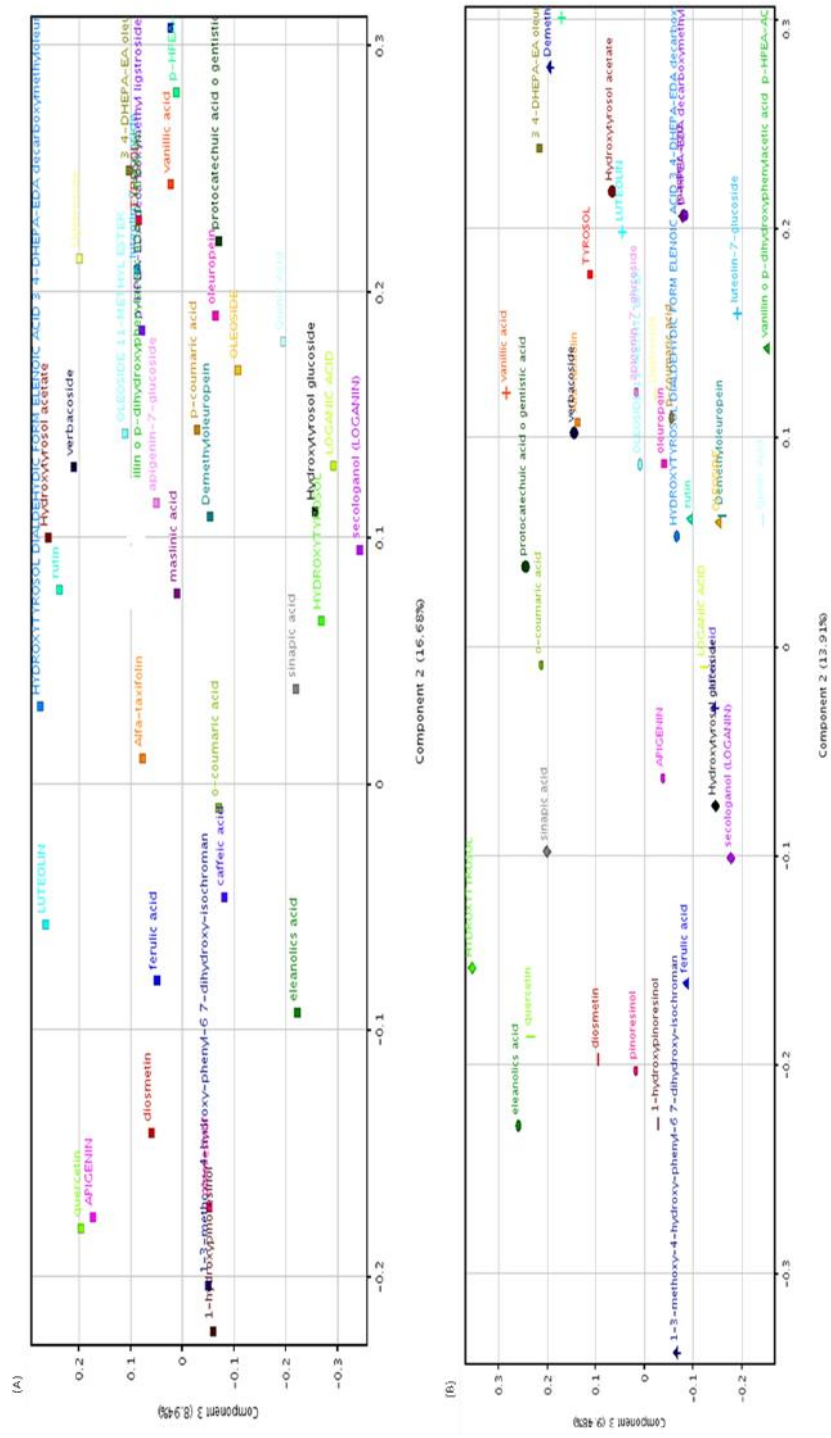
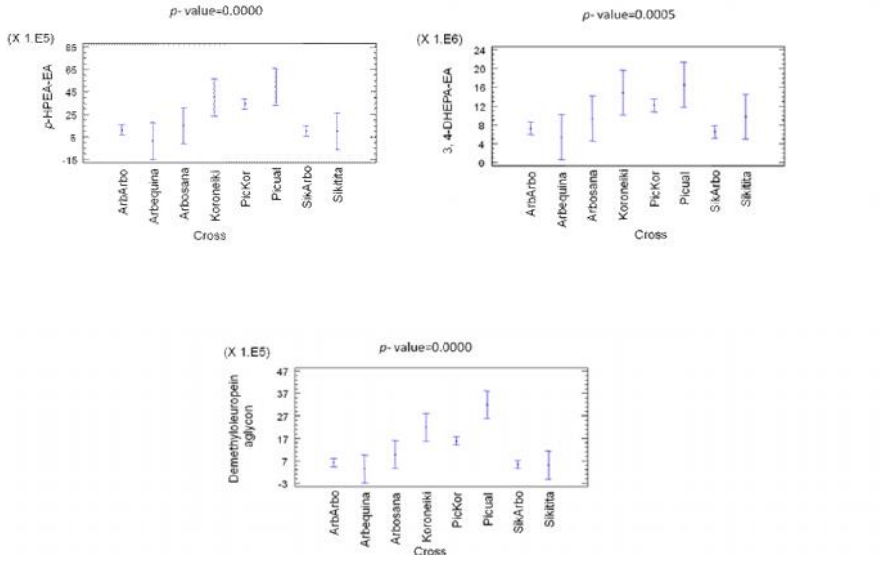
SUPPLEMENTARY MATERIAL



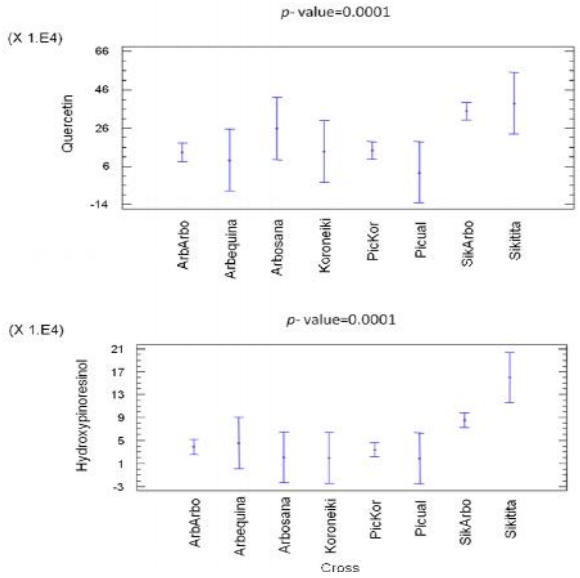
Supplementary Figure 1. Overlapped base peak chromatograms (BPCs) obtained by LC–QqTOF analysis and PCA scores plot associated to replicates of a phenolic extract from Arbosana VOO.



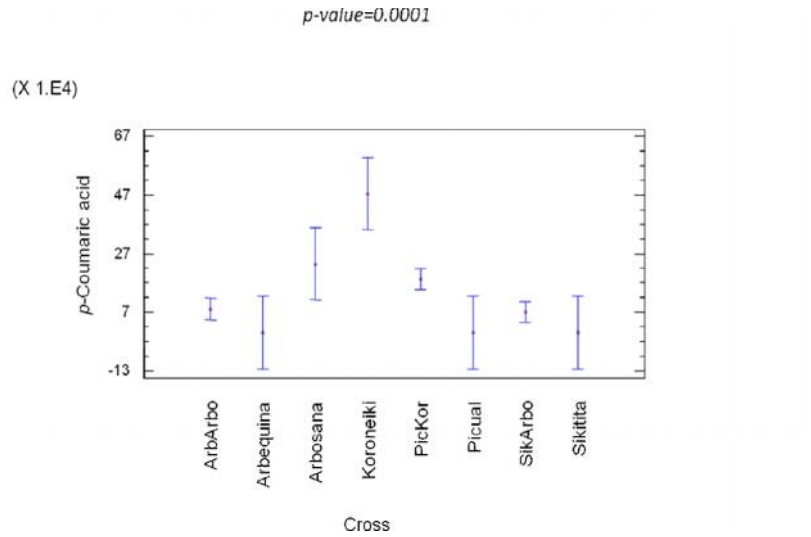
Supplementary Figure 2. PCA loadings plots corresponding to cross pairs: (A) Sikitita×Arbosana vs Picual×Koroneiki, (B) Sikitita×Arbosana vs Arbequina×Arbosana, (C) Picual×Koroneiki vs Arbequina×Arbosana.



Supplementary Figure 3. Average means plots for p-HPEA-EA, 3,4-DHPEA-EA and demethyloleuropein aglycon in each cross and their genitors. ArbArbo (Arbequina×Arbosana); PicKor (Picual×Koroneiki); SikArbo (Sikitita×Arbosana).



Supplementary Figure 4. Average means plots for quercetin and hydroxypinoresinol in each cross and their genitors. ArbArbo (Arbequina×Arbosana); PicKor (Picual×Koroneiki); SikArbo (Sikitita×Arbosana).



Supplementary Figure 5. Average means plots for p-coumaric acid in each cross and their genitors. ArbArbo (Arbequina×Arbosana); PicKor (Picual×Koroneiki); SikArbo (Sikitita×Arbosana).

SECCIÓN C: ESTUDIO DE LA INFLUENCIA DE LA MADURACIÓN Y EL GENOTIPO SOBRE LA COMPOSICIÓN DE ÁCIDOS GRASOS DE VOOS

La composición de la fracción lipídica de un aceite es su principal característica. Aunque en el VOO se considera que existe un buen equilibrio entre las concentraciones de ácidos grasos (FAs) saturados, monoinsaturados y poliinsaturados, la elección del grado de maduración del fruto idóneo para su recogida y la producción de nuevos cruces en que se mejore la composición lipídica son objeto de estudios. Para ello se ha aplicado una estrategia de análisis metabolómico orientado con el fin de demostrar su utilidad en programas de mejora de olivo. Dado el comportamiento analítico ampliamente conocido de los componentes de la fracción lipídica del VOO y la existencia de patrones comerciales tanto de los ácidos como de sus ésteres metílicos (forma en la que se determinan normalmente mediante GC), no se requiere una instrumentación sofisticada para realizar estudios en este caso, por lo que los recogidos en esta Sección C se han realizado por GC con detección mediante fotometría de ionización en llama (GC–FID).

El Capítulo 8 de esta sección recoge la investigación sobre la fracción de ácidos grasos del VOO obtenido de cruces de diferentes cultivares con respecto a sus genitores, en la que se han estudiado tanto ácidos grasos esterificados (EFAs) como no esterificados (NEFAs). El análisis de correlación entre pares de FAs permitió detectar conexiones metabólicas a través de procesos de desaturación y de elongación enzimática. Por otro lado, el análisis no supervisado ha puesto de manifiesto un solapamiento en los perfiles de FAs de los cruces con un genitor común y una discriminación clara en la composición de los FAs más significativos cuando los cruces no implican genitores comunes.

El binomio grado de maduración del fruto–genotipo resultante de cruces de cultivares es la base del estudio que recoge el Capítulo 9 de esta

Memoria. El análisis no supervisado de los datos de concentración de los principales FAs de los VOOs obtenidos de estos cruces a los diferentes tiempos de maduración ha permitido constatar que existe discriminación entre los tiempos de maduración cortos y los largos; mientras mediante el test ANOVA se explica la variabilidad en la concentración de EFAs y NEFAs.

CAPÍTULO 8:

Influence of the genotype on the fatty acids composition of virgin olive oils along the ripening process

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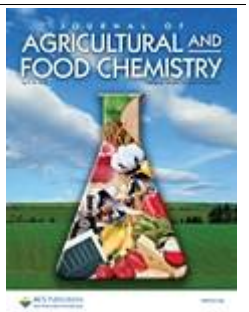

Influence of the genotype on the fatty acids composition of virgin olive oils along the ripening process

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Abstract

The composition of fatty acids (FAs) is one of the most critical aspects that affects the quality of virgin olive oil (VOO), which has been related to the balance of concentrations of saturated, monounsaturated and polyunsaturated FAs. The aim of this research was to study the influence of the ripening stage of olive fruits and genotype on the composition of esterified FAs (EFAs) and non esterified FAs (NEFAs) present in VOOs from advanced selections obtained by crosses between Arbequina, Picual and Frantoio cultivars. For this purpose a method based on gas chromatography with flame ionization detection (GC–FID) was used to estimate the percent of each type FAs. Statistical unsupervised analysis was carried out by Principal Component Analysis (PCA) to find clustering of samples attending to the ripening stages. Discrimination was observed for VOO samples associated to early and advanced ripening stages according to the concentration of FAs. Statistical analysis by ANOVA test allowed evaluating the contribution of ripening and genotype to explain the variability in the concentration of EFAs and NEFAs with p-value<0.05. Linoleic acid (C18:2) was the FA most influenced by the genotype; practically along the complete ripening process (from yellow or yellowish–green to black color). Furthermore, the highest genetic variability in FAs composition was observed in the ripening stage in which the fruit color is reddish or light violet, since five EFAs (C16:0, C16:1, C18:1, C18:2 and C18:3) were significant to explain this effect. Therefore, the interval that lasts this ripening stage is the most appropriate to compare genotypes in olive breeding programs according to FAs composition.

INTRODUCTION

Olive fruits for oil production can be harvested at different ripening stages according to their color (deep or dark green color, yellow or yellowish–green color, yellowish color with reddish spots, reddish or light violet color and black).[1] Fruit ripening exerts a critical influence on parameters such as pulp-to-stone ratio, fruit color and weight, oil content, chemical composition of the oil and enzyme activities.[2,3] This influence is directly related to quality, sensory characteristics, oxidative stability and nutritional value of olive oil.[4] Generally, early harvesting of the fruit, especially when the color is green to violet, produces oils with high phenolic content and low proportion of polyunsaturated fatty acids (PUFAs), representative of high-quality olive oils. However, a high concentration of certain phenolic compounds may enhance organoleptical properties such as bitterness and pungency, the effect of which is undesirable when in excess. Furthermore, olive fruits harvested at early ripening stages lead to low oil yield.[5,6] On the other hand, olive oils obtained from fruits harvested at early stages tend to be more stable to oxidation than those obtained from fruits harvested at an advanced maturation stage since the former are richer in phenols with recognized antioxidant properties. Additionally, fatty acids (FAs) composition is crucial for the stability of oils through the role PUFAs play on oil rancidity.[7] In fact, the quality of olive oils has been related with an optimal balance of saturated FAs (SFAs), monounsaturated FAs (MUFAs) and PUFAs.[8]

Olive oil is characterized by a high proportion of oleic acid (C18:1) as the most representative MUFA, which can range from 55 to 83% of total FAs.[9] A diet rich in MUFAs involves nutritional benefits such as reduced levels of low-density lipoprotein (LDL) cholesterol and total cholesterol without altering the levels of beneficial high density lipoprotein cholesterol.[10,11] As a result, the selection of olive oil as the primary fat source contributes to prevent cardiovascular disorders[12,13] and certain types of carcino-

genic diseases such as breast, ovary and prostate cancers.[14] Apart from C18:1, olive oil contains significant concentrations of palmitic (C16:0) and linoleic acids (C18:2), which are between 7.5–20% and 3.5–21%, respectively.[9]

There are many studies dealing with the influence of the ripening process in the quality of virgin olive oils.[4,15–17] Most of them have been focused on the effects of maturation on the quality of virgin olive oil (VOOs) obtained from different cultivars. Parameters such as the oil content, free acidity, peroxide value, UV absorption at 270 nm, phenolic content and FAs profile have been monitored to establish the optimum harvesting stage.[15–17] Nevertheless, external factors such as geographical origin as well as agronomic, pedo-climatic, technological and genetic factors could explain the differences observed in the obtained results. In the case of FAs, different trends have been observed for the most important FAs. Thus, some authors have reported that the content of C18:1 remains constant during the ripening process [15,18] or shows a slight decrease as ripening progresses.[4,19–21] However, this behavior can be affected by the previously indicated factors. Thus, Salvador *et al.* reported an increase in the concentration of C18:1 with ripening in one season, which was a part of a study involving four successive crop seasons.[16] Regarding C18:2, this FA increased its concentration with ripening, which could be ascribed to the continuous activity of oleate desaturase during the ripening process. Low temperatures have been reported to enhance desaturation of oleate to linoleate in oil seeds.[22,23] SFAs such as C16:0 and C18:0 decreased along the ripening process or remained constant as both FAs are precursors of C18:1.[4,15,21,24,25] Nevertheless, this trend could be altered when irrigation is adopted as agronomical practice since olive oils obtained from irrigated cultivars provided higher concentrations of SFAs than those provided by water-stressed cultivars.[23,26]

A general trend in all studies dealing with FAs is that their analyses were carried out after transterification, which means that only esterified FAs (EFAs) were considered; therefore, no attention was paid to non-esterified

FAs (NEFAs), which are markers of VOO degradation.[27,28] Usually, free acidity, expressed as percent in terms of C18:1 concentration, increases along ripening, fact ascribed to the increased enzymatic activity, especially from lipolytic enzymes present in olive fruits.[15] For this reason, mature fruits are more sensitive to pathogenic infections and mechanical damage, and the free acidity is frequently used to detect deterioration in olive fruits.[29]

The objective of the present research was to study the influence of ripening on the FAs composition (EFAs and NEFAs) of olive oils from advanced selections obtained by crosses between Arbequina, Picual and Frantoio cultivars. Olive fruits collected at five crop dates were used to obtain olive oil, which was analyzed for determination of EFAs and NEFAs in samples of the different genotypes. The influence of the genotype to explain the variability in the concentration of FAs along the ripening process has also been evaluated.

MATERIALS AND METHODS

Plant Material and Olive Oil Extraction

The selections evaluated in this research proceed from crosses between Arbequina, Frantoio and Picual cultivars carried out in 1991–1992 in the olive breeding program coordinated by the Department of Agronomy (University of Córdoba) and the Andalusian Institute of Agricultural Research and Training (IFAPA, Junta de Andalucía). Several genotypes were selected in the initial seedling population after three consecutive harvest seasons using as criteria their early bearing (short juvenile period) and high oil content. The selected genotypes were propagated by soft-wood cuttings and planted in 2001 in a comparative field trial together with the three genitors as control at 6 × 5 m spacing. Trees were trained as single-trunk vase, with three to four main branches, and minimal pruning was carried out to allow early bearing. Standard agronomical practices were followed including irrigation supply by in-line drips to avoid water stress of plants. The re-

sults of agronomic evaluation at the initial seedling stage[30] and comparative field trials[31,32] have so far been reported.

Olive fruits were manually collected from Arbequina, Frantoio and Picual cultivars as well as from nine advanced selections as follows: three from Arbequina×Picual, three from Picual×Arbequina and three from Frantoio×Picual (the first cultivar is the female genitor) at five ripening stages from 1st October to 26th November 2009 (every two weeks). Two trees-per-genotype were sampled each date to define the biological variability. No samples were available from Picual at the first date (1st October); therefore, the cohort was formed by 118 samples —(12 genotypes×2 replications×5 dates)–2=118 samples.

Determination of the Ripening Index

The ripening index (RI) of fruit samples was estimated as described by El Riachy et al.[33] Briefly, an aliquot of 100 randomly selected fruits was taken from samples obtained from each tree (2 kg). Fruits were classified into the following categories according to the skin color: 0= deep or dark green color; 1= yellow or yellowish–green color; 2= yellowish color and reddish spots; 3= reddish or light violet color; 4= black color.

The total number of olives in each category (n0, n1,…, n4) was recorded, and the following equation was applied to determine the RI:

$$RI = [(0\times n0) + (1\times n1) + (2\times n2) + (3\times n3) + (4\times n4)]/100$$

Then, the RI value was categorized as follows: RI≤0.5 (RI0); RI=0.5–1.5 (RI1); RI=1.5–2.5 (RI2); RI=2.5–3.5 (RI3) and; finally, RI≥3.5 (RI4).

An Abencor olive oil mill (MC2 Ingenierías y Sistemas, (Seville, Spain) was used to extract the oil. This system reproduces the industrial process at a laboratory scale, through three basic elements: a hammer mill, a thermobeater and a paste centrifuge. Approximately 1 kg of fruit was ground to a paste using the hammer mill; then, the paste was placed in the thermobeater and stirred for 30 min with the water bath set at 28±1 ºC with micronized talc and no water addition. Subsequently, vertical centrifugation

for 2 min permitted separation of the oily phase that was collected and left to decant for 24 h. Finally, the oil was separated, placed in glass vials without head space and stored at −18 ºC until analysis.[34]

Reagents

The reagents used for GC–FID analysis were chromatographic grade methanol and *n*-hexane from Scharlab (Barcelona, Spain). Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare water–methanol extractant mixtures. Anhydrous sodium sulfate from Sigma–Aldrich (Steinheim, Germany) was used as drying agent for the nonpolar phase in the derivatization process. Potassium hydroxide and 98% sulfuric acid from Panreac (Barcelona, Spain) were used to prepare solutions (2 M KOH and 1 M H_2SO_4, respectively) in methanol for derivatization of EFAs and NEFAs, respectively.

FA methyl esters (FAMEs) as analytical standards (purity≥98.5%) from Fluka (Steinheim, Germany) were used to prepare calibration multistandards. Methyl esters corresponding to the following FAs were acquired: C16:0; palmitoleic acid (C16:1); stearic acid (C18:0); C18:1, C18:2; linolenic acid (C18:3); eicosanoic acid (C20:0); eicosenoic acid (C20:1); behenic acid (C22:0) and tetracosanoic acid (C24:0). Nonadecanoic acid methyl ester (C19:0) used as internal standard (IS) in the determination step was also from Fluka.

Individual stock standard solutions and multistandard solutions were prepared by dilution of each compound in *n*-hexane. The solutions were stored in a freezer at −20 ºC.

Sample Preparation Protocol for Determination of EFAs and NEFAs in VOO

Prior to GC separation, EFAs and NEFAs from VOOs were converted into their FAMEs. With this objective, 0.1 g of VOO was diluted with 2 mL *n*-hexane and 200 μL of 2 M KOH methanolic solution was added. The biphasic system was shaked for 1 min in an MS2 minishaker, and

the resulting phases were separated after decantation. The mixture was left for 5 min and the *n*-hexane phase, containing FAMEs from EFAs, was transferred to a test tube and 1:50 (v/v) diluted with *n*-hexane containing 15 μg/mL of C19:0 as IS. Then, 1 μL of the resulting mixture was injected by triplicate into the GC for individual separation of EFA methyl esters.[35]

To obtain FAMEs from NEFAs, a small amount of anhydrous sodium sulfate was added to the remaining fraction from the previous step to remove residual water; then, 500 μL of 1 M H_2SO_4 methanolic solution was added and the tube was placed for 30 min into a water bath thermostated at 70 ºC. After cooling, 1 mL of *n*-hexane was added and shaked for 1 min in an MS2 minishaker. The biphasic system was left for 5 min and, after phases separation, the top *n*-hexane phase containing the FAMEs from NEFAs was transferred to a test tube. The step was repeated to ensure total conversion of NEFAs into NEFA methyl esters and the two extracts were mixed, 1:10 (v/v) diluted with *n*-hexane containing 15 μg/mL of C19:0 as IS, and 1 μL of this solution was injected into the GC for individual separation of NEFA methyl esters.[36]

Chromatographic Analysis

A thermostated water bath and an MS2 minishaker from Ika (Wilmington, USA) were used to assist the derivatization step. Separation of FAMEs was carried out using an Agilent 7820A GC equipped with an autosampler, a split/splitless injector and an FID. A SP™-2380 fused silica capillary column (60 m×0.25 mm I.D., 0.2 μm film thickness) provided by Supelco (Bellefonte, PA, USA) was used as analytical column.

The same chromatographic method was used for separation of the derivatized EFAs and NEFAs. Helium was used as carrier gas at 1.2 mL/min flow rate. The injection was in the splitless mode and the temperature of the chromatographic gradient was as follows: the initial oven temperature was kept at 120 ºC for 3 min and then, programmed to rise at 3 ºC/min up to 185 ºC, maintained for 2 min, and followed by a second gradient of 15

ºC/min to a final temperature of 250 ºC, which was held for 5 min. The equilibration time was 5 min. The injector and detector temperatures were 250 and 280 ºC, respectively. The signal from the FID was acquired and processed by EZ Chrom Elite Compact software (Version 3.3.2, Agilent Technologies, Santa Clara, CA, USA). The reference standard was used to identify and quantify individual FAMEs of VOO extracts from the olive breeding program. For this purpose, calibration models were built for FAs by using multistandard solutions at different concentrations spiked with the IS. The concentrations of EFAs and NEFAs were calculated as percentage.

Statistical Analysis

After GC–FID analysis, raw data files were used to create a data matrix with the concentrations of EFAs and NEFAs of each analytical sample (samples×variables). The file thus obtained was created in comma separated values files (.csv). The study was focused on a representative panel of FAs in VOO. Thus, the initial data set was formed by 118 samples×20 analytes (10 EFAs and 10 NEFAs).

The definitive files were exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. Normalization by logarithmic transformation was used as pre-processing step. Unsupervised statistical analysis was carried out by Principal Component Analysis (PCA) to find clustering of samples attending to RI. The ANOVA test was applied to evaluate the influence of maturation and genotype to explain the variability in the concentration of EFAs and NEFAs with p-value<0.05.

RESULTS AND DISCUSSION

Characterization of EFAs and NEFAs in VOO Obtained from Advanced Breeding Selections

The ten most important esterified and non-esterified forms of FAs present in VOO were monitored in this research as EFA and NEFA methyl esters, respectively. For this purpose, a sequential analysis using two derivatization protocols was planned for determination of EFAs and NEFAs by the same chromatographic method. Identification was supported on retention time by comparison with FAME standards. Quantitative analysis was carried out by using C19:0 as IS to generate data from twenty compounds (10 EFAs and 10 NEFAs) which were the variables considered for data treatment. The precision estimated as variability by analysis of replicates using the complete protocol was below 10% for all EFAs and NEFAs.

Tables 1 and **2** list the average concentrations of EFAs and NEFAs in VOO from advanced olive selections and the average concentrations for each considered RI group, respectively. As can be deduced, a high variability expressed as percent of relative standard deviation (%RSD) was observed for the analyzed FAs in the target cohort (**Table 1**). In general terms, most of the FAs reported the maximum variability at RI0, while the minimum observed variability for most FAs was found in VOOs obtained at advanced ripening stages (RI4). The EFAs presented RSD values between 19.9 and 113.2 %, which corresponded to C16:0 and C22:0, respectively. In the case of NEFAs, the variability was above 66.1% for all FAs, which was considerably higher than that observed for EFAs (**Table 1**). The influence of the RI depended on the particular FA. Thus, two of the most concentrated FAs in VOO (C16:0 and C18:1) presented the lowest variability range as a function of the RI (from 13.1 to 23.0% for C16:0 and from 10.5 to 23.8% for C18:1). On the other hand, C18:2 reported a high variability for the different RIs, which ranged from 58.6 to 87.3%.

in VOO from advanced olive selections.

EFA	Methyl ester IOOC (% w/w)	Min (%)	Max (%)	Mean (%)	RSD (%)	NEFA	Min (%)	Max (%)	Mean (%)	RSD (%)
C16:0	7.50–20.00	6.02	18.46	12.31	19.9	C16:0	0.44	10.64	2.82	71.8
C16:1	0.30–3.50	0.73	3.23	1.6	33.6	C16:1	0.04	1.34	0.36	73.5
C18:0	0.50–5.00	0.7	3.68	1.67	34.6	C18:0	n.q.	1.64	0.37	79
C18:1	55.00–83.00	23.26	73.42	57.37	18.4	C18:1	1.7	40.9	13.12	68.6
C18:2	3.50–21.00	1.33	19.19	6.3	71.9	C18:2	0.13	8.14	1.38	93.4
C20:0	≤ 0.60	0.17	0.82	0.36	39.3	C20:0	0.02	0.21	0.07	67.9
C18:3	≤ 1.00	0.25	1.41	0.72	27.4	C18:3	0.04	0.56	0.16	74.9
C20:1	≤ 0.40	0.1	0.62	0.32	33.3	C20:1	0.02	0.21	0.07	66.1
C22:0	≤ 0.20	0.08	2.54	0.37	113.3	C22:0	0.01	0.18	0.06	74.5
C24:0	≤ 0.20	n.q.	1.82	0.49	94.7	C24:0	n.q.	0.41	0.09	111.1

Table 2. Average concentrations of EFAs and NEFAs in VOO from advanced olive selection depending on the RI.

EFA	RI 0		RI 1		RI 2		RI 3		
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)
C16:0	12.36	22.9	12.71	22.3	11.82	21.2	12.3	17.2	12.88
C16:1	1.49	36.1	1.53	34.2	1.52	35.3	1.71	34.5	1.85
C18:0	1.38	32.5	1.64	24.3	1.6	39.1	1.79	30.9	1.97
C18:1	58.68	22.6	59.35	15.9	54.2	23.8	56.04	15.1	61.22
C18:2	5.6	87.3	5.74	77.5	5.61	72.9	7.55	58.6	8.11
C20:0	0.36	40	0.37	35.1	0.41	42.6	0.33	31.8	0.26
C18:3	0.75	29.5	0.81	30.5	0.72	25.4	0.67	23.3	0.66
C20:1	0.33	46	0.32	43.3	0.33	26.9	0.31	23.5	0.28
C22:0	0.46	145.9	0.3	60.8	0.54	94.3	0.3	114.9	0.23
C24:0	0.44	94.8	0.5	93.2	0.52	74.4	0.46	121.5	0.55

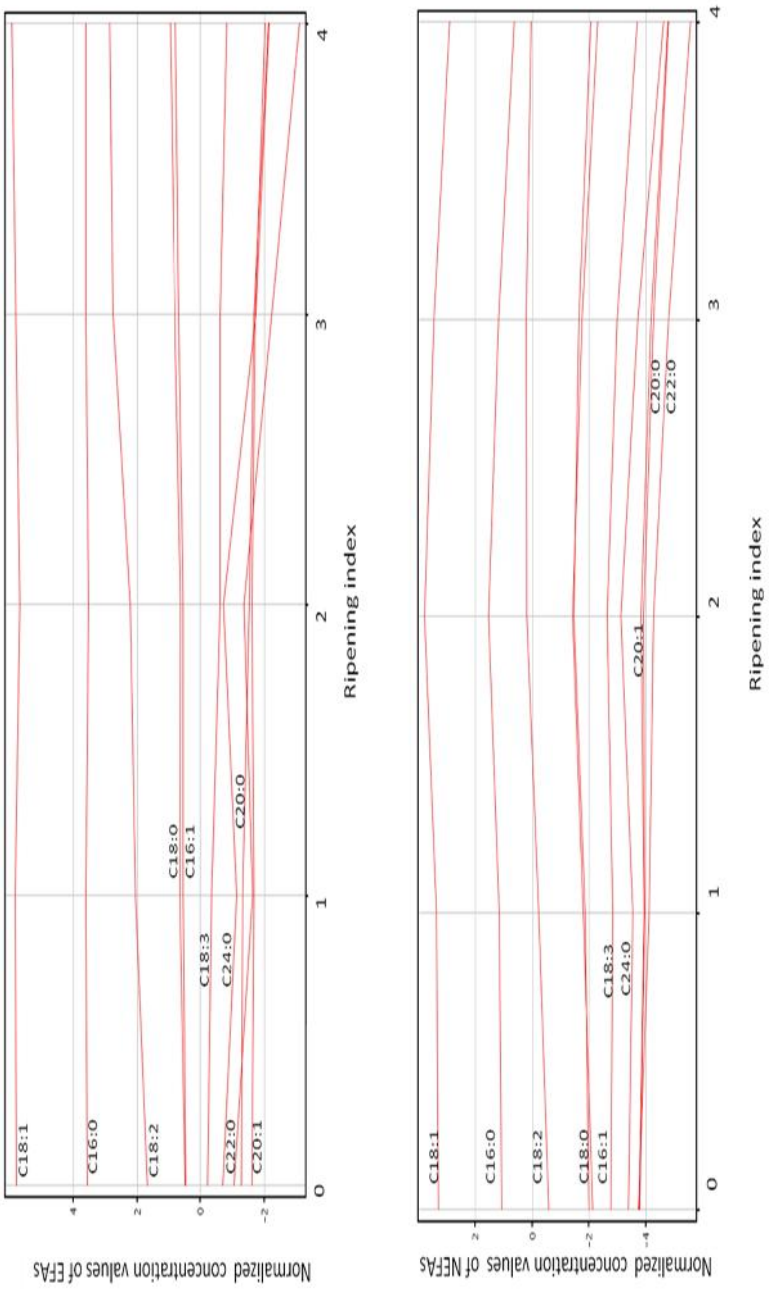

Figure 1. Evolution of the normalized concentrations of the monitored EFAs and NEFAs versus the RI.

Figure 1 shows the evolution of the normalized concentrations of the monitored FAs *versus* the RI with differentiation between EFAs and NEFAs. The evolution plots described different trends depending on the FA (EFA or NEFA), and the RI value. In the case of EFAs, the concentration of the main FAs present in VOO (C18:1, C16:0, C18:2, C18:0 and C16:1) increased slightly or remained constant during the ripening process. On the other hand, minor FAs (C18:3, C24:0, C22:0, C20:0 and C20:1) decreased their concentration with the RI. Therefore, the trend observed was different depending on the concentration of the FA in VOO. Taking into account the numerous factors contributing to alter the FAs profile in olive oil, it is quite complex to compare results with those published in previous studies. For this reason, the most interesting aspect deduced from this research is the differences observed for the main and minor FAs present in olive oil.

It is worth emphasizing that the main FAs (C16:0, C16:1, C18:0 and C18:1) were within the concentration range established by the IOOC for "virgin olive oil" qualification in all VOO samples of the cohort.[9] Only two fatty acids (C22:0 and C24:0) reported concentrations above the upper level defined by the IOOC (0.37 and 0.49%, respectively),[9] which were observed for all RI values. In fact, C22:0 and C24:0 provided the maximum variability ranges according to the RI (from 60.8 to 146.0% for C22:0 and from 74.4 to 121.5% for C24:0).

Concerning NEFAs, their evolution with ripening has not previously reported. In this study, the monitored NEFAs followed a trend similar to that observed for EFAs. Thus, differences were also observed between the main NEFAs of VOO and those present at minor concentration. C18:1, C16:0, C18:2, C18:0 and C16:1 NEFAs increased their concentration up to RI 2 (yellowish color with reddish spots) and, then, the concentration decreased up to RI 4 (violet color). C18:3 and C24:0 NEFAs followed the same trend observed for the most concentrated NEFAs. On the other hand,

C22:0; C20:0 and C20:1 NEFAs decreased their concentration during the ripening process up to minimum values achieved at RI4.

Influence of the Ripening Index on the Profile of Fatty Acids in VOOs from Advanced Selections

As previously mentioned, the chemical composition of VOO varies according to different factors such as genetic, agronomical, climatic, geographical and technological factors. However, this study was designed to elucidate the influence of fruit ripening on the FAs composition from VOOs obtained from advanced selections. The variability of the FAs profile was assessed as a function of the RI, which is a standardized parameter. Principal Component Analysis was applied to find grouping trends according to the RI.

Figure 2.A shows the PCA score plots associated to FAs as a function of the RI. As can be seen, a partial discrimination was observed in the 3D plot explaining 72.9% of the total variability. Two main sub-clusters can be clearly differentiated, as shows **Figure 2.B**. Thus, VOO samples from RI 0–1 (fruits with yellow or yellowish–green color) and 3–4 (fruits with color change from reddish or light violet to black) were separated along PC2, while RI 2 provided a wide variability with most of the samples distributed in the two sub-clusters. These results can be interpreted with the aid of **Figure 1**, since RI 2 coincided with the ripening stage at which the concentration of NEFAs changed from an upward trend (RI 0–1) to a downward trend (RI 3–4). The observed variability in the concentration of FAs would lead to divide the VOO samples in two ripening stages (early and advanced ripening).

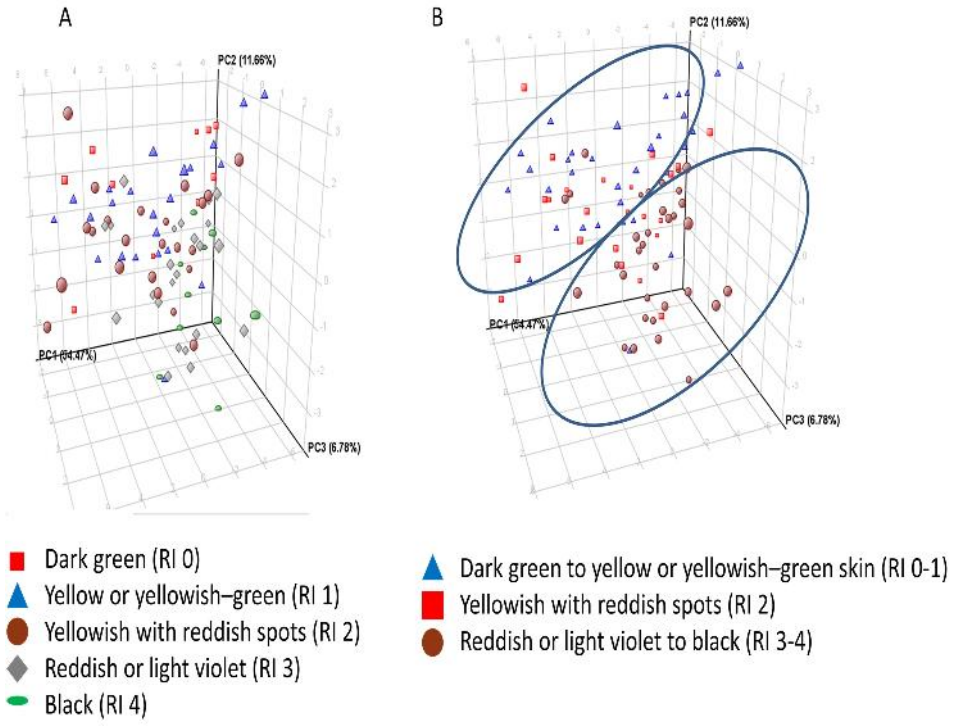

Figure 2. PCA score plots associated to the fatty acids in VOO (A) as a function of the ripening index or (B) by maturation of olive fruits in early harvested olive fruits (RI 0 and 1), intermediate harvested olive fruits (RI 2), and advanced harvested olive fruits (RI 3 and 4).

The influence of ripening on the FAs composition of VOO samples was evaluated by analysis of variance (ANOVA). **Table 3** shows the sum of squares between and within the considered groups (ripening indexes) and the *p*-value obtained after application of this test. As can be seen, the RI exerted no significant influence on the concentrations of FAs (95% confidence interval), although C18:3 EFA and C18:1 NEFA were close to be significant

Table 3. Sum of squares between and within groups (ripening indexes) and p-values obtained after application of ANOVA test.

EFA	Source	Sum of squares	*p*-Value
C16:0	Between groups	9.84	0.8103
	Within groups	532.72	
	Total (Corr.)	542.56	
C16:1	Between groups	1.29	0.353
	Within groups	24.82	
	Total (Corr.)	26.11	
C18:0	Between groups	2.40	0.1224
	Within groups	27.55	
	Total (Corr.)	29.95	
C18:1	Between groups	534.26	0.3147
	Within groups	9533.35	
	Total (Corr.)	10067.60	
C18:2	Between groups	103.78	0.2839
	Within groups	1742.11	
	Total (Corr.)	1845.89	
C18:3	Between groups	0.21	0.2562
	Within groups	3.29	
	Total (Corr.)	3.50	
C20:0	Between groups	0.18	0.0508
	Within groups	1.59	
	Total (Corr.)	1.77	
C20:1	Between groups	0.02	0.7719
	Within groups	0.99	
	Total (Corr.)	1.01	
C22:0	Between groups	1.19	0.1473
	Within groups	14.71	
	Total (Corr.)	15.91	
C24:0	Between groups	0.12	0.9717

Table 3. Sum of squares between and within groups (ripening indexes) and p-values obtained after application of ANOVA test (cont.).

NEFA	Source	Sum of squares	*p*-value
C16:0	Between groups	20.19	0.298
	Within groups	348.60	
	Total (Corr.)	368.78	
C16:1	Between groups	0.33	0.3179
	Within groups	5.96	
	Total (Corr.)	6.29	
C18:0	Between groups	0.28	0.5205
	Within groups	7.51	
	Total (Corr.)	7.79	
C18:1	Between groups	470.10	0.2138
	Within groups	6806.74	
	Total (Corr.)	7276.84	
C18:2	Between groups	14.22	0.0687
	Within groups	134.96	
	Total (Corr.)	149.18	
C18:3	Between groups	0.08	0.2361
	Within groups	1.25	
	Total (Corr.)	1.34	
C 20:0	Between groups	0.01	0.1446
	Within groups	0.18	
	Total (Corr.)	0.20	
C20:1	Between groups	0.02	0.1258
	Within groups	0.18	
	Total (Corr.)	0.20	
C22:0	Between groups	0.01	0.1344
	Within groups	0.14	
	Total (Corr.)	0.15	
C24:0	Between groups	0.05	0.2967

Influence of the Genotype on the Profile of Fatty Acids in VOOs from Advanced Selections According to the Ripening Index

The influence of genotype on the composition of FAs in VOOs was studied by including this factor in the ANOVA test together with the RI. For this purpose, an ANOVA study was planned for each RI group to analyze the effect of the genotype. As **Table 4** shows, the genotype was not an influential factor to explain the variability in the concentration of NEFAs. Concerning the evaluation of each RI, the genotype was not able to explain the levels of FAs in VOOs belonging to RI 0, while it contributed significantly (95 or 99% confidence interval) to explain the concentration of C18:2 EFA at RI values 1-to-4, with *p*-values 0.0043 (RI 1), 0.0014 (RI 2), 0.0151 (RI 3) and 0.0000 (RI 4). In fact, C18:2 was the FA more influenced by the genotype, which could justify the high variability of the content of this FA in **Table 1**. RI 3 was characterized as the ripening stage with the highest influence of the genotype, since five EFAs resulted significant to explain this effect —C16:0 and C16:1, which were highly significant (*p*-value 0.0059 and 0.0000, respectively), while C18:1, C18:2 and C18:3 were significant at 95% of significance (*p*-value 0.0289, 0.0151 and 0.0146, respectively). Therefore, RI 3 was identified as the ripening stage at which the maximum variability in the concentration of FAs can be explained by the genotype. With these premises, this ripening stage would be identified as the most interesting period to compare genotypes in olive breeding programs.

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group.

RI 0							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C16:0	Between groups	27.11	0.379	**C16:0**	Between groups	4.16	0.9127
	Within groups	61.66			Within groups	64.65	
	Total (Corr.)	88.77			Total (Corr.)	68.81	
C16:1	Between groups	1.72	0.0908	**C16:1**	Between groups	0.12	0.6753
	Within groups	1.49			Within groups	0.61	
	Total (Corr.)	3.21			Total (Corr.)	0.73	
C18:0	Between groups	1.07	0.13	**C18:0**	Between groups	0.08	0.9074
	Within groups	1.13			Within groups	1.23	
	Total (Corr.)	2.2			Total (Corr.)	1.31	
C18:1	Between groups	130.75	0.8982	**C18:1**	Between groups	126.47	0.8549
	Within groups	1806.29			Within groups	1316.24	
	Total (Corr.)	1937.05			Total (Corr.)	1442.71	
C18:2	Between groups	104.16	0.2343	**C18:2**	Between groups	0.29	0.9546
	Within groups	158.78			Within groups	7.26	
	Total (Corr.)	262.94			Total (Corr.)	7.54	
C18:3	Between groups	0.12	0.5328	**C18:3**	Between groups	0.02	0.8945
	Within groups	0.41			Within groups	0.23	
	Total (Corr.)	0.53			Total (Corr.)	0.25	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 0							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C20:0	Between groups	0.02	0.8862	**C20:0**	Between groups	0	0.8351
	Within groups	0.22			Within groups	0.03	
	Total (Corr.)	0.24			Total (Corr.)	0.04	
C20:1	Between groups	0	0.9856	**C20:1**	Between groups	0.003	0.8847
	Within groups	0.25			Within groups	0.04	
	Total (Corr.)	0.26			Total (Corr.)	0.04	
C22:0	Between groups	0.96	0.615	**C22:0**	Between groups	0.003	0.8515
	Within groups	4.05			Within groups	0.03	
	Total (Corr.)	5			Total (Corr.)	0.03	
C24:0	Between groups	0.18	0.8372	**C24:0**	Between groups	0.02	0.6087
	Within groups	1.71			Within groups	0.07	
	Total (Corr.)	1.89			Total (Corr.)	0.09	
RI 1							
EFA	**Source**	**Sum of Squares**	***p*-Value**	**NEFA**	**Source**	**Sum of Squares**	***p*-Value**
C16:0	Between groups	60.85	0.0954	**C16:0**	Between groups	12.04	0.539
	Within groups	125.23			Within groups	71.3	
	Total (Corr.)	186.08			Total (Corr.)	83.33	
C16:1	Between groups	2.12	0.0902	**C16:1**	Between groups	0.32	0.4318
	Within groups	4.26			Within groups	1.54	
	Total (Corr.)	6.38			Total (Corr.)	1.86	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 1							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C18:0	Between groups	1.69	0.0165	**C18:0**	Between groups	0.24	0.5037
	Within groups	2.02			Within groups	1.32	
	Total (Corr.)	3.71			Total (Corr.)	1.56	
C18:1	Between groups	214.61	0.6965	**C18:1**	Between groups	320.03	0.5205
	Within groups	1830.02			Within groups	1823.78	
	Total (Corr.)	2044.63			Total (Corr.)	2143.81	
C18:2	Between groups	230.69	0.0043	**C18:2**	Between groups	0.09	0.867
	Within groups	201.48			Within groups	0.31	
	Total (Corr.)	432.18			Total (Corr.)	6.18	
C18:3	Between groups	0.45	0.1165	**C18:3**	Between groups	0.03	0.6702
	Within groups	0.99			Within groups	0.23	
	Total (Corr.)	1.44			Total (Corr.)	0.26	
C20:0	Between groups	0.04	0.7553	**C 20:0**	Between groups	0.01	0.5853
	Within groups	0.38			Within groups	0.04	
	Total (Corr.)	0.42			Total (Corr.)	0.04	
C20:1	Between groups	0.06	0.5333	**C20:1**	Between groups	0.01	0.3619
	Within groups	0.34			Within groups	0.05	
	Total (Corr.)	0.4			Total (Corr.)	0.06	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 1							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C22:0	Between groups	0.13	0.4578	**C22:0**	Between groups	0.01	0.4048
	Within groups	0.67			Within groups	0.04	
	Total (Corr.)	0.8			Total (Corr.)	0.04	
C24:0	Between groups	1.54	0.1362	**C24:0**	Between groups	0.07	0.4259
	Within groups	3.68			Within groups	0.33	
	Total (Corr.)	5.22			Total (Corr.)	0.4	
RI 2							
EFA	**Source**	**Sum of Squares**	***p*-Value**	**NEFA**	**Source**	**Sum of Squares**	***p*-Value**
C16:0	Between groups	17.23	0.7817	**C16:0**	Between groups	17.66	0.7757
	Within groups	120.44			Within groups	121.34	
	Total (Corr.)	137.67			Total (Corr.)	139	
C16:1	Between groups	1.91	0.2485	**C16:1**	Between groups	0.22	0.8569
	Within groups	4.4			Within groups	1.94	
	Total (Corr.)	6.31			Total (Corr.)	2.16	
C18:0	Between groups	0.34	0.9806	**C18:0**	Between groups	0.58	0.6588
	Within groups	8.3			Within groups	2.98	
	Total (Corr.)	8.65			Total (Corr.)	3.56	
C18:1	Between groups	1065.72	0.2777	**C18:1**	Between groups	281.48	0.8235
	Within groups	2609.61			Within groups	2241.12	
	Total (Corr.)	3675.33			Total (Corr.)	2522.6	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 2							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C18:2	Between groups	242.62	0.0014	**C18:2**	Between groups	12.15	0.5765
	Within groups	125.29			Within groups	52.85	
	Total (Corr.)	367.91			Total (Corr.)	65	
C18:3	Between groups	0.24	0.1964	**C18:3**	Between groups	0.06	0.7666
	Within groups	0.49			Within groups	0.4	
	Total (Corr.)	0.73			Total (Corr.)	0.46	
C20:0	Between groups	0.12	0.5859	**C 20:0**	Between groups	0.01	0.7868
	Within groups	0.55			Within groups	0.06	
	Total (Corr.)	0.68			Total (Corr.)	0.07	
C20:1	Between groups	0.04	0.4669	**C20:1**	Between groups	0.01	0.7948
	Within groups	0.14			Within groups	0.05	
	Total (Corr.)	0.18			Total (Corr.)	0.06	
C22:0	Between groups	0.52	0.8768	**C22:0**	Between groups	0.004	0.8372
	Within groups	5.11			Within groups	0.03	
	Total (Corr.)	5.63			Total (Corr.)	0.04	
C24:0	Between groups	0.17	0.9666	**C24:0**	Between groups	0.004	0.9967
	Within groups	3.17			Within groups	0.24	
	Total (Corr.)	3.34			Total (Corr.)	0.24	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 3							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C16:0	Between groups	52.42	0.0059	**C16:0**	Between groups	17.43	0.0965
	Within groups	42.06			Within groups	31.65	
	Total (Corr.)	94.48			Total (Corr.)	49.08	
C16:1	Between groups	5.89	≤0.0001	**C16:1**	Between groups	0.24	0.3371
	Within groups	1.42			Within groups	0.83	
	Total (Corr.)	7.31			Total (Corr.)	1.07	
C18:0	Between groups	2.18	0.1177	**C18:0**	Between groups	0.25	0.2231
	Within groups	4.3			Within groups	0.67	
	Total (Corr.)	6.48			Total (Corr.)	0.92	
C18:1	Between groups	679.82	0.0289	**C18:1**	Between groups	170.04	0.1635
	Within groups	822.14			Within groups	388.02	
	Total (Corr.)	1501.95			Total (Corr.)	558.05	
C18:2	Between groups	204.43	0.0151	**C18:2**	Between groups	12.24	0.1905
	Within groups	206.68			Within groups	30.12	
	Total (Corr.)	411.11			Total (Corr.)	42.35	
C18:3	Between groups	0.25	0.0146	**C18:3**	Between groups	0.07	0.1859
	Within groups	0.25			Within groups	0.18	
	Total (Corr.)	0.51			Total (Corr.)	0.25	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 3							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C20:0	Between groups	0.02	0.7844	**C 20:0**	Between groups	0.01	0.1046
	Within groups	0.2			Within groups	0.02	
	Total (Corr.)	0.22			Total (Corr.)	0.03	
C20:1	Between groups	0.02	0.4794	**C20:1**	Between groups	0.004	0.3134
	Within groups	0.09			Within groups	0.01	
	Total (Corr.)	0.11			Total (Corr.)	0.02	
C22:0	Between groups	0.38	0.5639	**C22:0**	Between groups	0.01	0.1818
	Within groups	2.14			Within groups	0.02	
	Total (Corr.)	2.52			Total (Corr.)	0.03	
C24:0	Between groups	1.21	0.4579	**C24:0**	Between groups	0.04	0.0692
	Within groups	5.38			Within groups	0.06	
	Total (Corr.)	6.59			Total (Corr.)	0.09	
RI 4							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C16:0	Between groups	9.46	0.61	**C16:0**	Between groups	2.77	0.6699
	Within groups	16.26			Within groups	5.61	
	Total (Corr.)	25.72			Total (Corr.)	8.37	
C16:1	Between groups	0.95	0.2633	**C16:1**	Between groups	0.07	0.4364
	Within groups	0.66			Within groups	0.08	
	Total (Corr.)	1.61			Total (Corr.)	0.15	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 4							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C18:0	Between groups	4.56	0.1365	**C18:0**	Between groups	0.07	0.5409
	Within groups	1.96			Within groups	0.09	
	Total (Corr.)	6.52			Total (Corr.)	0.16	
C18:1	Between groups	240.25	0.2001	**C18:1**	Between groups	42.22	0.7136
	Within groups	134.14			Within groups	97.35	
	Total (Corr.)	374.39			Total (Corr.)	139.57	
C18:2	Between groups	265.09	≤0.0001	**C18:2**	Between groups	2.33	0.3869
	Within groups	2.89			Within groups	2.27	
	Total (Corr.)	267.98			Total (Corr.)	4.6	
C18:3	Between groups	0.07	0.0439	**C18:3**	Between groups	0.01	0.4828
	Within groups	0.02			Within groups	0.02	
	Total (Corr.)	0.09			Total (Corr.)	0.03	
C20:0	Between groups	0.02	0.2303	**C 20:0**	Between groups	0.001	0.7344
	Within groups	0.01			Within groups	0.002	
	Total (Corr.)	0.03			Total (Corr.)	0.003	
C20:1	Between groups	0.02	0.2622	**C20:1**	Between groups	0.001	0.7489
	Within groups	0.02			Within groups	0.002	
	Total (Corr.)	0.04			Total (Corr.)	0.003	

Table 4. Sum of squares between and within groups (genotype) and p-values obtained after application of ANOVA test for each RI group (cont.).

RI 4							
EFA	**Source**	**Sum of squares**	***p*-Value**	**NEFA**	**Source**	**Sum of squares**	***p*-Value**
C22:0	Between groups	0.15	0.8536	**C22:0**	Between groups	0.001	0.874
	Within groups	0.6			Within groups	0.003	
	Total (Corr.)	0.75			Total (Corr.)	0.003	
C24:0	Between groups	0.35	0.9112	**C24:0**	Between groups	0.01	0.1438
	Within groups	1.91			Within groups	0.01	
	Total (Corr.)	2.25			Total (Corr.)	0.02	

Fatty Acid Markers for Comparison of Advanced Selections Obtained in Olive Breeding Programs

The five FAs (C16:0, C16:1, C18:1, C18:2 and C18:3) that were significant (at least 95% confidence interval) to explain the influence of the genotype on the ripening process were subjected to a comparative study among cultivars. **Figure 3** shows the concentration profile obtained for each FA according to the RI. Those RI in which the genotype proved significance to explain the variability observed in the concentration of FAs are pinpointed as a function of the significance level (95%, 99% and 99.9% confidence intervals). Thus, C16:0, C16:1, C18:1 and C18:3 were significant at RI 3 to explain the influence of the genotype, while C18:2 was significant from RI 1 to RI4. The concentrations of these FAs in VOOs from advanced selections were compared with those provided by the analysis of VOOs from the cultivars used as genitors i.e. Arbequina, Picual and Frantoio. For this purpose, the comparison was focused on the specific RI groups on which the genotype was more influential, with special emphasis on RI 3.

The three advanced selections from the cross Arbequina×Picual provided the VOOs with the highest concentration of C16:0 and C16:1,

followed by Arbequina genitor (**Figure 3.A** and **B**); while Picual genitor had the VOOs with the lowest concentration of both FAs; therefore VOOs from Arbequina×Picual were more similar to the female genitor (Arbequina). Concerning the content of C18:1 FA, Arbequina×Picual crosses provided intermediate concentrations between the two genitors and also closer to the female genitor. The Picual×Arbequina cultivars provided VOOs with the highest concentration of C18:1 FA, followed by that in Picual genitor, the female genitor (**Figure 3.C**). Therefore, the selection of Picual or Arbequina cultivars as female genitor clearly influences the concentration levels of three important FAs in VOO such as C16:0, C16:1 and C18:1. Concerning Frantoio genitor and Frantoio×Picual cross, their VOOs were characterized by the lowest levels of these three relevant FAs.

C18:2 was the FA more influenced by the genotype along the ripening process, as previously described. At RI 1-to-3, VOOs from the Arbequina cultivar reported the highest concentration of C18:2, followed by Arbequina×Picual at RI 1 and 3, or Frantoio cultivar at RI 2. No Arbequina samples were collected with RI 4 since it is well-known that this cultivar reaches maturation later than the other cultivars.[37] In the case of RI 4, Arbequina×Picual cross provided the VOOs with the highest concentration of this FA (**Figure 3.D**). It is worth mentioning that Picual×Arbequina cross gave VOOs with the lowest concentration of C18:2, clearly below the concentration found for VOOs from Picual cultivar. In fact, VOOs obtained from this cross were characterized by reporting C18:2 concentrations at some RI values even below the levels specified by the IOOC for "extra-virgin olive oil" qualification.

Finally, the genotype also contributed to explain the variability observed in the concentration of C18:3 (**Figure 3.E**) with the ripening process, particularly, at advanced stages (RI 3 and 4). This PUFA also was present at the highest concentration in VOO from Arbequina×Picual cross, clearly more concentrated than in VOOs from the two genitors. As can be

seen, differences between Arbequina×Picual cross and the resting groups were higher for RI 3, the stage at which the significance of the genotype was more relevant.

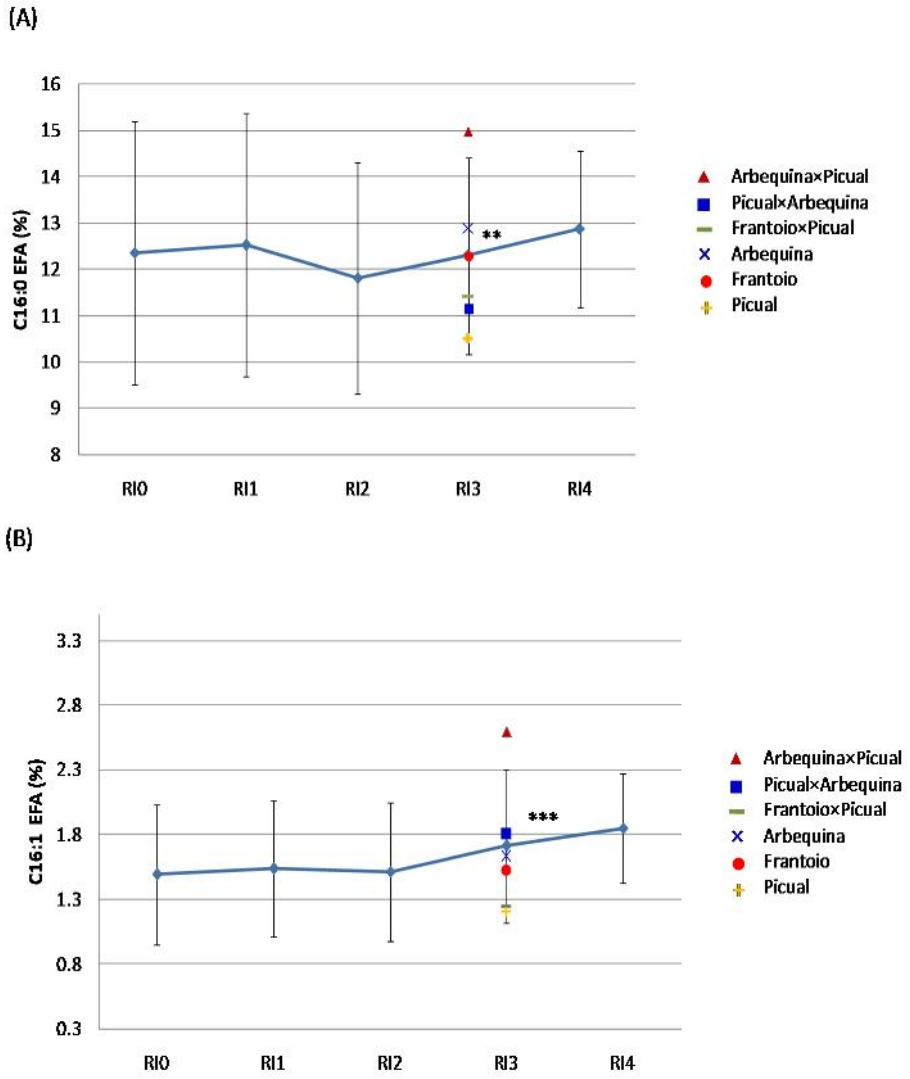

*Figure 3. Evolution plots for significant fatty acids (A) C16:0, (B) C16:1, (C) C18:1, (D) C18:2 and (E) C18:3, influenced by genotype. *: p-value<0.05; **: p-value<0.01; ***: p-value<0.0001.*

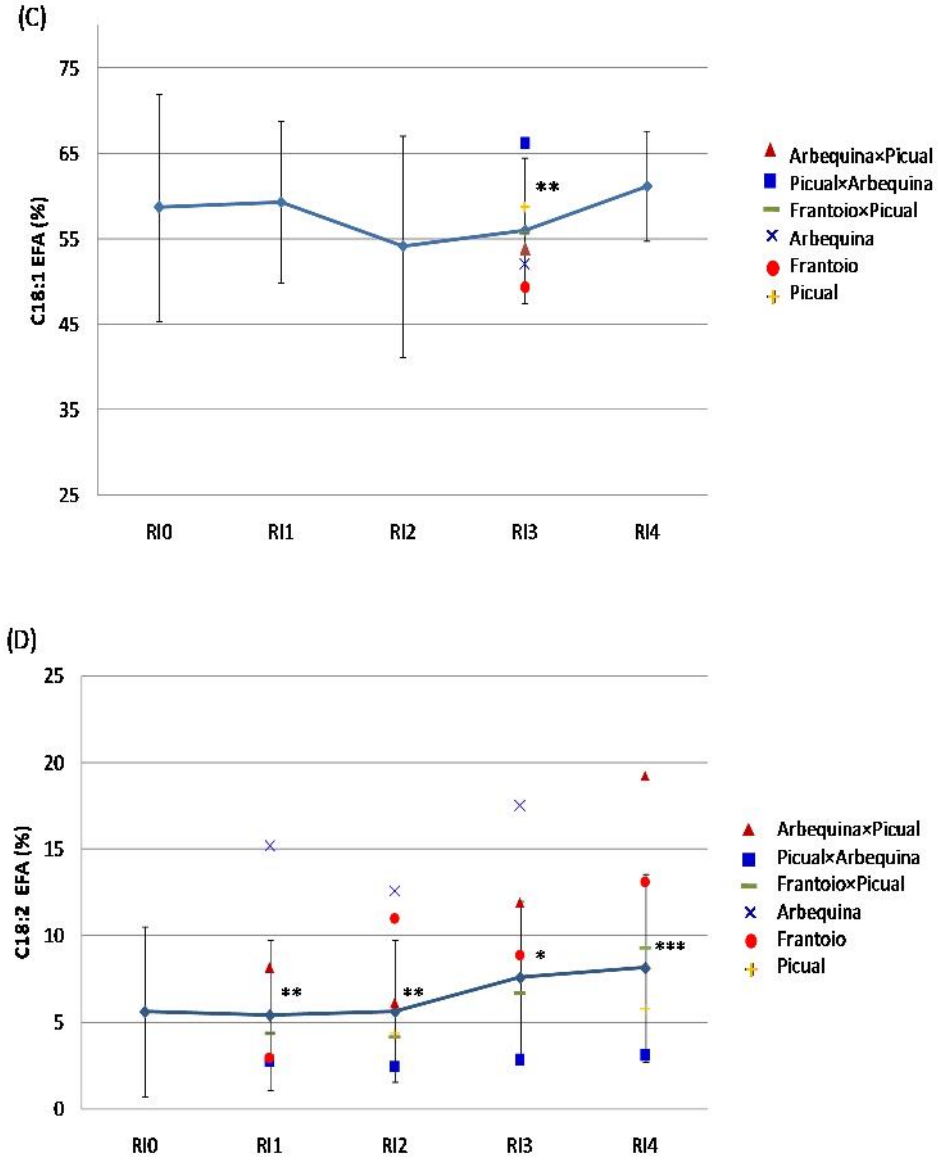

*Figure 3. Evolution plots for significant fatty acids (A) C16:0, (B) C16:1, (C) C18:1, (D) C18:2 and (E) C18:3, influenced by genotype. *: p-value<0.05; **: p-value<0.01; ***: p-value<0.0001 (cont.).*

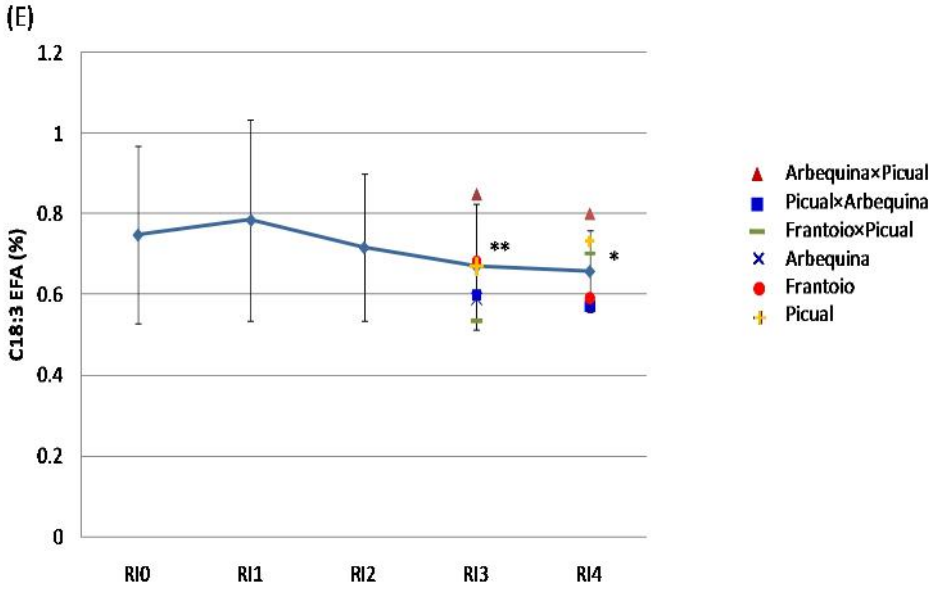

*Figure 3. Evolution plots for significant fatty acids (A) C16:0, (B) C16:1, (C) C18:1, (D) C18:2 and (E) C18:3, influenced by genotype. *: p-value<0.05; **: p-value<0.01; ***: p-value<0.0001 (cont.).*

In summary, the genotype is crucial to explain the concentrations of FAs in VOO obtained from olive fruits harvested along the ripening process. The influence of the genotype was more significant at RI 3, when olive fruits turn from red to violet color, due to the variability observed in the concentration of significant FAs.

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CAPÍTULO 9:

Composition of fatty acids in virgin olive oils from cross-breeding segregating populations by gas chromatography–flame ionization detection

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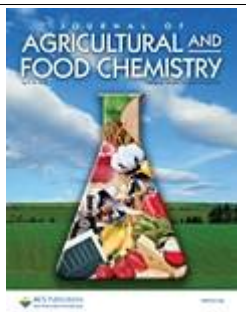

Composition of fatty acids in virgin olive oils from cross-breeding segregating populations by gas chromatography–flame ionization detection

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Abstract

Recent technological advances to improve virgin olive oil (VOO) quality have been focused on olive breeding programs, most of them among outstanding cultivars and selected progenies. Fatty acids (FAs) composition, with special emphasis on oleic (C18:1) and palmitic acids (C16:0), is one of the main characteristics considered in these programs, since FAs profile is one of the most critical aspects in evaluation of VOO quality. In this research, a method based on gas chromatography with flame ionization detection (GC–FID) was used to study the influence of genotype in the ten most important FAs in VOOs from target crosses Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana and their corresponding genitors, Arbequina, Arbosana, Koroneiki, Picual and Sikitita. For this purpose, a targeted metabolomics approach was selected for determination of esterified FA (EFAs) and non esterified FA (NEFAs), in a dual analysis with the same chromatographic method. A Pearson analysis revealed correlation between pairs of FAs, which allowed detecting metabolic connections through desaturation and elongation enzymes. An ANOVA test (with p-values<0.01) led to identifying C16:0 EFA, C16:1 EFA and C18:1 EFA as well as C16:1 NEFA and C18:0 NEFA as significant FAs to explain differences in the FAs profile of cross-breedings. Statistical analysis was carried out by unsupervised analysis using Principal Component Analysis (PCA) and Cluster Analysis (CA). Thus, crosses with a common genitor (Arbequina×Arbosana and Sikitita×Arbosana) were partially overlapped in PCAs using the complete profile of FAs. The CA results revealed clear differences in the composition of the most significant FAs between Sikitita×Arbosana and Picual×Koroneiki crosses while Arbequina×Arbosana was not properly discriminated from the other crosses.

INTRODUCTION

The olive oil industry has experienced technological advances intended to improve productivity and quality of virgin olive oil (VOO). One of the most recent advances in the main olive oil producing countries is the development of olive breeding programs, which are based on cross-breeding among the most outstanding cultivars and selection within the resulting progenies according to different parameters.[1,2] These programs have been considered the best strategy to provide new cultivars with improved characteristics. Due to the high level of heterozygosis of *Olea europaea*, any cross provides a wide variability range of agronomical or VOO quality parameters. Among agronomical parameters, factors such as productivity, vigor, resistance to diseases and shortening of the juvenile period have been emphasized.[3] On the contrary, few studies have been focused on parameters related to VOO quality for implementation in olive breeding programs. In these scant cases, the phenolic composition, the total fat content or the fatty acids (FAs) profile have been the target quality parameters.[1–5]

The composition of FAs —with special emphasis on oleic acid (C18:1) and palmitic acid (C16:0) as they are the most abundant FAs in VVO— is one of the most critical aspects of VOO quality.[6] An unfavorable composition of FAs has been reported as one of the main shortcomings in the quality of VOO. Thus, the FAs composition possesses a noticeable interest since it is crucial for stability of oils and responsible for the odors and flavors associated with oil quality.[7,8] Qualitative restrictions according to FAs composition are imposed by the International Olive Oil Council (IOOC) regulations. Thus, the allowed ranges for the two most important FAs, C18:1 and C16:0, are 55.0–83.0% and 7.5–20%, respectively (expressed as w/w).[6]

Among the few studies involving FAs composition and cross-breeding, it is worth mentioning those carried out by León et al.[9] and Ripa et

al.[10] The former research was a part of the olive breeding program initiated by the University of Córdoba and the Instituto Andaluz de Investigación y Formación Agraria, Pesquera, Alimentaria y de la Producción Ecológica (IFAPA) in 1991. The results obtained by these authors allowed detecting a strong genetic effect and significant differences between genotypes for all FAs evaluated (from C16:0 to C20:1). FAs composition also enabled classification of the genotypes into four groups with the highest contribution of C18:1, C18:2 and saturated FAs.[9] Ripa et al. studied the variability in the total phenolic content and in the concentration of C16:0, C18:1 and C18:2 according to the genotype and the environment (geographical location). The FA composition varied preferentially with the genotype but also with the environment. Few genotypes were identified as the best for each location, but they did not coincide in the three locations under study.[10] Nevertheless, it is worth mentioning that these studies were focused on esterified FAs (EFAs) and no attention was paid to non esterified FAs (NEFAs). The presence of NEFAs (free FAs) is a symptom of VOO deterioration by degradation of triglycerides and, therefore, they influence the organoleptic properties of oils.[11,12] The concentration of NEFAs, expressed as acidity index, should not surpass 2% (expressed as w/w of oleic acid) in VOOs according to the IOOC.[6]

Metabolomics seems to be specially suited to study FAs composition in VOO as a function of olive genotypes. From our knowledge, there are not available metabolomics studies on FAs composition to improve VOO quality in olive breeding programs. In this research, a method based on gas chromatography with flame ionization detection (GC–FID) was used to study the influence of genotype on the composition of FAs in VOOs from cross breeding segregating populations obtained by the Córdoba olive breeding program. This is the first time that a targeted metabolomics approach has been selected to study the FA composition of VOO aimed not only at EFAs determination, but also at that of NEFAs. VOOs extracted

from target crosses (Arbequina×Arbosana, Picual×Koroneiki and Sikitita×-Arbosana) and their corresponding genitors were used as sample cohort.

MATERIALS AND METHODS

Plant Material and Olive Oil Extraction

Healthy olive fruits were picked by hand, at turning color, from two trees of each of the cultivars: Arbequina, Arbosana, Koroneiki, Picual and Sikitita, and from 25 trees from each of the following crosses: Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana. Crosses were made in spring 2004–2005 and seedlings were planted in 2006–2007 in field trials in Córdoba, coordinated by the Department of Agronomy in the University of Córdoba and the IFAPA, Junta de Andalucía. Seedlings were subjected to a forcing growth protocol from seed germination to greenhouse and field growth, as described by Santos-Antunes et al.[13] Samples of olive fruits were collected at a similar ripening index —when fruits were at turning color— in the 2009/2010 harvest season (from 2nd November to 10th December) to avoid the influence of the ripening stage on the concentration of FAs in olive oil.[14]

An Abencor olive oil mill (MC2 Ingenierías y Sistemas, (Seville, Spain) was used to extract the oil. This system reproduces the industrial process (at a laboratory scale) through three basic elements: a hammer mill, a thermobeater and a paste centrifuge. Approximately 1 kg of fruit was ground to a paste using the hammer mill; then, the paste was placed in the thermobeater and stirred for 30 min with the water bath set at 28±1 ºC without micronized talc and water addition. Subsequently, a vertical centrifugation for 2 min permitted the separation of the oily phase that was collected and left to decant for 24 h. Finally, the oil was separated and stored in glass vials without head space and stored at −18 ºC until analysis.

Reagents

The reagents used for GC–FID analysis were LC grade methanol and *n*-hexane which were provided by Scharlab (Barcelona, Spain). Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare water–methanol extractant mixtures. Anhydrous sodium sulfate from Sigma–Aldrich (Steinheim, Germany) was used as drying agent for the nonpolar phase in the derivatization process. Potassium hydroxide and 98% sulfuric acid from Panreac (Barcelona, Spain) were used to prepare solutions (2 M KOH and 1 M H_2SO_4, respectively) in methanol for derivatization of EFAs and NEFAs, respectively.

FA methyl esters (FAMEs) as analytical standards (purity≥98.5%) were supplied by Fluka (Steinheim, Germany) to prepare calibration multistandards. Methyl esters corresponding to the following FAs were acquired: (C16:0, palmitoleic acid (C16:1), stearic acid (C18:0), C18:1, linoleic acid (C18:2), linolenic acid (C18:3), eicosanoic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0) and tetracosanoic acid (C24:0). Nonadecanoic acid methyl ester (C19:0) used as internal standard (IS) in the determination step was also from Fluka.

Individual stock standard solutions and multistandard solutions were prepared by dilution of each compound in *n*-hexane. These solutions were stored in a freezer at −20 ºC.

Sample Preparation Protocol for Determination of EFAs and NEFAs in VOO

Prior to GC separation, EFAs and NEFAs from VOOs were converted into their FAMEs. With this objective, 0.1 g of VOO was diluted with 2 mL *n*-hexane, and 200 μL of 2 M KOH methanolic solution was added. The biphasic system was shaked for 1 min in an MS2 minishaker, and the resulting phases were separated after decantation. The mixture was left for 5 min and the *n*-hexane phase, containing FAMEs from EFAs, was

transferred to a test tube and 1:50 (v/v) diluted with *n*-hexane containing 15 μg/mL of C19:0 as IS. Then, 1 μL of the resulting mixture was injected in triplicate into the GC for individual separation of EFA methyl esters.[16]

To obtain FAMEs from NEFAs, a small amount of anhydrous sodium sulfate was added to the remaining fraction from the previous step to remove residual water; then, 500 μL of 1 M H_2SO_4 methanolic solution was added and the tube was placed for 30 min into a water bath thermostated at 70 ºC. After cooling, 1 mL of *n*-hexane was added and shaked for 1 min in an MS2 minishaker. The biphasic system was left for 5 min and, after phases separation, the top *n*-hexane phase containing the FAMEs from NEFAs was transferred to a test tube. The step was repeated to ensure total conversion of NEFAs into NEFA methyl esters and the two extracts were mixed, 1:10 (v/v) diluted with *n*-hexane containing 15 μg/mL of C19:0 as IS, and 1 μL of this solution was injected into the GC for individual separation of NEFA methyl esters.[17,18]

Chromatographic Analysis

A thermostated water bath and an MS2 minishaker from Ika (Wilmington, USA) were used to assist the derivatization step. Separation of FAMEs was carried out using an 7820A GC (Agilent Technologies, Santa Clara, CA, USA) equipped with an autosampler, a split/splitless inyector and an FID. A SP™-2380 fused silica capillary column (60 m×0.25 mm I.D., 0.2 μm film thickness) provided by Supelco (Bellefonte, PA, USA) was used as analytical column.

The same chromatographic method was used for separation of the derivatized EFAs and NEFAs. Helium was used as a carrier gas at 1.2 mL/min flow rate. The injection was in the splitless mode and the temperature of the chromatographic gradient was as follows: the initial oven temperature was kept at 120 ºC for 3 min and then, programmed to rise at 3 ºC/min up to 185 ºC, maintained for 2 min, and followed by a second gradient of 15 ºC/min to a final temperature of 250 ºC, which was held for 5

min. The equilibration time was 5 min. The injector and detector temperatures were 250 and 280 ºC, respectively. The signal from the FID was acquired and processed by EZ Chrom Elite Compact software (Version 3.3.2, Agilent Technologies, Santa Clara, CA, USA). The reference standard was used to identify and quantify individual FAMEs in extracts from the VOO from the olive breeding program. For this purpose, calibration models were built for each FA by using multistandard solutions at different concentrations spiked with the IS. Concentrations of EFAs and NEFAs were calculated as percentages.

Statistical Analysis

After GC–FID analysis raw data files were used to create a data matrix with the concentrations EFA and NEFA of each analytical sample. The file obtained was created in comma separated values files (.csv). The study was focused on a representative panel of FAs in VOO. Thus, the initial data set was formed by 85 samples×20 analytes (10 EFAs and 10 NEFAs).

The definitive files were exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. Normalization by logarithmic transformation was used as pre-processing step. Statistical analysis was carried out by unsupervised analysis by Principal Component Analysis (PCA) to find clustering of samples attending to crosses. A Pearson analysis was carried out to evaluate correlations in each pair EFA/NEFA in VOOs. In addition, the ANOVA test was applied to evaluate the influence of the genotype to explain the variability in the concentration of EFAs and NEFAs with p-value<0.01. Then, a cluster analysis was applied to a reduced data set formed by the concentration of the FAs which were significant in the ANOVA test. This analysis allowed organizing samples into clusters based on similarity of their abundance profiles. The selected clustering algorithm was self-organization map (SOM).

RESULTS AND DISCUSSION

Characterization of EFAs and NEFAs in VOO Obtained from Olive Breeding Programs

The ten most important FAs present in VOO were monitored in this research as EFA and NEFA methyl esters in a dual analysis with the same chromatographic method. Identification was supported on retention time by comparison with FAME standards. Quantitative analysis was carried out by using C19:0 as IS to generate data of the twenty compounds (10 EFAs and 10 NEFAs) which were the variables considered for data treatment. The precision estimated as variability by analysis of replicates using the complete protocol was below 10% for all EFAs and NEFAs. **Tables 1** and **2** list the average concentrations of EFAs and NEFAs in VOO from the different varieties and crosses as well as the maximum and minimum values observed for each class. Relative standard deviation (RSD) values provide information about the variability in the concentration of FAs present in VOO samples from the same cross or variety. This information evidenced the biological variability by inclusion of different trees of the same genotype. As shown, high variability was observed for the analyzed FAs in genitors and corresponding segregating populations. The highest variability expressed as %RSD was observed for C24:0 EFA from the cross Picual×Koroneiki with 296%. On the other hand, the lowest variability (1.17%) was detected in C18:0 NEFA in VOOs from Picual variety. No discrimination was observed between varieties and crosses attending to the variability associated to each class. As can be seen, mean values of EFAs in VOOs from crosses and varieties were within the range established by IOOC for "*virgin olive oil*" qualification.[6] However, it is worth emphasizing that some of the studied oils exceeded the limits set by IOOC, as can be seen in **Table 1** for different genotypes. This behavior can also be visualized in **Figure 1** that shows the distribution of concentrations in crosses for EFAs which revealed anomalies according to the IOOC rules (viz. C18:1, C18:2 and C18:3). Concerning

C18:1, a significant proportion of VOO samples from the crosses Arbequina×Arbosana and Sikitita×Arbosana (44 and 47.8%, respectively) were below the limits set by the IOOC for "*virgin olive oil*" qualification (55%). The same two crosses reported samples with C18:3 values above the limits set by the IOOC for this FA (≤1%). These samples (4 and 5, respectively) should be qualified as "*ordinary olive oil*" according to IOOC regulations. In dealing with C18:2, only 72% of the samples from the cross Arbequina×Arbosana could be qualified as "*virgin olive oil*". In this particular case, two samples reported lower concentration of C18:2 than the limit set by IOOC (3.50%), while five of them surpassed the upper limit (21%). For the cross Sikitita×Arbosana, three samples were above the limit of the C18:2 concentration range. It is worth noting that only three samples from the cross Picual×Koroneiki could not be qualified as "*virgin olive oil*" for these three FAs.

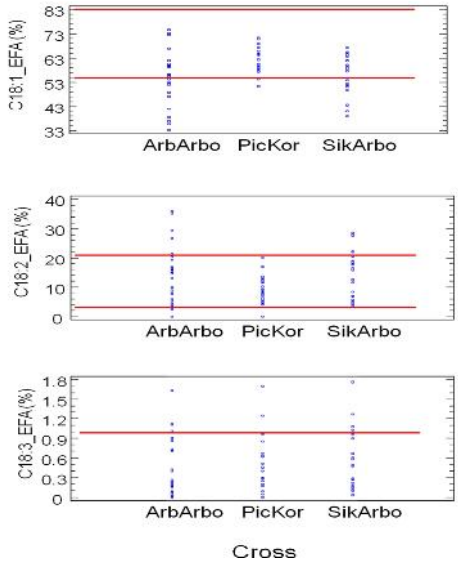


Figure 1. Distribution plots of FAs concentration corresponding to C18:1, C18:2 and C18:3 EFAs in different crosses. Horizontal line sets the limits established by the IOOC.

Table 1. Mean concentrations of EFAs and NEFAs in VOO from the different genitors.

Arbequina						
EFA	**Methyl ester IOOC (% w/w)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	18.5	42.4	C16:0	19.0	21.1
C16:1	0.30-3.50	2.27	41.1	C16:1	2.42	20.2
C18:0	0.50-5.00	1.35	38.0	C18:0	1.43	14.6
C18:1	55.00-83.00	61.6	22.5	C18:1	59.6	14.7
C18:2	3.50-21.00	15.7	41.8	C18:2	16.9	17.5
C20:0	≤ 0.60	0.005	37.9	C20:0	0.005	18.9
C18:3	≤ 1.00	0.588	81.5	C18:3	0.651	75.6
C20:1	≤ 0.40	0.004	60.6	C20:1	0.002	141.4
C22:0	≤ 0.20	0.003	67.6	C22:0	0.002	47.1
C24:0	≤ 0.20	0.004	47.1	C24:0	0.005	47.1
Arbosana						
EFA	**Methyl ester IOOC (% w/w)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	14.4	24.9	C16:0	14.5	37.4
C16:1	0.30-3.50	0.847	35.9	C16:1	0.625	129.6
C18:0	0.50-5.00	1.05	36.6	C18:0	3.35	81.8
C18:1	55.00-83.00	74.2	28.0	C18:1	72.0	41.4
C18:2	3.50-21.00	9.32	21.0	C18:2	9.36	49.4
C20:0	≤ 0.60	0.072	104.4	C20:0	0.102	121.4
C18:3	≤ 1.00	0.098	141.4	C18:3	n.q.	n.q.
C20:1	≤ 0.40	0.008	31.0	C20:1	0.008	22.5
C22:0	≤ 0.20	0.035	131.6	C22:0	0.005	44.0
C24:0	≤ 0.20	0.006	24.0	C24:0	0.006	0.792

Table 1. Mean concentrations of EFAs and NEFAs in VOO from the different genitors (cont.).

Koroneiki						
EFA	**Methyl ester IOOC (% w/w)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	12.8	11.0	C16:0	11.7	10.4
C16:1	0.30-3.50	0.661	17.4	C16:1	0.491	23.7
C18:0	0.50-5.00	1.717	21.4	C18:0	1.44	3.20
C18:1	55.00-83.00	77.0	9.88	C18:1	79.1	4.61
C18:2	3.50-21.00	7.48	18.8	C18:2	6.96	1.28
C20:0	≤ 0.60	0.013	31.3	C20:0	0.010	5.55
C18:3	≤ 1.00	0.346	25.7	C18:3	0.258	14.6
C20:1	≤ 0.40	0.007	8.52	C20:1	0.007	10.4
C22:0	≤ 0.20	0.004	34.7	C22:0	0.003	12.3
C24:0	≤ 0.20	0.008	41.1	C24:0	0.002	4.90
Sikitita						
EFA	**Methyl ester IOOC (% w/w)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	18.9	8.89	C16:0	18.9	20.9
C16:1	0.30-3.50	2.84	3.73	C16:1	2.63	19.0
C18:0	0.50-5.00	1.84	9.25	C18:0	1.51	28.5
C18:1	55.00-83.00	57.3	9.33	C18:1	58.1	21.6
C18:2	3.50-21.00	18.3	3.16	C18:2	18.8	16.5
C20:0	≤ 0.60	0.019	9.51	C20:0	0.021	42.6
C18:3	≤ 1.00	0.738	107.0	C18:3	<0.001	141.4
C20:1	≤ 0.40	0.014	11.0	C20:1	0.010	24.2
C22:0	≤ 0.20	0.004	119.2	C22:0	0.002	79.8
C24:0	≤ 0.20	<0.001	11.9	C24:0	0.001	38.4
Picual						
EFA	**Methyl ester IOOC (% w/w)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	14.8	40.2	C16:0	14.6	35.8
C16:1	0.30-3.50	1.76	53.6	C16:1	1.76	54.7
C18:0	0.50-5.00	1.95	15.5	C18:0	1.89	1.17
C18:1	55.00-83.00	74.1	33.4	C18:1	73.7	18.8
C18:2	3.50-21.00	5.70	47.9	C18:2	5.42	38.3
C20:0	≤ 0.60	0.331	13.9	C20:0	0.287	21.6
C18:3	≤ 1.00	0.551	57.2	C18:3	0.907	18.0
C20:1	≤ 0.40	0.225	68.8	C20:1	0.328	2.12
C22:0	≤ 0.20	0.238	54.1	C22:0	0.225	68.7
C24:0	≤ 0.20	0.304	94.4	C24:0	0.901	85.7

Table 2. Mean, minimum and maximum concentrations of EFAs and NEFAs in VOOs from the different cross breeding segregating populations.

Arbequina×Arbosana										
EFA	**Methyl ester IOOC (% w/w)**	**Min (%)**	**Max (%)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Min (%)**	**Max (%)**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	15.3	19.6	17.5	40.8	C16:0	11.2	18.9	17.8	39.3
C16:1	0.30-3.50	1.45	3.88	2.37	65.8	C16:1	0.210	3.35	2.19	65.2
C18:0	0.50-5.00	0.49	3.05	1.91	47.7	C18:0	0.520	2.85	1.67	45.1
C18:1	55.00-83.00	68.7	73.2	60.0	30.6	C18:1	3.630	79.9	63.7	52.3
C18:2	3.50-21.00	n.q	37.9	17.6	87.9	C18:2	n.q.	27.2	14.4	107.8
C20:0	≤ 0.60	<0.001	0.020	0.015	60.6	C20:0	0.001	0.030	0.017	46.4
C18:3	≤ 1.00	n.q	1.74	0.555	114.4	C18:3	n.q.	0.970	0.238	199.4
C20:1	≤ 0.40	n.q	0.021	0.011	55.8	C20:1	0.001	0.020	0.010	47.2
C22:0	≤ 0.20	<0.001	0.009	0.005	64.2	C22:0	0.003	0.010	0.007	51.7
C24:0	≤ 0.20	<0.001	0.020	0.003	245.7	C24:0	0.001	0.010	0.003	86.6
Picual×Koroneiki										
EFA	**Methyl ester IOOC (% w/w)**	**Min (%)**	**Max (%)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Min (%)**	**Max (%)**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	10.1	16.8	14.1	46.3	C16:0	9.94	16.8	13.8	40.7
C16:1	0.30-3.50	n.q	3.43	1.25	77.3	C16:1	n.q	3.21	1.03	74.5
C18:0	0.50-5.00	1.38	5.03	2.39	73.6	C18:0	1.24	4.9	2.09	80.3
C18:1	55.00-83.00	59.8	73.5	71.1	37.3	C18:1	64.5	81.6	73.2	42.1
C18:2	3.50-21.00	n.q	17.9	10.6	62.2	C18:2	n.q	15.1	9.47	73.9
C20:0	≤ 0.60	0.005	0.026	0.018	64.5	C20:0	0.010	0.030	0.017	62.1
C18:3	≤ 1.00	n.q	1.980	0.554	127.1	C18:3	n.q	1.94	0.366	196.1
C20:1	≤ 0.40	n.q	0.020	0.011	69.2	C20:1	n.q	0.010	0.010	41.7
C22:0	≤ 0.20	<0.001	0.005	0.004	77.8	C22:0	0.002	0.130	0.008	164.1
C24:0	≤ 0.20	<0.001	0.030	0.005	213.3	C24:0	0.001	0.260	0.008	296.7
Sikitita×Arbosana										
EFA	**Methyl ester IOOC (% w/w)**	**Min (%)**	**Max (%)**	**Mean (%)**	**RSD (%)**	**NEFA**	**Min (%)**	**Max (%)**	**Mean (%)**	**RSD (%)**
C16:0	7.50-20	15.3	20.5	17.8	42.2	C16:0	14.4	20.7	17.8	45.1
C16:1	0.30-3.50	1.00	3.55	2.45	62.8	C16:1	n.q	4.8	2.45	62.5
C18:0	0.50-5.00	1.52	1.91	1.42	49.2	C18:0	n.q	1.80	1.42	69.1
C18:1	55.00-83.00	56.9	73.9	63.4	33.8	C18:1	40.4	79.4	63.4	60.8
C18:2	3.50-21.00	4.81	31.1	14.6	82.6	C18:2	n.q	27.3	14.6	105.1
C20:0	≤ 0.60	<0.001	0.020	0.020	72.0	C20:0	0.010	0.100	0.020	67.2
C18:3	≤ 1.00	0.040	1.96	0.301	99.8	C18:3	n.q	0.870	0.301	185.8
C20:1	≤ 0.40	<0.001	0.010	0.009	68.3	C20:1	n.q	0.020	0.009	57.4
C22:0	≤ 0.20	<0.001	0.008	0.008	60.5	C22:0	<0.001	0.040	0.008	79.8
C24:0	≤ 0.20	<0.001	0.060	0.003	257.8	C24:0	0.001	0.020	0.004	67.2

As previously emphasized, one of the main aspects of this research is the inclusion of NEFAs in the evaluation of the genotype as a factor influencing the variability of FAs in VOO. However, correlation could exist between concentrations in VOO for the esterified and the nonsterified forms of the same FA, which was evaluated by Pearson analysis for each EFA/NEFA pair. **Supplementary Table 1** illustrates the results obtained by this test, which was applied to the complete data set. As can be seen, correlation with *p*-value below 0.01 was statistically proved for some EFA/NEFA pairs. However, correlation coefficients (*R*) were lower than 0.6 in all cases, which is indicative of a low correlation. Considering the complete data set, several correlations were found for the different quantified compounds with *p*-value below 0.01 and $R>0.65$, indicative of a close relationship. Positive correlations were detected for the following pairs: C16:0 EFA/C16:1 EFA ($R=0.81$) and C22:0 NEFA/C24:0 NEFA ($R=0.91$). C16:1 is synthesized through C16:0 by action of desaturase enzyme, while the correlation between C22:0 and C24:0 denotes the activity of the elongation enzymes. Additionally, positive correlation was also found for the C16:0 NEFA/C18:1 NEFA pair ($R=0.67$), which are the two most concentrated FAs present in VOO. Oleic acid is the main product of fatty acid synthesis in the plastids, which could explain this correlation.[19]

On the other hand, negative correlation was found for C18:1 EFA/C18:2 EFA ($R=-0.89$). The formation of linoleic acid (C18:2), the most abundant PUFA in vegetable oils, mainly occurs outside the plastid in contrast to the synthesis of oleic acid. The spatial differences between both pathways (synthesis of oleic acid and linoleic acid) could explain this negative correlation.[19]. Also negative correlation was detected for C16:0 EFA/C18:1 NEFA ($R=-0.69$), one EFA and one NEFA ascribed to the two most important FAs present in olive oil: C16:0 and C18:1. This negative correlation has not been previously described. Therefore, it could denote a link between the metabolism of EFAs and NEFAs in the synthesis of

triacylglycerols or in the hydrolysis of them by release of free fatty acids. This opposite behavior was clearly observed by monitoring the concentrations of these two FAs according to the genotype (see **Tables 1** and **2**). Thus, C16:0 EFA and NEFA were more concentrated in VOOs from the varieties Sikitita and Arbequina and VOOs from the crosses Sikitita×Arbosana and Arbequina×Arbosana. On the other hand, VOOs from the varieties Picual, Arbosana and Koroneiki and from the cross Picual×Koroneiki presented less percentage of C16:0. Opposite behavior was found for C18:1 EFA, so VOOs with the highest percentage of this FA were obtained from Koroneiki variety, followed by Arbosana and Picual and the cross Picual×Koroneiki. VOOs from the varieties Sikitita and Arbequina as well as from the crosses Sikitita×Arbosana and Arbequina×Arbosana presented the lowest percentage of C18:1. However, C18:1 NEFA behavior was not similar to C18:1 EFA since VOOs from the Arbosana variety were among the group of samples with the lowest concentration of C18:1 NEFA, while samples from the cross Arbequina×Arbosana was among the group of samples with the highest concentration of this NEFA.

Variability of FAs Composition in Cross Breeding Segregating Populations According to Genotype

It is worth emphasizing that the influence of fruit ripening in the samples cohort was non significant since most of the samples were collected at the same ripening stage (i.e. at turning color). As previously reported, this ripening stage had been identified as those with the lowest incidence on the FAs composition of VOO.[14] Therefore, the variability in FA composition of VOO should be ascribed to influence of genotype. This influence on the composition of FAs present in VOOs from olive trees obtained from cross breeding segregating populations was evaluated by analysis of variance (ANOVA). Thus, the genotype was an influential factor to explain the concentration of C16:0, C16:1 and C18:1 as EFAs; and C16:1 and C18:0 as NEFAs with the following *p*-value 0.0000, 0.0000, 0.0034, 0.0016, 0.0045,

respectively. Means average plots of concentration for each FA in the three cross breeding segregating populations are in **Figure 2**, which revealed the variability in concentration depending on the genotype. Apart from that, the results observed in the Pearson analysis were also confirmed, which indicates an opposite trend for saturated and monounsaturated C16 and C18 FAs.

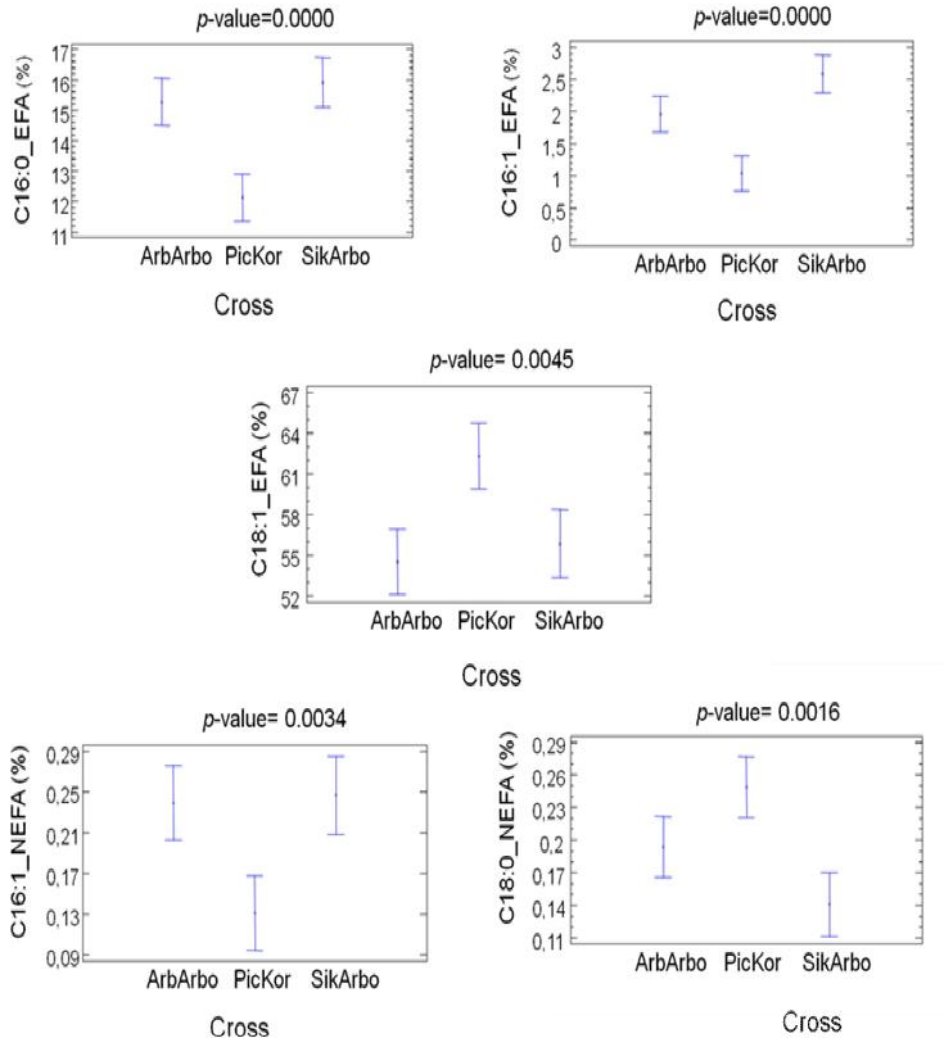


Figure 2. Average mean plots for the main significant FA in each cross. ArbArbo (Arbequina×Arbosana); PicKor (Picual×Koroneiki); SikArbo (Sikitita×Arbosana)

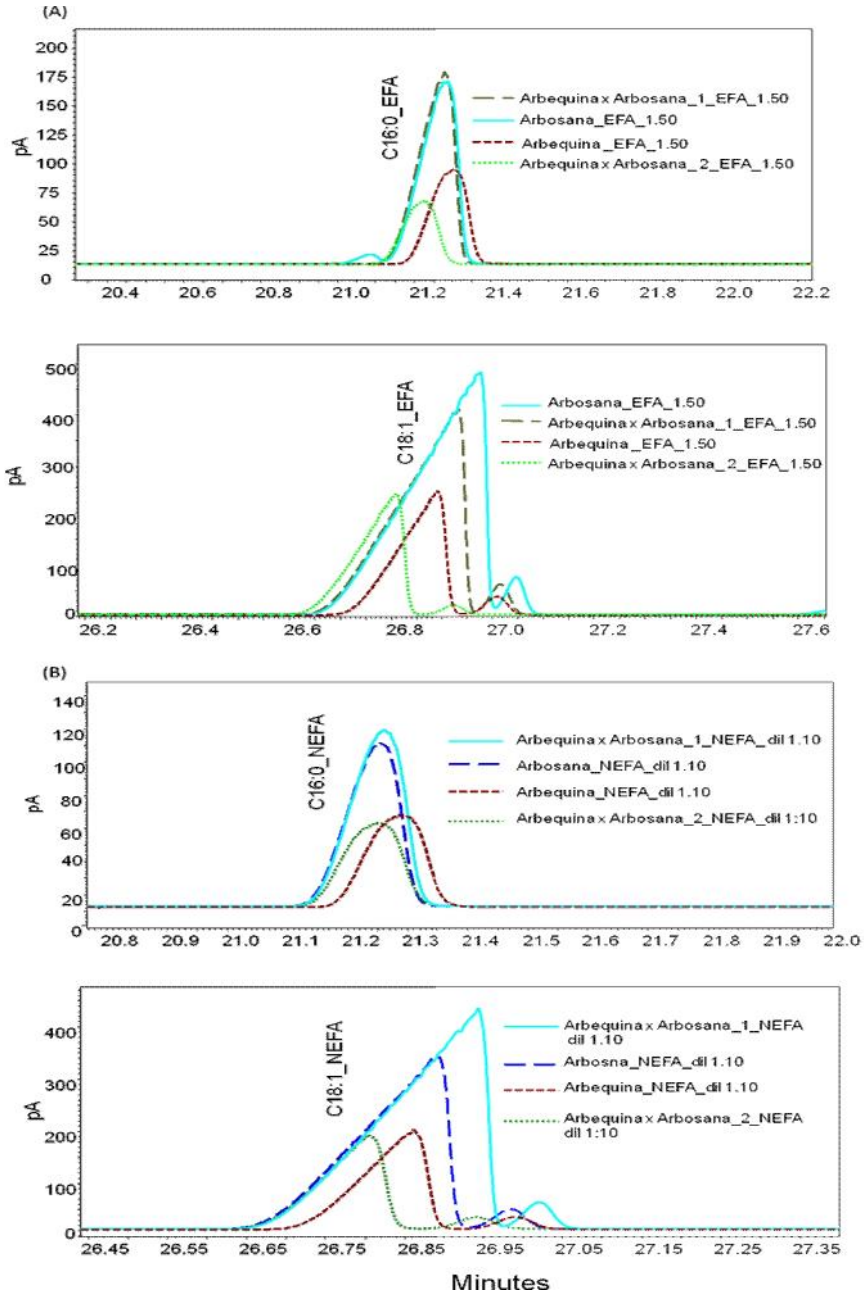


Figure 3. Chromatographic profiles obtained by GC–FID corresponding to (A) C16:0 and C18:1 EFAs and (B) C16:0 and C18:1 NEFAs from the cross Arbequina×Arbosana and the corresponding genitors. pA (pico amperios).

Variability in the concentration of FAs was also observed within genotypes, as can be deduced by comparing representative GC chromatograms from EFAs and NEFAs C16:0 and C18:1, provided by analysis of VOOs from the cross Arbequina×Arbosana as well as from genitors. Differences in the intensity of the chromatographic peaks associated to C16:0 and C18:1 in the cross Arbequina×Arbosana and the genitors are evident in **Figure 3.A** and **B**. As can be seen, VOO samples obtained by cross breeding presented concentrations of C18:1 and C16:0 similar to one or the other genitor, Arbequina or Arbosana. A similar behavior was found for NEFA forms of the same FAs. The variability in the content of both relevant FAs in VOOs from cross breeding samples is evident. The next step was to study the data set formed by 85 samples×20 variables to find the differences in the FAs profiles in the selected cohort.

Discrimination of VOOs from Cross Breeding Segregating Populations According to FAs Profiles

Once the data set was prepared, the next step was to study the variability of FAs composition in the sample cohort formed by cross-breeding genotypes selected from the olive breeding program. A PCA test was first applied to the resulting data set with the purpose of finding clusters and patterns dependent on the genotype. **Figure 4** shows the PCA scores plot corresponding to controlled crosses: Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana. A partial discrimination of the three groups of samples was observed in the 3D-plot explaining 49.79% of total variability. VOO samples from the cross Picual×Koroneiki were partially separated along PC1, whereas extracts from crosses Arbequina×Arbosana and Sikitita×Arbosana exhibited complete overlapping, which could be justified by their common genitor Arbosana as pollinator. Therefore, a certain variability associated to the composition of the main FAs present in VOOs can be explained by the genotype.

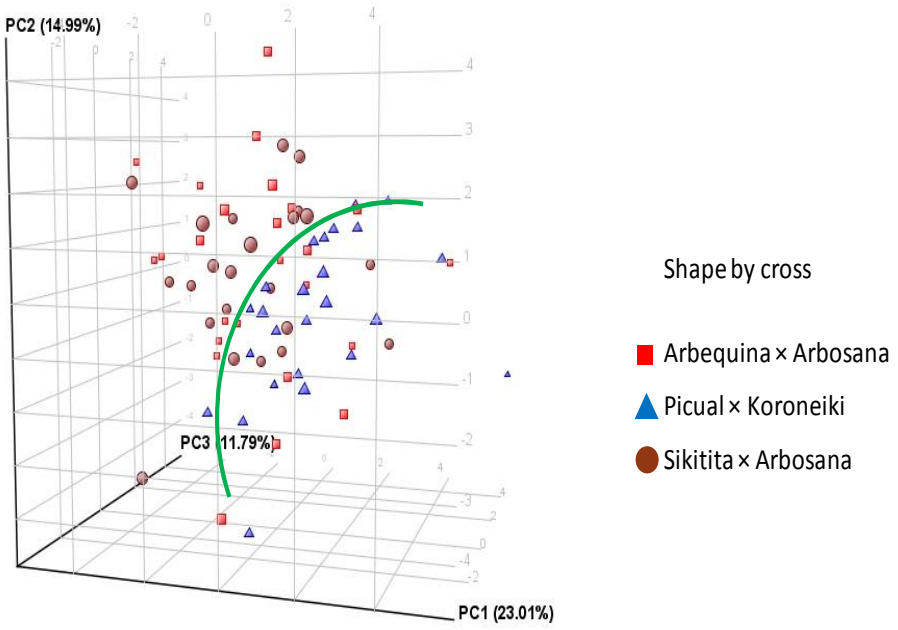


Figure 4. PCA score plots from controlled crosses: Arbequina×Arbosana, Picual×Koroneiki and Sikitita×Arbosana.

The influence of the genotype on the FA composition of the studied cross breeding segregating populations was evaluated by PCA for each pair of crosses to look for differences between them. **Figure 5** illustrates PCA scores plots provided by comparison of the pairs of cross breeding segregating populations: (A) Sikitita×Arbosana *vs* Picual×Koroneiki, (B) Sikitita×Arbosana *vs* Arbequina×Arbosana, (C) Picual×Koroneiki *vs* Arbequina×Arbosana. As can be seen, VOO extracts from crosses Sikitita×Arbosana *vs* Picual×Koroneiki and Picual×Koroneiki *vs* Arbequina×Arbosana show separation, whereas extracts from crosses Arbequina×Arbosana and Sikitita×Arbosana appear overlapped, which confirmed no discrimination between these two crosses according to the genotype.

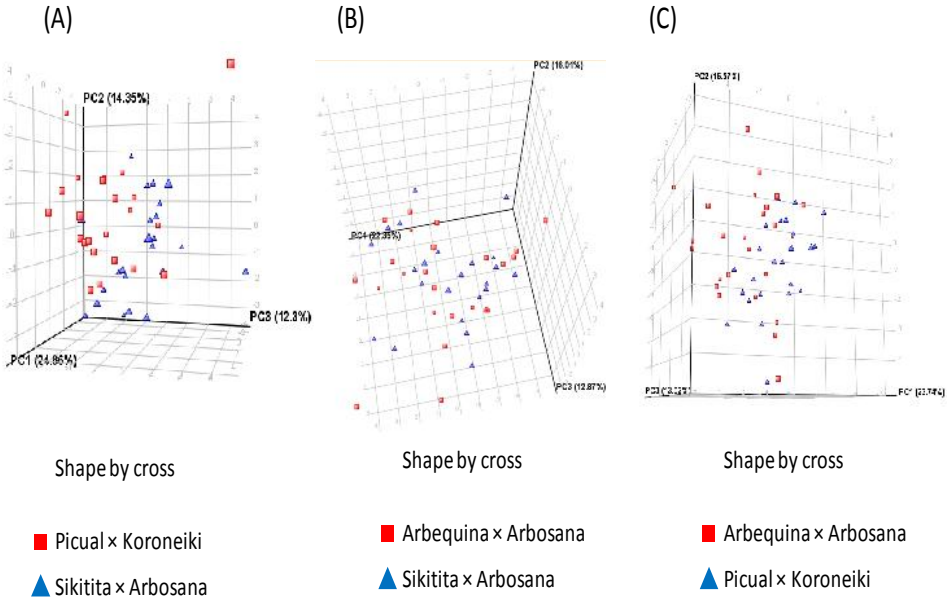


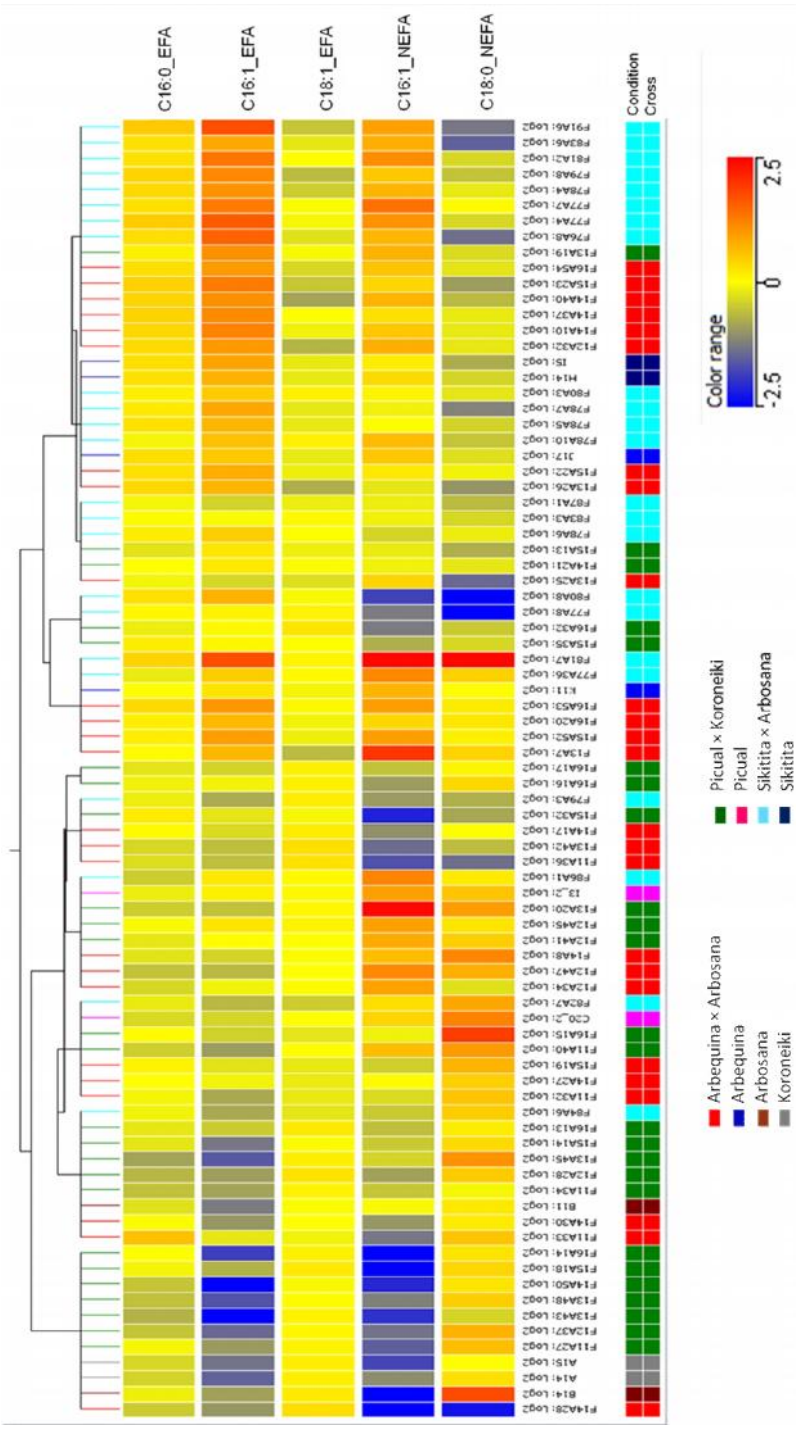
Figure 5. PCA scores plots from cross pairs (A) Sikitita×Arbosana vs Picual×Koroneiki, (B) Sikitita×Arbosana vs Arbequina×Arbosana, (C) Picual×Koroneik vs Arbequina×Arbosana.

Differences in the FA Profiles of the Cross Breeding Segregating Populations

The differences in the FAs profiles from the three cross breeding segregating populations with the associated varieties were established by cluster analysis to find similarities/dissimilarities. This analysis was carried out on FAs which resulted significant in the ANOVA test to study the influence of the genotype on the FAs profile (*viz.* EFAs C16:0, C16:1 and C18:1; and NEFAs C16:1 and C18:0). **Figure 6** shows the cluster graph obtained by comparing FAs profiles of the three crosses (Sikitita×Arbosana, Picual×Koroneiki and Arbequina×Arbosana) and the five varieties (Arbequina, Arbosana, Picual, Koroneiki, Sikitita), in which significant FAs revealed results similar to the PCA plot illustrated in **Figure 4**. Thus, two main clusters can be observed in the graph. Concerning cross breeding segregating

populations, the left cluster grouped 80% of Picual×Koroneiki VOOs, while the right cluster grouped 82% of Sikitita×Arbosana VOOs. Therefore, a clear distinction can be observed between both cross breeding segregating populations attending to this panel of significant FAs. On the other hand, in the third cross (Arbequina×Arbosana) VOOs were distributed in a similar proportion between both clusters. Two FAs were clearly responsible for this distribution according to the genotype: the EFA and NEFA forms of C16:1, which were identified as the two most critical FAs to discriminate VOOs from the three cross breeding segregating populations.

VOO samples from the genitors showed different behaviors. Thus, VOOs from Picual and Koroneiki were classified within the cluster containing most of the samples from Picual×Koroneiki, while those from Arbequina and Arbosana varieties were classified within different clusters, which explain the distribution of the VOO samples similarly classified in both clusters. Therefore, the influence of both genotypes (Arbequina and Arbosana) to explain the concentration of the most significant FAs in VOO from the cross Arbequina×Arbosana was similar. The influence of the genotype was more relevant in the cross Sikitita×Arbosana since VOOs from Sikitita were classified within the same cluster as this cross; while Arbosana samples were classified within the other cluster. Therefore, the influence of Sikitita as genitor was clearly higher than that of Arbosana in terms of FAs distribution. This study emphasizes the interest of FA profiles to characterize VOOs from cross breeding segregating populations. The selection of genitors in cross-breeding programs could influence the the FAs profile of VOOs obtained by crossings.


C16:0_EFA
C16:1_EFA
C18:1_EFA
C16:1_NEFA
C18:0_NEFA
Condition
Cross
Color range
2.5
0
-2.5
Picual × Koroneiki
Picual
Sikitita × Arbosana
Sikitita
Arbequina × Arbosana
Arbequina
Arbosana
Koroneiki

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. p-Value and correlation coefficients (R) obtained by Pearson correlational analysis of VOO levels for each EFA/NEFA pair.

	C16:0_EFA	C16:0_NEFA	C16:1_EEFA	C16:1_NEFA	C18:0_EFA	C18:0_NEFA	C18:1_EFA	C18:1_NEFA	C18:2_EFA	C18:2_NEFA
C16:0_EFA		-0.17	0.81	0.27	0.28	-0.37	-0.56	-0.69	0.47	-0.04
		0.15	0.00	0.02	0.02	0.00	0.00	0.00	0.00	0.71
C16:0_NEFA	-0.17		-0.15	0.63	-0.40	0.50	-0.30	0.67	-0.07	0.55
	0.15		0.22	0.00	0.00	0.00	0.01	0.00	0.56	0.00
C16:1_EFA	0.81	-0.15		0.53	0.02	-0.46	-0.46	-0.59	0.38	-0.13
	0.00	0.22		0.00	0.88	0.00	0.00	0.00	0.00	0.27
C16:1_NEFA	0.27	0.63	0.53		-0.33	0.05	-0.45	0.13	0.17	0.28
	0.02	0.00	0.00		0.00	0.65	0.00	0.27	0.15	0.02
C18:0_EFA	0.28	-0.40	0.02	-0.33		0.37	0.05	-0.33	-0.02	-0.31
	0.02	0.00	0.88	0.00		0.00	0.65	0.00	0.86	0.01
C18:0_NEFA	-0.37	0.50	-0.46	0.05	0.37		0.04	0.58	-0.26	0.16
	0.00	0.00	0.00	0.65	0.00		0.74	0.00	0.03	0.18
C18:1_EFA	-0.56	-0.30	-0.46	-0.45	0.05	0.04		0.34	-0.89	-0.63
	0.00	0.01	0.00	0.00	0.65	0.74		0.00	0.00	0.00
C18:1_NEFA	-0.69	0.67	-0.59	0.13	-0.33	0.58	0.34		-0.59	0.11
	0.00	0.00	0.00	0.27	0.00	0.00	0.00		0.00	0.36
C18:2_EFA	0.47	-0.07	0.38	0.17	-0.02	-0.26	-0.89	-0.59		0.51
	0.00	0.56	0.00	0.15	0.86	0.03	0.00	0.00		0.00
C18:2_NEFA	-0.04	0.55	-0.13	0.28	-0.31	0.16	-0.63	0.11	0.51	
	0.71	0.00	0.27	0.02	0.01	0.18	0.00	0.36	0.00	
C18:3_EFA	0.10	0.01	0.17	0.20	0.17	0.18	-0.20	-0.18	0.15	0.15
	0.39	0.95	0.16	0.09	0.14	0.13	0.09	0.13	0.20	0.20
C18:3_NEFA	-0.42	0.56	-0.29	0.31	-0.13	0.56	-0.02	0.55	-0.18	0.32
	0.00	0.00	0.01	0.01	0.28	0.00	0.89	0.00	0.14	0.01
C20:0_EFA	0.12	-0.22	-0.01	-0.22	0.56	0.20	0.19	-0.08	-0.19	-0.39
	0.30	0.06	0.92	0.06	0.00	0.09	0.10	0.50	0.11	0.00
C20:0_NEFA	-0.21	0.18	-0.27	-0.11	-0.07	0.20	0.19	0.30	-0.25	-0.01
	0.08	0.13	0.02	0.37	0.56	0.09	0.11	0.01	0.03	0.94
C20:1_EFA	0.14	-0.13	0.19	0.06	0.20	0.02	0.05	-0.16	-0.06	-0.12
	0.25	0.26	0.11	0.61	0.09	0.85	0.67	0.17	0.61	0.31
C20:1_NEFA	-0.06	0.31	-0.08	0.14	0.09	0.39	0.06	0.33	-0.25	-0.05
	0.64	0.01	0.51	0.23	0.46	0.00	0.61	0.00	0.03	0.69
C22:0_EFA	0.41	-0.21	0.38	0.06	0.19	-0.16	-0.13	-0.30	0.13	-0.28
	0.00	0.07	0.00	0.63	0.10	0.17	0.29	0.01	0.28	0.02
C22:0_NEFA	-0.21	-0.08	-0.15	-0.11	0.04	0.10	0.20	0.09	-0.15	-0.08
	0.07	0.48	0.20	0.34	0.72	0.38	0.10	0.47	0.21	0.48
C24:0_EFA	-0.18	0.10	-0.14	-0.01	-0.14	0.11	0.18	0.17	-0.19	-0.04
	0.12	0.42	0.24	0.95	0.23	0.37	0.14	0.14	0.11	0.75
C24:0_NEFA	-0.23	-0.09	-0.13	-0.07	0.05	0.10	0.19	0.08	-0.14	-0.06
	0.05	0.44	0.29	0.54	0.67	0.38	0.11	0.51	0.24	0.60

Supplementary Table 1. p-Value and correlation coefficients (R) obtained by Pearson correlational analysis of VOO levels for each EFA/NEFA pair (cont.).

	C18:3_EFA	C18:3_NEFA	C20:0_EFA	C20:0_NEFA	C20:1_EFA	C20:1_NEFA	C22:0_EFA	C22:0_NEFA	C24:0_EFA	C24:0_NEFA
C16:0_EFA	0.10	-0.42	0.12	-0.21	0.14	-0.06	0.41	-0.21	-0.18	-0.23
	0.39	0.00	0.30	0.08	0.25	0.64	0.00	0.07	0.12	0.05
C16:0_NEFA	0.01	0.56	-0.22	0.18	-0.13	0.31	-0.21	-0.08	0.10	-0.09
	0.95	0.00	0.06	0.13	0.26	0.01	0.07	0.48	0.42	0.44
C16:1_EFA	0.17	-0.29	-0.01	-0.27	0.19	-0.08	0.38	-0.15	-0.14	-0.13
	0.16	0.01	0.92	0.02	0.11	0.51	0.00	0.20	0.24	0.29
C16:1_NEFA	0.20	0.31	-0.22	-0.11	0.06	0.14	0.06	-0.11	-0.01	-0.07
	0.09	0.01	0.06	0.37	0.61	0.23	0.63	0.34	0.95	0.54
C18:0_EFA	0.17	-0.13	0.56	-0.07	0.20	0.09	0.19	0.04	-0.14	0.05
	0.14	0.28	0.00	0.56	0.09	0.46	0.10	0.72	0.23	0.67
C18:0_NEFA	0.18	0.56	0.20	0.20	0.02	0.39	-0.16	0.10	0.11	0.10
	0.13	0.00	0.09	0.09	0.85	0.00	0.17	0.38	0.37	0.38
C18:1_EFA	-0.20	-0.02	0.19	0.19	0.05	0.06	-0.13	0.20	0.18	0.19
	0.09	0.89	0.10	0.11	0.67	0.61	0.29	0.10	0.14	0.11
C18:1_NEFA	-0.18	0.55	-0.08	0.30	-0.16	0.33	-0.30	0.09	0.17	0.08
	0.13	0.00	0.50	0.01	0.17	0.00	0.01	0.47	0.14	0.51
C18:2_EFA	0.15	-0.18	-0.19	-0.25	-0.06	-0.25	0.13	-0.15	-0.19	-0.14
	0.20	0.14	0.11	0.03	0.61	0.03	0.28	0.21	0.11	0.24
C18:2_NEFA	0.15	0.32	-0.39	-0.01	-0.12	-0.05	-0.28	-0.08	-0.04	-0.06
	0.20	0.01	0.00	0.94	0.31	0.69	0.02	0.48	0.75	0.60
C18:3_EFA		0.29	-0.14	-0.05	0.25	0.11	-0.03	0.03	0.01	0.04
		0.01	0.25	0.67	0.03	0.35	0.80	0.83	0.95	0.73
C18:3_NEFA	0.29		-0.08	0.14	0.04	0.16	-0.27	0.04	0.16	0.07
	0.01		0.49	0.24	0.75	0.18	0.02	0.73	0.19	0.53
C20:0_EFA	-0.14	-0.08		0.08	0.25	0.27	0.33	0.17	0.16	0.15
	0.25	0.49		0.48	0.03	0.02	0.00	0.16	0.17	0.22
C20:0_NEFA	-0.05	0.14	0.08		0.05	0.35	-0.01	0.46	0.24	0.14
	0.67	0.24	0.48		0.65	0.00	0.95	0.00	0.04	0.24
C20:1_EFA	0.25	0.04	0.25	0.05		0.60	0.41	0.04	0.12	0.05
	0.03	0.75	0.03	0.65		0.00	0.00	0.73	0.32	0.66
C20:1_NEFA	0.11	0.16	0.27	0.35	0.60		0.25	-0.08	0.07	-0.18
	0.35	0.18	0.02	0.00	0.00		0.03	0.49	0.58	0.12
C22:0_EFA	-0.03	-0.27	0.33	-0.01	0.41	0.25		0.10	0.06	0.07
	0.80	0.02	0.00	0.95	0.00	0.03		0.42	0.62	0.54
C22:0_NEFA	0.03	0.04	0.17	0.46	0.04	-0.08	0.10		0.41	0.92
	0.83	0.73	0.16	0.00	0.73	0.49	0.42		0.00	0.00
C24:0_EFA	0.01	0.16	0.16	0.24	0.12	0.07	0.06	0.41		0.48
	0.95	0.19	0.17	0.04	0.32	0.58	0.62	0.00		0.00
C24:0_NEFA	0.04	0.07	0.15	0.14	0.05	-0.18	0.07	0.92	0.48	
	0.73	0.53	0.22	0.24	0.66	0.12	0.54	0.00	0.00	

SECCIÓN D: EVALUACIÓN CONJUNTA DE LA VARIABILIDAD DE LA COMPOSICIÓN FENÓLICA Y DE LOS FAS CON LA MADURACIÓN DEL FRUTO Y EL GENOTIPO

Esta sección recoge una ampliación de los estudios desarrollados en las Secciones B y C pues ha implicado el estudio conjunto de la fracción fenólica y del perfil de ácidos grasos. Esta estrategia conjunta ha permitido evaluar qué fracción se afecta en mayor o menor medida por la influencia del genotipo y del índice de maduración del fruto en los VOOs procedentes de los diferentes cruces de variedades de olivo. El estudio conjunto de ambos factores mediante los tratamientos quimiométricos adecuados arroja un tipo de información que pone de manifiesto la importancia del análisis integrado con el fin de establecer los cambios que se producen debido a dos de los factores con mayor influencia en la composición del VOO.

CAPÍTULO 10:

Influence of genotype and ripening process on fatty acid and phenolic profiles of virgin olive oils from advanced selections

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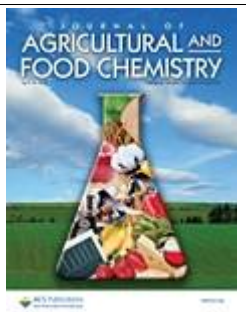

Influence of genotype and ripening process on fatty acids and phenolic profiles of virgin olive oils from advanced selections

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Abstract

Olive breeding programs are supported on the monitoring of agronomical and/or quality parameters of virgin olive oil (VOO). Composition of phenols and fatty acids (FAs) are key aspects in programs aimed at improving VOOs quality. The objective of the present study was the combined assessment of changes resulting in phenols and FAs (both esterified and non esterified forms) in VOO by the effect of genotype and ripening of olive fruits. For this purpose, phenols and FAs profiles of VOOs from advanced selections (*viz.* Arbequina × Picual, Picual × Arbequina and Frantoio × Picual) and their respective genitors were obtained by LC–QqTOF and GC–FID, respectively. Principal Component Analysis (PCA) provided discrimination according to both genotype and standardized parameter ripening index (RI). Significant phenols and FAs with high discrimination capability between pairs of cross-breedings and RI were detected by ANOVA test (with p-values<0.01). Partial Least Squares (PLS) regression model was used to evaluate quantitative relationship between RI and FAs and phenols concentrations and between total phenols content and phenol profiles in VOOs samples.

INTRODUCTION

The growing demand for high quality virgin olive oils (VOOs) has increased the interest in olive breeding programs, in which the high level of heterozygosity in *Olea europaea* cross-breeding is considered the best strategy. Thus, new genotypes can be generated to take profit from the good qualities of the genitors to get improved cultivars.[1] These programs are supported on the monitoring of agronomical and/or VOO quality parameters. Among agronomical parameters, productivity, vigor, resistance to diseases and shortening of the juvenile period have been evaluated.[2] Regarding VOO, quality generic parameters according to the official methods described in the Regulation EC 2568/91 of the Commission of the European Union such as free acidity, peroxide value, UV spectrophotometric indices (K232, K270) and sensory analysis have been taken into account.[3] However, the composition of VOO is another aspect considered in breeding programs aimed at obtaining new olive cultivars. Fatty acids (FAs) , phenolic profiles,[4,5] α-tocopherol, pigments [6] or volatile compounds[7] of VOO have been studied in olive breeding programs.

Phenols composition and profile of FAs have been the main quality parameters used in breeding programs.[2,4,6,8,9] Phenolic compounds have been widely studied because of their nutraceutical effects, relevant contribution to the sensory properties of VOO, with special emphasis on bitterness and pungency, and stabilizing role to ensure long shelf-life of VOOs as compared to other vegetable oils.[10] On the other hand, an unfavorable composition of FAs has been reported as one of the main shortcomings in the quality of VOOs due to the essential role of this fraction in stability of oils with direct responsibility on undesired odors and flavors.[11,12] It is worth mentioning that qualitative restrictions according to FAs composition are imposed by International Olive Oil Council (IOOC) regulations. Thus, the allowed ranges

for the two most concentrated FAs, oleic (C18:1) and palmitic (C16:0), are 55.0–83.0% and 7.5–20% (expressed as w/w), respectively.[13]

The composition of FAs is also relevant from a nutritional point of view. A diet rich in monounsaturated FAs (MUFAs), specially C18:1, introduces important cardiovascular benefits by reduction of low-density lipoprotein (LDL) cholesterol levels and total cholesterol in plasma without altering the levels of beneficial high density lipoprotein (HDL) cholesterol.[14,15] In turn, saturated FAs (SFAs) are nutritionally unfavorable since they increase the amount of LDL in human blood.

Several studies on olive breeding programs have been focused on analyses of FAs or phenols.[1,6,7,16,17] Thus, El Riachy *et al.* evaluated the influence of the ripening process, genotypes and planting system on the phenolic composition of VOO from advanced and segregating populations to determine the optimum time for comparison of genotypes with the aim of improving olive oil quality.[17] On the other hand, the genotype and ripening were identified as the main contributors of the composition of FAs in segregating populations obtained by cross-breeding.[1] Nevertheless, no studies dealing with a combined assessment of these two families in VOO have been carried out. The aim of the present research was to study the influence of two variability sources such as the genotype and ripening of olive fruits on the composition of phenols and FAs, esterified (EFAs) and non esterified (NEFAs). These factors have been identified as two of the main variables to explain the quality of VOOs.

MATERIALS AND METHODS

Samples

The selections evaluated in this research come from crosses between 'Arbequina', 'Frantoio' and 'Picual' cultivars carried out in the olive cross-breeding program of Córdoba in 1991–1992 coordinated by the Department of Agronomy in the University of Córdoba and the Andalusian Institute of

Agricultural Research and Training (IFAPA, Junta de Andalucía). Several genotypes were selected in the initial seedling population after three consecutive harvest seasons using as criteria their early bearing (short juvenile period) and high oil content. The selected genotypes were propagated by soft-wood cuttings and planted, in 2001, in a comparative field trial together with the three genitors as control at 6 × 5 m spacing. Trees were trained as single-trunk vase, with three to four main branches, and minimal pruning was carried out to allow early bearing. Standard cultural practices, including irrigation supply by in-line drips to avoid water stress of plants, were followed. Results of their agronomic evaluation at the initial seedling stage[18] and comparative field trials[19,20] have previously been reported.

Olive fruits were manually collected from 'Arbequina', 'Frantoio' and 'Picual' cultivars as well as from nine selections as follows: three from 'Arbequina' × 'Picual', three from 'Picual' × 'Arbequina' and three from 'Frantoio' × 'Picual' at five ripening stages from 1st October to 26th November 2009 (every two weeks) and two trees-per-genotype were sampled each date. No samples were available from 'Picual' at the first date (1st October); therefore, the cohort was formed by 118 samples**.**

Determination of the Ripening Index (RI)

The RI of fruit samples was recorded as described by El Riachy *et al.*[4] Briefly, an aliquot of 100 randomly selected fruits was taken from each fruit sampling, and fruits were classified into the following categories of skin color: 0= deep or dark green; 1= yellow or yellowish–green; 2= yellowish with reddish spots; 3= reddish or light violet; 4= black.

The total number of olives in each category (n0, n1,…, n4) was recorded, and the following equation was applied to determine the RI:

$$RI = ((0\times n0) + (1\times n1) + (2\times n2) + (3\times n3) + (4\times n4)) \, 100–1.$$

Then, the RI was categorized as follows: RI<0.8 (RI0); RI=0.8–1.5 (RI1); RI=1.5–2.1 (RI2); RI=2.1–2.95 (RI3) and, finally, RI≥2.95 (RI4). This

categorization was set in order to obtain five groups of samples with a similar size.

Olive Oil Extraction

Olive fruit samples were processed using an Abencor system (MC2 Ingenierías y Sistemas, Sevilla, Spain), which consists of three essential elements: the mill, the thermo-malaxer and the olive paste centrifuge. VOO was extracted according to the manufacturer protocol, which is schemed as: (i) olives were crushed with the hammer mill; (ii) the olive paste thus obtained was malaxated for 30 min with the water bath set at 28±1 ºC; (iii) olive oil was separated from the paste by centrifugation for 2 min; finally, (iv) the oil was separated from the wastewater by decantation and collected in dark brown glass bottles and stored at –20 ºC until analysis.

Reagents

The reagents used for characterization of VOOs were LC grade methanol and *n*-hexane which were provided by Scharlab (Barcelona, Spain). LC–MS grade acetonitrile and formic acid for preparation of chromatographic mobile phases were also from Scharlab. Folin–Ciocalteu (F–C) reagent and anhydrous sodium carbonate, for total phenols determination, were purchased from Panreac (Barcelona, Spain). Deionized water (18 MΩ • cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare water–methanol extractant mixtures and chromatographic phases for analysis of phenols. Anhydrous sodium sulfate from Sigma–Aldrich (Steinheim, Germany) was used as drying agent for the non-polar phase in the derivatization process of EFAs. Potassium hydroxide and 98% sulfuric acid from Panreac (Barcelona, Spain) were used to prepare solutions (2 M KOH and 1 M H_2SO_4) in methanol for derivatization of EFAs and NEFAs, respectively.

Fatty acid methyl esters (FAMEs) as analytical standards (purity ≥ 98.5%) were supplied by Fluka (Steinheim, Germany) to prepare calibration multistandards. Methyl esters corresponding to the following FAs were

acquired: C16:0, palmitoleic acid (C16:1), stearic acid (C18:0), C18:1, linoleic acid (C18:2), linolenic acid (C18:3), eicosanoic acid (C20:0), eicosenoic acid (C20:1), behenic acid (C22:0) and tetracosanoic acid (C24:0). Nonadecanoic acid methyl ester (C19:0) used as internal standard (IS) in the determination step was also from Fluka.

Individual stock standard solutions and multistandard solutions were prepared by dilution of each compound in *n*-hexane. These solutions were stored in a freezer at −20 ºC.

Apparatus and Instruments

A thermostated water bath and an MS2 minishaker from Ika (Wilmington, USA) were used to assist the derivatization step in FAs analysis and also to enhance the transfer of phenols from oil to a methanol–water solution for individual quantification of the phenolic compounds.

Separation of FAMEs was carried out using an Agilent 7820A GC equipped with an autosampler, a split/splitless injector and an FID. A SP™-2380 fused silica capillary column (60 m × 0.25 mm I.D., 0.2 μm film thickness) provided by Supelco (Bellefonte, PA, USA) was used as analytical column.

A ThermoSpectronic Helios UV–vis spectrophotometer was used for determination of total phenol concentration by the Folin-Ciocalteu method. Target phenols were analyzed by an 1200 Series LC system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6540 QTOF hybrid mass spectrometer with a dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected m/z ratios.

Protocol for Analysis of EFAs and NEFAs in VOO

Prior to GC separation, EFAs and NEFAs from VOOs were converted into their FAMEs by a two-step derivatization procedure. With this objective, 0.1 g of VOO was diluted with 2 mL *n*-hexane and 200 μL of 2 M KOH methanolic solution was added. The biphasic system was shaked

for 1 min and the resulting phases were separated after decantation. The mixture was left for 5 min and the *n*-hexane phase, containing the FAMEs from EFAs, was transferred to a test tube and 1:50 (v/v) diluted with *n*-hexane containing 15 μg/mL of C19:0 methyl ester as IS. Then, 1 μL of the resulting mixture was injected in triplicate into the GC for individual separation of EFA methyl esters.[21]

To obtain FAMEs from NEFAs, a small amount of anhydrous sodium sulfate was added to the remaining fraction from the previous step to remove residual water; then, 500 μL of 1 M H_2SO_4 methanolic solution was added and the tube was placed into a water bath thermostated at 70 ºC for 30 min. After cooling, 1 mL of *n*-hexane was added and shaked in an MS2 minishaker for 1 min. The biphasic system was left for 5 min and, after phases separation, the top *n*-hexane phase containing the FAMEs from NEFAs was transferred to a test tube. The step was repeated to ensure total conversion of NEFAs into NEFA methyl esters and the two extracts were mixed, 1:10 (v/v) diluted with *n*-hexane containing 15 μg/mL of C19:0 methyl ester as IS, and 1 μL of this solution was injected into the GC for individual separation of NEFA methyl esters.[22]

The same chromatographic method was used for separation of the derivatized EFAs and NEFAs. Helium was used as carrier gas at 1.2 mL/min flow rate. The injection was in the splitless mode and the temperature of the chromatographic gradient was as follows: the initial oven temperature was kept at 120 ºC for 3 min and then, programmed to rise at 3 ºC/min up to 185 ºC, maintained for 2 min, and followed by a second gradient of 15 ºC/min to a final temperature of 250 ºC, which was held for 5 min. The equilibration time was 5 min. The injector and detector temperatures were 250 and 280 ºC, respectively. The signal from the FID was acquired and processed by EZ Chrom Elite Compact software (Version 3.3.2, Agilent Technologies, Santa Clara, CA, USA). The reference standards were used to identify and quantify individual FAMEs in VOOs from the olive breeding

program. For this purpose, calibration models were built for each FA by using multistandard solutions at 11 different concentration levels (from 0.5 μg/mL to 100 μg/mL) spiked with the IS. The concentrations of EFAs and NEFAs were calculated as percentages.

Protocol for Analysis of Phenolic Compounds in VOO

Phenolic compounds were extracted by shaking 1 g of each oil with 1 mL of hexane and 1 mL of a 60:40 methanol–water (v/v) mixture for 1 min. The hydroalcoholic phase, isolated after centrifugation, was directly injected into the LC–QqTOF. Liquid–liquid extraction has been widely validated in previous publications and is accepted as preparation strategy for analysis of phenols in VOO.[9,23,24]

Olive phenols analysis was conducted by LC–QTOF in accurate mode. The analytical column was a C18 Pursuit XRs Ultra (50 × 2.0 mm I.D, 2.8 μm particle size) from Varian (Walnut Creek, CA, USA). The mobile phases were: A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). The gradient program, at 0.4 mL/min constant flow rate, was as follows: initially 96% A and 4% B; 0–8 min, 96–0% A and 4–100% B; 8–10 min, 0% A and 100% B. After analysis, the column was equilibrated for 5 min.

Five μL of extract was injected in the LC system without any additional pretreatment. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3500 V in negative ionization mode; skimmer, 65 V; octopole radiofrequency voltage, 750 V; fragmentor voltage, 175 V. Data were acquired in centroid mode in high resolution (2 GHz). Full scan was carried out at 1 spectrum/s within the m/z range 100–1700 with subsequent activation of the three most intense precursor ions per scan (only single or double charged ions were allowed) by MS/MS using a 20 eV collision energy. MS/MS scanning was carried out at 1 spectrum/s within the m/z range 100–

1700. An active exclusion window was programmed after one MS/MS spectrum and released after 0.75 min to avoid repetitive fragmentation of the most intense precursor ions and, in this way, increase the detection coverage. Before experiments, the instrument reported mass detection resolution of 25000 FWHM (Full Width at Half Maximum) at m/z 119.0362 (proton abstracted purine) and 45000 FWHM at m/z 966.0007 (formate adduct of hexakis (1H,1H,3H-tetrafluoropropoxy)-phosphazine). To assure the desired mass accuracy of recorded ions, internal calibration was performed during analyses with the dual ESI source by continuous infusion of a solution containing both standards.

MassHunter Workstation software (version B.05.00 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing the raw LC−QTOF data files. Phenols were searched in the raw data files using a home-made database containing information of the most representative phenols found in VOO (molecular formula, monoisotopic molecular weight and retention time). As search criteria, the allowed negative ions were deprotonated species and formate adducts. Dehydratation neutral losses were also included. The search algorithm limited extraction to ions exceeding 1000 counts and present in all VOO samples. Tolerance parameters for phenols detection were 0.5 min in retention time and 10 ppm in mass accuracy.

The extracted information for each sample was exported as compound exchange format files (.cef) that contained the peak areas for each monitored phenol. **Table 1** lists the phenols found in VOO samples with identification parameters. In addition to phenols, other compounds involved in biosynthetic pathways for production of phenols such as secologanol, loganic acid or elenolic acid were included in this study. Therefore, the term "phenolic compounds" is used in this research to define both phenols as such but also these compounds that are directly involved in their biosynthesis.

Table 1. Phenolic compounds and derivatives detected in VOO samples and identification parameters by LC–QTOF.

Compound	Theoretical *m/z*	Experimental *m/z*	Error (ppm)	Adduct	Retention time (min)	Fragments
Simple phenols						
Hydroxytyrosol	153.0557	153.0559	-1.3067	$[M-H]^-$	2.60–3.00	123.0450 105.0339
Hydroxytyrosol glucoside	315.1085	315.1082	0.9520	$[M-H]^-$	3.08–3.48	123.0446 153.0570
Tyrosol	137.0604	137.0609	-3.6480	$[M-H]^-$	3.80–4.20	119.0505 111.0098
Hydroxytyrosol acetate	195.0663	195.0659	2.0505	$[M-H]^-$	5.70–6.10	123.0450 105.0339
Phenylethanoids						
Verbascoside	623.1981	623.1959	3.5302	$[M-H]^-$	4.77–5.17	461.1744 161.0254
Phenolic acids and derivatives						
Quinic acid	191.0561	191.0558	1.5702	$[M-H]^-$	0.26–0.66	-
Ferulic acid	193.0506	193.0513	3.6260	$[M-H]^-$	5.50–5.90	134.0377
***p*-Coumaric acid**	163.0401	163.0388	7.9735	$[M-H]^-$	5.70–6.10	119.0504
***o*-Coumaric acid**	163.0401	163.0401	0	$[M-H]^-$	5.90–6.50	119.0503
Protocatechuic acid	153.0193	153.0181	7.8421	$[M-H]^-$	5.89–6.29	109.0295
Sinapic acid	223.0612	223.0598	6.2763	$[M-H]^-$	5.90–6.30	-
Vanillic acid	167.035	167.0334	9.500	$[M-H]^-$	5.98–6.38	-
Caffeic acid	179.035	179.0343	3.9098	$[M-H]^-$	6.40–6.80	135.0449
Secoiridoids and derivatives						
Demethyloleuropein	525.1614	525.1605	1.7137	$[M-H]^-$	4.50–4.90	-
Oleuropein	539.177	539.176	1.85467	$[M-H]^-$	5.20–5.60	377.1306 307.0874 275.0962
Ligstroside	523.1821	523.1812	1.7202	$[M-H]^-$	5.00–5.40	-
Demethyloleuropein aglycon	363.1085	363.1078	1.9278	$[M-H]^-$	5.82–6.22	-
3,4-DHPEA-EDA	319.1187	319.1185	0.6267	$[M-H]^-$	5.70–6.10	123.0446 139.0769
***p*-HPEA-EA**	361.1293	361.1287	1.6614	$[M-H]^-$	5.90–6.30	-
***p*-HPEA-EDA**	303.1238	303.1235	0.9897	$[M-H]^-$	6.30–6.70	-
3,4-DHPEA-EA	377.1242	377.1241	0.2652	$[M-H]^-$	6.50–6.90	123.0446 255.0869
Pinoresinols						
Pinoresinol	357.1344	357.1335	2.5201	$[M-H]^-$	5.90–6.30	-
1-Hydroxypinoresinol	373.1293	373.1272	5.6281	$[M-H]^-$	6.70–7.10	-

Table 1. Phenolic compounds and derivatives detected in VOO samples and identification parameters by LC–QTOF (cont.)

Compound	Theoretical *m/z*	Experimental *m/z*	Error (ppm)	Adduct	Retention time (min)	Fragments
Flavonoids						
Rutin	609.1461	609.1459	0.3283	$[M-H]^-$	4.79–4.19	301.0391
Luteolin-7 glucoside	447.0933	447.0928	1.1183	$[M-H]^-$	4.85–5.25	285.0423
Apigenin-7-glucoside	431.0984	431.1098	-3.5259	$[M-H]^-$	5.10–5.50	269.0450
Luteolin	285.0405	285.0414	-3.1574	$[M-H]^-$	5.80–6.20	-
Quercetin	301.0354	301.0351	0.9966	$[M-H]^-$	5.90–6.30	-
Apigenin	269.0455	269.0448	2.6018	$[M-H]^-$	6.20–6.60	-
α-Taxifolin	303.051	303.0511	-0.3300	$[M-H]^-$	6.00–6.40	-
Diosmetin	299.0561	299.0546	5.0158	$[M-H]^-$	6.30–6.70	-
Phenol aldehydes						
Vanillin	151.0401	151.0401	0	$[M-H]^-$	4.40–4.80	-
Hydroxy-isocromans						
1, 3 Methoxy 4-hydroxy-phenyl-6, 7-dihydroxy-isochroman	287.0925	287.0909	5.5731	$[M-H]^-$	6.40–6.80	-
Non-phenolic compounds						
Eleanolic acid	241.0718	241.0716	0.8296	[M-H]-	5.80–6.20	-
Loganic acid	375.1297	375.1296	0.2666	[M-H]-	3.38–3.78	151.0776
						113.0248
Secologanol	389.1453	389.145	0.7709	[M-H]-	3.42–3.82	151.0776
						113.0248
Oleoside	389.1089	389.1089	0	[M-H]-	4.00–4.40	121.0660
						101.0243
						209.0477
						345.1253
Oleoside 11-methylester	403.1246	403.1243	0.7442	[M-H]-	4.60–5.00	101.0244
						119.0350
						223.0626
						179.0707

Folin–Ciocalteu Test for Measurement of Total Phenols Concentration

Briefly, 20 μL of sample (with prior 1:50 dilution with water) was, in this order, mixed with 1.58 mL of water, 0.3 mL of 20% (w/v) Na_2CO_3 aqueous solution, and 0.1 mL of F–C reagent and heated in an oven for 5

min at 50 ºC. Then, the resulting solution was allowed to stand for 30 min. The reaction product was photometrically monitored at 765 nm. Gallic acid was used as standard for calibration, so the results were expressed as equivalent of gallic acid (GAE) per gram of oil.[25]

Data Pretreatment

After GC–FID analysis, raw data files were used to create a data set with the concentrations of EFAs and NEFAs of each analytical sample (samples × variables). This data set, formed by 118 samples × 20 analytes (10 EFAs and 10 NEFAs), was exported as comma separated values files (.csv) into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. On the other hand, the .cef files were exported into the MPP software package to create the data set for further processing (118 samples × 36 phenols). The two data sets (FAs and phenolic compounds) were grouped into a unique matrix formed by 118 samples × 56 quantitative variables.

Normalization by logarithmic transformation was used as pre-processing step taking into account that data from two different analyses were used. Unsupervised statistical analysis was carried out by Principal Component Analysis (PCA) to find clustering of samples attending to any variability source by MPP software package. Supervised statistical analysis by Partial Least Squares (PLS) was employed with Unscrambler X 10.1 (CAMO, Oslo, Norway).

RESULTS AND DISCUSSION

Genotype Effect on Phenolic and FA Profiles

There are numerous factors that affect the production and quality of VOO such as climate, soil properties, agronomical practices, production cycles and technological factors. This research was focused on the influence of the genotype and ripening in the composition of two critical families of compounds (phenols and FAs) present in VOO. For this purpose, an

experimental set including advanced selections obtained by cross-breeding was defined. The set of VOO samples corresponded to advanced selections obtained by cross-breeding cultivated in the same field; thus, the same agronomical practice was used, which means that the main variability sources were limited to genotype and fruit ripening.

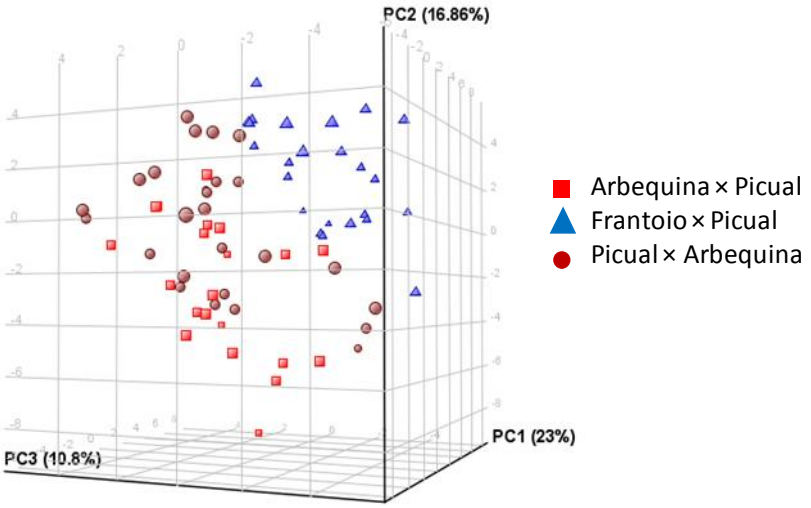

Figure 1. PCA scores plot provided by VOO samples from the crosses Arbequina × Picual, Picual × Arbequina and Frantoio × Picual.

Taking into account that data provided by two independent analyses were combined in a unique data set, a normalization pre-processing step was required. **Supplementary Figure 1** shows the effect of the normalization step by comparison of the variability estimated for each quantified compound. As a result, a normalized data set was obtained for statistical analysis, which was initiated by PCA in order to find groupings of VOO samples from cross-breedings attending to any variability source. **Figure 1** shows a clear discrimination according to the genotype with a 3-D scores plot explaining a 50.66% of the total variability. VOO samples from the Frantoio × Picual cross-breeding were separated from Arbequina × Picual

and Picual × Arbequina crosses along PC2 and PC3. However, Arbequina × Picual and Picual × Arbequina samples were overlapped, which could be ascribed to their common genitors. No discrimination trends between VOOs were detected according to the ripening state of olive fruits. Therefore, the genotype contributes to explain the variability associated to the composition of phenols and FAs present in VOO, but different variability levels could be set according to differences between genotype. This was proved by comparison of pairs of crosses to detect discrimination patterns but in this case the genitors were included in the analysis. **Figure 2** illustrates the PCA scores plots provided by comparison of pairs of advanced selections: (A) Arbequina × Picual *vs* Picual × Arbequina, (B) Arbequina × Picual *vs* Frantoio × Picual, (C) Frantoio × Picual *vs* Picual × Arbequina. As can be seen, VOO samples were clearly separated, even Arbequina × Picual *vs* Picual × Arbequina, which confirmed the discrimination between these crosses as a function of the genotype. In all cases, VOOs from cross-breedings were similar to those from their genitors according to the phenol profile and FAs composition. Concerning Arbequina × Picual *vs* Picual × Arbequina, VOOs from crosses were more similar to those provided by the female genitors, Arbequina and Picual, respectively.

After discrimination, an ANOVA test (with p-values < 0.01) was applied to identify significant compounds contributing to explain differences in the FAs and phenol profiles of VOOs. **Table 2** lists the panel of compounds with the highest discrimination capability between pairs of cross-breedings, which emphasizes the strong connection between genotype and composition of these two families of compounds in VOOs. Generally, it is assumed that the compounds responsible for pungency and bitterness properties are secoiridoids and derivatives. It is well-know that Picual VOO is characterized by an intense bitterness, which has been frequently attributed to the concentration of 3,4-DHPEA-EA. On the other hand, pungency has been frequently associated to the concentration of *p*-HPEA-EDA

(oleocanthal). The main compounds that contribute to explain differences between Frantoio × Picual VOOs as compared to Picual × Arbequina and Arbequina × Picual VOOs were phenolic compounds and, among them, secoiridoids and derivatives played a critical role. Thus, Frantoio × Picual VOOs contained significantly higher concentrations of *p*-HPEA-EA than the other two cross-breedings, while *p*-HPEA-EDA showed the opposite effect, which would enhance the pungent attribute of Picual × Arbequina and Arbequina × Picual VOOs. One other phenol present at high concentration in VOO such as 3,4-DHPEA-EDA, which is recognized as a potent oxidation inhibitor, was also more concentrated in the two cross-breedings involving Arbequina and Picual genitors. On the other hand, 3,4-DHPEA-EA, characterized by a direct correlation with bitterness attribute, was more concentrated in Frantoio × Picual VOOs than in Arbequina × Picual VOOs. However, no statistical differences were observed in the concentration of this compound in VOOs from Frantoio × Picual VOOs and Picual × Arbequina VOOs. One other remark-able aspect was that tyrosol was more concentrated in Frantoio × Picual VOOs than in Arbequina × Picual VOOs, which allowed setting differences between both genotypes for this relevant phenol.

Statistical differences were also observed in the levels of phenolic acids. Caffeic acid and protocatechuic acid were more concentrated in Frantoio × Picual VOOs, while ferulic acid, *p*-coumaric acid and *o*-coumaric acid were more concentrated in cross-breedings obtained from Arbequina and Picual genitors. Additional significant differences were found for minor families such as flavonoids, pinoresinols and isochroman derivatives.

Concerning the two closest genotypes, Picual × Arbequina and Arbequina × Picual, the main differences were ascribed to *p*-HPEA-EA and 3,4-DHPEA-EA, which would enhance the bitter character of Picual × Arbequina VOOs. Similarly to previous comparisons, other differences were detected for minor compounds. As an example, vanillin was detected at

higher concentration in Arbequina × Picual crosses, which could be associated to the predominant role of the female genitor since Arbequina VOO is described as fruity and softly bitter.

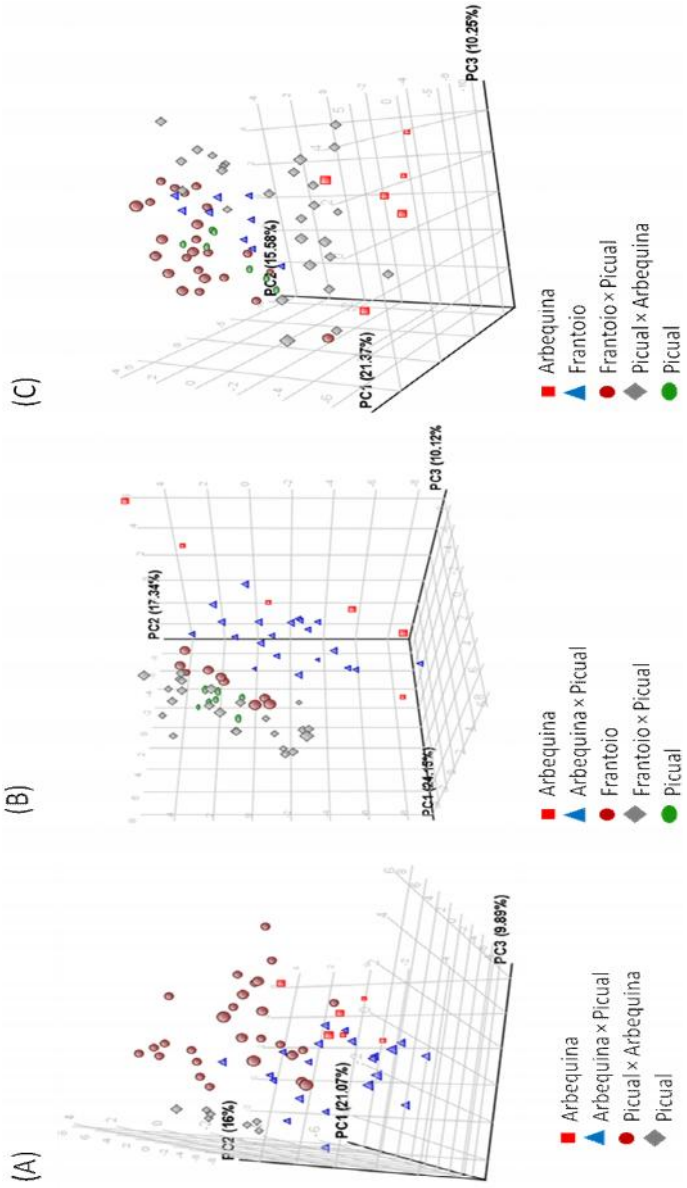

Figure 2. PCA scores plots provided by VOO samples from pairs of crosses: (A) Arbequina × Picual vs Picual × Arbequina; (B) Arbequina × Picual vs Frantoio × Picual; (C) Frantoio × Picual vs Picual × Arbequina.

Table 2. Significant compounds with p-value < 0.01 identified by comparison between pairs of cross-breedings.

Arbequina × Picual[1] *vs* Picual × Arbequina[2]			
Compound	***p*-Value**	**Regulation**	**Fold change**
1-Hydroxypinoresinol	<0.0001	up	3.2
***p*-HPEA-EA**	<0.0001	down	-2.5
3,4-DHEPA-EA	0.0020	down	-2.3
Apigenin-7-glucoside	<0.0001	down	-2.7
Diosmetin	<0.0001	up	2.8
Ferulic acid	0.0065	up	2.1
***p*-Coumaric acid**	0.0080	up	2.3
Protocatechuic acid	0.0037	down	-2.0
Vanillin	<0.0001	up	2.5
Oleoside	0.0006	down	-2.7
C16:0_EFA	0.0005	up	2.3
C18:2_EFA	<0.0001	up	3.0
C18:3_EFA	0.0057	up	1.8
Arbequina × Picual[1] vs Frantoio × Picual[2]			
Compound	***p*-Value**	**Regulation**	**Fold change**
1, 3-Methoxy-4-hydroxy-phenyl-6, 7-dihydroxy-isochroman	<0.0001	up	2.5
1-Hydroxypinoresinol	<0.0001	up	3.9
Pinoresinol	<0.0001	up	2.9
Tyrosol	0.0011	down	-2.4
Demethyloleuropein aglycon	<0.0001	up	2.1
***p*-HPEA-EA**	<0.0001	down	-3.8
***p*-HPEA-EDA**	0.0031	up	2.2
3,4-DHEPA-EA	<0.0001	down	-3.2
3,4-DHEPA-EDA	0.0011	up	2.1
Apigenin-7-glucoside	<0.0001	down	-3.7
Diosmetin	<0.0001	up	4.3
Caffeic acid	<0.0001	down	-2.8
Ferulic acid	<0.0001	up	3.0
***p*-Coumaric acid**	<0.0001	up	2.0
***o*-Coumaric acid**	0.0011	up	2.2
Protocatechuic acid	<0.0001	down	-3.8
C16:1_EFA	0.0031	up	2.5

Table 2. Significant compounds with p-value < 0.01 identified by comparison between pairs of cross-breedings (cont.).

Frantoio × Picual1 vs Picual × Arbequina2			
Compound	p-Value	Regulation	Fold change
1, 3-Methoxy-4-hydroxy-phenyl-6, 7-dihydroxy-isochroman	0.0010	down	-2.3
Pinoresinol	<0.0001	down	-3.8
Demethyloleuropein	0.0002	down	-2.5
Demethyloleuropein aglycon	0.0001	down	-2.4
3, 4-DHEPA-EDA	<0.0001	down	-2.7
Oleoside-11-methyl ester	0.0058	up	2.1
***p*-HPEA-EA**	0.0033	up	1.6
***p*-HPEA-EDA**	0.0029	down	-2.1
Caffeic acid	<0.0001	up	1.8
Protocatechuic acid	0.0048	up	1.9
Vanillin	0.0025	up	2.0
C18:2_EFA	0.0002	up	2.0

Up: increased concentration in 1 as compared to 2.
Down: decreased concentration in 1 as compared to 2.

In relation to fatty acids, no significant trends were observed. Thus, C16:0, C16:1, C18:2 and C18:3 were the FAs with statistical differences in terms of concentration between VOOs from different crosses. High concentrations of PUFAs, which are more susceptible to oxidation than monounsaturated FAs, were found in Arbequina × Picual VOOs. Saturated C16:0 and monounsaturated C16:1 were also more concentrated in this cross as compared to the other crosses. In summary, the genotype exerted a highly-significant effect on the concentrations of phenolic compounds, while less relevant changes were observerd in FAs.

Maturation Effect on Phenolic Profile and Fatty Acids Composition

Color variation is one of the most significant observable changes during fruit maturation. The initial green fruits undergo color changes and associated physiological modifications leading to purplish-black fruits, which is indicative of the end of morphological development. Color change is

associated with the decline in chlorophyll and oleuropein levels and appearance of anthocyanins that endow olive fruits with a purplish-black tone.[26]

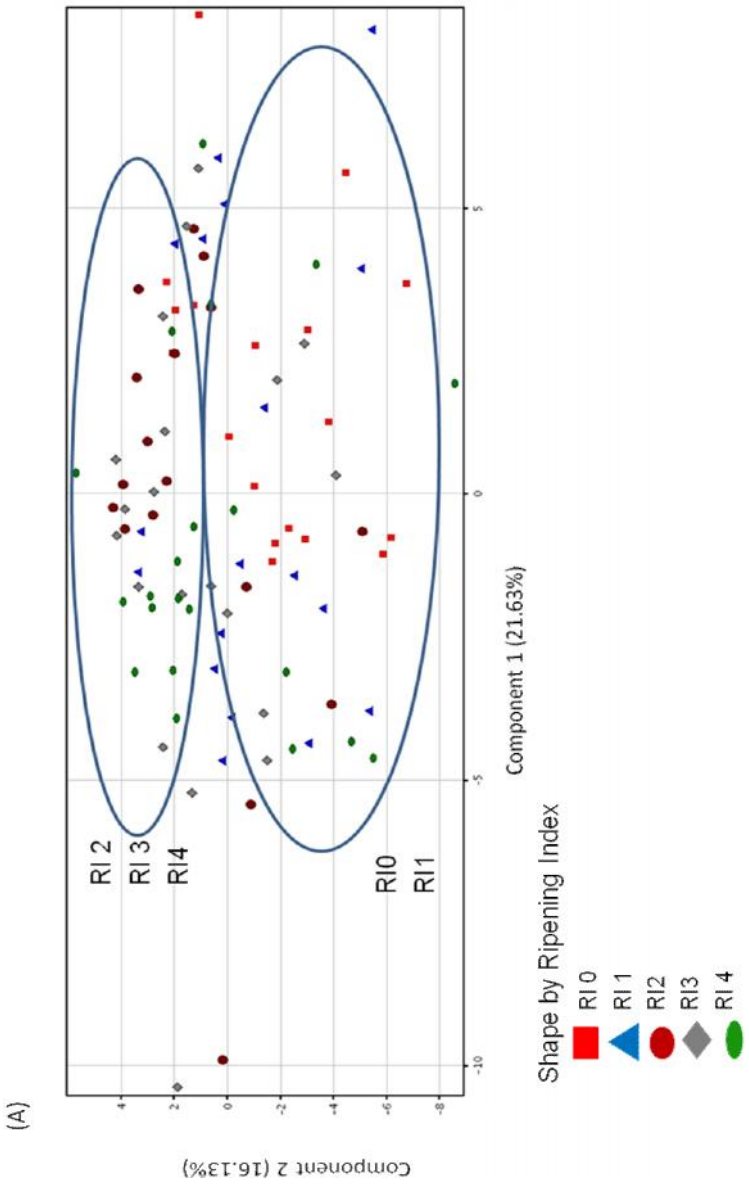

Figure 3. PCA scores (A) and loadings plots (B) associated to the composition of phenolic compounds and FAs as a function of the ripening index (as categorical variable) for the three advanced selections obtained by crosses.

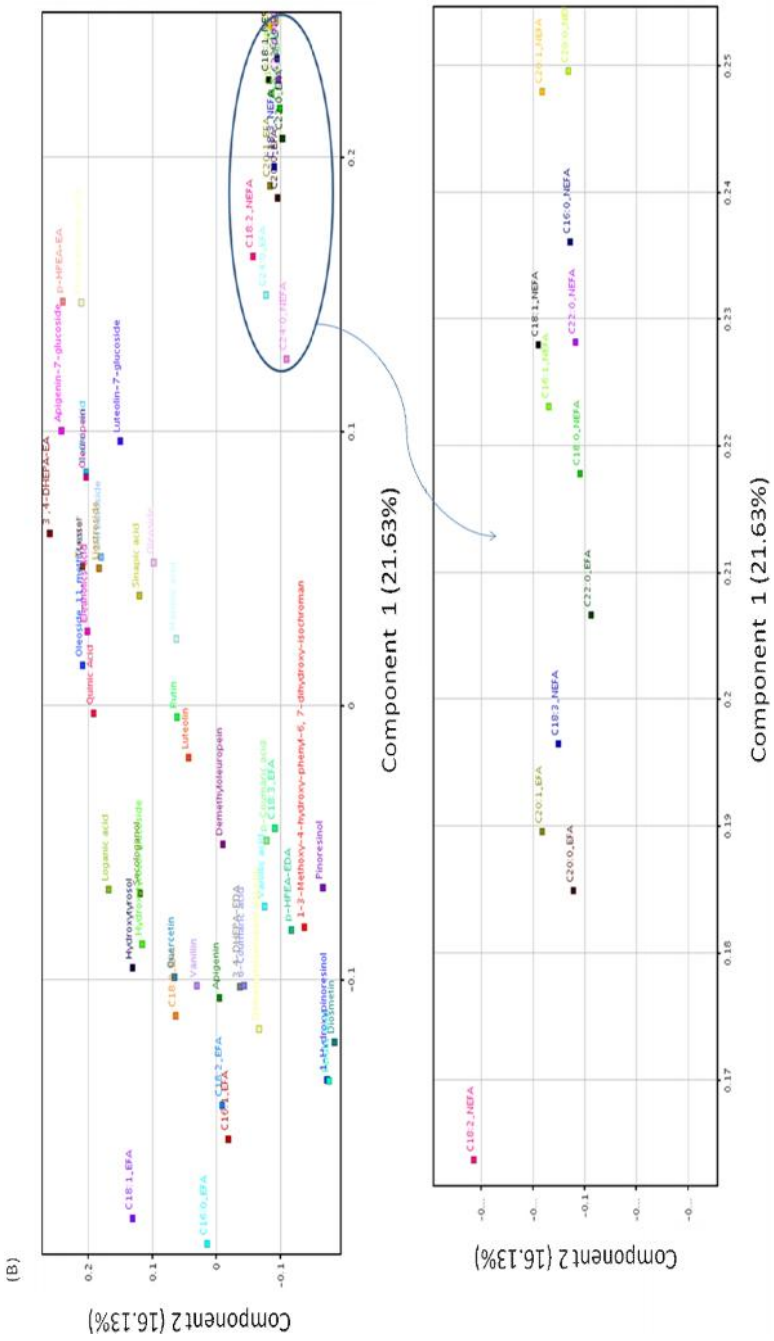

Figure 3. PCA scores (A) and loadings plots (B) associated to the composition of phenolic compounds and FAs as a function of the ripening index (as categorical variable) for the three advanced selections obtained by crosses (cont.).

The variability in the composition of FAs and phenolic compounds was assessed as a function of the RI, which is a standardized parameter scaled as a function of fruit color. PCA was applied to find grouping trends according to the RI. **Figure 3.A** shows the PCA score plots associated to FAs and phenols as a function of the RI. As can be seen, no clear discrimination was observed among the five groups of RI considered in the 2D plot. However, certain trends can be appreciated for initial and late maturation stages. Thus, two main sub-clusters can be partially differentiated along PC2 that explained 16.1% of the total variability. Olive oil samples from RI 0–1 (fruits with yellow or yellowish–green color) and 2–4 (fruits with color from yellowish with reddish spots, reddish or light violet to black) were separated in the two clusters. **Figure 3.B** shows the loadings plot with information about the main compounds contributing to explain the variability associated to ripening. Thus, most of monitored FAs (all NEFAs and most EFAs) were grouped on the right side of the lower part of the PCA, except for EFAs such as C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3. Other significant compounds contributing to explain the observed variability were pinoresinol derivatives, the unique detected isochroman, and glucoside flavonoids, which had an opposite effect to aid in the discrimination of early and advanced ripening stages. Apart from these results, it is worth emphasizing the opposite effect of two critical components in the sensory properties of VOO: 3,4-DHPEA-EA and *p*-HPEA-EDA, directly correlated to the bitterness and pungency attributes of VOO.

Quantitative Relationship between the RI and the Concentrations of Fatty Acids and Phenols in VOOs

Qualitative influence of the RI on the concentration of FAs and phenolic compounds has been evaluated in the previous section. This study allowed identifying the change from yellowish with reddish spots fruits to reddish or light violet fruits as the turning point to explain the variability in the concentrations of phenols and FAs. In this section, the objective was to

find a quantitative regression model using as response factor the numerical RI. The data set was formed by the concentrations of FAs and phenolic compounds. Cross-validation was used to validate internally the obtained models. **Figure 4** shows the PLS regression model as well as statistical parameters such as the root-mean square error (RMSE) and the regression coefficient (R^2). The RMSE was 0.73, while the R^2 coefficient was 0.892, which characterizes the regression model with good precision according to the criteria established by Shenk and Westerhaus.[27] Therefore, the concentration of phenolic compounds and FAs is strongly dependent on the RI value as numerical variable, which is a direct measurement of fruit maturation.

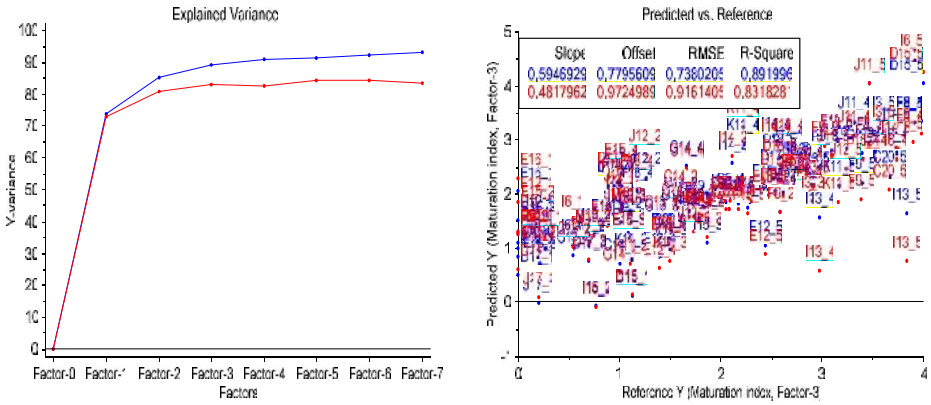

Figure 4. PLS regression model of the quantitative relationship between ripening index (as numerical variable) and the concentrations of FAs and phenols in VOOs.

Once the model was characterized, statistical significance of the concentration of phenolic compounds and FAs to explain the RI was evaluated by ANOVA test with p-value < 0.01. Numerous compounds, FAs and phenols, were highly-significant as a function of the variability of the RI. Among the seven significant FAs, six of them corresponded to NEFAs, while only the C18:2 EFA varied significantly its concentration with the RI. Therefore, the RI was better explained by monitoring NEFAs than EFAs, probably because the variability of RI affects more the concentration of

NEFAs. On the other hand, the most concentrated EFAs present in VOO (C16:0, C16:1, C18:0, C18:1) were not statistically influenced by the ripening process at 99% confidence level.

Concerning phenols, six compounds were significantly affected by the RI with a strong association (*p*-value < 0.01). These were hydroxy-pinoresinol (*p*-value 0.0052), hydroxytyrosol glucoside (0.0017), *p*-coumaric and *o*-coumaric acids (0.0001 and 0.0007, respectively), and two non-phenolic compounds involved in the synthesis of secoiridoids such as loganic acid (0.0000) and secologanol (0.0001). Therefore, there is not a direct significant effect of RI on secoiridoids and derivatives, which are the most concentrated phenolic compounds present in VOO, but two critical compounds in the biosynthesis of oleosides were strongly altered during maturation. It is worth mentioning that three relevant phenols with important nutraceutical and organoleptical properties present in VOO, such as hydroxytyrosol (0.0170), tyrosol (0.0125) and *p*-HPEA-EDA (0.0383), showed a high correlation with the RI. **Supplementary Figure 2** shows the dependence of the relative concentration of the mentioned phenolic compounds or derivatives on the RI parameter.

Quantitative Relationship between Total Phenolic Concentration and the Phenolic Profile in VOO

The Folin–Ciocalteu test is widely used to estimate the total concentration of phenolic compounds present in VOO by application of a fast and high-throughput methodology. However, no direct association between the response provided by the Folin-Ciocalteu test and individual concentrations of phenolic compounds has been described. A PLS regression model was here developed using the total concentration of phenols measured by the Folin-Ciocalteu test as response variable. The data set was formed by the complete group of samples and the 36 phenolic compounds and derivatives monitored by LC–MS/MS. The statistical parameters defining the PLS model were RMSE (128.99 and 157.10) and the R^2 was 0.85 and 0. 79

for the training and validation sets, respectively. **Figure 5** shows the regression curve obtained by PLS regression analysis, which proves the direct association between total phenolic concentration and the profile of phenolic compounds. The application of ANOVA test with 99% confidence level (*p*-value < 0.01) revealed high significance of certain phenolic families to explain the quantitative response measured by the Folin–Ciocalteu test, as listed in **Table 3**. As can be seen, lignane derivatives (isochroman and pinoresinols), secoiridoids (oleuropein and ligstroside) and those formed by condensation of hydroxytyrosol or tyrosol with elenolic acid (3,4-DHPEA-EA and *p*-HPEA-EA), tyrosol, phenolic acids (ferulic acid and protocatechuic acid), and flavonoids (diosmetin and glucoside apigenin and luteolin) were significant to explain the total phenolic concentration by the Folin-Ciocalteu test, which is based on antioxidant capability. Among them, only four phenolic compounds reported a moderate correlation with the total phenolic concentration. These were ferulic acid, which provided a negative moderate correlation (R= –0.51), while protocatechuic acid, *p*-HPEA-EA and oleuropein (although this was detected at trace levels) reported a positive moderate correlation (R= 0.58, 0.54, 0.62, respectively). Therefore, the total concentration of phenolic compounds, as measured by the Folin–Ciocalteu test, is the result of the combination of all phenolic compounds, which explains the good correlation between the total phenolic concentration and the monitored phenolic compounds. This means that there is not a strong direct relationship between the total phenolic concentration and levels of relevant phenols with nutraceutical or organoleptical interest.

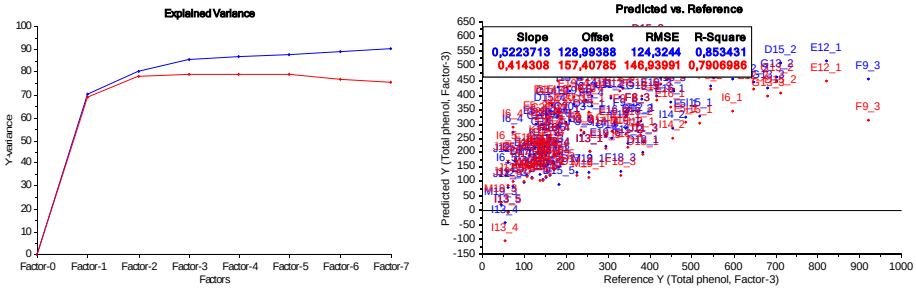

Figure 5. PLS regression model of the total phenolic concentration measured by the Folin–Ciocalteu test and the profile of phenolic compounds in VOO obtained by LC–MS/MS.

Table 3. Significant compounds with p-value < 0.01 to explain the quantitative response measured by the Folin–Ciocalteu test.

Compound	***p*-Value**
1, 3-Methoxy-4-hydroxy-phenyl-6, 7-dihydroxy-isochroman	0.0037
1-Hydroxypinoresinol	0.0001
Pinoresinol	0.0222
Tyrosol	0.0183
p-HPEA-EA	0.0001
3, 4-DHEPA-EA	0.0001
Ligstroside	0.0001
Oleuropein	0.0001
Apigenin-7-glucoside	0.0001
Luteolin-7-glucoside	0.0059
Diosmetin	0.0067
Rutin	0.0008
Ferulic acid	0.0001
Protocatechuic acid	0.0001
Vanillin	0.0004
Verbascoside	0.0001

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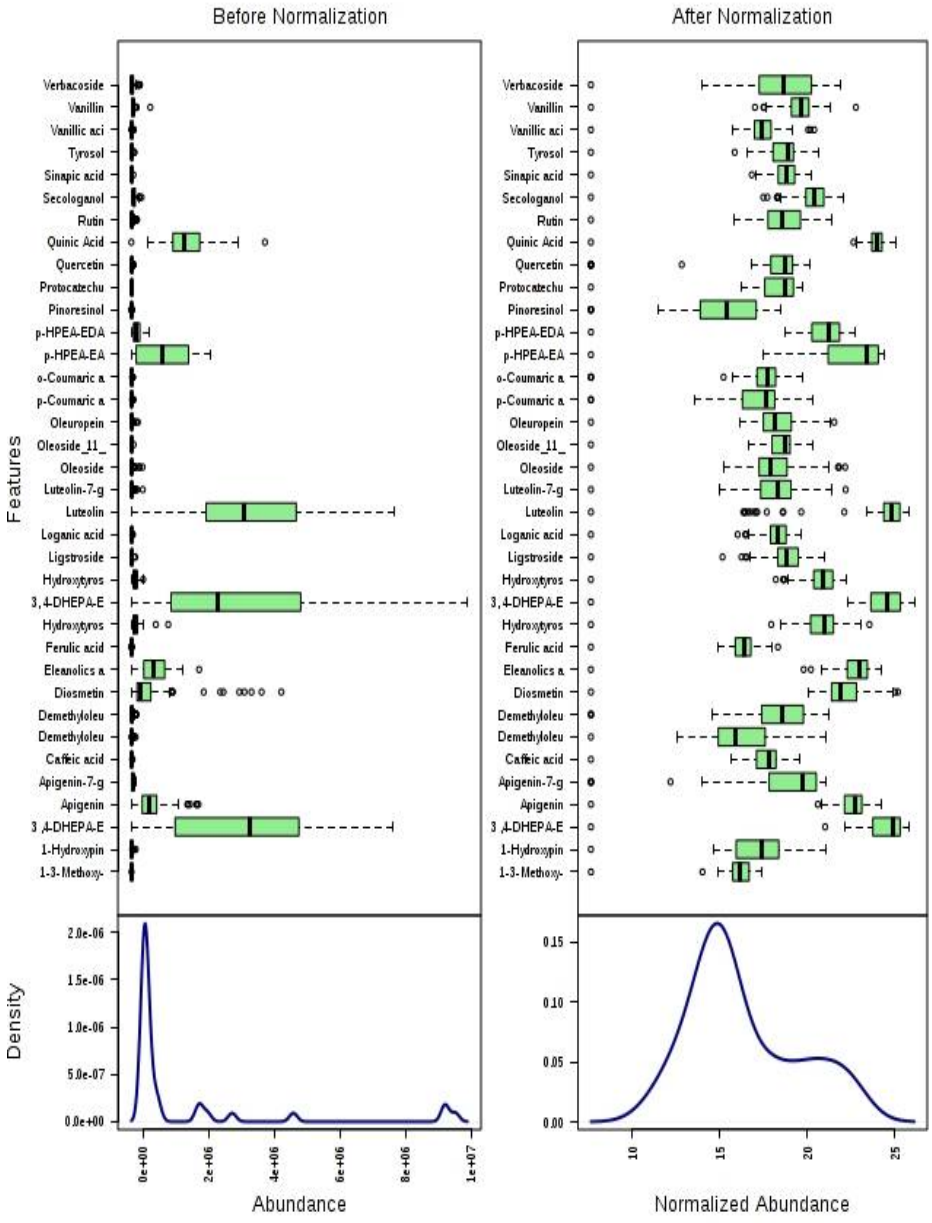
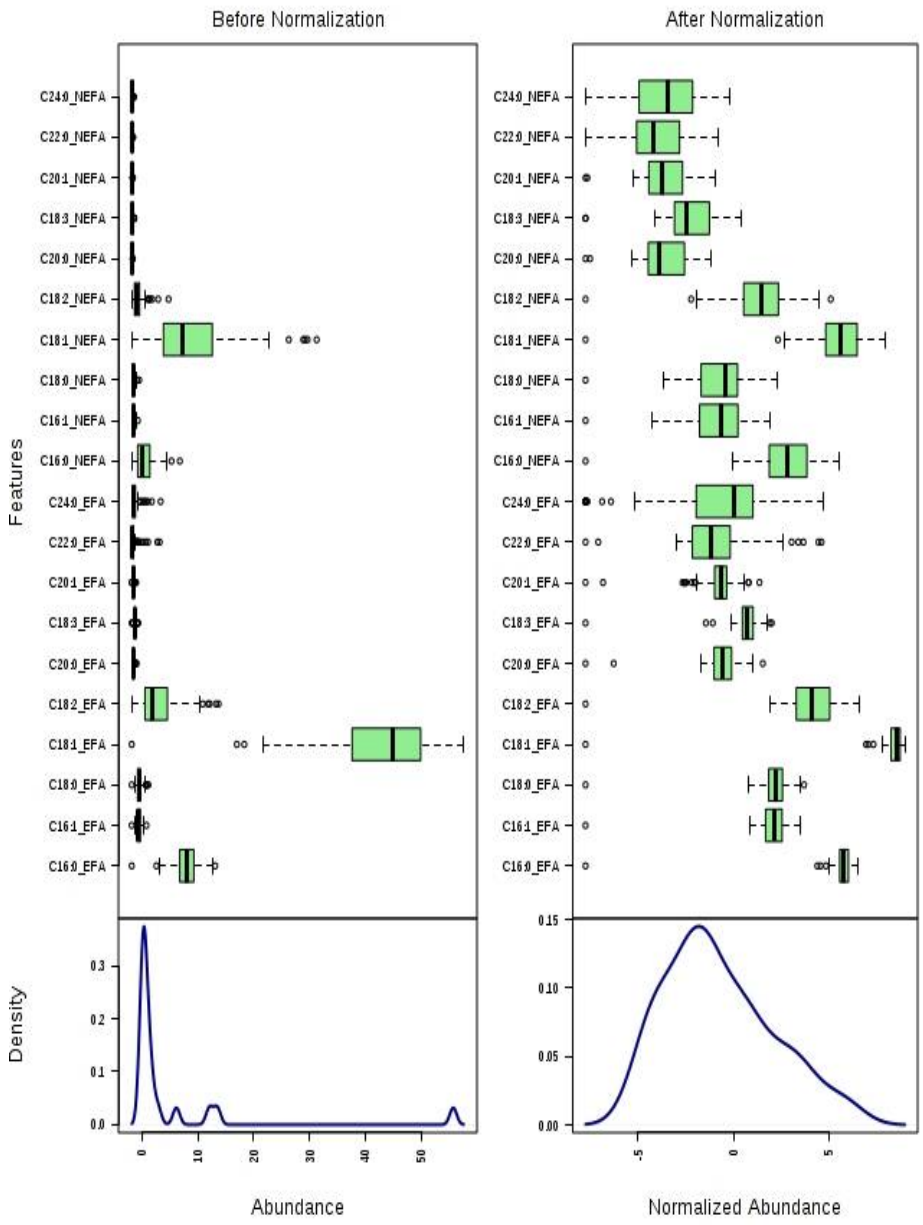
SUPPLEMENTARY MATERIAL

(A)

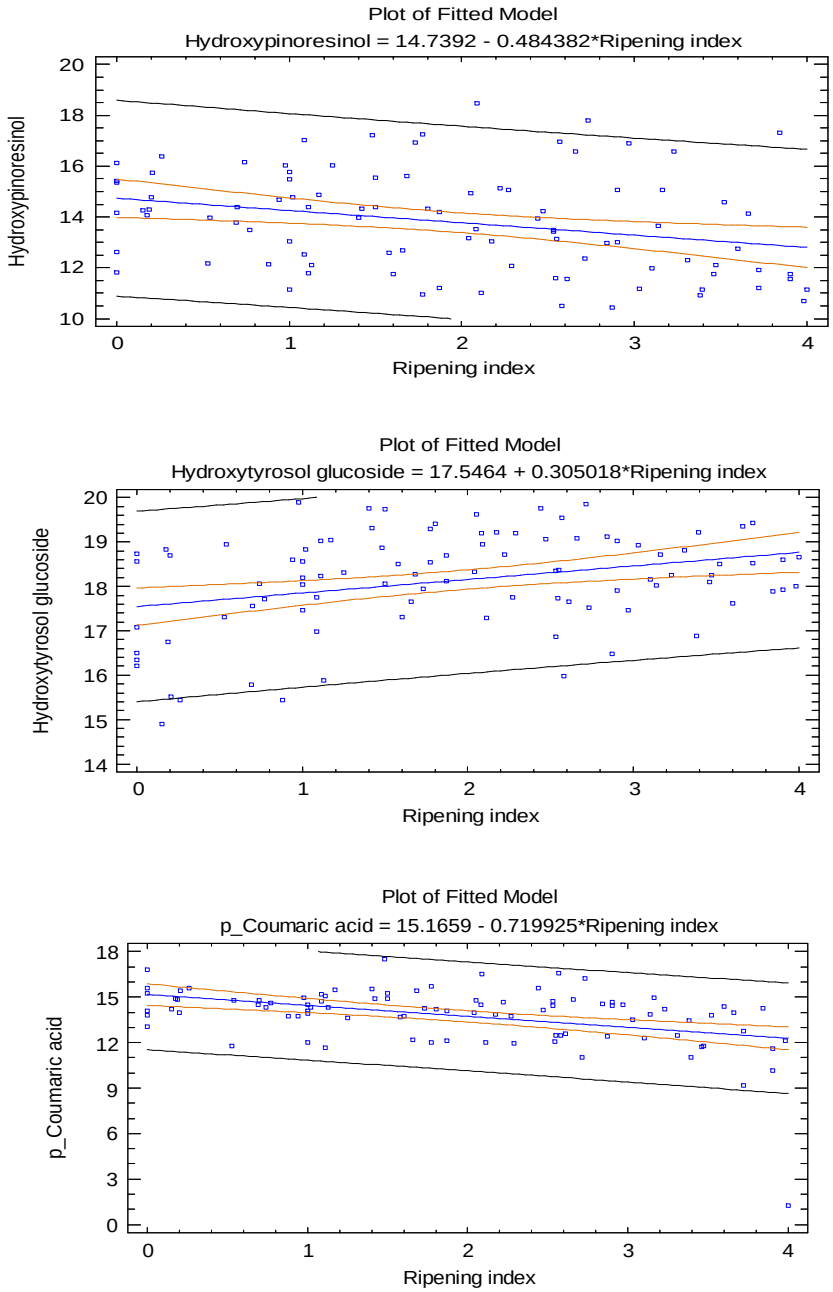


Supplementary Figure 1. Effect of the normalization step on the observed variability of (A) phenols and (B) FAs.

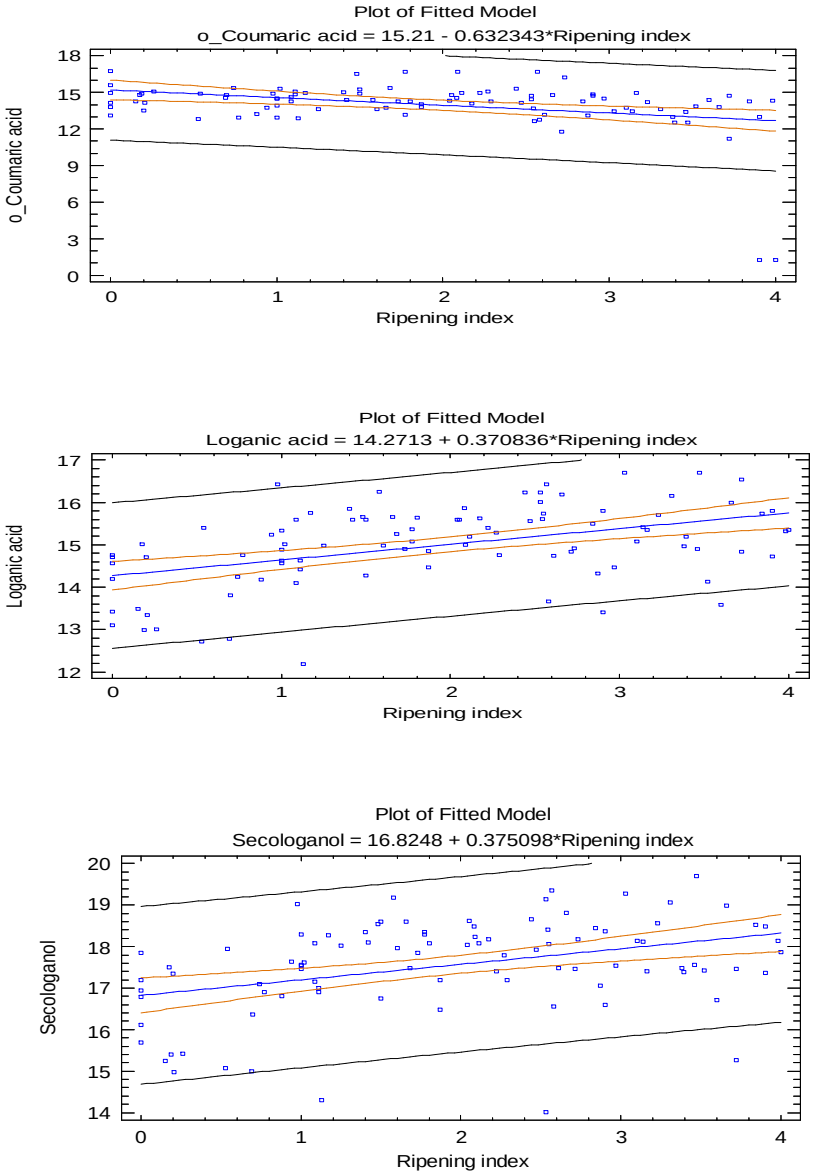
(B)



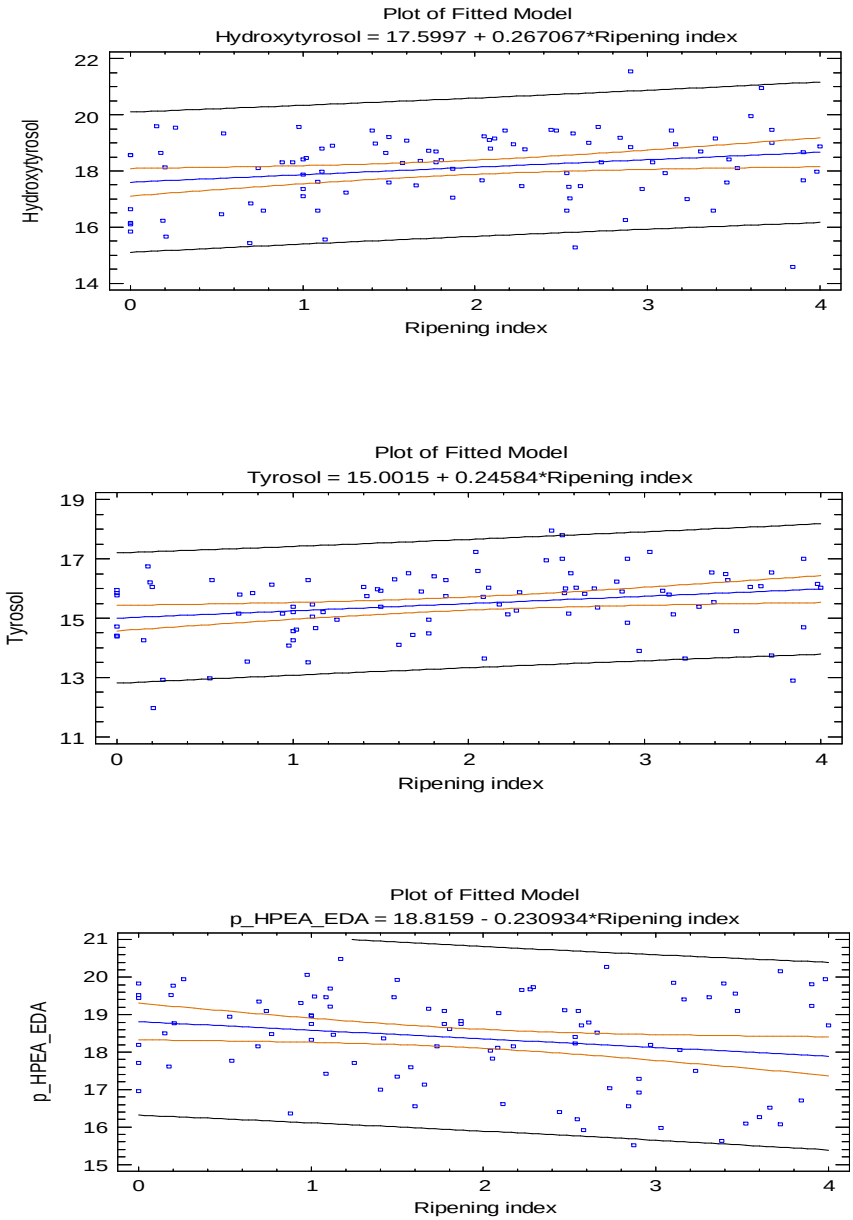
Supplementary Figure 1. Effect of the normalization step on the observed variability of (A) phenols and (B) FAs (cont.).



Supplementary Figure 2. Correlation between the relative concentration of the RI and significant phenolic compounds or derivatives.



Supplementary Figure 2. Correlation between the relative concentration of the RI and significant phenolic compounds or derivatives(cont.).



Supplementary Figure 2. Correlation between the relative concentration of the RI and significant phenolic compounds or derivatives (cont.).

SECCIÓN E: ENRIQUECIMIENTO DE ACEITES REFINADOS DE DIFERENTES SEMILLAS CON DESECHOS DE LA INDUSTRIA OLIVARERA Y COMPARACIÓN DE LOS PERFILES FENÓLICOS

Esta Sección E recoge un estudio que entronca directamente con una de las líneas de investigación del grupo en el que se integra la doctoranda (el aprovechamiento de residuos de la industria agroalimentaria) y constituye una nueva aportación para reforzar las aproximaciones a la explotación de los residuos y desechos de la industria agroalimentaria, de la cuenca mediterránea en general, de la española en particular y, en referencia concreta al olivo/aceite, de la andaluza de forma especial.

Para esta investigación se utilizaron dos tipos de desechos del olivo/aceite: Las hojas y el alperujo, de los que sus extractos ya habían puesto de manifiesto su semejanza en composición de compuestos antioxidantes característicos y su discordancia en la concentración relativa de estos compuestos.

En el Capítulo 11, primero de esta sección, se recoge el enriquecimiento de aceites con ambos tipos de extractos, de forma individual para cada uno de los fenoles característicos del olivo, cada aceite y cada tipo de extracto. La positiva influencia del enriquecimiento en los parámetros típicos de cada aceite (índices de acidez y de peróxidos y estabilidad según el método Rancimat —todos ellos expresados en valor absoluto y relativo al respectivo aceite no enriquecido) constituye un firme soporte para un potencial enriquecimiento a escala industrial.

El estudio del perfil fenólico de cada uno de los aceites enriquecidos en comparación con el VOO es el contenido del Capítulo 12. En él se recoge el análisis de todos estos aceites mediante LC–QTOF y el tratamiento quimiométrico apropiado de los perfiles obtenidos, que pone de manifiesto el grado de semejanza del aceite resultante de cada enriquecimiento con el patrón al que pretende asemejarse: El VOO.

CAPÍTULO 11:

Quality and stability of edible oils enriched with hydrophilic antioxidants from olive tree: the role of enrichment extracts and lipid composition

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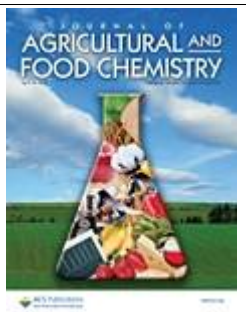

Quality and stability of edible oils enriched with hydrophilic antioxidants from olive tree: the role of enrichment extracts and lipid composition

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Quality and stability of edible oils enriched with hydrophilic antioxidants from olive tree: the role of enrichment extracts and lipid composition

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Abstract

Phenolic extracts from olive-tree leaves and olive pomace were used to enrich refined oils (*viz.* maize, soy, high-oleic sunflower, sunflower, olive, and rapeseed oils) at two concentration levels (200 and 400 μg/mL, expressed as gallic acid). The concentration of characteristic olive phenols in these extracts together with the lipidic composition of the oils to be enriched influenced the mass transfer of the target antioxidants, which conferred additional stability and quality parameters to the oils as a result. In general, all the oils experienced either a noticeable or dramatic improvement of their quality–stability parameters (*e.g.* peroxide index and Rancimat) as compared with their non-enriched counterpasses. The enriched oils were also compared with extra-virgin olive oil with a natural content in phenols of 400 μg/mL. The healthy properties of these phenols and the scarce or nil prices of the raw materials used can convert oils in supplemented foods or even nutra-ceuticals.

INTRODUCTION

Extra-virgin olive-oil, EVOO, is the most demanded liquid fat in the Mediterranean basin thanks to the nutraceutical properties of its components, divided into two groups as a function of their concentration: major and minor compounds. The first group, known as the saponifiable fraction, represents more than 98% of the total weight of the oil and consists of triacylglycerols, diacylglycerols, monoacylglycerols and free fatty acids. The second group, the unsaponifiable fraction, comprises about 2% of the total weight, and it is constituted by a great variety of compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants —most of the last being carotenes, tocopherols and phenols *(1)*. Contrarily to the majority of edible oils, EVOO can be consumed in crude form, conserving all beneficial properties typical of its minor components, the excellences of which have been discussed somewhere *(2, 3)*.

Within the unsaponifiable fraction, interest on olive phenols (OPs) has increased in recent decades, thus stimulating multidisciplinary research on their composition, histological distribution and histochemical localization to determine their biomolecular functions *(4)*. Nevertheless, the main reason of the growing research in this field lies in the antioxidant properties of OPs from clinical and pharmacological points of view. These proved excellent properties of OPs have promoted active research on raw materials for their isolation *(5–11)*. The two main sources of OPs are olive leaves and the pomace waste generated in the olive oil industry, known as alperujo. This is a polluting semisolid residue resulting from the two-phase olive oil extraction method, presently the method most frequently used in this industry. Alperujo is a cheap source of natural antioxidants, in concentrations up to 100-times higher than in olive oil *(8)* —which results from the polar nature of both alperujo and OPs and the low-polar nature of oil—; however, olive leaves have the highest antioxidant and scavenging power between the different parts of the olive three (*e.g.* taking oleuropein as an OP model, its content in

olive oil ranges between 0.005–0.12%; in alperujo up to 0.87% and in olive leaves between 1–14%) *(8, 12)*. Thus, both agricultural residues are well-characterized sources for extraction of OPs to take benefits from their antioxidant properties. The food industry is an active area with possibilities to exploit the high content of phenols present in olive-tree materials.

The addition of synthetic oxidation inhibitors to refined edible oils to improve their stability-related properties is a common practice. However, the reported deleterious effects on human health of these synthetic additives such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, or tertiary-butyhydroquinone have decreased their use and promoted the general consumers' rejections of synthetic food additives *(13, 14)*. As a result, enrichment of edible oils with natural antioxidants to inhibit or suppress oil oxidation becomes of great interest. These substances ought to be cheap and do not produce any deleterious compounds under oxidation conditions such as deep frying.

As EVOO contains a high amount of natural antioxidants, addition of artificial antioxidants is unnecessary, in general. Nevertheless, in many countries the market for olive oil is limited; thus, it is replaced by a mixture of refined olive oil, seed oil or others with minimum or no phenols contents. Taking into account the high price of olive oil and the antioxidant and nutraceutical properties of olive phenols, there is a growing interest in the use of these compounds to enrich low-priced oils *(15, 16)* in order to obtain a healthy added-value product.

Conferring to other oils oxidative stability properties similar to EVOO involves two main steps: extraction of the target compounds from the raw material, either leaves or alperujo, and enrichment of the oil with the extract. The extraction step is a cheap task because of the low-price of the raw materials from which OPs can be obtained, facilitated by the number of methods accelerated by auxiliary energies developed in the last decade *(5–11)*. Previous laboratory-scale methods for OPs extraction used extractants such

as methanol–water mixtures *(12)* or hexane *(17)*, but the increased human use of these compounds made mandatory the development of methods based on non toxic extractants; so water or ethanol–water mixtures as extractants helped by some type of energy is the present trend. Thus, methods for extraction of OPs from either leaves or alperujo with assistance by ultrasound *(5)*, superheated extractants *(6)*, or microwaves *(7)* have endowed this task with rapidity and automation, two key characteristics to facilitate industrial implementation. Microwaves have shown to be the fastest and easiest alternative *(18)*, so it was the type of energy selected to accelerate the obtainment of the extracts used in the present study.

Concerning the enrichment step, there are three alternatives in the literature for oil enrichment with these valuable compounds from the olive tree: 1) liquid–liquid extraction *(19)*, in which the oil is put into contact with an alcoholic extract of phenols, which are transferred to the oily phase as a function of their distribution factor removing the alcoholic phase by centrifugation; 2) solid–liquid extraction *(20)*, in which the purified phenolic extract is dried under appropriate conditions and the paste obtained is partially dissolved into the oil as a function of the solubility of the different paste components in the oily phase; and, 3) a combination of these procedures, in which the alcoholic extract and the oil are put into contact and the two-phase system is subject to alcohol removal in a rotary evaporator. This last has been the procedure used for enrichment of the target oils used in the present research.

The improvement of healthy-related properties of oils is the main final goal pursued with the present and similar studies *(21)*, but also enlargement of stability properties, usually determined by a series of well-established tests (*e.g.* Rancimat test, peroxide index), which also provide information about resistance to changes during frying *(22– 24)*.

The present research is focused on the assessment of quality and stability properties of refined edible oils enriched with phenolic antioxidants

from olive materials. With this aim, several refined oils (that is, oils in which polar antioxidants are absent as they are massively removed during the refining process) have been enriched with OPs extracts from both olive leaves and alperujo and at two different levels of total antioxidants: 200 and 400 μg/mL. The stability conferred to the oils by the presence and amount of these OPs has been checked by using the established methods for these studies as a function both of the lipid composition of the oils and the relative concentration of the individual phenols in the extracts, which is different for the extract from each raw material.

MATERIALS AND METHODS

Samples

Alperujo obtained during the 2009/2010 crop season was taken directly from the production line in Núñez de Prado, C.B. (Córdoba, Spain) and stored at –20 ºC until use. Olive leaves from the Picual, Picudo and Hojiblanca cultivars were selected for this research, collected at the end of October, dried at 35 ºC for 60 h, milled by a cyclonic mill up to homogeneous particle size (diameter ≤ 0.5 mm) and kept at 4 ºC until use.

The vegetable edible oils used in this research were refined maize oil (RMO), refined soy oil (RSoO), refined high-oleic sunflower oil (RHSO), refined sunflower oil (RSO), refined olive oil (ROO), refined rapeseed oil (RRO) and extra virgin olive oil (EVOO). All of them were provided by Carbonell (SOS Cuétara S.A, Madrid). The criteria for selection were low-price oils (less than 1 euro/Kg), and wide range of lipid composition.

Reagents

The reagents used for characterization of vegetable oils were: LC grade methanol, acetonitrile, hexane and absolute ethanol from Scharlab (Barcelona, Spain); sodium carbonate, sodium chloride and Folin–Ciocalteu (F–C) reagent from Panreac (Barcelona, Spain); ortophosphoric acid for acidification of mobile phases in liquid chromatography and gallic acid, as standard for quantification in the F–C test, from Merck (Darmstadt,

Germany). Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford) was used to prepare mobile chromatographic phases. 0.2 N sodium methylate in methanol (Panreac) was used as derivatization reagent to hydrolyse and transform the fat into fatty-acid methyl-esters (FAMEs).

The reagents used for quality studies were: analysis-grade ethanol, ethyl ether, acetic acid, chloroform, potassium hydroxide, potassium iodide and sodium thiosulfate, all them from Panreac; phenolphthalein and potato starch from Panreac for acidity test and peroxide index, respectively; and cyclohexane from Prolabo (Geldenaa Ksebaan, Leuven) used for spectrophotometric tests.

The most abundant phenolic compounds in olive oil were purchased from Extrasynthese (Genay, France) in the case of hydroxytyrosol, tyrosol, oleuropein, apigenin and luteolin; meanwhile vanilline, vanillic acid, *p*- and *o*-coumaric acids, ferulic acid and the external standard *p*-cresol were from Merck. The stock standard solution of each phenol was prepared at 1000 μg/mL by dissolving 10-mg each phenol in 10 mL methanol. The multistandard solutions containing 10 phenols were prepared by mixing the appropriate volume of each stock solution and diluting them as required in a 60:40 methanol–water solution. All these solutions were stored in the dark at −20 ºC in glass vials until use. *p*-Cresol from Merck was used as external standard in the quantification of phenolic compounds.

Apparatus and Instruments

A Tecator Cyclotec cyclonic mill (Hoganas, Sweden) was used to grind the leaves. Microwave irradiation was applied by means of a MIC-II focused-microwave extraction system of 400 W maximum power (Puebla, Mexico) furnished with a manual power control unit. A Selecta Mixtasel centrifuge (Barcelona, Spain) was used to remove solid particles from the extract. A Büchi R-200 rotary evaporator (Postfach, Switzerland) furnished with a B-490 heating bath was used to concentrate the phenol extracts after

microwave-assisted extraction (MAE), and to evaporate traces of ethanol in the enriched oils. A Selecta Vibromatic electrical stirrer (Barcelona, Spain) was used to favour the liquid–liquid extraction of phenols. An MS2 minishaker from Ika (Wilmington, USA) was used to favour phenols transfer from oil to methanol for individual quantification of the target compounds.

A Varian ProStar liquid chromatograph (Walnut Creek, CA), consisting of a ProStar 240 pump, a ProStar 330 diode array detector (DAD) and a ProStar 410 Autosampler, was used for individual determination of antioxidant compounds. The analytical column was a C18 Inerstil ODS-2 (250×4.6 mm i.d. 5 μm) from GL Sciences Inc (Tokio, Japan).

A Perkin-Elmer Clarus 500 gas chromatograph (Massachussetts, USA) with a flame ionization detector (FID) equipped with a programmable-temperature injector and a Phenomenex BPX70 analytical column (50 m, i.d 0,22 mm, film 0,25 μm) was used for determination of FAMEs.

A ThermoSpectronic Helios UV/vis spectrophotometer was used for determination of total phenols concentration by the F–C method, and a Lambda 25 Perkin-Elmer single-beam UV/vis spectrophotometer was used to monitor K232 and K270 parameters.

A Metrohm 679 Rancimat (Herisau, Switzerland) was used to determine the stability of vegetable oils.

Confirmatory analysis for identification of olive phenols were carried out with an Agilent 1200 Series LC system interfaced to an Agilent 6540 UHD Accurate-Mass TOF LC/MS detector (Palo Alto, USA), equipped with an Agilent Jet Stream Technology electrospray ion source operating in the negative ion mode.

Procedure for Extraction of Phenols from Alperujo or Leaves

The procedure was similar to that proposed by Girón *et al. (14)* and Japón *et al. (19)*. Briefly, 12 g of alperujo or leaves and 100 mL ethanol were placed into the quartz extraction vessel located in the zone of focused microwave irradiation of the extractor (a total of 500 g of alperujo and 300 g

of leaves were used for enrichment of the target oils). After extraction (10-min microwave irradiation at 400 W), the suspension was centrifuged at 855 *g* for 5 min for phases separation. This process was repeated as many times as required to obtain the necessary extract for the subsequent enrichment step after the extract had been concentrated in a rotary evaporator at 35 ºC to reduce ten times its initial volume. The extract thus obtained was reconstituted in 200 mL ethanol prior to measurement of total phenols concentration by the F–C test.

Folin–Ciocalteu Test for Measurement of Total Phenols Concentration

Briefly, 20-μL sample (with prior 1:50 dilution with water) was, in this order, mixed with 1.58 mL water, 0.3 mL 20% (w/v) Na_2CO_3 aqueous solution, 0.1 mL F–C reagent, and heated in an oven for 5 min at 50 ºC. Then, the resulting solution was allowed to stand 30 min. The reaction product was photometrically monitored at 765 nm. Gallic acid was used as standard for calibration, so the results were expressed as equivalent of gallic acid (GAE) per gram or milliliter *(25)*.

Enrichment of Edible Vegetable Oils with Phenols Extracts from Alperujo or Leaves

The enrichment was carried out at two concentration levels (200 and 400 μg/mL of phenols according to the F–C test) per each oil and with each of the two extracts (from alperujo and from leaves); that is, four enriched oils were obtained from each original oil. In all cases, an aliquot of the corresponding ethanolic extract was put into contact with 200 mL oil, and the ethanol in the two-phase system was evaporated in the rotary evaporator at 30 ºC. Then, the mixture was shaken in the electrical stirrer at 700 U/min to favour enrichment. This process was repeated as many times as required until the 200 mL oil portions were enriched in phenols from each of the extracts up to 200 or 400 μg/mL (as determined by the F–C method). Distinction between the different oils, extract for enrichment and enrichment

degree is as follows: abbreviation as under "Samples" —capital letters— is used for 400 μg/mL enrichment, followed by point and the initial of the raw material to prepare the extract (A for alperujo and L for leaves). Similar nomenclature, but case letters, is used for oils enriched with 200 μg/mL phenols. As example, RMO.A and rmo.a correspond to refined maize oil enriched with 400 μg/mL and 200 μg/mL, respectively, of phenols from alperujo; while RMO.L and rmo.l correspond to the high and low enrichment, respectively, from leaves.

Characterization of Phenols and Fatty Composition of Pure and Enriched Oils

Phenols were photometrically determined by DAD after individual separation by HPLC. For doing this, 0.5 g of either pure or enriched oil was shaken with 1 mL hexane and 1 mL 60:40 methanol–water mixture for 1 min in the MS2 minishaker at 1200 U/min. The polar phase contained *p*-cresol as internal standard (IS), at 5 μg/mL as final concentration. The hydroalcoholic phase was injected into the chromatograph. The mobile phases were: A (0.2% H_3PO_4 in water) and B (methanol). The gradient program, at 1 mL/min constant flow-rate, was as follows: initially 96% A and 4% B; 0–40 min, 96–50% A and 4–50% B; 40–45 min, 50–40% A and 50–60% B; 45–60 min, 40–0% A and 60–100% B; 60–70 min, 0% A and 100% B. After analysis, the column was reequilibrated for 9 min. The chromatograms were acquired at 230, 280, 325, and 350 nm (wavelengths of maximum absorption for the different phenols). LC–DAD quantitative analysis was expressed as area of the target analyte/area of ES for all analytes. Compounds with no calibration standards were quantified by the calibration curve of the phenol with a more similar structure. Thus, aglycon secoiridoids were quantified by the oleuropein calibration curve.

Fatty acids were individually separated and quantified by GC–FID after derivatization by methylation to more volatile compounds. With this aim, 0.5 g of oil was mixed with 6-mL sodium methylate in a test tube, which

was introduced in a sand bath at 150 ºC for 10 min and then cooled. After cooling, 2 mL hexane and 4 mL of a saturated sodium chloride solution were added to the test tube, which was shaken for 5 s. The upper phase was collected, and 1 μL of this phase was injected into the gas chromatograph. The injector and detector temperature was 290 ºC. The initial oven temperature was 175 ºC, maintained for 24 min, then risen at 10 ºC/min to 240 ºC, at which it was kept constant for 7.5 min.

LC–TOF/MS Confirmatory Analysis of Olive Phenols

Olive phenols identification was conducted by LC–TOF/MS confirmatory analysis in accurate mode due to the complexity of OPs extracts from EVOO. The extraction and separation conditions were identical to those for the LC–DAD determination, except for the use of the respective LC–MS/MS grade solvents.

The phenolic fraction present in aliquots of EVOO and enriched oils was extracted and the extracts injected into the LC–TOF/MS system without additional pretreatment. The injected extract volume was 20 μL. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes were governed via the Agilent MassHunter Workstation software. The mass range and detection window were set at m/z 100–1100 and 100 ppm, respectively. Reference mass correction on each sample was performed with a continuous infusion of Agilent TOF biopolymer analysis mixture containing purine (m/z 121.0508) and hexamethoxyphosphazyne (m/z 322.0481) with resolution of 45 000. Analytes were identified by accurate mass detection.

Molecular features were extracted from raw data files prior formula generation. Two or more ions were used as compound ion count threshold. The isotope model corresponded to common organic molecules with peak

spacing tolerance of m/z 0.0025 ± 7.0 ppm. Identification of the compounds proceeded by generation of candidate formulae with a mass accuracy limit of 4 ppm. The contribution to mass accuracy, isotope abundance and isotope spacing scores was 100.00, 60.00 and 50.00, respectively. Retention times, formulae, experimental and theoretical masses, and errors, in ppm, obtained by accurate mass measurements of secoiridoids compounds are shown in **Supplementary Table 1**. After confirmation, LC–DAD chromatographic peaks were assigned to OPs by retention times (both absolute and relative to external standard and other phenols monitored).

Quality Evaluation

Different tests were carried out to compare the quality of the edible oil before and after enrichment. Thus, the acidity index was calculated by titration with 0.1 M KOH and 2% phenolphthalein as indicator *(26)*.

This evaluation was completed with the determination of K232 and K270 parameters by spectrophotometric absorption of cyclohexane oil solutions at 232 and 270 nm, respectively (*27)*. Since these parameters are exclusive to evaluate the quality of olive oils, they were only measured in refined olive oil, then compared with those of EVOO.

Stability Study

Two complementary tests, peroxide index and Rancimat test, were carried out to compare the stability of the vegetable oils. The peroxide index was estimated by titration of 0.1 N KI saturated solutions of each oil with 0.1 N $Na_2S_2O_3$ and starch as indicator according to the official method (*24)*.

The Rancimat test was employed to evaluate the stability of target oils by conductimetry measurements of an aqueous solution where the volatile compounds were collected after heating each oil at 120 ºC *(28)*. The concentration of polar compounds formed by oil oxidation increased the conductivity with time. During the induction period, the conductivity of the water increases very slightly; then, a high increase of conductivity indicates a massive oxidation: formation of a number of volatile polar compounds

which are soluble in water. The stability of the oil is given by the induction period.

RESULTS AND DISCUSSION

Comparison of Phenol Extracts from Alperujo or Olive Leaves

According to previous studies developed with olive-tree biomass, alperujo and olive leaves were selected as raw materials for isolation of OPs since they provide different phenolic profiles, as reported by Japón-Luján *et al.(6, 29)*. This was confirmed in this research, as **Table 1** shows, for the concentration of the main phenols present in extracts from alperujo and olive leaves. As can be seen, alperujo is richer in secoiridoid hydrolysis compounds than leaves, while the latter are highly concentrated in oleuropein. Thus, hydroxytyrosol was found in alperujo extracts at concentrations four times higher than in leaves, and tyrosol was found in alperujo at concentrations around 20 μg/g, and non- detected in leaf extracts. These two compounds, hydroxytyrosol and tyrosol, are well-known because of their powerful antioxidant properties *(2, 3, 30)*. Similar results were obtained for other hydrolysis products from oleuropein and ligstroside such as the dialdehydic forms of secoiridoid aglycons, which were exclusively detected in alperujo extracts. Aglycon forms in EVOO included interesting compounds from an organoleptical point of view such as dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA), dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol (*p*-HPEA-EDA) and the aglycon of the dialdehydic form of ligstroside (*p*-HPEA-FA). Following with extracts comparison, two flavonoids such as luteolin and apigenin were also more concentrated in alperujo extracts. In fact, apigenin was not detected in extracts from olive leaves.

Table 1. Concentration of the main olive phenols found in the extracts from leaves and alperujo.

Compound	Leaf extract Concentration (μg/g)	Alperujo extract Concentration (μg/g)
Hydroxytyrosol	27.3	106.7
Tyrosol	< LOD	18.5
Vanillic acid	51.4	< LOD
Vanillin	7.2	< LOD
***p*-Coumaric acid**	< LOD	< LOD
Ferulic acid	10.0	< LOD
***o*-Coumaric acid**	< LOD	< LOD
3,4,DHPEA-EDA[a]	< LOD	2288.0
Oleuropein	2479.0	820.0
***p*-HPEA-EDA**[b]	< LOD	495.0
***p*-HPEA-FA**[c]	< LOD	372.0
Luteolin	11.9	56.3
Apigenin	< LOD	13.4

[a]dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol; [b]dialdehydic form of decarboxymethyl elenolic acid linked to tyrosol; [c]aglycon of the dialdehydic form of ligstroside.

On the contrary, the secoiridoid precursor of compounds previously described, oleuropein, was three times more concentrated in olive leaves than in alperujo (2480 μg/g *versus* 820 μg/g). Thus, alperujo extracts are well-balanced in the concentration of powerful antioxidants such as hydroxytyrosol, tyrosol and other secoiridoid hydrolysis products, while leaf extracts are highly concentrated in oleuropein as one of the main precursors of these compounds. This different composition is a consequence of the conditions under which the raw materials are obtained. Alperujo was

obtained from olive fruits at their optimum ripening stage with an advanced hydrolysis state of secoiridoid precursors such as oleuropein and ligstroside, thus reducing their presence in EVOO and their contribution to undesired bitterness of this oil. To finalize this comparison, simple phenols such as vanillic or ferulic acids and vanillin were not detected in alperujo extracts.

Discussion of the richness of each raw material in each of the most valuable phenols was based on the identical procedure for extraction followed in all cases: the same weight of raw material, type and volume of extractant and time and working conditions for extraction, as well as degree of evaporation of the extractant for extract concentration.

Enrichment of Refined Edible Oils with Phenol Extracts from Alperujo or Olive Leaves

Refining of edible oils allows the maximum amount of oil to be extracted from seeds by an economical process. By this process, all impurities and natural flavours are removed and free-fatty acids neutralized. This is carried out by subjecting oils to heat, solvent extraction, filtering, neutralization, distilling, degumming, bleaching and high-heat deodorization. As a result of the operational conditions of these steps, polar compounds, including phenolic antioxidants, are completely removed. This can be checked in **Supplementary Figure 1** that illustrates chromatograms obtained by analysis of polar phenolic extracts from some of the target oils. As can be seen, no phenolic compounds were detected in these oils as compared to EVOO, which justify the purpose of enrichment of these refined oils with OPs to improve their quality and stability.

The two levels, 200 and 400 μg/mL, for enrichment of target oils with phenols extracts from alperujo and olive leaves (two levels per extract) were selected as representative values because 200-to-400 μg/mL is the usual concentration range of phenols in EVOO, the model oil naturally containing phenols similar to those used for enrichment of the other oils.

The enrichment step was similarly performed for the different oils and degree of enrichment as programmed. Distinction among the different oils, different degree of enrichment and different extract used for enrichment, are discussed using the abbreviations under experimental. The discussions exposed below concern first to the degree of individual enrichment of the most important phenols as a function of the extracts and, then, to the fatty acids profile in the monitored oils.

Mass-Transfer of Phenols to the Oils as a Function of the Extract. The richness of the extracts in the target phenols explains the mass-transfer behaviour of them and allows distinguishing between those with higher transfer from alperujo extracts (a), those with higher transfer from olive leaf extracts (b) and, those which only are transferred from alperujo extracts (c). **Supplementary Table 2** lists the concentration of OPs in the target oils before and after enrichment revealing changes ascribed to the latter. **Supplementary Figure 2**, which plots in a comparative way by bar diagrams the concentration of each individual phenol in each of the oils, and **Figure 1**, which plots the behaviour in the enrichment process of the most representative phenols, supports the discussion below:

(a) Alperujo extracts transfer to all target oils amounts of hydroxytyrosol, tyrosol, 3,4-DHPEA-EDA and luteolin that widely surpass the content of these compounds in EVOO (*e.g.* between 15 μg/g and 40 μg/g in the case of hydroxytyrosol, while in EVOO the usual content does not surpass 10 μg/g) (**Figure 1**). This behaviour was foreseeable as the massive hydrolysis of secoiridoid precursors such as oleuropein occurs at the optimum ripening stage for collection of olive fruits. Additionally, the rich-in-water content of alperujo after oil separation also favours secoiridoid hydrolysis to release hydroxytyrosol and tyrosol as well as aglycon forms. The high content of hydroxytyrosol in all oils after enrichment with alperujo extracts increases their antioxidant power and endows them with the healthy properties attributed to this phenol *(2, 3, 30)*. The decarboxymethyl oleuro-

pein aglycon was also highly enriched in all oils by using alperujo, which could be justified by its less polar character. This phenol was concentrated up to values close to 600 μg/g expressed as oleuropein. This compound is frequently associated to a positive organoleptic property of EVOO being also a significant source of hydroxytyrosol. A lower enrichment factor was found for *p*-HPEA-EDA, which was lower than that observed in EVOO, and undetectable in the case of RMO and RSoO.

Concerning luteolin, especially noticeable is its transfer to RSoO, in the order of 12 μg/g. Vanillic acid, that seems to be absent from alperujo extracts (see **Table 1**), is in fact in these extracts, but the high dilution required for their chromatographic analysis lowers its concentration below its detection limit. Nevertheless, extractant evaporation increases the concentration of all phenols in the extract during oils enrichment, thus promoting a significant mass transfer of this acid to the oils, as listed in **Supplementary Table 2**. Similar behaviour is observed for *p*-coumaric acid, but in a lesser extension as it does not appear in the oils subjected to the lower-concentration enrichment.

(b) Olive leaf extracts, because of their higher content in oleuropein, perform better mass-transfer of this phenol to the target oils, as compared with alperujo extracts, all widely surpassing the natural content of oleuropein in EVOO, which is practically undetectable because of the ripening state of the fruit for oil production (**Figure 1**). Ferulic acid, only present in olive leaf extracts, is transferred to RMO and RRO in a quantifiable concentration only in the higher enrichment degree (400 μg/mL total enrichment).

(c) Phenols only transferred from alperujo extracts are tyrosol (either surpassing or equalizing the natural content in EVOO), *p*-HPEA-FA —in this case never surpassing the natural content in EVOO, see **Figure 1**—, and apigenin (equalizing the content in EVOO in the enrichment up to 400 μg/mL in total phenols).

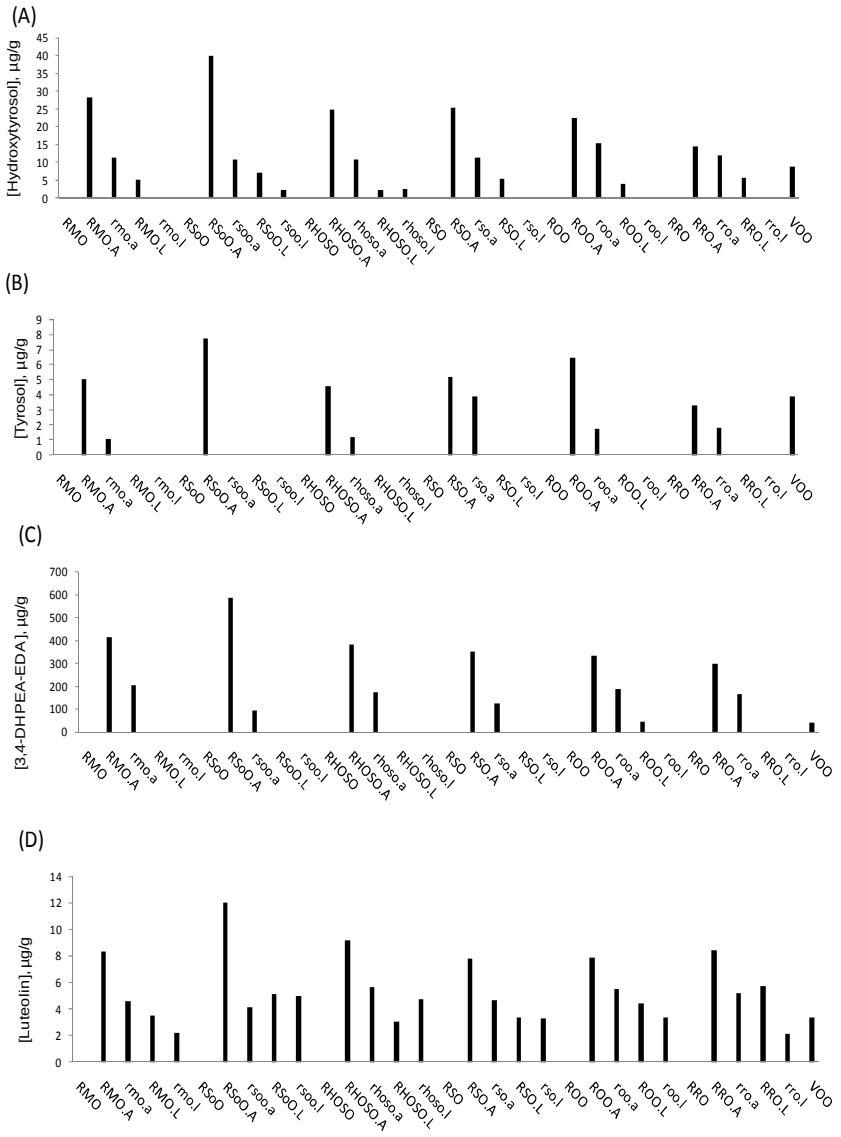

Figure 1. Oils enrichment in: (A) hydroxytyrosol, (B) tyrosol, (C) 3,4-DHPEA-EDA and (D) luteolin.

Mass-Transfer of Phenols to the Oils as a Function of their Fatty Acid Composition. The mass transfer of phenols to the target oils was related to their fatty acid composition, which is exposed in **Table 2** (concentrations expressed as percentage). Thus, the most polar OP and that with higher

antioxidant power, hydroxytyrosol, experienced a higher transfer to oils with lower content in oleic acid and higher concentration of polyunsaturated acids. For this reason, the oil with the highest mass transfer was RSoO, with the lowest content of oleic acid and the highest content of polyunsaturated acids. RMO, the second oil with higher enrichment in hydroxytyrosol, is also the second oil with lower content in oleic acid and with a similar content in polyunsaturated acids to RsoO. This trend was not followed for the rest of the oils and similar enrichment was observed for RHOSO, RSO and ROO. The lowest enrichment in hydroxytyrosol was found in RRO. Exactly the same trend was observed for an intermediate in the pathway from oleuropein to hydroxytyrosol such as 3,4-DHPEA-EDA.

The behaviour of tyrosol in mass-transfer from extracts to oils was similar to that of hydroxytyrosol. Thus, RSoO was the most enriched, followed by ROO; while the other oils experienced lower enrichment, but similar among them, except for RRO, which repeated as the lowest oil enriched by this phenol. Only alperujo extracts are able to enrich oils with this phenol as this was not detected in leaf extracts.

Ferulic acid only enrichs RRO and RMO, both with a linolenic acid content similar to EVOO. No mass transfer of this phenol was observed with alperujo extracts. Similarly behaves *p*-HPEA-EDA, but in this case the most enriched oil was RSoO.

Oleuropein enrichs preferably RMO, then RSoS both clearly surpassing the enrichment experienced by the other oils. *p*-HPEA-EDA is preferably transferred to RHOSO, then to ROO and RRO: the oils with higher content in oleic acid. *p*-HPEA-FA mainly enrichs RsoO; then, and in a similar way, enrichs RHOSO, ROO RRO and RMO; finally, RSO, with a composition less similar to EVOO, is the lowest enriched.

Table 2. Percent of fatty acids in the pure oils used in this study.

Fatty acid	Formula	RMO	RSoO	RHSO	RSO	ROO	RRO	VOO
Miristic acid	C14:0	0.03	0.07	0.04	0.06	0.01	0.05	0.01
Palmitic acid	C16:0	10.86	10.88	4.06	6.49	10.48	4.59	10.50
Palmitoleic acid	C16:1	0.13	0.10	0.13	0.17	0.86	0.24	0.76
Margaric acid	C17:0	0.07	0.08	0.03	0.04	0.09	0.11	0.10
Margaroleic acid	C17:1	0.03	0.05	0.03	0.03	0.16	0.06	0.14
Stearic acid	C18:0	2.07	3.05	3.86	3.44	3.05	1.64	3.53
trans-Oleic acid	C18:1n9t	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Oleic acid	C18:1n9c	32.69	26.00	76.42	32.64	76.86	64.09	77.49
trans-Linoleic acid	C18:2n9,12t	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Linoleic acid	C18:2	52.28	54.11	13.50	55.70	7.31	18.71	6.05
Linolenic acid	C18:3	0.73	4.48	0.11	0.09	0.65	8.49	0.61
Arachidic acid	C20:0	0.45	0.29	0.32	0.24	0.24	0.50	0.39
Gadoleic acid	C20:1n9	0.26	0.22	0.17	0.14	0.18	1.08	0.25
Behenic acid	C22:0	0.18	0.47	1.01	0.69	0.08	0.33	0.11
Lignoceric acid	C24:0	0.22	0.19	0.32	0.25	0.03	0.12	0.06

Luteolin is transferred to all oils at higher concentrations from alperujo extracts, which quintuplicates the content of this phenol as compared with leaf extracts. All oils, when enriched at 400 μg/mL, clearly surpass the concentration of luteolin in EVOO.

Apigenin, only present in alperujo extracts, provides oils with a concentration similar to that naturally present in EVOO when they are enriched with 400 μg/mL of total phenols.

In the light of **Figure 1**, **Supplementary Fig. 2**, and, specially, **Supplementary Table 2**, the decision of preparing tailor-made enriched oils with high content of given olive-tree phenols can be adopted as a function of both type of extract and oil. Thus, according to previous research *(14)*, corroborated by the present study, (i) oils with high content of saturated fatty acids are less prone to enrichment, particularly with the most polar phenols in the target extracts; (ii) oils with high content of monounsaturated fatty acids are easier enriched with the target phenols as the mass transfer is facilitated by these type of acids; (iii) oils with high content of polyunsaturated acids have even easily enriched and with higher content of the most polar phenols that monounsaturated ones, but the difference of the behaviour between oils with high content of mono- and polyunsaturated fatty acids is not so important as between these two (mono and polyunsaturated) and those oils with high content of unsaturated fatty acids.

Quality Tests

Common tests used in the industry and standardization organisms to evaluate the quality of olive oil were applied to compare the results obtained for the target oils, before and after enrichment, to those of EVOO. In this way, the effect of enrichment on quality characteristics can be assessed.

Acidity Index. This quality parameter, which is an indicator of the concentration of free fatty-acids (usually expressed as percent of free oleic acid), decreases with increased quality of the raw material for oil production. According to legislation *(26)*, its concentration, in the case of olive oil, should not surpass 0.8%, being lower than 0.3% when obtained from high-quality olive fruits collected at their optimum ripening stage.

As can be concluded from **Figure 2.A**, the acidity index increased slightly by the mass transfer of phenols to the target oils with a maximum

increase of 0.15 units. The increase was particularly significant in oils enriched at higher phenols concentration with both types of extracts, except for RMO enriched with leaf extracts. Nevertheless, the change of acidity index was within an acceptable range. Therefore, there was not a critical contribution to this parameter caused by the use of extracts from alperujo or olive leaves for oil enrichment.

Determination of K232 and K270 Parameters. These parameters are only measured in olive oils with different quality grade being one of the main characteristics for classification. For this reason, these parameters were only measured in pure and enriched refined olive oil to be compared with EVOO. Despite K232 and K270 parameteres are used to classify the quality of olive oils, they are strongly related to the stability and purity of oils, storage and technological aspects for oils extraction. In fact, K232 enables to evaluate the oxidative deterioration of oils at short-term (primary oxidation of conjugated dienes) while K270 does at long-term by contribution of other species (secondary oxidation of trienes). Thus, EVOO reported acceptable values of 1.74 and 0.18 absorbance arbitrary units (AU) for K232 and K270 indexes, respectively, *versus* ROO that gave values of 2.22 and 0.33 AU. Therefore, the difference between EVOO and ROO is clear attending to these parameters; however, the enrichment process modified positively these values. Thus, K270 decreased to 0.30 and 0.27 AU for enrichment of ROO with antioxidants from alperujo at 400 and 200 μg/mL, respectively. In case of ROO enriched with antioxidants from leaves at 400 and 200 μg/mL, K270 was 0.34 and 0.28, respectively. A similar behaviour was found for K232, which decreased to 1.94 and 1.89 AU for ROO enriched at 400 and 200 μg/mL with alperujo phenols and to 1.80 and 1.76 AU for ROO enriched at 400 and 200 μg/mL with leaf phenols. It is worth emphasizing that there is a substantial improvement of both parameters in ROO enriched with hydrophilic antioxidants, although this effect is not linked to phenols enrichment degree. 7

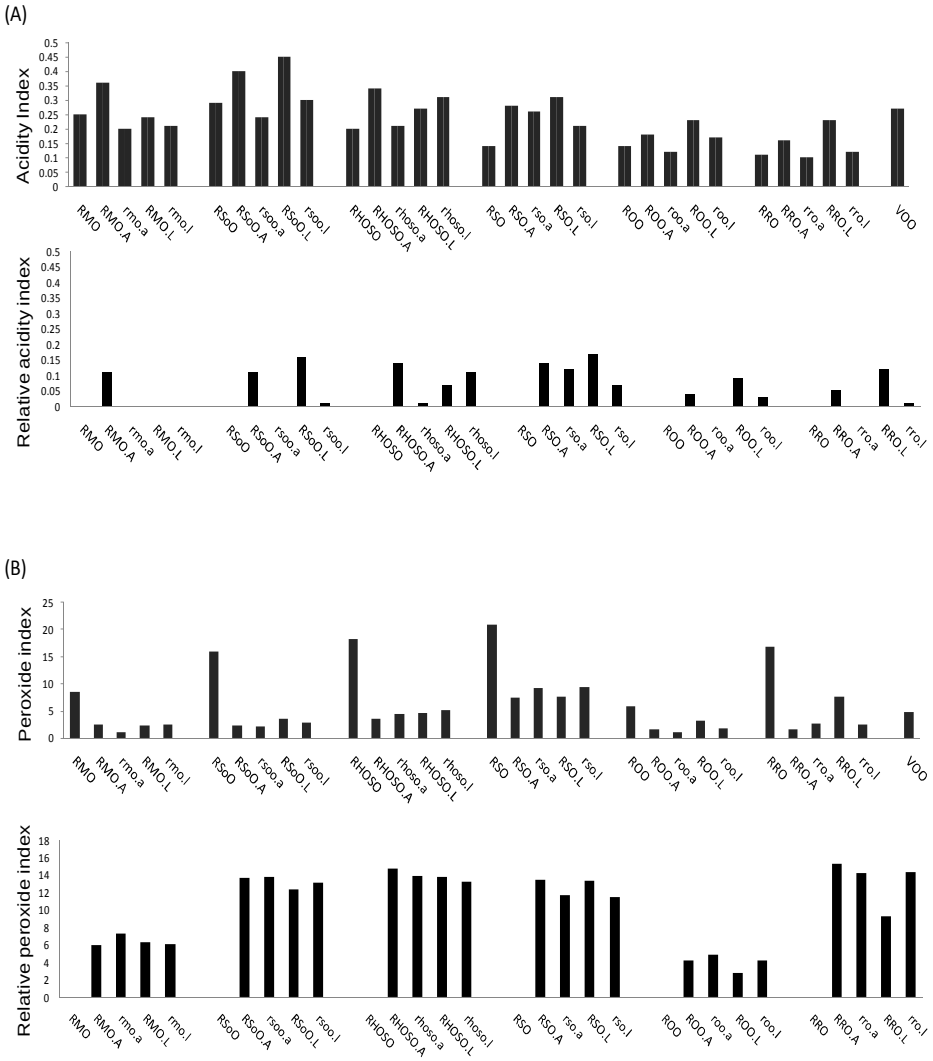

Figure 2. Target oils: Absolute and relative, (A) acidity indexes; (B) peroxide indexes.

Stability Tests

The stability of oils is related to their resistance to degradation, which can be evaluated through the peroxide index, the Rancimat test or both.

*Peroxide Index.*_This quality parameter is related to the primary oxidative stability of the given oil to avoid rancidity. It is expressed as

miliequivalent-oxygen/Kg-oil. All oxidation products usually produced by oxygen in the air in contact with the oil are considered under the umbrella of "peroxides". **Figure 2.B** shows a significant increase of the stability of all enriched oils (from 60 to 90%) as compared with their non enriched counterparts, which even became more stable than the reference EVOO in terms of primary oxidation excepting for enriched RSO versions. This behaviour justifies by itself the enrichment of oils with the target phenolic antioxidants in the light of the enlarged storage period thus achieved. No significant differences between enrichment with extracts from alperujo or leaves were observed for the same degree of enrichment.

Rancimat Test. The objective of this test is to evaluate the chemical quality of oils, so extreme conditions of temperature and ventilation are applied and the evolution of this dynamic parameter, which indicates deterioration of the oil, is monitored. The data obtained from the Rancimat study are plotted in **Figure 3. A**, which shows the hours each oil expends in the induction period, before starting massive oxidation that marks quality deterioration; while **Figure 3. B** plots the ratio between the hours required by the given enriched oil and the pure oil, with no enrichment, thus evidencing clearly the improvement of resistance to oxidative processes, attributable to the enrichment.

Thus, both types of extracts confer to refined maize oil similar stability, which doubles that of the pure oil when the enrichment in phenols is 400 μg/mL.

Refined soy oil is more stable when enriched with 400 μg/mL of alperujo extracts (its stability becomes up to 5 times that of the pure oil). The enrichment with 200 μg/mL alperujo extracts and any enrichment with leaf extracts does not double the stability of pure oil.

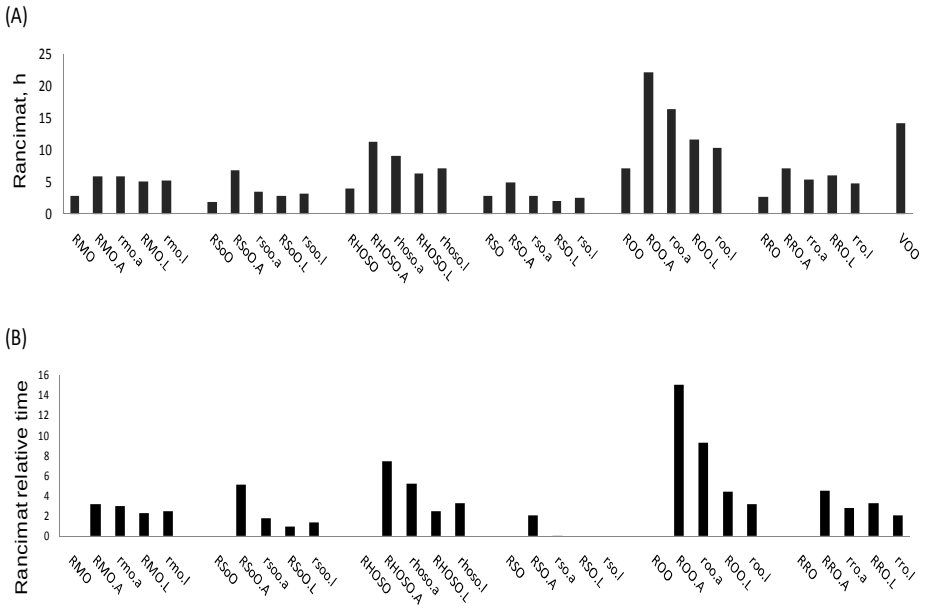

Figure 3. Rancimat values for, (A) all enriched oils and EVOO; (B) each enriched oil divided by its pure counterpart.

Refined high-oleic sunflower oil achieves the highest stability (8 times higher than pure oil) by enrichment with 400 μg/mL phenols, in this case from alperujo extracts. The stability of RHOSO.A is similar to that of EVOO. Also rhoso.a achieved a good stability (5 times higher than that of the pure oil). When leaf extracts are used, the stability increases between 2 and 3 times.

The only extract that increases the stability of RSO (and only at the highest concentration, 400 μg/mL) is alperujo, doubling, under these conditions, the stability of the pure oil. The stability of refined olive oil when enriched with alperujo extracts at 400 μg/mL increases 15 times, thus surpassing EVOO stability.

Refined rapeseed oil is better stabilised with alperujo extracts, surpassing 4.5 times that of the pure oil when the total phenols content in it reaches 400 μg/mL; while for 200 μg/mL the increase of stability is 2.5 times. RRO.L and rro.l increase their stability 5 and 2 times, respectively.

In the light of both main phenols transferred and the characteristics of the given oils, it can be concluded that the increase of stability for oil storage conferred by enrichment justifies, in general, the implementation of this step. In some cases, as for ROO —and, in a lesser extension for RRO— the enrichment is highly recommendable.

In short, all the parameters of all oils under study improved —some of them in a dramatic manner— by the presence of OPs; so the obtainment of oils with better stability and quality (and with proved healthy properties — as widely assessed in nutritional studies *(31)*) is an easy, cheap and highly recommendable process.

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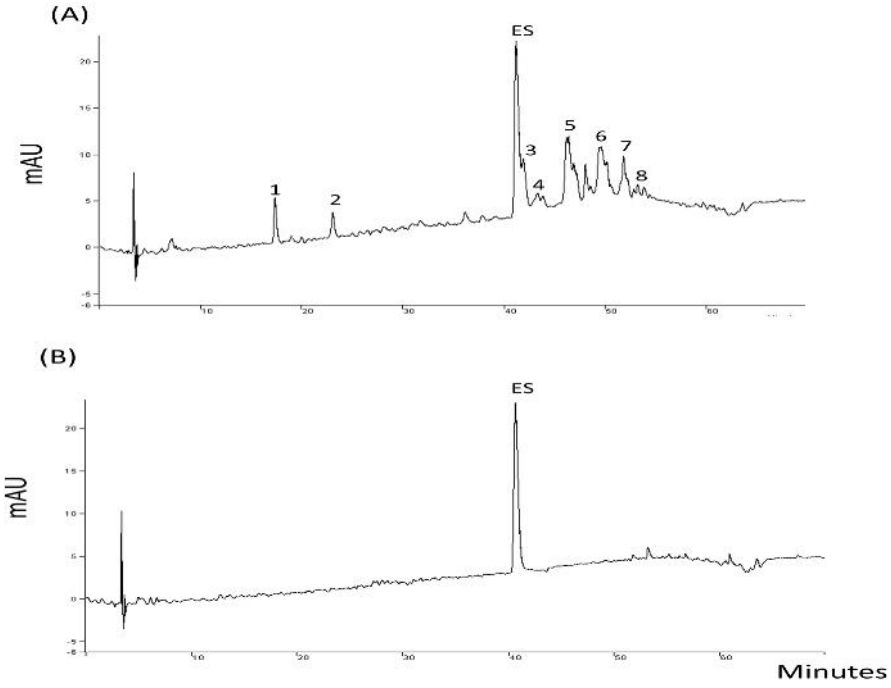
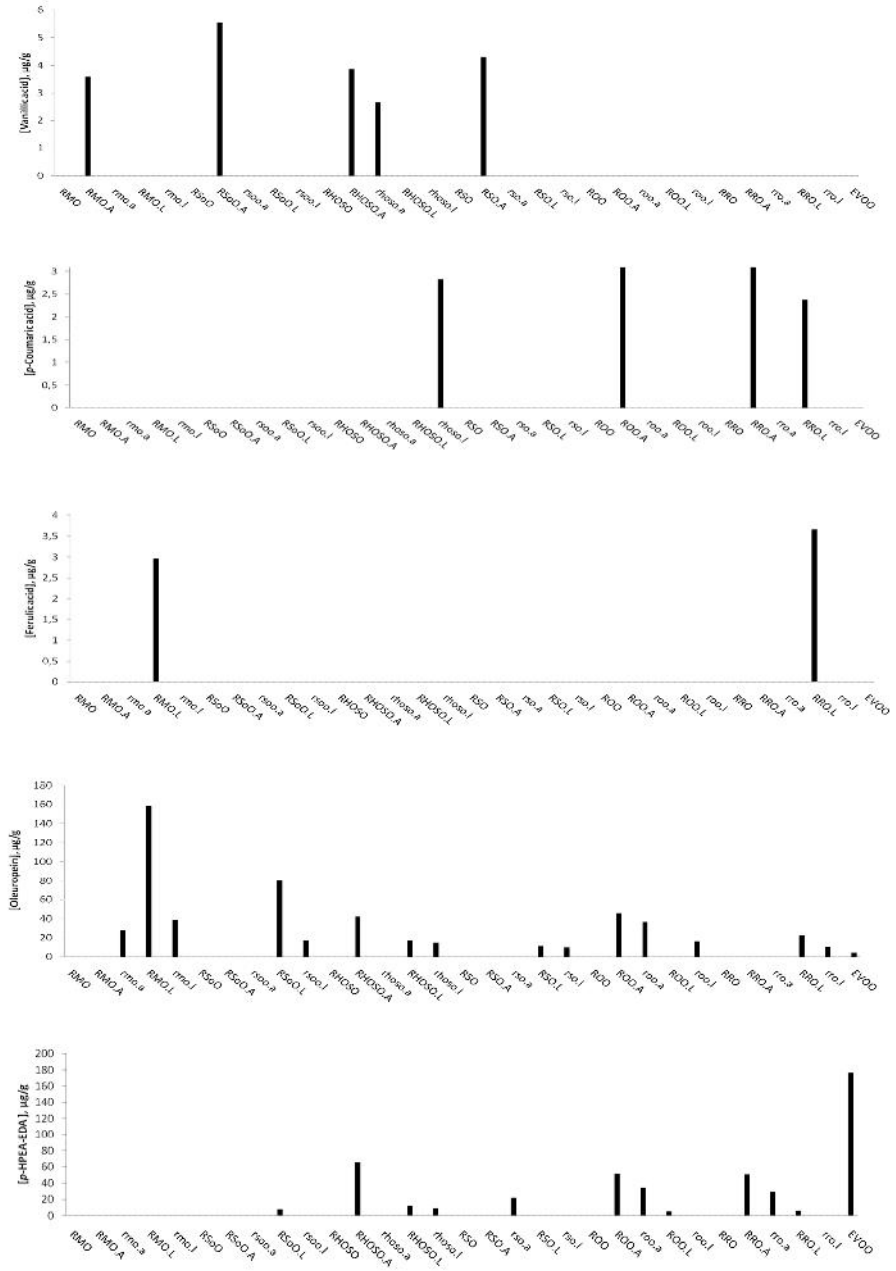
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Chromatograms, at λ=280 nm, provide by analysis of an antioxidants extract from extra-virgin olive oil (A) and pure sunflower oil (B). Peak identification: (A) 1, hydroxytyrosol; 2, tyrosol; ES, external standar at 280 nm, p-cresol; 3, 3,4-HPEA-EDA; 4, oleuropein; 5, p-DHPEA-EDA; 6, p-HPEA-FA; 7, luteolin; 8, apigenin. (B) ES, external standar at 280 nm, p-cresol.



Supplementary Figure 2. Oil enrichment in phenols not included in Fig. 1 in the article.

Supplementary Table 1. Concentration of the main olive phenols found in the extracts from leaves and alperujo.

Compound	Retention time (min)	Ion	Actual mass (*m/z*)	Formula	Experimental mass (*m/z*)	Error (ppm)
Hydroxytyrosol	12.8	[M-H]-	153.0562	$C_8H_{10}O_3$	153.0563	0.6533
Tyrosol	26.8	[M-H]-	137.0614	$C_8H_{10}O_2$	137.0617	2.1887
3,4-DHPEA-EDA	26.5	[M+CHO2]	365.1241	$C_{17}H_{20}O_6$	365.1236	–1.3693
Oleuropein	29.8	[M-H]-	539.1795	$C_{25}H_{32}O_{13}$	539.1801	1.1128
p-HPEA-EDA	37.0	[M-H]-	303.1256	$C_{17}H_{20}O_5$	303.1253	–0.9896
p-HPEA-FA	33.0	[M-H]-	377.1236	$C_{19}H_{22}O_8$	377.1245	2.3864
Luteolin	36.5	[M-H]-	285.0398	$C_{15}H_{10}O_6$	285.0388	–3.5082

Supplementary Table 2. Concentration of each phenol in the oils both with and without enrichment.

Enri-ched Oil	Hydro-xytyrosol	Ty-rosol	Vani-llic acid	*p*-Cou-maricacid	Feru-lic acid	3,4-DHPEA-EDA	Oleuropein	*p*-*HPEA*-EDA	*p*-*HPEA*-FA	Lute-olin	Apige-nin
RMO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RMO.A	28.21	5.05	3.58	0.00	0.00	411.04	0.00	0.00	29.01	8.32	1.97
rmo.a	11.25	1.08	0.00	0.00	0.00	205.89	27.68	0.00	15.99	4.60	0.00
RMO.L	5.02	0.00	0.00	0.00	2.97	0.00	158.92	0.00	0.00	3.49	0.00
rmo.l	0.00	0.00	0.00	0.00	0.00	0.00	38.56	0.00	0.00	2.26	0.00
RSoO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RSoO.A	39.86	7.81	5.55	0.00	0.00	585.07	0.00	0.00	56.41	12.02	2.40
rsoo.a	10.86	0.00	0.00	0.00	0.00	91.73	0.00	0.00	0.00	4.13	0.00
RSoO.L	7.03	0.00	0.00	0.00	0.00	0.00	80.08	7.57	0.00	5.14	0.00
rsoo.l	2.30	0.00	0.00	0.00	0.00	0.00	17.08	0.00	0.00	4.99	0.00
RHOSO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RHOSO.A	24.89	4.62	3.86	0.00	0.00	379.99	42.35	65.61	41.21	9.14	2.41
rhoso.a	10.95	1.21	2.66	0.00	0.00	174.38	0.00	0.00	7.75	5.65	0.00
RHOSO.L	2.41	0.00	0.00	0.00	0.00	0.00	17.47	11.87	0.00	3.03	0.00
rhoso.l	2.48	0.00	0.00	2.83	0.00	0.00	14.95	8.96	0.00	4.76	0.00
RSO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RSO.A	25.33	5.23	4.30	0.00	0.00	350.41	0.00	0.00	0.00	7.84	1.92
rso.a	11.27	3.89	0.00	0.00	0.00	125.56	0.00	21.62	8.36	4.70	0.00
RSO.L	5.41	0.00	0.00	0.00	0.00	0.00	11.15	0.00	0.00	3.38	0.00
rso.l	0.00	0.00	0.00	0.00	0.00	0.00	9.74	0.00	0.00	3.31	0.00
ROO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ROO.A	22.54	6.49	0.00	3.28	0.00	330.77	45.98	51.31	36.15	7.89	2.52
roo.a	15.33	1.76	0.00	0.00	0.00	188.18	36.67	34.49	15.85	5.48	0.00
ROO.L	4.00	0.00	0.00	0.00	0.00	42.95	0.00	5.34	0.00	4.47	0.00
roo.l	0.00	0.00	0.00	0.00	0.00	0.00	16.55	0.00	0.00	3.34	0.00
RRO	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RRO.A	14.67	3.34	0.00	3.22	0.00	298.31	0.00	50.74	34.64	8.40	2.23
rro.a	11.95	1.80	0.00	0.00	0.00	166.09	0.00	29.29	15.68	5.18	0.00
RRO.L	5.65	0.00	0.00	2.38	3.66	0.00	22.63	5.85	0.00	5.70	0.00
rro.l	0.00	0.00	0.00	0.00	0.00	0.00	10.46	0.00	0.00	2.15	0.00
EVOO	8.95	3.94	0.00	0.00	0.00	40.30	4.38	176.87	58.42	3.35	2.53

CAPÍTULO 12:

Characterization of refined edible oils enriched with phenolic extracts from olive leaves and pomace

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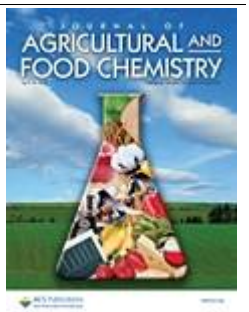

Characterization of refined edible oils enriched with phenolic extracts from olive leaves and pomace

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Characterization of refined edible oils enriched with phenolic extracts from olive leaves and pomace

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Abstract

Refined edible oils (*viz.* oils from maize, soya, high-oleic sunflower, sunflower, olive and rapeseed) enriched at two concentration levels (200 and 400 μg/mL total phenolic content) with phenolic extracts isolated from olive pomace and leaves have been characterized and compared with non-enriched oils and extra virgin olive oil (EVOO). Enriched oils were analysed by LC–TOF/MS to generate representative fingerprints and compared with non-enriched oils and EVOO by unsupervised principal component analysis (PCA). The two raw materials reported enriched oils with profiles which were compared with those provided by EVOOs. Correlation analysis enabled to establish the enriched oils with a composition more similar to EVOO. Discrimination according to the enrichment level depending on the raw material for extracts, and a global discussion about the enrichment on relevant phenolic compounds present in EVOO has reported quantitative results concerning the enrichment level for those significant compounds with known nutraceutical properties.

INTRODUCTION

Extra-virgin olive-oil (EVOO) is highly-appreciated thanks to the organoleptical properties and healthy benefits associated to its balanced composition. Among the different components present in EVOO, the unsaponifiable fraction is comprised by a great variety of compounds such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants —most of the last being carotenes and phenols.[1] Among the different families of compounds forming the unsaponifiable fraction olive phenols include a major group of secondary metabolites that display a wealth of structural variety and diversity of key activities. The healthy effects of EVOO —mainly due to the presence of these particular phenols— have been widely studied in the last decades.[2,3] Oleuropein, the most abundant phenol in olive leaves and also at high concentrations in olive pomace, has been used in a number of medical treatments since its first reference in the literature.[4] Hydroxytyrosol, an oleuropein derivative, gets better cardiac and tumoral diseases with similar effects to those of oleuropein; in addition, it protects against atherosclerosis[5] and is closely related to protection of low-density lipoprotein particles from oxidative damage.[6] Also, the nutraceutical utility of other phenols present in olive leaves and pomace at high amounts such as verbascoside, apigenin-7-glucoside, and luteolin-7-glucoside has been studied.[7–10]

The unsaponificable fraction is almost completely eliminated in refined edible oils from seeds. Refining of edible oils is a common strategy to maximize the amount of oil extracted from seeds by an economical process. The purpose of oils refining is the removal of impurities and natural flavours (compounds that can be easily oxidized) and neutralization of free-fatty acids. This is carried out by subjecting oils to heat, solvent extraction, filtering, neutralization, distilling, degumming, bleaching and high-heat deodorization.

As a result of the operational conditions of these steps, polar compounds, including phenolic antioxidants, are completely removed.[11]

Conferring to refined oils the benefits associated to the unsaponifiable fraction can be partially attained by an enrichment process with suited extracts of these components isolated from vegetal raw materials. Particularly, two different raw materials from *Olea europaea* cultivars such as olive pomace and leaves (in fact, the two main sources of olive phenols) have been used for enrichment of refined edible oils with phenolic compounds.[12] Olive pomace is a polluting semisolid residue resulting from the two-phase olive oil extraction method, which is at present the most implemented in this industry. This pomace is a cheap source of natural antioxidants, in concentrations up to 100-times higher than in olive oil,[13] which results from the polar nature of both this residue and olive phenols, but also from the low–polar nature of oil. On the other hand, leaves posses the highest antioxidant and scavenging power between the different parts of the olive tree. As an example, taking oleuropein as a model phenol, its content in olive oil ranges between 0.005–0.12%; in pomace up to 0.9% and in olive leaves between 1–14%.[13,14] Attending to the differences in composition of extracts from olive pomace and leaves obtaining tailor-made enriched oils up to the desired level of certain compounds could be perfectly accessible.

The protocol for enrichment of edible oils with phenolic extracts involved two main steps: (1) extraction of the target compounds from the raw material, either leaves or pomace; and, (2) enrichment of the oil with the extract. The benefits in terms of stability and quality of refined edible oils after enrichment with phenolic extracts have been previously evaluated. [12] However, no qualitative studies have been carried out to compare the composition of enriched oils with that of EVOO. The aim of this research was to compare qualitative fingerprints provided by analysis of EVOOs and enriched refined edible oils with extracts from olive pomace and alperujo.

For this purpose, LC–TOF/MS analyses of the target oils were used to obtain representative profiles of their composition.

MATERIALS AND METHODS

Samples

Olive pomace obtained during the 2009/2010 crop season was taken directly from the production line of a mill in Núñez de Prado, C.B. (Baena, Spain) and stored at –20 ºC until use. Olive leaves selected for this research were collected in June 2010 (Baena), dried at 35 ºC for 60 h, milled by a cyclonic mill up to homogeneous particle size (diameter ≤ 0.5 mm) and kept at 4 ºC until use.

Vegetable edible oils were selected for this research according to criteria such as low-price (less than 1 euro/Kg) and wide variability in the profile of fatty acids. The target oils were refined maize oil (RMO), refined soy oil (RSoO), refined high-oleic sunflower oil (RHSO), refined sunflower oil (RSO), refined olive oil (ROO), refined rapeseed oil (RRO) and extra virgin olive oil (EVOO). All of them were provided by Carbonell (SOS Cuétara S.A, Madrid). Extra virgin olive oils sampled from local mills located at different places in the South of Spain (Jaén, Sevilla, Málaga, Córdoba, Ciudad Real, Badajoz) were provided by Carbonell.

Reagents

The reagents used for characterization of vegetable oils were: LC grade methanol, acetonitrile, hexane and absolute ethanol from Scharlab (Barcelona, Spain). Deionized water (18 MΩ•cm) from a Millipore Milli-Q water purification system (Bedford, MA, USA) was used to prepare mobile chromatographic phases. The most abundant phenolic compounds in olive oil were purchased from Extrasynthese (Genay, France) in the case of hydroxytyrosol, tyrosol, oleuropein, apigenin and luteolin; meanwhile vanilline, vanillic acid, *p*- and *o*-coumaric acids and ferulic acid were from Merck. The stock standard solution of each phenol was prepared at 1000

μg/mL by dissolving 10-mg each phenol in 10-mL methanol. The multistandard solutions containing 10 phenols were prepared by mixing the appropriate volume of each stock solution and diluting them as required in a 60:40 methanol–water solution. All these solutions were stored in the dark at −20 ºC in glass vials until use.

Apparatus and Instruments

Microwave irradiation was applied in the extraction step by means of a MIC-II focused-microwave extraction system of 400 W maximum power (Puebla, Mexico) furnished with a manual power control unit. A Selecta Mixtasel centrifuge (Barcelona, Spain) was used to remove solid particles from the extract. A Büchi R-200 rotary evaporator (Postfach, Switzerland) furnished with a B-490 heating bath was used to concentrate the phenol extracts after microwave-assisted extraction (MAE), and to evaporate traces of ethanol in the enriched oils. A Selecta Vibromatic electrical stirrer (Barcelona, Spain) was used to favour the liquid–liquid extraction of phenols to refined oils. An MS2 minishaker from Ika (Wilmington, USA) was used to favour phenols transfer from oil to methanol for individual quantification of the target compounds.

Profiling analysis for identification of olive metabolites was carried out with an Agilent 1200 Series LC system interfaced to an Agilent 6540 UHD Accurate-Mass TOF LC/MS detector (Palo Alto, USA), equipped with an Agilent Jet Stream Technology electrospray ion source operating in the negative ion mode.

Procedure for Extraction of Phenols from Alperujo or Leaves

The procedure was similar to that proposed by Girón *et al.*[15] and Japón *et al.*[16] Briefly, 12 g of alperujo or leaves and 100-mL ethanol were placed into the quartz extraction vessel located in the zone of focused microwave irradiation of the extractor. After extraction (10-min microwave irradiation at 400 W), the suspension was centrifuged at 855 *g* for 5 min for phases separation. This process was repeated as many times as required to

obtain the necessary extract for the subsequent enrichment step after the extract had been concentrated in a rotary evaporator at 35 ºC to reduce ten times its initial volume. The extract thus obtained was reconstituted in 200-mL ethanol prior to measurement of total phenols concentration by the F–C test. [17]

Enrichment of Edible Vegetable Oils with Phenols Extracts from Alperujo or Leaves

The enrichment was carried out at two concentration levels (200 and 400 μg/mL of phenols according to the F–C test) per oil with extracts from alperujo and from leaves. In all cases, an aliquot of the corresponding ethanolic extract was put into contact with 200-mL oil, and the ethanol in the two-phase system was evaporated in the rotary evaporator at 30 ºC. Then, the mixture was shaken in the electrical stirrer at 700 U/min to favor enrichment. This process was repeated as many times as required until the 200-mL oil portions were enriched in phenols from either of the extracts up to 200 or 400 μg/mL (as determined by the F–C method using caffeic acid as standard). Distinction between the different oils, extract for enrichment and enrichment degree is as follows: abbreviation as under "Samples" —capital letters— is used for 400 μg/mL enrichment, followed by point and the initial of the raw material to prepare the extract (A for alperujo and L for leaves). Similar nomenclature, but case letters, is used for oils enriched with 200 μg/mL phenols. As example, RMO.A and rmo.a correspond to refined maize oil enriched with 400 μg/mL and 200 μg/mL, respectively.

LC–TOF/MS Confirmatory Analysis of Olive Phenols

Olive phenols identification was conducted by LC–TOF/MS confirmatory analysis in accurate mode due to the complexity of phenolic extracts from EVOO. The analytical column was a C18 Inerstil ODS-2 (250×4.6 mm i.d. 5 μm) from GL Sciences Inc (Tokio, Japan). The hydroalcoholic phase was injected into the chromatograph. The mobile phases were: A (0.1% formic acid in water) and B (0.1% formic acid in

acetonitrile). The gradient program, at 0.9 mL/min constant flow-rate, was as follows: initially 96% A and 4% B; 0–44 min, 96–50% A and 4–50% B; 44–54 min, 50–40% A and 50–60% B; 54–74 min, 40–0% A and 60–100% B; 74–85 min, 0% A and 100% B. After analysis, the column was reequilibrated for 15 min.

The phenolic fraction present in aliquots of EVOO and enriched oils was extracted for subsequent analysis by injecting the extracts into the LC–TOF/MS system without additional pretreatment. The injected extract volume was 20 μL. The operating conditions were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes were governed via the Agilent MassHunter Workstation software. The mass range and detection window were set at m/z 100–1100 and 100 ppm, respectively. Reference mass correction on each sample was performed with a continuous infusion of Agilent TOF biopolymer analysis mixture containing purine (m/z 121.0508) and hexamethoxyphosphazyne (m/z 322.0481) with resolution of 45 000. Analytes were identified by accurate mass detection.

Mass Hunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing raw LC–TOF/MS data. Molecular features were extracted using the molecular feature extraction algorithm (MFE) from raw data files prior to formula generation. Ions with identical elution profiles and related m/z values (representing different adducts, ions generated after specific neutral losses or isotopic forms from the monoisotopic ions) were extracted as molecular features (MFs) in a matrix characterized by retention time (RT) and accurate mass and containing intensity in apex of chromatographic peaks as data. The isotope model corresponded to common organic molecules with peak

spacing tolerance of m/z 0.0025 ± 7.0 ppm. The MFE algorithm limited extraction to ions exceeding 1000 counts with charge state limited to a maximum of two. The allowed negative ions were deprotonated species and formate adducts. Dehydratation neutral losses were also allowed. The generated raw data files in compound exchange format (.cef files) were created for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) to create the data matrix for further processing. Stepwise protocols for reduction of MFs number based on frequency filters and fold-change analysis were followed. Principal component analysis was used to find clustering of samples attending to level of enrichment and material used for enrichment.

Identification of the compounds proceeded by generation of candidate formulae with a mass accuracy limit of 5 ppm. The contribution to mass accuracy, isotope abundance and isotope spacing scores was 100.00, 60.00 and 50.00, respectively. Retention times, formulae, experimental and theoretical masses, and errors, in ppm, obtained by accurate mass measurements of a panel of phenolic compounds were considered in the identification step. This confirmatory analysis enabled to predefine the cut-off value for accuracy in the study.

RESULTS AND DISCUSSION

Qualitative Fingerprinting of Refined Oils After Enrichment

As a result of the operational conditions of the steps involved in refining oils, polar compounds, including phenolic antioxidants, are completely removed. The enrichment process was planned to supply the phenolic fraction isolated from raw materials in refined oils. The mass transfer occurred in the enrichment process was checked by analysis of the hydroalcoholic extracts from both the refined oils and the same oils after enrichment with the two types of raw materials. LC–TOF/MS chromatograms can be used as representative fingerprints of the content in polar and

mid-polar compounds. The results of the enrichment step with the two extracts can be seen in **Figure 1**. This figure illustrates the base peak chromatograms (BPC) obtained by LC–TOF/MS analysis of hydroalcoholic extracts from refined olive oil (ROO, **Figure 1.A**), EVOO (**Figure 1.F**) and those corresponding to enriched ROO with extracts from olive pomace (**Figure 1.D** and **E**) and leaves at both enrichment levels (**Figure 1.B** and **C**). As above exposed, TOF chromatograms reveals the absence of phenolic compounds in pure ROO owing to the refining process. In fact, practically none of these compounds are detected in this analysis. On the opposite side, EVOO analysis reports a representative profile that could be considered like a fingerprint of the polar and mid-polarity fraction. In between, the contribution of the enrichment process with both extracts, with clear differences associated to their composition, can be observed. Thus, the enrichment with extract from olive leaves is particularly significant in the first part of the chromatogram (up to 30 min elution time), where the polar phenolic fraction is eluted. On the other hand, the enrichment with extract from olive pomace is more pronounced in the elution window from 26 to 50 min, which could be directly related to secoiridoids. Additionally, two characteristic chromatographic peaks are detected at 13 and 17 min that could be identified as hydroxytyrosol and tyrosol (confirmed in subsequent sections). An additional effect can be visualized in the BPC corresponding to enriched ROO, different depending on the concentration level, 200 and 400 μg/mL. Thus, this effect can be clearly visualized if the y-axis scale is compared for BPC obtained from ROO enriched with extracts from the same raw material. These preliminary analyses open a discussion to the possibility of preparing tailor-made enriched oils by optimum selection of the raw material (single or a mixture) to be extracted and the enrichment concentration. Thus, oils can be prepared either with the desired organoleptical and healthy properties to attain the EVOO benefits associated to these olive phenols (OPs) or other modified characteristics depending on the final aim.

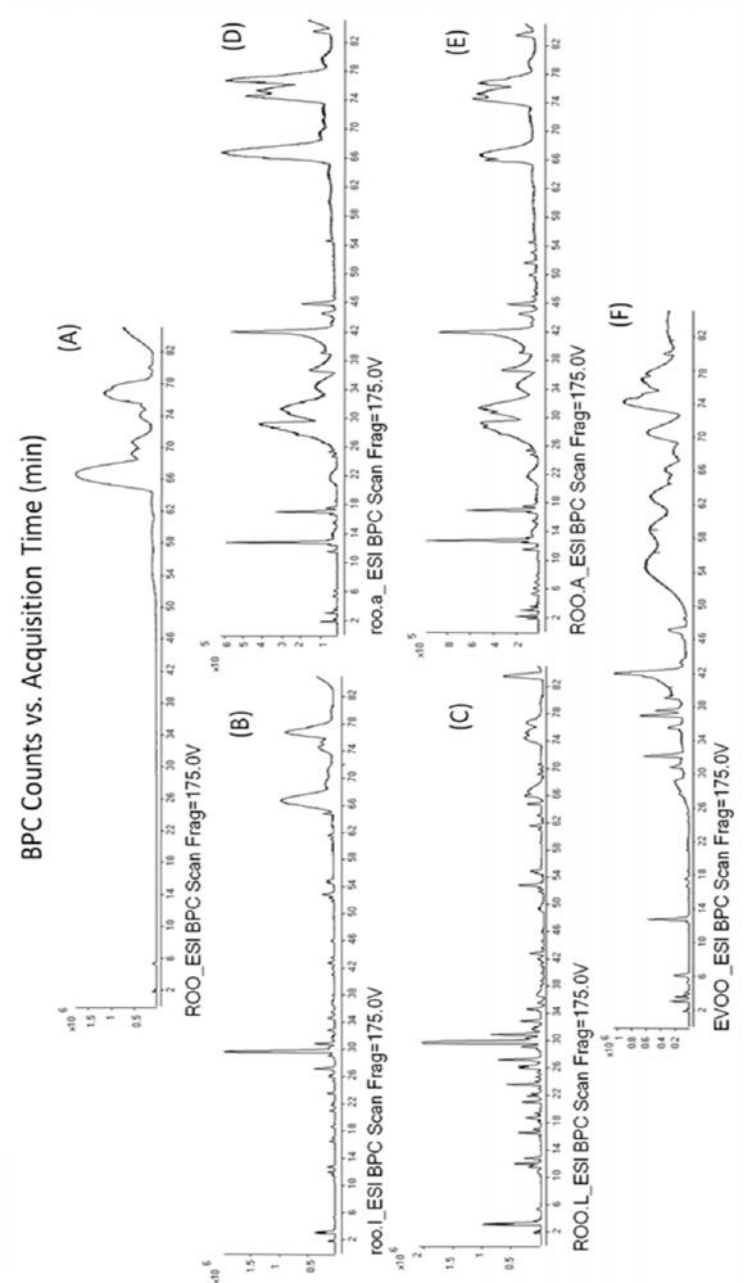

Figure 1. Base peak chromatograms (BPC) obtained by LC–TOF/MS analysis of hydroalcoholic extracts from: pure refined olive oil —ROO— (A); ROO enriched with leaf extract at 200 µg/mL (B), and 400 µg/mL (C); ROO enriched with extract from olive pomace at 200 µg/mL (D), and 400 µg/mL (E), and extra-virgin olive oil (F).

Mass Transfer of Phenols to Refined Oils as a Function of the Extract

Distinction among the different oils, different degree of enrichment and different extracts used for enrichment are discussed using the abbreviations under experimental. The discussions exposed below concern firstly to the degree of individual enrichment of the most important phenols detected in EVOO as a function of the extracts. These results were supported by LC–TOF/MS analyses in accurate mode for the tested oils. **Supplementary Fig. 1** illustrates extracted ion chromatograms for representative compounds of different families after analysis of the hydroalcoholic extracts of enriched ROO and EVOO. The trends discussed here were found in the resting enriched oils. The behaviour of the different OPs is exposed as follows:

- Hydroxytyrosol and tyrosol: oils were preferentially enriched with these simple phenols by extract from olive pomace. In fact, a clear difference in the concentration of hydroxytyrosol was found in the case of ROO enriched with OPs from olive pomace at 400 μg/mL with respect to all enriched oils and EVOO, while the enrichment at 200 μg/mL reported a level of hydroxytyrosol similar to that of EVOO (**Supplementary Fig. 1.A**). In the case of leaf extracts, lower hydroxytyrosol concentrations than in EVOO were found for all enriched oils. A similar behaviour was found for tyrosol, except for ROO enriched with extract from olive leaves in which tyrosol was below the detection limit.

- Secoiridoids: a different behaviour was found as a function of the monitored compounds (*viz.* decarboxymethyl aglycon or EDA derivatives, aglycons or EA forms, and oleuropein). Thus, oils were substantially enriched with decarboxymethyl oleuropein aglycon (3, 4-DHEPA-EDA) by extracts from olive pomace and leaves at 400 μg/mL (superior for extracts from olive pomace).

Intermediate enrichment was attained at 200 μg/mL as compared to EVOO, which did not provide detectable levels of this secoiridoid despite its organoleptical and healthy contribution (**Supplementary Fig. 1.B**). The decarboxymethyl ligstroside aglycon (*p*-HPEA-EDA) was detected at trace level for all ROO enrichments (**Supplementary Fig. 1.C**); therefore, the concentration of this secoiridoid was significantly lower in enriched oils than in the EVOO used as reference. The concentration of oleuropein aglycon (in equilibrium with its aldehydic form) in ROO enriched with extract from olive pomace was considerably higher than ROO enriched with leaf extract and EVOO, with also a clear difference between the two enrichment degrees (**Supplementary Fig. 1.D**). Ligstroside aglycon (in equilibrium with the aldehydic form) was only detected in ROO enriched with extracts from olive pomace but at low concentrations as compared with those in EVOO (**Supplementary Fig. 1.E**). Finally, it is worth mentioning the presence of oleuropein in ROO enriched with extract from olive leaves (**Supplementary Fig. 1.F**). Additional research is here demanded for guarantying the null contribution of oleuropein to food taste if this oil is employed for frying.

- Flavonoids: the two main flavonoids detected in EVOO, luteolin and apigenin, were also detected in enriched ROO. In the case of luteolin, the enrichment was higher when it was performed with extract from olive pomace. In fact, practically the same enrichment degree was found for ROO enriched with extract from olive pomace at low concentration than with extract from olive leaves at the higher enrichment concentration (**Supplementary Fig. 1.G**). The situation was different for apigenin, which was found at higher concentration in ROO enriched with extract from leaves (**Supplementary Fig. 1.H**).

- Pinoresinols: The enrichment process was studied for this family of compounds with nutraceutical interest. 1-Hydroxy-pinoresinol was detected in ROO enriched with both raw materials and at the two enrichment concentrations. Similar concentrations were found in enriched ROO at low concentration and EVOO, being at higher concentration for ROO prepared at the superior OPs concentration (**Supplementary Fig. 1.I**).
- Other compounds: two triterpenes such as oleanolic and ursolic acids were monitored as representative of enrichment of ROO in this family. In fact, EVOO did not report detectable levels of both acids, which appeared at significant concentration in the oil when enriched with extract from olive leaves (**Supplementary Fig. 1.J**).

Statistical Analysis based on LC–TOF/MS Data

The purpose of the enrichment of refined edible oils with compounds present in EVOO was to enhance oils stability and improve their healthy properties. The composition of the oils enriched with extract from the two raw materials has been tested and compared in this study. For this purpose, statistical analysis was carried out using as data set that composed by molecular features extracted from LC–TOF/MS analysis. Prior to analysis, the possibility of filtering molecular features in the original data set according to frequency in the variability of samples included in the study should be discussed. For this purpose, the samples were classified in four classes: pure refined oils, oils enriched with extract from olive pomace, oils enriched with extract from olive leaves and EVOOs. Attending to this preclassification, two different filters were tested, namely: (i) restrictive analysis, which means a filtration of the data matrix to include only those molecular features present in all samples belonging to one of the four classes and, (ii) non-restrictive analysis, which involves a filtration of the data matrix to include those molecular features present in at least 40% of all samples

belonging to one of the four classes. One of the characteristic aspects of EVOO is the significant influence on its composition of factors such as maturation of olive fruits and genetic variability (cultivars). Additionally, there is another group of factors encompassing agropedoclimatic conditions that contribute considerably to the composition of EVOO. [18,19] The purpose of the first filter was to reduce the data matrix only to those molecular features which are representative of the four predefined classes. On the opposite side, the non-restrictive filtration was focused on removing those molecular features present in a reduced number of samples of each class. After both operations, PCA was carried out resulting in the score graphs in **Figure 2**. As can be seen, similar results are obtained with both the restrictive and the non-restrictive analysis since a clear discrimination is observed in the four groups formed by: (i) pure oils, (ii) oils enriched with extract from olive pomace (iii) oils enriched with extract from olive leaves and (iv) extra-virgin olive oils. The grouping of the four classes is perfect attending to the symbols used to label each class. Also, the samples representing EVOOs are separated from the group of pure oils being closer to both groups of enriched oils.

The similarity between samples integrating each class can also be detected with the heat map shown in **Figure 3**, based on correlations between each pair of samples. Apart from the diagonal line exposing the evident maximum correlation between equal samples, two main areas can be discriminated. The first area corresponds to the pure oils that are not practically correlated except for: (i) RHOSO, RSoO and RMO that are highly correlated; (ii) RRO that shows an intermediate positive (established in the scale shown in the figure) correlation to RSO and EVOOs; and (iii) RSO that is softly correlated to EVOOs. The heat diagram also reveals interesting associations between EVOOs and enriched oils. Thus, there is a low correlation between EVOOs and oils enriched with extract from olive leaves. By contrast, oils enriched with extract from olive pomace showed a high

correlation to EVOO. Therefore, the enrichment of refined oils with extracts from olive pomace is the suited strategy to obtain oils with similar composition of phenolic compounds to EVOO. Apart from these results, this study confirms a different profile for oils enriched with both types of extracts.

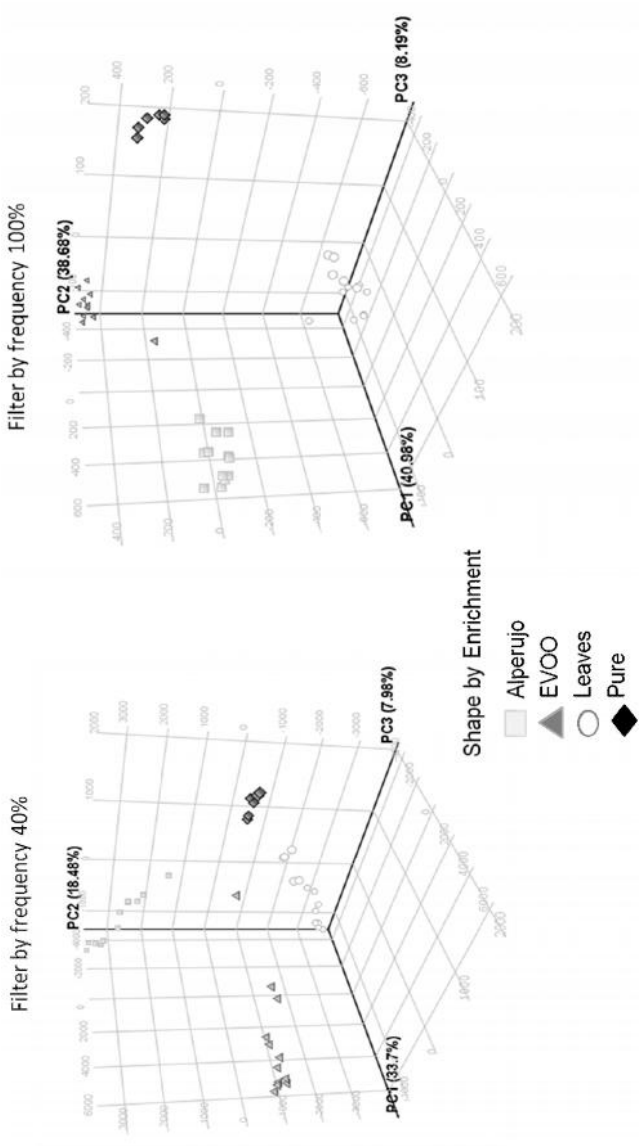

Figure. 2. Scores plots for PCA of oils enriched with the two types of extracts and pure oils depending on the filter used for reduction of molecular features.

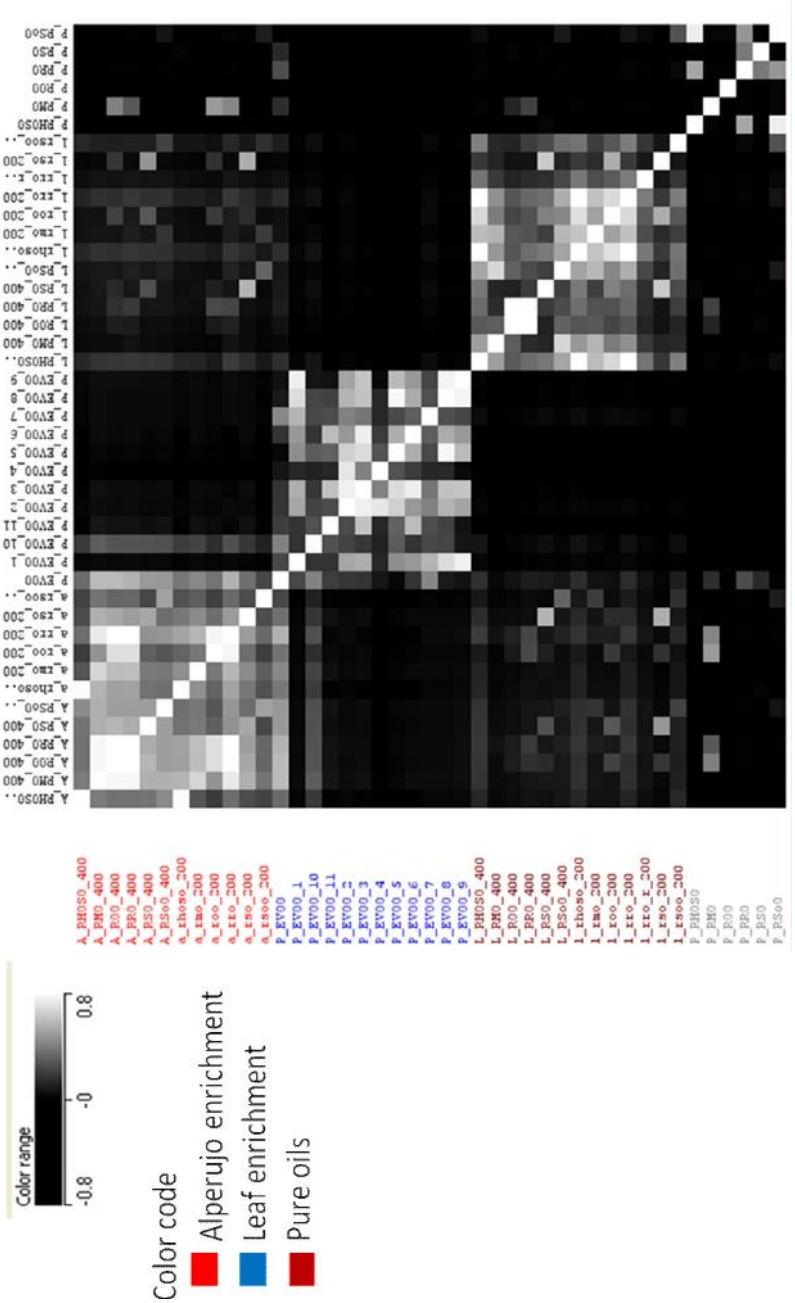

Figure 3. Heat map based on correlation analysis between refined oils, EVOOs and enriched oils.

Evaluation of Significant Molecular Features for each Enrichment

After these statistical tests, a stepwise strategy was followed to assess the influence of molecular features on the PCA analysis, and also to identify those molecular features in which the enrichment process was more significant in relative terms. Thus, a fold change analysis was applied to molecular features included in the data set. This algorithm enabled to isolate those ions with more impact to explain the variability observed in previous PCA tests. **Table 1** summarizes the number of molecular features after the different operations both in the restrictive and non-restrictive analysis. As can be seen, the number of molecular features was considerably reduced for higher fold change ratios. Additionally, **Figure 4** represents the PCA tests applied to the data matrix generated after fold change analysis setting ratio at 4.0 both in the restrictive and non-restrictive analysis. This cut-off value was set according to the fold change ratio obtained for two representative compounds such as hydroxytyrosol and 3,4-DHPEA-EDA, known because of their antioxidant properties. [20] In both cases, there is a clear discrimination between oils enriched with extract from olive pomace and those enriched with extract from olive leaves, but also from pure oils. The only difference between restrictive and non-restrictive analysis is the dispersion within groups. Thus, samples pertaining to a specific group are more dispersed in the case of non-restrictive analysis. In fact, the samples corresponding to EVOO are close to enriched oils and practically separated from the rest of pure oils that are quite grouped between them.

A more advanced level of information can be attained by including in the PCA the enrichment concentration. The inclusion of this variable in the PCA analysis reveals an additional discrimination based on concentration of enrichment, apart from that observed for type of raw material for enrichment. Thus, **Figure 5** represents the graphs of scores corresponding to PCAs for non-restrictive and restrictive analysis. As can be seen, in both

cases, a gradual discrimination between oils enriched at low and high concentration of phenols can be observed. This graduation is perfectly visualized in both types of enrichment in the restrictive analysis including just those molecular features only detected in all samples belonging to a specific group. The discrimination in terms of concentration enrichment is less perceptible in the case of non-restrictive analysis for oils enriched with extract from olive leaves. In fact, two oils enriched at high concentration such as RHOSO and RSO remain overlapped with oils enriched at low concentration. The number of molecular features considered in each study were 1785 and 178 for the non-restrictive and restrictive analysis.

Table 1. Number of Molecular Features along Statistical Tests Both in the Restrictive and Non-Restrictive Analysis.

	Original data set	**Frequency filter**	**Fold change analysis (cut-off 4.0)**
Restrictive (Frequency filter 100% samples of one condition)	12336	178	113
Non-restrictive (Frequency filter at least 40% samples of one condition)		1785	119

Table 2 lists the fold change ratios for characteristic compounds in olive oil that confirms the results exposed in **Supplementary Fig. 1**. As can be seen, the relative concentration of hydroxytyrosol in enriched oils was affected as a consequence of the raw material used with a higher increase for those oils enriched with olive pomace. A more critical effect was observed in the case of tyrosol, which was only enriched in oils prepared with extract from olive pomace. These results were also found for secoiridoids (3,4-DHPEA-EDA, 3,4-DHPEA-EA and *p*-HPEA-EA) with higher relative concentrations in refined oils enriched with olive pomace extract. Particularly, the secoiridoid aglycon forms were not detected or slightly detected in oils enriched with extracts from leaves. Transfer to oils of one simple phenol such as *p*-coumaric acid was similar with extracts from both vegetal

materials, while vanillic acid and elenolic enriched preferentially oils prepared with extract from olive pomace. On the other hand, flavonoids enriched preferentially oils prepared with extract from olive leaves, except for apigenin that was similarly distributed in oils independently of the raw material from which the extract was obtained. A similar behavior to apigenin was found with a lignan derivative such as 1-hydroxypinoresinol. Finally, two terpenic compounds such as oleanolic and maslinic acids were significantly more enriched in oils prepared with extracts isolated from olive leaves. Attending to these results, the relevance of the vegetal raw material used for enrichment is critical in the composition of prepared oils. The elevated antioxidant potential of secoiridoids and two simple phenols such as hydroxytyrosol and tyrosol are highly-responsible for the higher correlation between oils enriched with olive pomace extract and extra virgin olive oil.

Table 2. Fold Change Ratio Found for a Panel of Representative Compounds.

Compound	Fold Change Ratio [Alperujo]vs [Pure]	Fold Change Ratio [Leaves]vs [Pure]	Adduct	Mass (database)
Hydroxytyrosol	4.11	4.00	M-H [1-]	154.063
Tyrosol	-	4.41	M-H20-H [1-]	138.0681
3,4-DHPEA-EDA	4.00	2.53	M+FA-H [1-]	320.126
3,4-DHPEA-EA	3.90	2.75	M-H [1-]	378.1315
p-HPEA-EA	3.48	3.01	M-H [1-]	362.1366
Apigenin	4.08	4.02	M-H [1-]	270.0528
Rutin	2.09	2.48	M-H [1-]	610.1534
Quercetin	-	2.61	M-H [1-]	302.0427
Diosmetin	2.05	4.21	M-H [1-]	300.0634
Taxifolin	3.20	4.29	M-H [1-]	304.0583
Catechin	2.15	3.27	M-H20-H [1-]	290.079
Apigenin-7-glucoside	-	4.42	M-H [1-]	432.1056
Luteolin-7-glucoside	3.11	3.97	M-H [1-]	448.1006
Vanillic acid	4.01	-	M-H20-H [1-]	168.0423
p-Coumaric acid	3.60	-	M-H [1-]	164.0463
Elenolic acid	3.34	-	M-H [1-]	242.079
1-Hydroxypinoresinol	3.09	-	M-H [1-]	374.1366
Maslinic acid	-	2.71	M+FA-H [1-]	472.3553
Oleanolic acid	2.37	3.10	M-H [1-]	456.3603

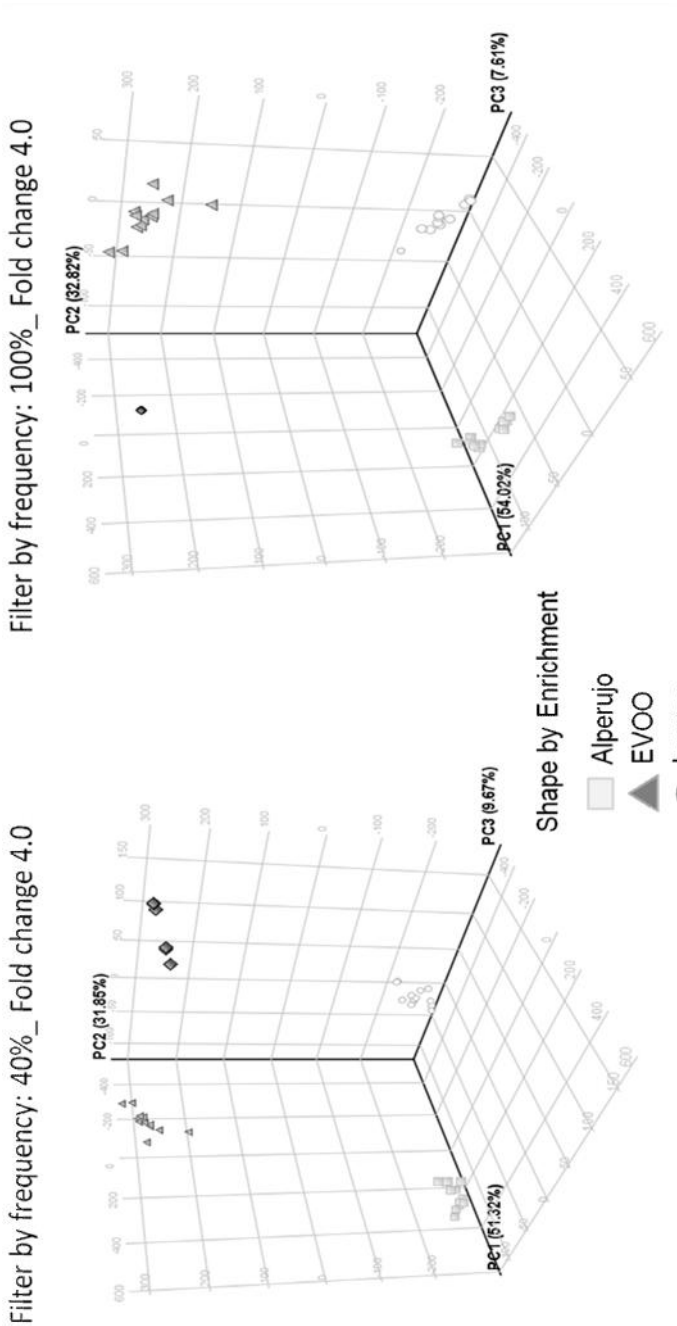

Figure 4. Scores plots for PCA tests applied to the data matrix generated after fold change analysis setting ratio at 4.0 both in the restrictive and non-restrictive analysis.

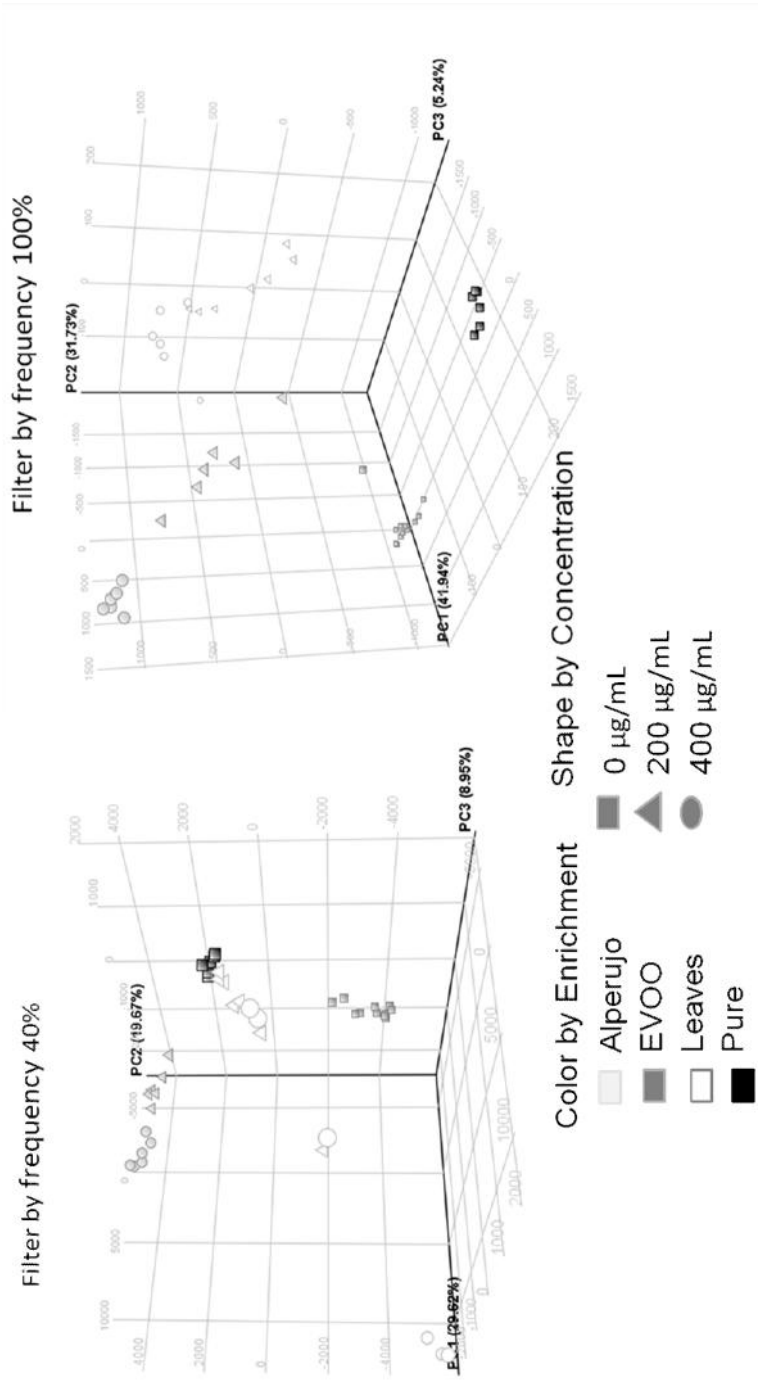

Fig. 5. Scores plots for PCA tests corresponding to non-restrictive and restrictive analysis including enrichment concentration.

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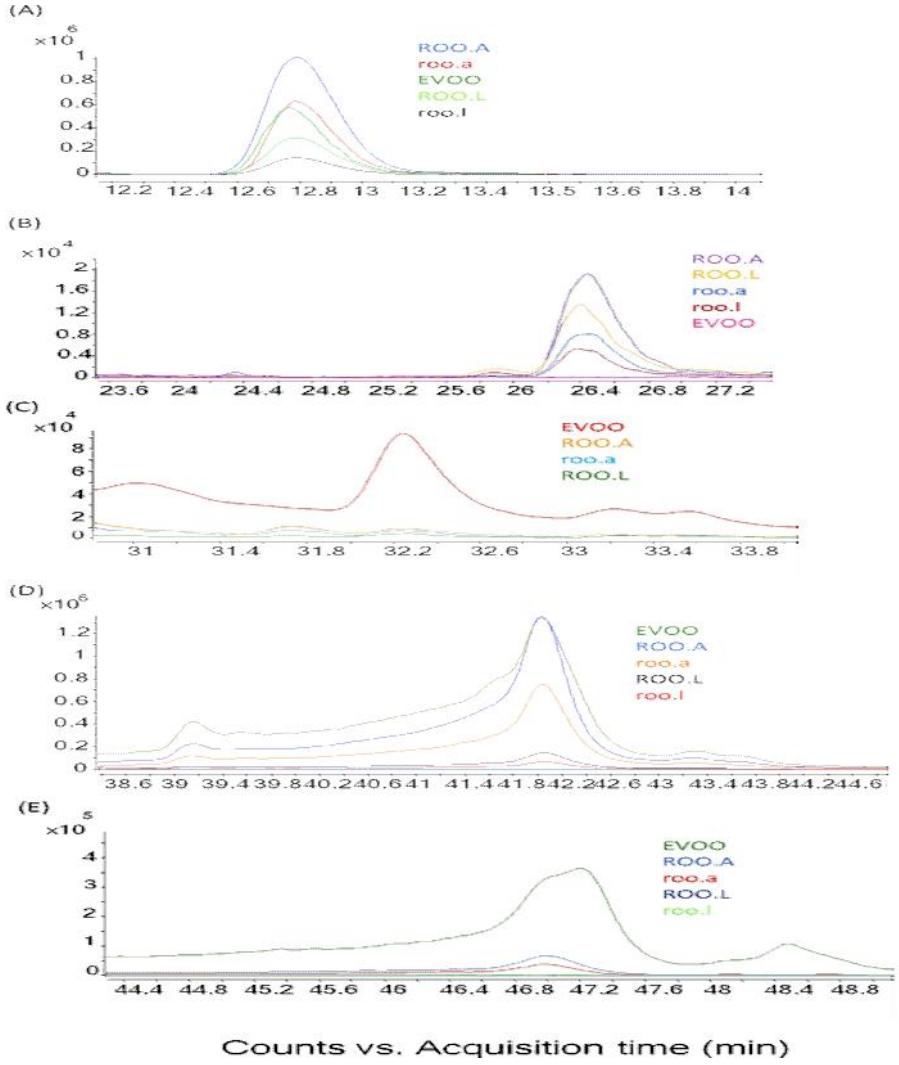
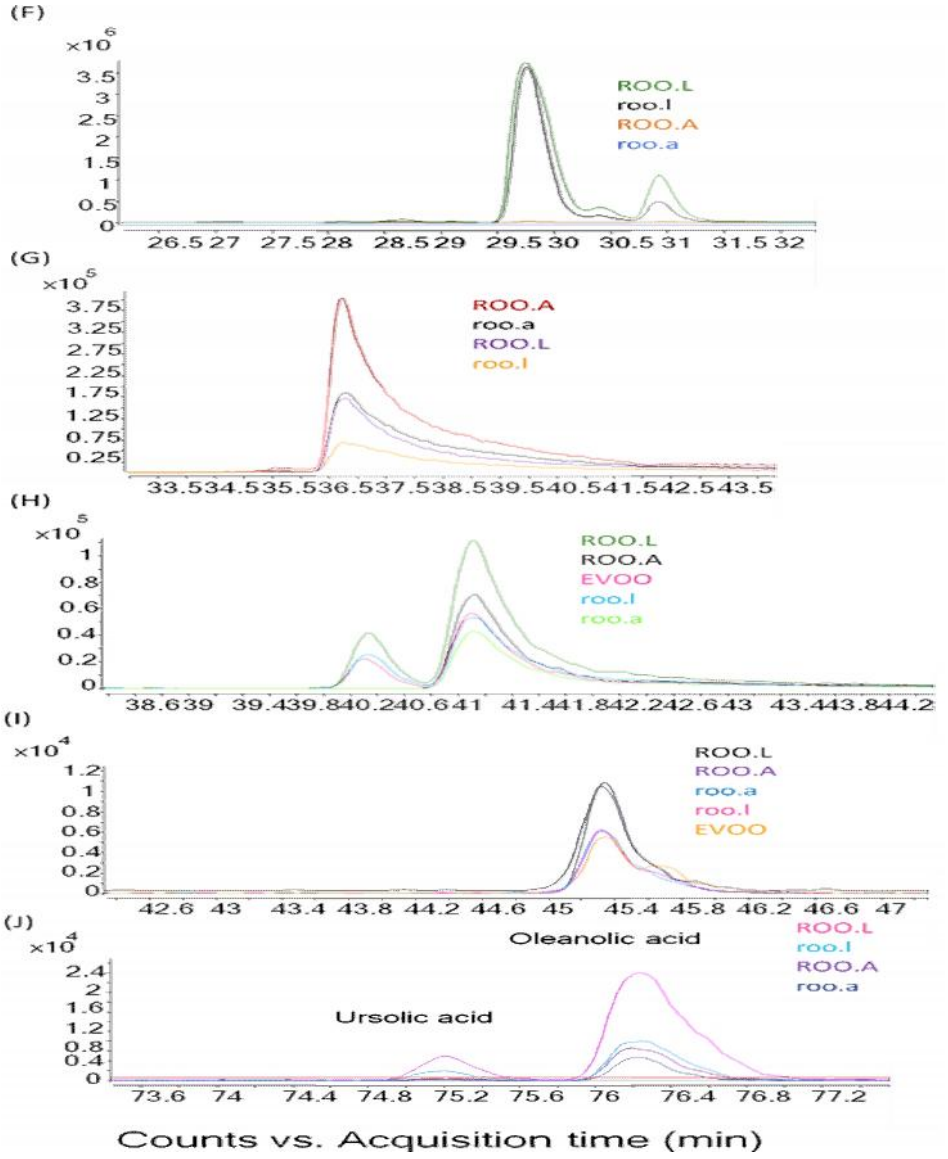
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Extracted ion chromatograms for representative compounds of different families after analysis of the hydroalcoholic extracts of enriched ROO: (A) hydroxytyrosol, (B) 3,4-DHEPA-EDA, (C) p-HPEA-EDA, (D) 3,4-DHEPA-EA, (E) p-HPEA-EA, (F) oleuropein, (G) luteolin, (H) apigenin, (I) 1-hydroxypinoresinol, (J) oleanolic and ursolic acids.



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DISCUSIÓN DE LOS RESULTADOS

La normativa vigente en la Universidad de Córdoba para la elaboración de una Memoria de Tesis Doctoral en la modalidad en la que se incluyen los artículos (publicados o próximos a su publicación) como tales requiere una discusión conjunta de los resultados de la investigación realizada.

La Memoria en la que se recoge la investigación se ha dividido en 5 secciones, de las cuales las comprendidas de la A a la D tienen como denominador común el desarrollo y la aplicación de plataformas analíticas para el estudio de la calidad del aceite de oliva virgen (VOO), referido a las tres familias de compuestos presentes en el aceite de oliva que caracterizan su calidad: Fenoles, ácidos grasos (FA) y fracción insaponificable no polar. La importancia de estas familias estriba en sus propiedades nutricionales y organolépticas (con especial énfasis en el amargor y el picor), y en el papel que desempeñan en la estabilidad del VOO. Se ha dado una mayor importancia a la fracción fenólica y la de ácidos grasos, a tener muy en cuenta en los programas de mejora de cultivo del olivo orientados a la calidad y estabilidad del producto final, el VOO. La fracción insaponificable no polar, compuesta por fitoesteroles, hidrocarburos y tocoferoles, entre otras familias, está menos estudiada que la de los fenoles o los ácidos grasos, pero puede aportar nuevos criterios a la hora de seleccionar variedades. Es importante resaltar que la investigación desarrollada en esta Tesis Doctoral ha tenido un doble objetivo: Por un lado el desarrollo de plataformas analíticas orientadas al análisis de las familias de compuestos de interés en VOO y, por otro, la aplicación de los métodos en el programa de mejora para evaluar qué se

puede aportar desde una perspectiva metabolómica a la mejora de las propiedades del VOO.

La Sección D se ha dedicado al enriquecimiento de aceites con extractos de compuestos provenientes de los residuos generados en la industria olivarera, con los que se ha pretendido mejorar las propiedades nutricionales y la estabilidad de los aceites. El escaso o nulo coste de estos residuos, tan abundantes en la cuenca mediterránea, y la facilidad de extracción de los compuestos de interés pueden resultar en una interesante fuente de ingresos. Se podría de esta forma compensar en alguna proporción el bajo precio del VOO. La investigación que se recoge en esta Sección ha pretendido cerrar el ciclo del cultivo del olivo con el desarrollo de estrategias que permitan un aprovechamiento de los principales residuos generados en la producción del aceite de oliva, cuya gestión será sin duda uno de los temas de actualidad del sector en los próximos años.

Teniendo en cuenta la independencia de los diferentes bloques se ha planteado una discusión de los resultados obtenidos en esta investigación por secciones, aunque con referencias cruzadas entre secciones debidas a su relación.

Sección A: Estudio de la fracción insaponificable del VOO y desarrollo de métodos analíticos para su caracterización

Esta Sección engloba 4 capítulos en los que la investigación que recogen ha tenido un doble objetivo: Un conocimiento exhaustivo de las características de la fracción insaponificable y de los estudios realizados hasta la fecha sobre ella. Con este propósito se realizó una revisión bibliográfica en la que las características y propiedades de las diferentes familias que componen esta fracción minoritaria del VOO se recogen en el Capítulo 1. En él se discuten las características químicas y propiedades beneficiosas tanto de los compuestos insaponificables no relacionados químicamente con los ácidos grasos (hidrocarburos, alcoholes, esteroles, fenoles, pigmentos y la fracción volátil) como de los derivados de los ácidos grasos (fosfolípidos,

ceras y ésteres de esteroles). Se hace hincapié en este estudio bibliográfico en que algunos de los compuestos minoritarios no relacionados con los ácidos grasos (*e.g.* esteroles y tocoferoles) pueden encontrarse en otros aceites vegetales; mientras que existen algunos exclusivos de las *Oleaceas (e.g.* hidroxitirosol y secoiridoides). De esta forma, el conocimiento de las características de esta fracción puede contribuir a una mejor explotación de estos metabolitos excepcionales. Aunque los efectos beneficiosos de algunos de estos compuestos minoritarios han sido reconocidos por la Autoridad Europea de Seguridad Alimentaria (EFSA) —como es el caso del hidroxitirosol—, existen otros muchos de los que el reconocimiento de sus efectos sobre la salud es todavía una asignatura pendiente. Uno de los objetivos indirectos de la investigación realizada es proporcionar soporte para el reconocimiento de esos efectos que, por otra parte, han formado parte de la cultura popular de la región mediterránea desde tiempos inmemoriables.

En el Capítulo 2 se realiza una discusión crítica de la gran cantidad de métodos existentes para el análisis de la fracción insaponificable. Debido a la variedad de compuestos que la constituyen son necesarios diferentes métodos tanto para la preparación de muestra como para el análisis. Se requieren equipos de alta resolución para llevar a cabo la identificación de compuestos desconocidos pertenecientes a estas familias teniendo en cuenta que la mayoría de ellos no está disponible comercialmente, o para la cuantificación individual de concentraciones bajas de estos compuestos (especialmente cuando se estudia su efecto en el organismo a través de algún fluido biológico). Tradicionalmente, la etapa de preparación de muestra para el análisis de la fracción insaponificable del VOO ha sido larga y tediosa, siendo ésta la etapa la más crítica del análisis global por el enorme número de errores a que puede dar lugar. Actualmente las investigaciones se centran en mejorar, acelerar o evitar esta etapa haciendo uso de energías auxiliares y equipos analíticos de última generación, respectivamente.

Como resultado de esta revisión bibliográfica se evaluó la importancia de familias de compuestos como los fenoles o los compuestos insaponificables no polares que, junto con la fracción de ácidos grasos, han sido el objetivo de las innovaciones realizadas en esta Tesis, tanto metodológicas como de aplicaciones. La importancia de estas tres familias de compuestos en la evaluación de la calidad del VOO permite considerarlas las de mayor interés para el seguimiento de los cambios obtenidos en los programas de mejora del olivo.

La realización de esta revisión ha proporcionado a la doctoranda una puesta al día sobre lo publicado sobre la materia en cuestión, adquiriendo una base sólida en la que soportar la investigación a desarrollar, así como una formación para la interpretación crítica de la investigación publicada por otros autores. Cabe señalar que el punto de vista analítico de esta revisión puede ser de interés para científicos que trabajen en esta área pero cuya formación no sea analítica.

La segunda parte de la Sección A se dedicó al desarrollo de metodologías analíticas para la caracterización de la fracción insaponificable no polar y su aplicación al VOO y a aceites refinados de diferentes semillas. La experiencia adquirida en los estudios teóricos que dieron lugar a los Capítulos 1 y 2 proporcionó la base para la investigación que se recoge en el Capítulo 3: Desarrollo de una metodología para la caracterización de la fracción insaponificable, en la que se dio especial importancia a una etapa crítica del análisis de esta fracción minoritaria como es la saponificación. Con el objetivo de utilizar la mejor opción, se compararon cuatro formas de llevar a cabo la saponificación considerando la información disponible en la bibliografía consultada: (i) En frío, en la que no se usa ningún tipo de energía auxiliar; (ii) en caliente, usada por el método oficial de la "American Oil Chemists' Society" (AOCS): (iii) asistida por microondas; (iv) asistida por ultrasonidos. Mediante el análisis de la fracción orgánica por cromatografía de líquidos y espectrometría de masas (LC–MS) de alta resolución y con el

uso de herramientas estadísticas multivariantes se comprobó que el desarrollo en caliente proporciona la mayor eficiencia en la etapa de saponificación en términos cualitativos y cuantitativos, debido, probablemente, a que cuando se utilizan energías auxiliares se produce degradación, o a que la eficiencia del proceso es menor. Mediante análisis no supervisado de componentes principales (PCA), se comprobó que la saponificación en frío y la asistida por microondas proporcionan composiciones similares, mientras que la asistida por ultrasonidos es la que da lugar a una fracción con una composición más diferente a la obtenida con el protocolo en caliente propuesto por la AOCS.

Los resultados de este estudio comparativo proporcionan la base para el desarrollo adecuado de un método apto para su aplicación en programas de mejora del olivo. Su importancia radica en el interés que presenta esta fracción, hasta el momento la menos estudiada desde un punto de vista analítico y, por tanto, no evaluada en los programas de mejora.

Una vez seleccionado el método de saponificación más adecuado, se aplicó a la obtención de la fracción insaponificable en diferentes VOO monovarietales y en aceites refinados procedentes de diferentes semillas, tal como se recoge en el Capítulo 4. El análisis de esta fracción, llevado a cabo por LC–MS de alta resolución, junto con el uso de herramientas estadísticas multivariantes, puso de manifiesto que la fracción insaponificable de los VOOs proporciona un perfil más completo que el de los aceites refinados. Por otro lado, se observó que el aceite que mayor similitud presenta con el VOO es el aceite de soja, seguido del aceite de orujo y aceite de oliva refinado. Finalmente, se estudiaron algunos compuestos representativos de la fracción insaponificable pertenecientes a diferentes familias (tales como el Δ-5-avenasterol, stigmastatrianol, ácidos oleanólico y ursólico, 11-tocoferol, diapo-zeta-caroteno, luteína, 2,4-δ-*cis*-retinol, octadienal, octanal, N-acetilesfingosina y oleamida). Los resultados mostraron que los procesos de refinado reducen considerablemente el contenido de estos compuestos en los aceites comestibles. El análisis de esta fracción puede ser útil desde un punto

de vista global para identificar aceites refinados con una composición de la fracción insaponificable similar a la del VOO.

Sección B: Caracterización de la fracción fenólica de los VOOs por espectrometría de masas. Estudio de la influencia de la maduración y el genotipo en esta fracción

Esta sección y la siguiente recogen la investigación realizada sobre dos de las fracciones de mayor interés del VOO resultante de los programas de mejora del olivo: La fracción fenólica (Sección B) y la de los ácidos grasos (Sección C). Las herramientas desarrolladas para la caracterización de ambas fracciones se aplicaron a un conjunto de muestras de VOO obtenidas a partir del programa de mejora del olivo coordinado por la Universidad de Córdoba y el IFAPA, con el fin demostrar la utilidad de estas herramientas para la selección de variedades de olivo.

En concreto, la fracción fenólica, la más estudiada en los programas de mejora, se ha evaluado en esta Sección B mediante espectrometría de masas (MS) utilizando dos espectrómetros de masas complementarios por sus propiedades analíticas: El de triple cuadrupolo (QqQ) en modo selección de las reacciones monitorizadas (SRM), que constituye una herramienta excelente para la cuantificación y la confirmación, gracias a su sensibilidad y selectividad. El detector híbrido cuadrupolo tiempo de vuelo (QTOF), que resulta muy adecuado para el análisis cualitativo y semicuantitativo por su buen nivel de sensibilidad y elevada exactitud en la medida de masas y es crucial para la identificación de compuestos y en análisis comparativo.

La investigación que se recoge en el Capítulo 5 se realizó tras comprobar los escasos estudios existentes en bibliografía sobre caracterización de fenoles en VOOs monovarietales. Para ello se seleccionaron siete VOOs monovarietales (Arbequina, Arbosana, Cornicabra, FS- 17, Hojiblanca, Picual y Sikitita) y se caracterizaron mediante LC–MS/MS con detector QqQ. Para ello se hizo uso de la versatilidad de este detector de masas en análisis cuantitativo y confirmatorio. En primer lugar, se

desarrollaron métodos basados en el barrido de iones precursores, de iones producto o de fragmentos neutros que permitieron confirmar la presencia de derivados secoiridoides, la familia con mayor importancia en la fracción fenólica de los VOOs. Estos compuestos son los que se encuentran en mayor concentración en esta fracción y los que diferencian al aceite de oliva de cualquier otro aceite vegetal. La aplicación de estas metodologías resultó de gran utilidad para confirmar la presencia de las agliconas del ligstrósido (*p*-HPEA-EA) y de la oleuropeína (3,4-DHPEA-EA) y sus formas dialdehídicas (*p*-HPEA-EDA y 3,4-DHPEA-EDA) que no están disponibles comercialmente.

Posteriormente se llevó a cabo el análisis mediante SRM para obtener la concentración de los principales compuestos que constituyen el perfil fenólico. Los perfiles fenólicos obtenidos se utilizaron para buscar correlaciones entre pares de fenoles, así como semejanzas y diferencias entre los diferentes VOOs. EL análisis de Pearson reveló las siguientes correlaciones positivas: (i) Entre *p*-HPEA-EA y 3,4-DHPEA-EA, con valores de $p = 0.002$ y coeficientes de correlación (R) = 0.92; (ii) entre *p*-HPEA-EDA y 3,4-DHPEA-EDA, con R = 0.89 y $p = 0.007$; (iii) entre los glucósidos de la apigenina y de la luteolina, con R= 0.91 y $p = 0.003$. Por el contrario, entre la quercetina y el ácido vainíllico se encontró una fuerte correlación negativa (R = -0.91 y $p = 0.004$). Todas estas correlaciones se explican por la conexión entre las principales rutas de biosíntesis de los compuestos fenólicos, lo que puede ser de gran utilidad a la hora de interpretar las propiedades sensoriales y nutracéuticas de un VOO determinado. Las correlaciones entre los diferentes VOOs se pusieron de manifiesto mediante análisis de Clúster (CA), en el que se distinguieron claramente dos grupos principales entre los VOOs, el primero formado por Arbequina, Hojiblanca, Cornicabra y Picual y el segundo por Sikitita, Arbosana y FS-17. Se observaron correlaciones altas (R > 0.7) para los siguientes pares de VOOs Arbequina/Hojiblanca (R = 0.77), Cornicabra/FS-17 (R = 0.81), Picual/FS-17 (R = 0.79). La mejor correlación se observó para el par Picual/Cornicabra (R = 0.99), lo que da idea de la

similitud entre ambos aceites. Estos resultados ponen de manifiesto la aplicabilidad de estos estudios a programas de mejora del olivo con el fin de obtener VOOs con una composición fenólica deseada.

La utilización de la espectrometría de masas de alta resolución permitó buscar un segundo enfoque en el análisis de la fracción fenólica. En concreto, hizo posible el desarrollo de métodos de análisis comparativo destinado a evaluar la variabilidad de la fracción fenólica en VOO. Sin duda, supone una prueba de las posibilidades de la metabolómica en programas de mejora de olivo para obtener beneficios desde un punto de vista agronómico.

Los factores que más afectan a la composición química y a la calidad del VOO son los edafoclimáticos, genéticos, geográficos, agronómicos (riego, fertilización, tiempo de cosecha), y tecnológicos (proceso de extracción y almacenamiento). En los programas de mejora del olivo se evalúan tanto parámetros agronómicos –vigorosidad, resistencia a enfermedades, reducción del periodo juvenil–, como parámetros químicos, entre los que destacan la composición fenólica y el perfil de ácidos grasos. De la variedad de factores que influyen en la composición del VOO destacan el genotipo y la maduración. El estudio de la influencia de ambos en la composición fenólica fue el objetivo de la investigación que recogen los Capítulos 6 y 7.

Se utilizó un enfoque global para estudiar la influencia del proceso de maduración de la aceituna en la fracción fenólica del VOO. Para ello, utilizando LC–QTOF y mediante análisis no orientado, se analizaron los extractos fenólicos de VOOs obtenidos de 12 genotipos seleccionados en el programa de mejora ya comentado, tres provenientes de las variedades Arbequina, Picual y Frantoio y 9 de selecciones avanzadas seleccionadas de la siguiente manera: Tres de Arbequina×Picual, tres de Picual×Arbequina y tres de Frantoio×Picual. De todas ellas se recogieron los frutos en diferentes estados de maduración.

Se estudió la variación de la composición fenólica, tanto con la fecha de recogida de la aceituna como con el índice de maduración (RI), que se

estima en función de la evolución del fruto con la variación del color (RI0= verde intenso, RI1= verde amarillento, RI2= enverado, RI3= violeta, RI4= negro). Mediante herramientas de análisis multivariante, tales como (PCA) y Mapas de calor, se compararon los perfiles obtenidos de los diferentes VOOs. En ambos casos (la fecha de recogida y el RI), la discriminación sólo se observó para determinados estados de maduración, lo que se explica por la fuerte influencia del genotipo. Esta influencia se evaluó comparando el número de entidades moleculares (potenciales metabolitos fenólicos) que cambiaba su concentración relativa con pares de RIs. Las mayores variaciones se produjeron en las etapas tempranas y avanzadas de maduración (entre los pares RI 0–1 y 3–4). Si todos los genotipos hubieran presentado un comportamiento similar, la concentración de compuestos fenólicos podría modelarse en función del índice de maduración; pero al evaluarse los genotipos de forma independiente se observó que no todos seguían esta tendencia. Así, en el cruce Picual×Arbequina los mayores cambios en la composición fenólica se observaron en estados avanzados de maduración; mientras que en el cruce Frantoio×Picual fue en los estados de maduración temprana donde el cambio fue más acusado. Esta misma metodología de análisis se aplicó a un panel compuesto por los fenoles representativos del VOO observándose que la evolución de algunos fenoles en función de la fecha de recogida y del RI tenía un comportamiento similar en algunos de los cruzamientos estudiados. Éste fue el caso del hidroxitirosol y del 3,4-DHPEA–EDA en los cruzamientos Arbequina×Picual y Picual×Arbequina, respectivamente. Por el contrario, la tendencia no se mantuvo para algunos compuestos como el *p*-HPEA-EDA en el cruce Arbequina×Picual o el tirosol en el Picual×Arbequina.

La enorme influencia del genotipo en la composición de la fracción fenólica puesta de manifiesto en este estudio condujo a la investigación recogida en el Capítulo 7, que abarcó el estudio de la influencia del genotipo en la composición de la fracción fenólica de los VOOs procedentes de poblaciones segregantes (reproducción sexual, cultivadas en el mismo campo

y en las mismas condiciones agronómicas) formadas por los cruzamientos Arbequina×Arbosana, Picual×Koroneiki y Sikitita×Arbosana y sus correspondientes genitores. La influencia de la maduración se eliminó, ya que todos los aceites procedían de aceitunas recogidas entre los índices de maduración 2 y 3. Se obtuvieron, mediante LC–QTOF, los perfiles fenólicos de 37 fenoles+compuestos que participan en las rutas principales de su biosíntesis tales como secologanol o ácido eleanólico. Haciendo uso de análisis estadístico multivariante, mediante PCA, *t*-test y análisis de cambio relativo, se evaluó la influencia del genotipo en la fracción fenólica. En el análisis no supervisado de los tres cruzamientos se observó que el cruce Picual×Koroneiki se separa de los de Arbequina×Arbosana y Sikitita×Arbosana, que presentaron un solapamiento parcial atribuible al genitor común. El siguiente paso fue la comparación por pares de cruzamientos, en la que se observó una clara separación entre las dos poblaciones comparadas en cada caso, siendo mayor entre los cruzamientos que implicaron los genitores Picual y Koroneiki frente a los otros dos; mientras que Sikitita×Arbosana y Arbequina×Arbosana tampoco se discriminaron completamente mediante el perfil fenólico. Estos resultados permitieron establecer la influencia del genotipo como fuente de variabilidad de la fracción fenólica de los VOOs. Por último, se evaluó la significancia de los fenoles frente al genotipo, de manera independiente. Un total de nueve fenoles (a saber, quercetina, *p*-HPEA-EA, la aglicona de la dimetiloleuropeína, 3,4-DHPEA-EA, hidroxypinoresinol, hidroxitirosol, ácido *p*-cumárico, ácidos ferúlico y protocatético), resultaron significativos y contribuyeron a explicar la variabilidad observada con el genotipo en un intervalo de confianza del 99% ($p < 0.01$).

Los resultados obtenidos de estos estudios sobre los cambios producidos en la fracción fenólica por los procesos de maduración o relacionados con el genotipo pueden ser de interés para los programas de mejora del olivo como base para mejorar la calidad del aceite de oliva. Para ello, el desarrollo de herramientas de análisis comparativo ha demostrado su

utilidad en este tipo de programas agronómicos enfatizando el potencial de la metabolómica en este contexto.

Sección C: Estudio de la influencia de la maduración y el genotipo en la composición de ácidos grasos de VOOs

Como se comentó en la Sección B, los parámetros con mayor interés en los programas de mejora del olivo son los agronómicos y los químicos, y en estos últimos la composición del VOO. Por tanto, tras el estudio de la fracción fenólica (Sección B), la Sección C recoge el estudio de otra de las fracciones de mayor interés en estos programas: La fracción de FAs, ya que su composición es uno de los factores más determinantes de la calidad del aceite de oliva. Al igual que la fracción fenólica, la composición de FAs se afecta por diferentes factores, siendo también en este caso los más relevantes el estado de maduración del fruto y el genotipo. Para el análisis de esta fracción se desarrolló un método basado en cromatografía de gases (GC) con detector de ionización de llama (FID), mediante el cual se analizaron muestras de VOO de diferentes genotipos procedentes del programa de mejora del olivar.

En el Capítulo 8 se recoge la evaluación de la influencia de la maderación y el genotipo en la composición de 10 de los FAs más significativos del VOO, teniendo en cuenta tanto sus formas esterificadas (EFAs) como no esterificadas (NEFAs). Se utilizaron VOOs de poblaciones avanzadas con reproducción asexual, cultivadas en una parcela dada y en las mismas condiciones agronómicas, obtenidas por cruzamientos entre las variedades Arbequina, Picual y Frantoio. Tras el análisis cuantitativo de los EFAs por GC–FID, se observó que presentaban diferente comportamiento dependiendo de su concentración en el VOO. Así, los EFA mayoritarios (C18:1, C16:0, C18:2, C18:0 y C16:1) incrementaban ligeramente su concentración con la maduración o la mantenían constante; los FAs minoritarios (C18:3, C24:0, C22:0, C20:0 y C20:1) experimentaron una disminución de su concentración

con la maduración. Esta misma tendencia se observó en los NEFAs: Los mayoritarios incrementaron su concentración hasta el RI 2, a partir del cual comenzaron a disminuir. Los NEFAs minoritarios disminuyeron su concentración a lo largo del proceso de maduración.

La aplicación de análisis multivariante con PCA sólo permitió distinguir entre estados de maduración temprana (RI 0 y 1) y avanzada (RI 3 y 4) —comportamiento similar al encontrado en la fracción fenólica. Para evaluar la influencia de la maduración en la composición de los FAs se realizó un análisis de varianza (ANOVA), que puso de manifiesto que el RI no ejerció influencia significativa (intervalo de confianza del 95%) en las concentraciones de FAs. De hecho, la variabilidad observada entre muestras pertenecientes a un mismo RI fue mayor que la que se observó entre grupos de muestras de diferente RI. Este comportamiento se justificó por la influencia de un factor externo como el genotipo. Para demostrar la influencia de este factor se realizó un ANOVA para cada RI teniendo en cuenta el efecto del genotipo; lo que puso de manifiesto que este efecto no influye en la concentración de NEFAs, ni es capaz de explicar los niveles de FAs en VOOs de frutos de RI 0. No obstante, sí contribuyó de manera significativa (95 ó 99% de nivel de confianza) a explicar la concentración de EFAs tales como C16:0, C16:1, C18:1, C18:2 y C18, y a identificar el RI 3 como la fase de maduración en la que más se influencia por el genotipo la composi-ción de FAs en el VOO. Por lo tanto, este RI es el más representativo para la comparación de genotipos en programas de mejora del olivo. Asimismo, se evaluaron las concentraciones de los FAs que resultaron significativos (con un nivel de confianza del 99%) para explicar la influencia del genotipo en el proceso de maduración, y se sometieron a un estudio comparativo entre los diferentes cultivos. Se observó una clara influencia del genotipo en la concentración de cada FA. Así, en algunos cruzamientos las concentraciones de determinados FAs eran mayores que en sus dos genitores. Tal es el caso del C16:0 y el C16:1 en los VOOs procedentes del cruzamiento Arbequina×Picual, que se asemejan más a la concentración del genitor

Arbequina. En otros casos la concentración del FA en el cruzamiento se encontraba a un valor intermedio entre ambos genitores, como en el cruzamiento Picual×Arbequina para el C18:1, que se aproxima más a Picual. Por tanto, en ambos caso existe una mayor influencia del genitor en el que se produjo la polinización.

El Capítulo 9 se dedicó al estudio de la variación de la concentración de FAs del VOO con el genotipo. Para ello se estudiaron también los 10 FAs más importantes del VOO en poblaciones segregantes (reproducción sexual, cultivadas en el mismo campo y en las mismas condiciones agronómicas) de los cruzamientos Arbequina×Arbosana, Picual×Koroneiki y Sikitita× Arbosana, y en sus correspondientes genitores. El método analítico utilizado fue el mismo que en el capítulo anterior.

Los valores de desviación estándar relativa (DER) obtenidos del análisis cuantitativo proporcionaron información de la elevada variabilidad en las concentraciones de EFAs y NEFAs. La inclusión de los NEFAs se realizó tras observar que no existían correlaciones entre los pares EFA/NEFA de un mismo FA: Todos los coeficientes de correlación R obtenidos de un análisis de Pearson fueron menores de 0.60. Por el contrario, sí se encontraron correlaciones entre los pares de FAs C16:0 EFA/C16:1 EFA (R = 0.81); C16:0 NEFA/C18:1 NEFA (R = 0.67) o C22:0 NEFA/C24:0 NEFA (R = 0.91); correlaciones que se explican mediante las rutas de biosíntesis de los FAs.

Se estudió la influencia del genotipo en cada FA realizando un ANOVA, en el que resultaron significativas (p<0.01) las formas EFAs de C16:0, C16:1, C18:1 y el NEFA C18:0. También se observó variabilidad en las muestras pertenecientes a la misma variedad, asemejándose algunas muestras más al genitor "femenino" y otras más al "masculino".

El análisis mediante PCA de la matriz de muestras se aplicó a los tres cruzamientos y también a cada par de cruzamientos. En ambos casos se obtuvo discriminación de las muestras en función del genotipo. Los VOOs

procedentes del cruzamiento Picual×Koroneiki fueron los que presentaron una mejor discriminación. Sin embargo, las muestras procedentes de los cruzamientos Arbequina×Arbosana y Sikitita×Arbosana presentaron solapación, tanto en los PCAs en los que incluyeron los 3 cruzamientos, como en los que se realizaron por pares de cruzamientos.

Con los compuestos que resultaron significativos tras la realización del ANOVA, se llevo a cabo un CA. Los VOOs se agruparon en dos conjuntos, uno de los que provenían de los cruzamientos Picual×Koroneiki y otro de los de Sikitita×Arbosana; mientras que los que provenían de Arbequina×Arbosana presentaron una distribución cuyas proporciones participaban parcialmente de las de ambos grupos. Por tanto, con este panel de FAs significativos fue posible la discriminación entre los cruzamientos sin genitor común.

Se identificó el C16:1 —tanto en su forma EFA como NEFA— como el principal responsable de la discriminación de VOOs provenientes de diferentes poblaciones segregantes. Es importante resaltar pues la información complementaria que aporta el análisis de la fracción de ácidos grasos en VOO para su implementación en programas de mejora.

Sección D: Evaluación conjunta de la variabilidad de la composición fenólica y de los FAs con la maduración del fruto y el genotipo

En esta sección se recoge le evaluación conjunta de la variabilidad en la composición de las dos fracciones de mayor interés en los programas de mejora del olivo, la composición fenólica y la de los FAs, con el genotipo y la maduración del fruto. Se pretendió así establecer qué familia es más significativa con cada uno de estos factores. En concreto, se evaluaron 36 fenoles o compuestos derivados pertenecientes a las familias más importantes en VOO y en 10 de los FAs más representativos (en formas esterificada y no esterificada) en muestras provenientes de poblaciones avanzadas formadas por los cruzamientos Arbequina×Picual, Picual×Arbequina y Frantoio×Pi-

cual y sus respectivos genitores. Los árboles seleccionados fueron cultivados en la misma parcela y con las mismas prácticas agronómicas, de forma que las fuentes de variación fueron el genotipo y la maduración del fruto. En el estudio se utilizó una estrategia combinada de análisis no supervisado mediante PCA y supervisado mediante Regresión Parcial de Mínimos Cuadrados (PLS-R). El análisis mediante PCA permitió una clara discriminación de los VOOs pertenecientes al cruzamiento Frantoio×Picual, existiendo un solapamiento entre los cruzamientos Arbequina×Picual, Picual×Arbequina debido a sus genitores comunes y no se pudo discriminar respecto al estado de maduración. Este comportamiento demuestra la influencia del genotipo en la composición del VOOs, que se ratificó mediante PCAs por pares de cruzamientos, que mostró en los tres casos una clara separación en función del genotipo. Para evaluar qué compuestos son los que más se influencian por el genotipo se realizó un ANOVA a cada par de cruzamientos, proporcionando valores de $p<0.01$. Los resultados mostraron que el genotipo influye de manera significativa en los compuestos fenólicos, mientras que los cambios son menos relevantes en el caso de los FAs. Por otro lado, se evaluó la influencia del parámetro estandarizado RI en el perfil fenólico y de los FAs, que mostró discriminación entre los VOOs obtenidos con frutos pertenecientes a los RI 0–1y los que pertenecían a los RI 2–3–4.

El análisis por PCA también permitió identificar los compuestos con mayor contribución para explicar esta variabilidad, destacando el comportamiento opuesto encontrado para dos de los secoiridoides directamente relacionados con dos propiedades sensoriales como el amargor (3,4-DHPEA-EA) y el picor (*p*-HPEA-EDA) de los VOOs.

Un estudio complementario consistió en el desarrollo de modelos de regresión cuantitativos mediante Regresión Parcial de Mínimos Cuadrados (PLS-R) utilizando la serie completa de datos (FAs y fenoles) y como factor de respuesta el valor numérico del RI. El modelo obtenido presentó una muy buena precisión —R^2 = 0.892, raíz cuadrada del error cuadrático medio

(RMSE) = 0.73—, por lo que se concluyó que la concentración de fenoles y la de FAs de los VOOs está fuertemente relacionada con RI del fruto. Un test ANOVA permitió la identificación de compuestos que contribuyen en mayor medida a explicar la variabilidad observada. Entre estos compuestos destacan la mayoría de los NEFAs, seis de los compuestos fenólicos y dos de los principales compuestos implicados en las rutas de síntesis de los secoiridoides (ácido logánico y secologanol). Además, presentaron correlación con el RI tres de los fenoles con importantes propiedades nutracéuticas y organolépticas, como son el hidroxitirosol (p=0.0170), el tirosol (p=0.0125) y el *p*-HPEA-EDA (p=0.0383).

Se desarrolló otro modelo de regresión cuantitativo utilizando la concentración total de fenoles (determinados mediante el método de Folin–Ciocalteu) como variable de respuesta y el conjunto de datos formado por los compuestos fenólicos. Los parámetros estadísticos obtenidos, R^2 = 0.853, RMSE = 0.791, pusieron de manifiesto la buena precisión del modelo y, por tanto, la asociación directa entre la concentración de fenoles totales y los compuestos fenólicos individuales. Un test de ANOVA permitió la identificación de los siguientes fenoles, como los más significativos: El isochroman, el hidroxipinoresinol, el pinoresinol, la oleuropeína, el ligstrósido, las agliconas de la oleuropeína (3, 4-DHPEA-EA) y del ligstrósido (*p*-HPEA-EA), el tirosol, los ácidos ferúlico y protocatéquico, la diosmetina y los glucósidos de la apigenina y la luteolina. Aunque sólo 4 (ácidos ferúlico y protocatéquico, *p*-HPEA-EA y oleuropeína) presentaron correlaciones moderadas con la concentración total de fenoles.

Sección E: Enriquecimiento de aceites refinados de diferentes semillas con desechos de la industria olivarera y comparación de los perfiles fenólicos

Esta sección engloba dos capítulos dedicados al aprovechamiento de residuos de la industria olivarera y aceitera: Las hojas de olivo y el alperujo.

Estos residuos, ya caracterizados por el grupo de investigación al que pertenece la doctoranda, habían mostrado poseer una cantidad de antioxidantes polares muy superior a la existente en el aceite.

En el estudio que se recoge en el Capítulo 11 se llevó a cabo el enriquecimiento de aceites refinados de maíz, soja, girasol, girasol alto contenido en ácido oleico, oliva y colza, con extractos de fenoles de hojas y alperujo, a dos niveles de concentración (200 y 400 μg/mL). El proceso de extracción de fenoles en ambos casos se realizó con auxilio de microondas focalizadas (MAE). Se evaluó el enriquecimiento de cada aceite mediante cromatografía líquida con detector de diodos en fila (LC–DAD) y el método de Folin-Ciocalteu.

Las características y la proporción de los ácidos grasos presentes en cada aceite refinado establecen el coeficiente de partición de cada fenol entre el extracto y el aceite en cuestión. En líneas generales, los fenoles más polares, como el hidroxitirosol, el tirosol, el 3,4-DHPEA-DA y la oleuropeína se transfirieron en mayor proporción a los aceites con bajo contenido de ácido oleico (C18:1) y elevado contenido en ácidos grasos poliinsaturados (PUFAS), tales como los de soja y maíz. Por el contrario, el *p*-HPEA-EDA se transfirió preferiblemente a aceites con elevado contenido de C18:1, como es el caso del de oliva o con alto contenido en oleico.

En lo que se refiere a la composición de los extractos, los de alperujo son más ricos en antioxidantes como el hidroxitirosol y el tirosol, en productos de hidrólisis de los secoiridoides (como el 3,4-DHPEA-EDA) y en flavonoides (como luteolina y apigenina). Por tanto, el enriquecimiento con extractos del alperujo condujo a una transferencia de fenoles a los aceites refinados que igualaba o superaba los niveles de algunos de estos compuestos presentes de forma natural en el VOO —tal es el caso del 3,4-DHPEA-EDA, el hidroxitirosol y el tirosol, en ambos niveles de enriquecimiento. Por otra parte, los extractos de hojas presentaron elevados niveles de oleuropeína, por lo que los aceites enriquecidos con estos extractos superaron amplia-

mente el nivel de este fenol en el VOO, que es prácticamente indetectable debido al estado de maduración en el que se recoge la aceituna para la producción del aceite.

Para comparar la calidad y la estabilidad del VOO con las de los aceites antes y después del enriquecimiento, se realizaron los tests de calidad (índice de acidez) y de estabilidad (índice de peróxidos y Rancimat) usuales en la industria aceitera. El índice de acidez, un indicador de la cantidad de ácidos grasos libres expresada en porcentaje de oleico (índice que disminuye con la calidad del aceite), estuvo en el rango 0.3–0.8% en el VOO y aumentó ligeramente en los aceites enriquecidos, pero en ningún caso superó el límite establecido por el Comité Oleícola Internacional (IOC) para el VOO. La determinación de los parámetros K232 y K270, usados para la clasificación de aceites de oliva, y directamente relacionados con la estabilidad y la pureza, miden el deterioro por oxidación de los aceites. Se comprobó una mejora sustan-cial de ambos parámetros en el aceite de oliva enriquecido, aunque este pará-metro sólo es representativo para el aceite de oliva y no se relaciona con el grado de enriquecimiento de otros aceites.

El índice de peróxidos es un parámetro de calidad relacionado con la estabilidad a la oxidación de los aceites; o lo que es igual, su resistencia al enranciamiento. La estabilidad de los aceites enriquecidos mejoró respecto a la de los no enriquecidos aumentando de un 60 a un 90%. No se observaron diferencias significativas de este parámetro entre los dos tipos de extractos para el mismo grado de enriquecimiento.

Por último, se evaluó la estabilidad oxidativa de los aceites mediante el ensayo de Rancimat (alta temperatura en corriente de aire). El periodo de inducción —definido como el tiempo en el que la oxidación se produce de manera relativamente lenta — de cada aceite aumentó en todos los casos, siendo más largo para los enriquecimientos mayores (400 μg/mL). Este comportamiento permite augurar una buena respuesta de los aceites enriquecidos en los procesos de fritura.

En general todos los parámetros estudiados mejoraron como consecuencia de la presencia de fenoles en los aceites, aumentando así no sólo su calidad y estabilidad sino también sus propiedades saludables. El proceso de enriquecimiento (rápido y poco costoso) permite aumentar las propiedades nutracéuticas de los aceites enriquecidos.

Tras la evaluación de la calidad y la estabilidad oxidativa de los aceites enriquecidos la siguiente etapa (Capítulo 12) consistió en su caracterización mediante LC–QTOF, con generación del perfil fenólico característico de cada uno de ellos. Los cromatogramas obtenidos revelaron la ausencia de compuestos fenólicos en los aceites refinados y una representativa huella dactilar de compuestos polares y de polaridad media en el VOO. Los perfiles obtenidos para los enriquecimientos con hojas y con alperujo revelaron diferencias significativas, proporcionando los primeros una mayor cantidad de picos significativos en la primera parte del cromatograma, donde eluyen los compuestos más polares. Los enriquecimientos con alperujo proporcionaron mayor cantidad de picos cromatográficos en la zona de elución de los secoiridoides. Los dos niveles de enriquecimiento también permitieron diferenciar claramente los cromatogramas.

Tras la extracción de cromatogramas de iones (EIC) de compuestos representativos —tales como el hidroxitirosol, el tirosol, la oleuropeína, el 3,4-DHPEA-EDA, el p-HPEA-EDA, el 3,4-DHPEA-EA, la luteolina, la apigenina y el hidroxipinoresinol— de las diferentes familias, se puso de nuevo claramente de manifiesto la influencia del coeficiente de partición de cada fenol en función del tipo de aceite y de la concentración relativa del fenol dado en el extracto. Hay que destacar el siguiente comportamiento: (i) Los aceites enriquecidos con extractos de alperujo superaron la concentración de hidroxitirosol de los VOOs; (ii) tanto con los extractos de hojas como con los de alperujo el contenido de 3,4-DHPEA-EDA en los aceites enriquecidos fue superior a los de los VOOs; (iii) la concentración de oleuropeína fue mayor en todos los aceites enriquecidos que en los VOOs;

(iv) los niveles de *p*-HPEA-EDA no alcanzaron en ninguno de los enriquecimientos los de este fenol en los VOOs.

Haciendo uso de herramientas quimiométricas se llevó a cabo la comparación cualitativa de aceites enriquecidos con los no enriquecidos y con el VOO. Las muestras se dividieron en cuatro grupos: Aceites puros (los refinados no enriquecidos), aceites enriquecidos con extractos de hojas, aceites enriquecidos con extractos de alperujo y VOOs. Se llevaron a cabo dos tipos de análisis, uno restrictivo en el cual la matriz estaba formada por 178 entidades moleculares que estaban presentes en el 100% de los aceites, y otro no restrictivo donde en la matriz se consideraban aquellas entidades moleculares presentes al menos en el 40% de los aceites. El análisis no supervisado mediante PCA para ambas matrices permitió una clara discriminación entre los 4 grupos de aceites (puros, enriquecidos con extractos de alperujo, enriquecidos con extractos de hojas y VOOs), en la que los VOOs estaban más cercanos a los aceites enriquecidos que a los puros. Se estudió la semejanza entre aceites pertenecientes a un mismo grupo mediante mapas de correlación. Los aceites puros prácticamente no presentaron correlaciones entre ellos ni con otros aceites, mientras los enriquecidos con el mismo extracto mostraron altas correlaciones y los enriquecidos con extractos diferentes presentaron bajas correlaciones. Por otro lado, a pesar de los factores que influyen en la composición de los diferentes VOOs, la correlación entre ellos y con los aceites enriquecidos con extractos de alperujo fue significativa. Por el contrario, las correlaciones entre VOOs y los aceites enriquecidos con extractos de hojas fueron bajas.

Este estudio abre la posibilidad de preparación de aceites "a la carta" con unas propiedades —asociadas a los compuestos fenólicos— organolépticas o saludables concretas si se lleva a cabo una selección adecuada de la materia prima usada para preparar los extractos usados para el enriquecimiento. De esta forma se podría incrementar de manera notable la

calidad de los aceites refinados dándoles un valor añadido al mejorarse sus propiedades mediante el enriquecimiento fenólico.

CONCLUSIONES

La investigación realizada para optar al grado de Doctora en Ciencias ha dado lugar a las siguientes conclusiones:

- El estudio en profundidad de los métodos existentes para el análisis de la fracción minoritaria del VOO, así como de las características y propiedades de las diferentes familias que la componen ha permitido:

  - El desarrollo de la metodología más apropiada para la caracterización de esta fracción, con especial atención a la etapa de saponificación, ha llevado a demostrar que: a) La saponificación en caliente proporciona la eficiencia más alta en términos cualitativos y cuantitativos; b) la saponificación en frío y la asistida por microondas presentan perfiles similares —y esta última es la más similar a la saponificación en caliente—; c) la saponificación asistida por ultrasonidos da lugar a los perfiles más diferentes.
  - La comparación de la fracción insaponificable de diferentes VOOs con la de otros aceites vegetales tras saponificación en caliente, ha llevado a la conclusión de que: a) El VOO proporciona perfiles con una composición más compleja que el resto de los aceites; b) el aceite de soja tiene el perfil más similar a los VOOs seguido de los aceites de orujo y oliva refinados.

- El análisis de la fracción fenólica de diferentes VOOs monovarietales mediante un espectrómetro de masas de triple cuadrupolo (QqQ), con sus diferentes modalidades de trabajo, junto con el uso de las herramientas quimiométricas apropiadas, han llevado a las siguientes conclusiones:

- La espectrometría de masas, gracias a sus propiedades analíticas tales como sensibilidad, selectividad y precisión, es una técnica muy adecuada para el análisis confirmatorio y cuantitativo de los compuestos fenólicos del VOO, especialmente los secoiridoides y sus derivados. Es importante destacar la versatilidad del triple cuadrupolo en sus diferentes modalidades de trabajo ("*data dependent methods*") para esta finalidad.
- Existen correlaciones entre los diferentes VOOs monovarietales, lo que ha permitido establecer similaridades en base al perfil fenólico, así como entre los diferentes compuestos fenólicos — estas últimas explicadas a través de sus rutas biosintéticas.

- El análisis de la fracción fenólica de VOOs procedentes de poblaciones segregantes y selecciones avanzadas del programa de mejora del olivar mediante un equipo cuadrupolo-tiempo de vuelo (Qq-TOF), junto con las herramientas quimiométricas apropiadas, han mostrado el potencial de la metabolómica en esta parcela agronómica. En concreto, se ha estudiado la variación de esta fracción con el genotipo (en poblaciones segregantes) y el estado de maduración del fruto (en selecciones avanzadas), llevando a concluir que:

- El índice de maduración (RI) no es suficiente para explicar la variabilidad encontrada en la composición fenólica de los VOOs. La discriminación de los VOOs obtenidos de frutos con diferentes RI, sólo ha sido posible para VOOs procedentes de frutos de recolección temprana y de recolección tardía; lo que pone de manifiesto la influencia del genotipo en la fracción fenólica. Los VOOs procedentes de diferentes cruzamientos muestran una clara separación, que es mayor en cruzamientos sin genitores comunes.

- Los fenoles que contribuyen de manera significativa a explicar la variabilidad observada con el genotipo son la quercetina, las agliconas del ligstrósido (*p*-HPEA-EA), de la oleuropeína (3,4-DHPEA-EA) y de la dimetiloleuropeína, el hidroxypinoresinol, el hidroxitirosol y los ácidos *p*-cumárico, ferúlico y protocatéquico.

- Otra fracción interesante en el VOO, que se ha estudiado con el mismo planteamiento que la fenólica, ha sido la compuesta por los ácidos grasos. También se ha demostrado la utilidad de este tipo de estrategias de análisis comparativo basado en metabolómica para obtener conclusiones de interés agronómico en un programa de mejora. Así, el genotipo y la maduración afectan también a la composición de los FAs –en sus formas esterificadas (EFAs) y no esterificadas (NEFAs)– presentes en los VOOs procedentes de poblaciones segregantes y selecciones avanzadas. El desarrollo de un método basado en cromatografía de gases (GC) con detección mediante ionización en llama (FID) ha permitido el estudio de la variación de la composición de los FAs con estos dos factores. Las conclusiones de este estudio son las siguientes:

- No existen correlaciones significativas entre los pares EFA y NEFA de un mismo FA. Sin embargo, se ha visto significancia estadística para ciertos NEFAs en algunos estudios, lo que demuestra su interés a pesar de ser una fracción escasamente considerada. Se han observado correlaciones entre diferentes FAs que se explican mediante sus rutas de biosíntesis.
- Los cambios en las concentraciones de los FAs con la maduración no siguen un mismo patrón para todos ellos, ya que mientras los mayoritarios incrementan o mantienen constante su concentración con la maduración, los minoritarios la disminuyen.
- Al igual que ocurre con la fracción fenólica, el RI no afecta de manera significativa a la composición de los FAs. La variabilidad

observada entre VOOs procedentes de frutos con el mismo RI es mayor que la observada entre VOOs que proceden de frutos de RI diferentes, lo que confirma la influencia de un factor externo como es el genotipo.

- El estudio del genotipo para cada RI ha demostrado que: a) La variación de la composición de los FAs en los VOOs de RI 0 no se explica por el genotipo; b) el RI 3 es el índice de maduración en el que la composición de los FAs experimenta mayor variación; por tanto, es el índice más adecuado para la comparación de genotipos en los programas de mejora del olivo; c) los FAs que han contribuido de manera significativa a explicar la variabilidad debida al genotipo son los siguientes: C16:0, C16:1, C18:1, C18:2 y C18:3; d) el genotipo no es un factor influyente en la composición de los NEFAs.
- Los VOOs se discriminan claramente en función del genotipo. Al igual que en el estudio de la fracción fenólica, la discriminación es más clara en los VOOs que no tienen genitores comunes. El EFA C16:1 se ha identificado como el ácido graso con mayor contribución para explicar esta discriminación.

- Una evaluación conjunta de la influencia del genotipo y del proceso de maduración en los perfiles de ácidos grasos y de fenoles de los VOOs, procedentes de selecciones avanzadas, junto con el uso de las herramientas quimiométricas adecuadas, han demostrado que:

- La fracción fenólica mostró una mayor variabilidad que la de los FAs debida a la maduración del fruto y a la influencia del genotipo. Por tanto, refleja mayores diferencias en función de estos dos factores.
- Al igual que en los estudios independientes de fenoles y de FAs: a) Los VOOs procedentes de cruzamientos diferentes pueden

discriminarse, lo que confirma la influencia del genotipo en la variabilidad de la composición de FAs y de fenoles; b) los VOOs de cruzamientos con genitor común presentan solapación parcial; c) los estudios por pares de cruzamientos presentan discriminación total respecto al genotipo.

- El RI tiene un comportamiento similar al mostrado en los estudios independientes de fenoles y de FAs; sólo se observa discriminación en los VOOs procedentes de RI 0-1 y de RI 2-3-4.
- Las concentraciones de fenoles y de FAs están estrechamente relacionadas con el RI. Los compuestos que influyen de manera significativa y presentan una fuerte correlación con este parámetro son: hidroxitirosol, tirosol y su derivado secoiridoide (*p*-HPEA-EDA).
- El contenido total de fenoles (determinado mediante el método de Folin-Ciocalteu) se relaciona directamente con el de compuestos fenólicos individuales. Los fenoles que explican esta relación de manera más significativa son: El derivado isocromano, el hidroxipinoresinol, el pinoresinol, oleuropeína, ligstrósido, las agliconas de la oleuropeína (3, 4-DHPEA-EA) y del ligstrósido (*p*-HPEA-EA), el tirosol, los ácidos ferúlico y protocatéquico, la diosmetina y los glucósidos de la apigenina y la luteolina.

- La evaluación de los parámetros de calidad y estabilidad de aceites refinados enriquecidos con extractos fenólicos procedentes de residuos del olivo y de la industria aceitera en comparación con los del VOO ha mostrado que:

- La composición en FAs de cada aceite refinado da lugar a un coeficiente de reparto de los fenoles característico, lo que resulta en que: a) Los aceites con elevada concentración de FAs

saturados (SFAs) son menos propensos al enriquecimiento con los fenoles más polares; b) los aceites con elevada concentración de FAs monoinsaturados (MUFAs) experimentan un mayor enriquecimiento; c) los PUFAs se enriquecen más en fenoles más polares que los MUFAs.

- La composición fenólica de cada extracto también ha influido en el coeficiente de reparto de los fenoles, por lo que: a) Los extractos fenólicos procedentes de alperujo, ricos en fenoles polares, secoiridoides y flavonoides, se transfieren en tal proporción los aceites refinados que en algunos casos superan los niveles presentes en el VOO; b) todos los aceites enriquecidos con extractos de hojas han superado el contenido de oleuropeína del VOO debido a que es el fenol mayoritario en estos extractos y a su baja concentración en el VOO.
- La presencia de estos fenoles en los aceites refinados mejora de forma significativa o extraordinaria su calidad y estabilidad oxidativa.

- La caracterización mediante LC–TOF/MS, de aceites refinados puros (sin enriquecer), enriquecidos (a dos niveles 200 y 400 μg/mL) y de VOOs, junto con el uso de las herramientas quimiométricas adecuadas, lleva a concluir que:

- Los perfiles fenólicos obtenidos en el enriquecimiento revelan diferencias significativas. Los aceites enriquecidos con extractos fenólicos procedentes de hojas proporcionan perfiles con una mayor cantidad de picos en la parte inicial del cromatograma, en la que eluyen los compuestos más polares. Por el contrario, los enriquecidos con extractos de alperujo proporcionan más picos en la zona de elución de los secoiridoides y sus derivados, asemejándose más a los perfiles de los VOOs.

- El coeficiente de reparto de cada fenol entre el extracto y el aceite es función de este último y de la concentración relativa del fenol dado en el extracto.
- Los aceites enriquecidos muestran más semejanza en su composición polar con los VOOs que con los aceites no enriquecidos. Los enriquecidos con el mismo extracto presentan buenas correlaciones, mientras que las correlaciones son menos favorables entre los que se enriquecen con extractos diferentes.

ANEXOS

ANEXO I:

ANEXO II:

XII Reunión del Grupo Rregional Aandaluz de la Sociedad Española de Química Analítica, Córdoba (España), junio 2010.

1. **"Métodos y dispositivo experimental para el seguimiento de la evolución de un biorreactor de degradación de cianuro"**. M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Póster.

The international Conference on Natural Products, Castres Cedex (Francia), mayo 2011.

2. **"Quality parameters of edible oils enriched with antioxidants from olive tree: the role of enrichment extracts and lipid composition"**. . M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Póster.

5th International Conference on Polyphenols and Health, Sitges (España), octubre 2011.

3. **"Olive phenols for enhancement stability of refined edible oils and improvement metabolism of essential fatty acids"**. M. D. Luque de Castro, F. Priego-Capote, C. Ferreiro-Vera, V. Sánchez de Medina. Ponencia en congreso.

5th International conference on polyphenols and health, Sitges (España), octubre 2011.

4. **"Tailor-made enriched oil with olive phenols: improvement of stability/quality properties and health benefits"**. M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Póster.

V Jornadas de divulgación de la investigación en biología molecular, celular, genética y biotecnología. Córdoba (España), marzo 2012.

5. **"Tailor-made enriched oil with olive phenols: improvement of stability/quality properties and health benefits"**. M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Póster.

I Congreso científico de investigadores en formación en agroalimentación del eida3. Córdoba (España), mayo 2012.

6. **"Nuevos estudios sobre la mejora de la calidad del aceite de oliva y el aprovechamiento de los residuos generados en el cultivo del olivo"**. M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Comunicación en congreso..

Reunión del grupo regional andaluz de la sociedad española de química analítica. Málaga (España), junio 2012.

7. **"Aprovechamiento de desechos de la agricultura y de la industria agroalimentaria andaluzas"**. M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Póster.

XXIII Reunión nacional-VII congresso iberico espectroscopía. Córdoba (España), septiembre 2012.

8. "Mass spectroscopy applied to olive breeding programs". M. D. Luque de Castro, F. Priego-Capote, V. Sánchez de Medina. Póster.

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Métodos y dispositivo experimental para el seguimiento de la evolución de un biorreactor de degradación de cianuro

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El cianuro es un compuesto nitrogenado que inhibe las metaloproteínas que participan en los procesos respiratorios de los seres vivos. Aunque el cianuro tiene un origen natural, ya que algunos animales o plantas lo producen como mecanismo de defensa, provoca problemas medioambientales debido a la producción masiva en minas y en industrias de manufactura de metales nobles. Una de las industrias más importantes en Córdoba y su provincia es la joyería, que genera aproximadamente 5 toneladas de un residuo que contiene cianuro libre y complejos cianometálicos de Fe, Co, Ni, Ag, V, y Au. A pesar de la alta toxicidad del cianuro, existen microorganismos capaces de degradarlo usándolo como fuente de nitrógeno. La bacteria cianotrófica *Pseudomonas pseudoalcaligenes* tolera altas concentraciones de cianuro, cianatos, nitrilos y complejos estables cianuro–metal como fuente de nitrógeno. El hecho de que la bacteria crezca en un medio de pH alcalino, entre 9 y 9.5, minimiza la volatilización del cianuro libre como ácido cianhídrico (HCN), importante en el proceso degradativo del biorreactor debido al carácter aerobio de la biotransformación. Para el control del funcionamiento del biorreactor es necesario el seguimiento de una serie de parámetros analíticos como cianuro libre, complejado y total, iones metálicos y yodo. Asimismo, se requiere establecer un sistema de eliminación de los iones metálicos que quedan libres tras la degradación bacteriana del cianuro.

Se ha desarrollado un dispositivo experimental basado en los siguientes métodos:

Para la determinación del cianuro libre se emplea un método fotométrico basado en la reacción de Asmus y Garschagen y desarrollado en un sistema de inyección en flujo (FI), que consta de una bomba peristáltica, un baño termostatizado y un detector de diodos en fila. El intervalo de cuantificación se sitúa entre 0.5 y 2 mM en cianuro.

El cianuro total se mide, también en un sistema FI, tras una etapa de pervaporación del HCN, mediante un electrodo potenciométrico selectivo para cianuro frente al de referencia de Ag/AgCl. El uso de una lámpara UV actuando sobre el serpentín de transporte de la muestra hasta el pervaporador permite la ruptura de los complejos cianurados. El método hace posible la determinación de cianuro en concentraciones del orden de 10^{-6} M. La concentración de cianuro complejado se obtiene por diferencia entre el cianuro libre y el total.

Los metales se determinan por espectrometría de absorción atómica con llama; para el iodo se utiliza la espectrometría de fluorescencia de rayos-X. Para concentraciones bajas de yodo es necesaria una etapa de preconcentración en fase sólida.

La eliminación de los iones metálicos libres que se generan como consecuencia del consumo bacteriano del cianuro se realiza mediante un "bypass" en el biorreactor por el que se lleva la disolución a un sistema con dos columnas de intercambio iónico que funcionan de forma alternativa, una reteniendo los metales y otra regenerándose con una corriente ácida circulando en sentido opuesto al de la retención para evitar el incremento de compactación del sorbente.

P1-J

QUALITY PARAMETERS OF EDIBLE OILS ENRICHED WITH ANTIOXIDANTS FROM OLIVE TREE: THE ROLE OF ENRICHMENT EXTRACTS AND LIPID COMPOSITION

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Extra-virgin olive-oil, EVOO, is the most demanded liquid fat in the Mediterranean basin thanks to the nutraceutical properties of its components, divided into two groups as a function of their concentration: major and minor compounds. The first group, known as the saponifiable fraction, represents more than 98% of the total weight. The second group, unsaponifiable fraction, is constituted by aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, carotenes and phenols. Interest on olive phenols (OPs) has increased in recent decades because of their antioxidant properties, very appreciated from the clinical, pharmacological cosmetic and nutritional points of view. These proved excellent properties of OPs have promoted active research on raw materials for their isolation.

Given the high price of EVOO and the excellent properties of its phenols, there is a growing interest in the use of these compounds to enrich low-priced oils in order to obtain a healthy added-value product.

The present research was focused on the assessment of quality and stability properties of refined edible oils enriched with phenols from olive-tree materials. With this aim, extracts from olive-tree leaves and olive pomace (the semisolid waste from the two-phases production of EVOO termed alperujo) were used to enrich different refined oils (*viz.* maize oil, soy oil, high-oleic sunflower oil, sunflower oil, olive oil, rapeseed oil) with natural phenolic antioxidants at two concentration levels (200 and 400 μg/mL, expressed as gallic acid according to the Folin-Ciocalteu test). The different ratios of characteristic OPs in these extracts together with the lipidic composition of the oils to be enriched gave place to different mass transfer of the target antioxidants, which conferred additional stability to —and improved quality parameters of — oils as checked by subjecting them to typical tests such as Rancimat.

In general, all the oils experienced an improvement of their quality–stability parameters after enrichment as compared with their non-enriched counterpart and also by comparison to EVOO with a natural content in phenols of 400 μg/mL. Taking into account the scarce or nil prices of the raw materials for extraction of OPs, considered as agricultural waste, and the sustainable methods both for extraction of the target antioxidants and oil enrichment, these processes can be an alternative to endow refined oils with better characteristics from the industry and health points of view.

The healthy properties of phenols from olive-tree materials can convert oils into supplemented foods or even into nutraceuticals.

P427

PHARMACEUTICAL ASSISTANCE IN SYNTHETIC DRUGS AND HERBAL MEDICINES IN PREGNANCY IN ARARAQUARA, SÃO PAULO STATE, BRAZIL

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Objectives: Pharmaceutical Assistance can be understood as a set of actions with a multidisciplinary focus on promotion, protection and restoration of health, both individually and collectively, focusing mainly on the rational use of drugs as a major focus. Among the tools we can mention the Pharmaceutical Assistance to Health Education with the practices of educational groups. In this work, meetings were held with pregnant participants of the NGO baby aboard of Araraquara, SP state, Brazil, with practices educational and informative about the differences between the actions of the synthetic drugs prescribed by physicians and herbal medicines purchased at pharmacies and drugstores.
Results: The results revealed that pregnant women don't know the difference between these two types of synthetic and herbal medicines. They also reported that if herbal medicines are prepared from medicinal plants did not cause any harm to their health, to health of the fetus and baby during breastfeeding, and then can be freely purchased at pharmacies and drugstores without a report to your doctor. Reported to use of the medicines prescribed by doctor in clinic for pregnant women with other herbal medicines purchased at pharmacies and drugstores, especially for treatment of the constipation and flatulences.
Conclusions: This study showed the importance of deploying groups in health education for pregnant women and infants, with the purpose of informing, and might reduce the potential health risks to pregnant, fetus and infants, resulting from possible drug interactions and adverse reactions, arising from the concurrent use these two types of drugs.
Support Fundunesp, PROEX, PADC-FCF-UNESP

P428

ANTIINFLAMMATORY ACTIVITY OF PRENYLATED FLAVANONES NATURAL AND DERIVATIVES STUDIED IN THE EXPERIMENTAL MODEL OF INFLAMMATION OF EAR OF MOUSE INDUCED BY TPA

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Inflammation is caused by a variety of stimuli including physical damage, ultra violet irradiation, microbial invasion, and immune reactions. The classical key features of inflammation are redness, warmth, swelling, and pain. Inflammation cascades can lead to the development of diseases such as rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, and psoriasis. Natural products or natural product-derived compounds represent great structural diversity, which is not commonly seen in synthetic compounds. Natural products play a dominant role in the discovery of leads for the development of drugs for treating human diseases. The major anti-inflammatory targets include enzymes, COX-1 and COX-2; between others. Of the various flavonoid conjugates known to accumulate in plants, the occurrence of isoprenoids as natural plant constituents came to be recognized fairly recently. In this work, we prepared by structure modulation desing, the derivatives modulated: Acethylated (1a, 2a), methylated (1b, 2b), cyclyzed (1c, 2c) and vinyloge cyclyzed (1d, 2d) derivates by natural prenylated flavanones: 5, 7-dihydroxy-6-methyl-8-prenylflavanone (1) and 5, 7-dihydroxy-6-methyl-8-prenyl-4'-methoxi-flavanone (2). The compounds were studied in the experimental model of inflammation of ear of mouse induced by TPA. Natural flavanonas (1) and (2) showed 12.24 % and 68.35% of reduction of inflammation, respectively. A significant improvement was obtained in the antiinflammatory activity on having prepared the acethylated and cyclyzed derivatives (1c, 2a, 2c) with reduction percent of 71.64 %, 100.00% and 98.62 %, respectively.

Olive Oil

P429 (See Poster Talks, PT17)

SYNTHESIS OF OLIVE OIL PHENOL GLUCURONIDES AND BIOLOGICAL ACTIVITY ASSESSMENT

P430 (See Poster Talks, PT18)

PHENOL-ENRICHED OLIVE OIL IMPROVES ENDOTHELIAL DYSFUNCTION IN HYPERTENSIVE PATIENTS

P431 (See Poster Talks, PT18.1)

OLIVE PHENOLS FOR ENHANCEMENT STABILITY OF REFINED OILS AND IMPROVEMENT METABOLISM OF ESSENTIAL FATTY ACIDS

P431.2

TAILOR-MADE ENRICHED OILS WITH OLIVE PHENOLS: IMPROVEMENT OF STABILITY/QUALITY PROPERTIES AND HEALTH BENEFITS

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Extra-virgin olive-oil, EVOO, is the most demanded liquid fat in the Mediterranean basin thanks to the nutraceutical properties of its components, mainly to the minor, unsaponifiable fraction, constituted by aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds, carotenes and phenols. Given the high price of EVOO and the excellent properties of its phenols, there is a growing interest in the use of these compounds to enrich low-priced oils in order to obtain a healthy added-value product and also to study their effect on health.
The assessment of quality and stability properties of refined edible oils enriched with phenols from olive-tree materials —leaves and alperujo— at two concentration levels (200 and 400 µg/mL, expressed as gallic acid) has been the aim of part of the developed research. The different ratios of characteristic phenols in the extracts, together with the lipidic composition of the oils (six refined oils) to be enriched, gave place to different mass transfer of the target antioxidants, which conferred additional stability to —and improved quality parameters of — oils as checked by subjecting them to typical tests.
On the other hand, the effect of fried edible oils ingested by twenty-six obese individuals on the metabolism of essential fatty acids —*viz.* w-3 and w-6, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3)— as precursors of three different series of eicosanoids localized hormones, which regulate the adhesion of blood platelets, the constriction and dilation of blood vessels and smooth muscles, has been studied
A clear separation was observed in the panel of compounds for individuals before and after intake of the oils. Additionally, it is worth emphasizing that significant differences were found between individuals after intake of oils with phenols and after intake of sunflower oil subjected to a refining process. The panel of eicosanoids provided by individuals after intake of virgin olive oil or seed oils enriched with antioxidants was close to that provided by individuals before oils intake. Finally, MANOVA analysis revealed that the concentration of eicosanoids at different sampling periods can be particularly useful to explain the incidence of different oils on the metabolism of essential fatty acids.

Neuroscience

P432 (See Poster Talks, PT26)

COCOA FLAVANOL INTERVENTION ACUTELY IMPROVES EPISODIC MEMORY AND EXECUTIVE FUNCTION IN HEALTHY OLDER ADULTS

P433 (See Poster Talks, PT27)

FLAVONOID METABOLITES TRANSPORT ACROSS BBB

Ferreiro-Vera C, Priego-Capote F, Luque de Castro MD (2011) Automated method for targeting analysis of prostanoids in human serum by on-line solid-phase extraction and liquid chromatography–mass-spectrometry in selected reaction monitoring. J Chromatogr A. 1218:2848-2855

León-González L, Ferreiro-Vera C, Priego-Capote F, Luque de Castro MD (2011) Bioaccumulation assessment of the sunscreen agent 2.ethylhexyl 4-(N,N-dimethylamino)benzoate in human semen by automated online SPE–LC–MS/MS. Anal Bioanal Chem. 401:1003-1011

Orozco-Solano, MI, Priego-Capote F, Luque de Castro DM (2011) Influence of simultaed deep frying on the antioxidant fraction of vegetable oils after enrichment with extracts from olive oil pomace. J Agric Food Chem.

Rojano-Delgado A, Priego-Capote F, Luque de Castro MD, de Prado R (2010) Screening and confimatory analysis of glyoxilate: a biomarker of plants resistance against herbicides. Talanta. 82:1757-1762

PONENCIA:

CONTRIBUCIONES DEL GRUPO FQM-227 EN METABOLÓMICA Y EN EL APROVECHAMIENTO DE RESIDUOS DE LA VID/VINO Y DEL OLIVO/ACEITE

RESUMEN

La investigación actual del grupo FQM-227 se centra en dos líneas de investigación: (1) Desarrollo de plataformas analíticas en metabolómica/proteómica, con aplicación en tres vertientes, (1.i) metabolómica nutricional, fundamentalmente dirigida a la evaluación del efecto metabólico de la ingesta de diferentes dietas o tipos de alimentación, así como a la búsqueda de nutraceúticos y complementos dietéticos extraídos del olivo o de la vid; (1.ii) metabolómica clínica, orientada a la búsqueda de herramientas analíticas que auxilien en la prognosis y diagnosis de diferentes patologías como enfermedades cardiovasculares, diabetes y ciertos tipos de cáncer (pulmón, mama y colon); (1.iii) metabolómica vegetal, en la que se desarrollan herramientas para la selección avanzada en el programa de mejora genética del olivo

y para la identificación de las rutas metabólicas que diferencian variedades de plantas resistentes y sensibles a herbicidas para la interpretación de este comportamiento. (2) La explotación de los residuos agroalimentarios, que abarca, (2.i) los procedentes del olivo y de la industria del aceite de oliva (hoja y alperujo, respectivamente); (2.ii) los derivados de la vid (hojas y sarmientos) y de la industria vinícola (orujo de vino –hollejos, pepitas, raspón y restos de industrias alcoholeras y lías de vinificación).

INTRODUCCIÓN

El grupo FQM-227 tiene una antiguedad de más de 20 años, buena parte de los cuales se ha dedicado a la automatización de sistemas dinámicos especialmente a la aceleración y mejora de la preparación de la muestra. Paralelamente a esta investigación desarrollo estudios metabolómicos aun antes de que la disciplina metabolómica se designara como tal. Con las plataformas instrumentales con las que cuenta actualmente el Grupo (cromatografías de gases y de líquidos acopladas a espectrómetros de masas de trampa de iones, de triple cuadrupolo y de tiempo de vuelo), la investigación que desarrolla abarca 2 líneas principales de las que se expondrán ejemplos de trabajos ya completados o que actualmente se están desarrollando

(1) Plataformas analíticas en metabolómica/proteómica

(1.i) Área nutricional: En el área de la nutrimetabolomica se han desarrollado plataformas para el *análisis orientado (targeted analysis)* de las diferentes familias de lípidos derivadas de los ácidos grasos poli-insaturados omega-6 y omega-3 a través de sus rutas biosintéticas – la de la ciclooxigenasa (COX), la de la lipoxigenasa (LOX) y la del citocromo P-450—, con especial énfasis en el metabolismo del ácido araquidónico. Para ello se ha utilizado como técnica analítica la cromatografía de líquidos–espectrometría de masas en tándem (LC–MS/MS) con analizador de triple cuadrupolo y como muestra analítica suero humano. Las plataformas se han aplicado conjuntamente al estudio de la modificación de estas rutas (estrechamente implicadas en los mecanismos que provocan la inflamación) causada por la ingesta de alimentos cocinados con aceites de fritura que contenían antioxidantes naturales o artificiales. (Forma parte de la tesis doctoral de C. Ferreiro Vera –fecha de lectura: 20/12/2011).

En *análisis global* se han obtenido las *huellas dactilares (fingerprints)* mediante LC–MS con detector de tiempo de vuelo (LC–TOF/MS) de muestras de suero tomadas a diferentes tiempos de pacientes sometidos a dietas ricas/pobres en ácidos grasos saturados o monoinsaturados, o ricas/pobres en

UNIVERSIDAD D CÓRDOBA

El Vicerrector de Estudios de Posgrado y Formación Continua de la Universidad de Córdoba **ACREDITA** que :

VERÓNICA SÁNCHEZ DE MEDINA BAENA

ha presentado la comunicación oral que lleva por título .

" NUEVOS ESTUDIOS SOBRE LA MEJORA DE LA CALIDAD DEL ACEITE DE OLIVA Y EL APROVECHAMIENTO DE LOS RESIDUOS GENERADOS EN EL CULTIVO DEL OLIVO"

en el **I Congreso Científico de Investigadores en Formación en Agroalimentación** de la eidA3 y **II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba** celebrado en Córdoba los días 8 y 9 de Mayo de 2012.

Y para que así conste, se expide y firma este certificado en Córdoba, a 9 de Mayo de 2012

Fdo: JOSE CARLOS GÓMEZ VILLAMANDOS

Vicerrector de Estudios de Posgrado y Formación Continua

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Mass spectroscopy applied to olive breeding programs

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Olive oil, particularly extra-virgin olive oil (EVOO), is gaining consumers around the world after its beneficial nutritional properties have been widely proved. The chemical composition and quality of virgin olive oil are influenced by a variety of factors such as environmental conditions (soil, climate), agronomic factors (irrigation, fertilization), cultivation (harvesting time, ripening index), and technological factors (extraction process, post-harvest storage).

Harvesting time influences not only fruit weight, pulp-to-stone ratio, color, oil content, and chemical composition, but also changes the sensory characteristics of EVOO (1); so, oils resulting from an early olive harvesting, especially during green-to-violet evolution, are characterized by a higher quality as olive fruit (or drupes) has both lower free fatty acid content and peroxide values, which define high-quality olive oil. In addition, early-harvest olive oils are more stable to oxidation than mature oils because they are richer in antioxidant and aromatic compounds, which endow them with longer shelf-life —thus increasing their commercial value. Nevertheless, aromatic compounds, mainly phenols, also contribute to bitterness and pungency that are undesirable organoleptic properties (2). On the other hand, early harvesting negatively influences oil yield. The effect of drupes ripeness on oil quality is supported on a number of studies (5–8). However, the study of the maturation process from a global point of view, which involves all compounds experiencing a significant change with maturation has not been studied yet.

Different studies confirm a strong influence of genetics on olive oil quality and the results from all of them showed high degree of variability between genotypes with a higher range of variation than that observed for genitors. Most of the observed variability was attributable to differences in genotypes within crosses rather than among crosses (3,4); therefore, the crop season is not a critical variable in quality of oil; therefore, harvest date is not a parameter that may be used with normalization purposes to compare genotypes within a breeding program (5).

The objective of the present research was to study, from an overall point of view, how the composition of olive oil from advanced breeding selections is affected by the maturation index and the crosses between different genitors; objective which is considered under the umbrella of metabolomics. Mass spectrometry has been used to analyze olive oil from 'Arbequina', 'Frantoio' and 'Picual' cultivars as well as from crosses between them carried out in the olive cross-breeding program of Córdoba in the 1991–1992 period.

After LC–TOF/MS analysis, processing of raw data was performed with the XCMS package and annotated using the CAMERA workflow. This workflow allowed detection of molecular entities, peak finding, alignment of chromatograms and assignation of the isotopic distribution. This pre-processing step allowed to prepare a two-dimensional data matrix created by extraction of molecular features detected in each analyzed sample. The data set (116 samples × 705 molecular features) was exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA). Statistical analysis was carried out by Principal Component Analysis (PCA) to find clustering of samples as a function of their phenolic composition, while supervised analysis by Partial Least Squares–Discrimination Analysis (PLS–DA) allowed building predictive set to model the maturation process according to crop date or ripening index. Cross-validation was used as validation test.

Figure 1 shows the PLS-DA scores plots obtained by correlation of the global phenolic composition with the crop date (A) and ripening index (B). As can be seen, the crop date enabled to explain better the maturation process by monitoring the phenolic fraction. The prediction model for ripening index, based on color observation of the olive fruit, failed to discriminate EVOO from fruits collected with yellowish skin color (ripening index 1 and 2). This was associated to the influence of the genotype since previous studies have identified these ripening states as those in which the genotype variability exerts the maximum contribution on the total phenolic content of EVOO measured by the Folin-Ciocalteu test (3). This result allows confirming these ripening states as the preferred to compare genotypes in a olive breeding program.

International Congress on
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-A two-fold benefit from extraction of olive pomace: de-contamination of an undesirable residue and production of hydrophilic antioxidants for food enrichment

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Olive oil industry is especially important in the Mediterranean area, representing 70% of global production and a very significant percent of the agrifood economy. However this industry produces large volumes of an undesirable byproduct named alperujo —the harmful semisolid waste resulting from the two-phase olive oil extraction method. Alperujo is a source of natural antioxidants (olive phenols, OP) at concentrations up to 100 times higher than olive oil is because of the polar nature of alperujo and antioxidants and the non-polar nature of oil. In fact, while the natural content of OP in olive oil ranges between 0.005 and 0.12%, in alperujo the content is up to 0.87%.

Natural antioxidants from this byproduct are endowed with healthy properties, the cost for extraction is very low and, in addition, after antioxidants extraction the resulting alperujo is less contaminant. As antioxidants from alperujo do not require an acceptance period from food security organisms —these compounds have been ingested for millenia by Mediterranean people in olives or "aceitunas"— the extracts from alperujo can be used for food enrichment and nutraceuticals production, in addition to their use in cosmetics.

We have used alperujo extracts for enrichment of oils obtained by solvent extraction, as they lack of these polar compounds. The effect of OP in the oils subjected to frying is spectacular as oil resistance to degradation undergoes an enormous increase, as can be seen in the figure, even in comparison with the well known resistance of virgin olive oil (VOO).

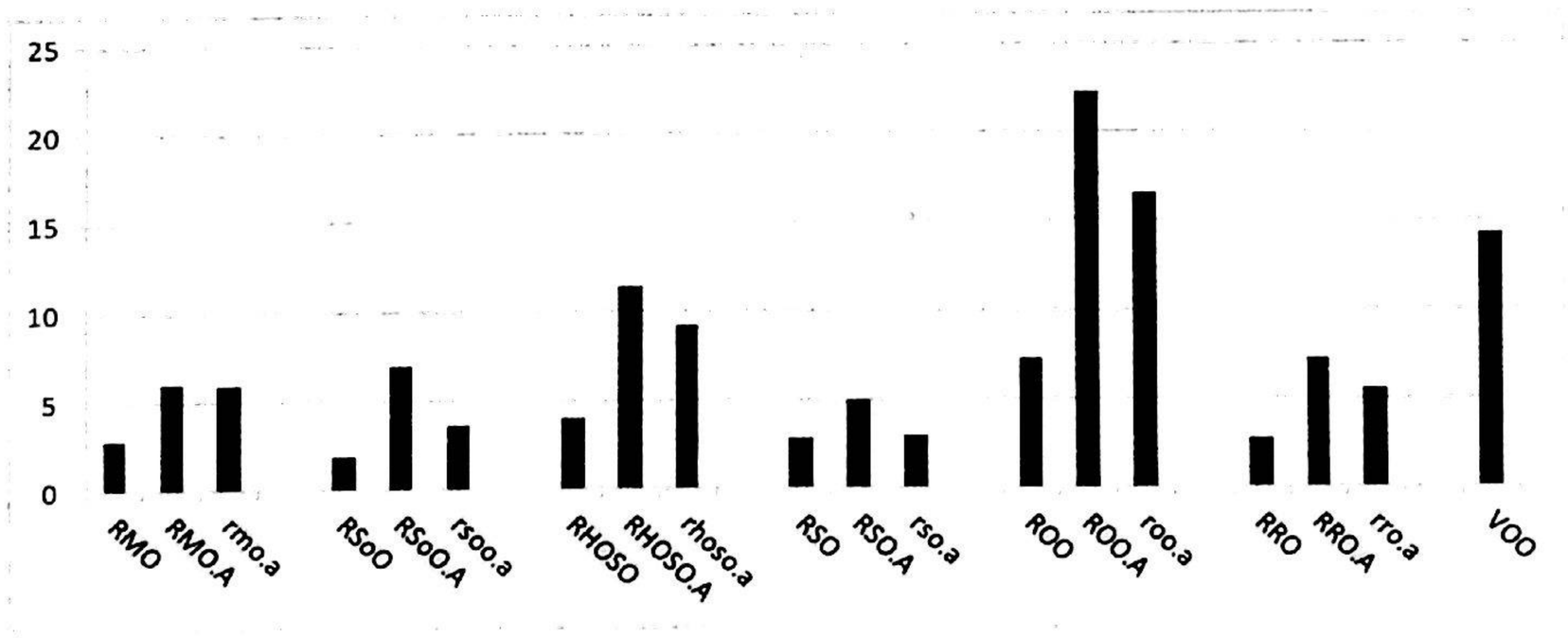

Figure 1. Rancimat values for different enriched oils and VOO

ALI-24

EFFECT OF FRUITS RIPENING ON THE PHENOLIC PROFILE OF VIRGIN OLIVE OILS FROM OLIVE BREEDING PROGRAMS

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Olive oil, particularly virgin olive oil (VOO), is gaining consumers around the world after its beneficial nutritional properties have been widely proved. The chemical composition and quality of virgin olive oil are influenced by a variety of factors such as environmental conditions (soil, climate), agronomic factors (irrigation, fertilization), cultivation (harvesting time, ripening index), and technological factors (extraction process, post-harvest storage). Harvesting time influences not only fruit weight, pulp-to-stone ratio, color, oil content, and chemical composition, but also changes the sensory characteristics of VOO; so, oils resulting from an early olive harvesting, especially during green-to-violet evolution, are characterized by a higher quality as olive fruit (or drupes) has both lower free fatty acid content and peroxide values, which define high-quality olive oil. In addition, early-harvest olive oils are more stable to oxidation than mature oils because they are richer in antioxidant and aromatic compounds, which endow them with longer shelf-life —thus increasing their commercial value. Nevertheless, aromatic compounds, mainly phenols, also contribute to bitterness and pungency that are undesirable organoleptic properties. On the other hand, early harvesting negatively influences oil yield. The effect of drupes ripeness on oil quality is supported on a number of studies. However, the study of the maturation process from a global point of view, which involves all compounds experiencing a significant change with maturation has not been studied yet.

Different studies confirm a strong influence of genetics on olive oil quality and the results from all of them showed high degree of variability between genotypes with a higher range of variation than that observed for genitors. Most of the observed variability was attributable to differences in genotypes within crosses rather than among crosses; therefore, the crop season is not a critical variable in quality of oil; therefore, harvest date is not a parameter that may be used with normalization purposes to compare genotypes within a breeding program. The objective of the present research was to study, from an overall point of view, how the composition of olive oil from advanced breeding selections is affected by the maturation index and the crosses between different genitors; objective which is considered under the umbrella of metabolomics. Mass spectrometry has been used to analyze olive oil from 'Arbequina', 'Frantoio' and 'Picual' cultivars as well as from crosses between them carried out in the olive cross-breeding program of Córdoba in the 1991–1992 period.

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LISTA DE ABREVIATURAS

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ANOVA, análisis de la varianza
AOCS, American oils chemists' society
ACN, acetonitrilo
APCI, ionización química a presión atmosférica
API, ionización a presión atmosférica
APPI, fotoionización a presión atmosférica
BPC, base peak chromatograms
BSTFA, bis(trimetilsilil)-trifluoroacetamida
C14:0, ácido mirístico
C16:0, ácido palmítico
C16:1, ácido palmitoleico
C18:0, ácido esteárico
C18:1, ácido oleico
C18:2, ácido linoleico
C18:3, ácido linolénico
C19:0, ácido nonadecanoico
C20:0, ácido eicosanoico
C20:1, ácido eicosenoico
C22:0, ácido behénico
C24:0, ácido tetracosanoico
CA, análisis de clúster
CAR, carboxeno
CDB, doble enlace conjugado
CE, electroforesis capilar
cGC, cromatografía de gases capilar

CI, ionización química

Da, Dalton

DAG, diacilglicérido

DAD, detector de diodos en fila

3,4-DHPEA, (3,4-dihidroxifenil)-etanol

3,4-DHPEA-AC, acetato de (3,4-dihidroxifenil)-etanol

3,4-DHPEA-EA, aglicona de la oleuropeína

3,4-DHPEA-EDA, aglicona de la descarboximetiloleuropeína

DMF, N,N-dimetilformamida

2D GC, cromatografía de gases bidimensional

DER, desviación estándar relativa

DVB, divinilbenceno

EFA, ácido graso esterificado

EFSA, European Food Safety Authority

EIC, extracted ion chromatogram

EI, impacto electrónico

ELSD, detector de dispersión de luz evaporativa

ESI, ionización por electrospray

EVOO, aceite de oliva virgen extra

FA, ácido graso

FAME, éster metílico de ácido graso

F–C, Folin–Ciocalteu

FD, desorción de campo field desorption

FFA, ácido graso libre

FI, field ionization

FID, detector de ionización de llama

FTIR, espectrometría de reflectancia en el infrarrojo con transformada de Fourier

FWHM, ancho de pico en la semialtura

GAE, equivalente en ácido gálico
GC, cromatografía de gases
p-HPEA, (*p*-hidroxifenil)-etanol
p-HPEA-EA, aglicona del ligstrósido
p-HPEA-EDA, aglicona del descarboximetilligstrósido
HRGC, cromatografía de gases de alta resolución
HS, espacio de cabeza
ID, diámetro interno
IFAPA, Instituto andaluz de investigación y formación agraria, pesquera, alimentaria y de la producción ecológica
IOC, Consejo Oleícola Internacional
IS, estándar interno
IT, trampa de iones
LC, cromatografía líquida
LC–MS/MS, cromatografía de líquidos-espectrometría de masas en tándem
LC–TOF MS, cromatografía de líquidos-espectrometría de masas con analizador de tiempo de vuelo
LDL, lipoproteína de baja densidad
LLE, extracción líquido–líquido
MAG, monoacilglicérido
MFE, extracción de entidades moleculares
MFs, entidades moleculares
MIM, multiple ion monitoring
MPP, Mass Profiler Professional
MS, espectrometría de masas
MW, microondas
MUFA, ácido graso monoinsaturado
NEFA, ácido graso no esterificado
NIR, infrarrojo cercano

NMR, resonancia magnética nuclear

NP, fase normal

PCA, análisis por componentes principales

PDMS, polidimetilsiloxano

PLS-R regresión parcial de mínimos cuadrados

POO, aceite de orujo de oliva

PTV, vaporizador de temperatura programada

PUFA, ácido graso poliinsaturado

R, coeficiente de correlación

R^2, coeficiente de regresión

RHSO, aceite de girasol refinado rico en ácido oleico

RI, índice de refracción

RI, índice de maduración

RMO, aceite de maíz refinado

ROO, aceite de oliva refinado

RRO, aceite de colza refinado

RSD, desviación estándar relativa

RSO, aceite de girasol refinado

RSoO, aceite de soja refinado

P&T, purga y trampa

RMSE, raíz cuadrada del error cuadrático medio

RP, fase reversa

RT, tiempo de retención

SOM, mapa auto-organizativo

SFA, ácido graso saturado

SFC, cromatografía de fluidos supercríticos

SFE, extracción con fluidos supercríticos

SHS, espacio de cabeza estático

SIM, single ion monitoring

SPE, extracción en fase sólida

SPME, micro extracción en fase sólida

SMR selection monitoring reaction

TAG, triacilglicérido

TCCs, total chromatograms compounds

TLC, cromatografía en capa fina

TMS, trimetilsilil

TOF, tiempo de vuelo

TOTAD, transferencia adsorción-desorción mediante horno

TPI, índice de fenoles totales

US, ultrasonidos

UV, ultravioleta

VLCFA, ácidos grasos de cadena larga

VOO, aceite de oliva virgen