UNIVERSIDAD DE CÓRDOBA
FACULTAD DE CIENCIAS
DEPARTAMENTO DE QUÍMICA ANALÍTICA

DESARROLLO DE MÉTODOS PARA EVALUAR EL POTENCIAL SALUDABLE DEL ACEITE DE OLIVA VIRGEN Y ESTUDIAR LA ASOCIACIÓN ENTRE CALIDAD Y COMPUESTOS MINORITARIOS

DEVELOPMENT OF METHODS TO EVALUATE THE HEALTHY VALUE OF VIRGIN OLIVE OIL AND TO STUDY THE ASSOCIATION BETWEEN QUALITY AND MINOR COMPOUNDS

TESIS **DOCTORAL**
CÓRDOBA 2021

INMACULADA
CRIADO NAVARRO

TITULO: *Development of methods to evaluate the healthy value of virgin olive oil and to study the association between quality and minor compounds*

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Desarrollo de métodos para evaluar el potencial saludable del aceite de oliva virgen y estudiar la asociación entre calidad y compuestos minoritarios

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Inmaculada Criado Navarro

Córdoba, 2021

Desarrollo de métodos para evaluar el potencial saludable del aceite de oliva virgen y estudiar la asociación entre calidad y compuestos minoritarios

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

Fdo. Inmaculada Criado Navarro

Feliciano Priego Capote, Profesor Titular del Departamento de Química Analítica de la Facultad de Ciencias, y **Carlos Augusto Ledesma Escobar**, Investigador Postdoctoral del Departamento de Química Analítica de la Facultad de Ciencias, ambos de la Universidad de Córdoba, en calidad de Directores de la Tesis Doctoral presentada por Inmaculada Criado Navarro, con el título "Desarrollo de métodos para evaluar el potencial saludable del aceite de oliva virgen y estudiar la asociación entre calidad y compuestos minoritarios",

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 trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, a 14 de mayo de 2021.

Fdo. Feliciano Priego Capote   Fdo. Carlos A. Ledesma Escobar

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

1. Informes favorables de dos doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:
    - Prof. Tea Bilušić, Department of Food Technology and Biotechnology, Faculty of Chemistry and Technology, University of Split, Croacia.
    - Prof. Mohamed Bouaziz, Department of Food and Technology, Higher Institute of Biotechnology of Sfax, University of Sfax, Túnez.
2. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de Enseñanza Superior de otro país:
    - Prof. Prokopios Magiatis, Department of Pharmacognosy, Faculty of Pharmacy, National and Kapodistrian University of Athens, Grecia.
3. La exposición y la defensa de parte de esta Tesis se realizarán en una lengua diferente a la materna: inglés.
4. Estancia de tres meses en un centro de investigación de otro país:

Faculty of Science, Department of Chemistry at National University of Singapore, bajo la supervisión del Profesor Sam Li Fong Yau.

TÍTULO DE LA TESIS: Desarrollo de métodos para evaluar el potencial saludable del aceite de oliva virgen y estudiar la asociación entre calidad y compuestos minoritarios

DOCTORANDA: Inmaculada Criado Navarro

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

La doctoranda Inmaculada Criado Navarro ha completado el trabajo planificado para la defensa de su Tesis Doctoral de manera muy solvente. Desde su incorporación al grupo de investigación en 2017 ha mostrado gran interés por el análisis metabolómico y su potencial aportación para la evaluación de la calidad y el potencial saludable del aceite de oliva virgen. Durante su formación predoctoral, se ha implicado en dos proyectos de investigación, Aristoil y Nutradaf. El proyecto Aristoil, del programa europeo Interreg-Med, le ha permitido contribuir al conocimiento sobre la fracción fenólica del aceite de oliva virgen, su valor saludable y la variabilidad asociada a diferentes factores. El proyecto Nutradaf, de la convocatoria CDTI-Interconecta, ha contribuido a su formación en análisis no dirigido de componentes bioactivos a lo largo del proceso de extracción del aceite de oliva virgen extra. Ambos proyectos se han caracterizado por tener una relevante actividad de transferencia gracias a la implicación de productores y empresas implicadas en el aprovechamiento de residuos derivados de la industria del olivar.

El trabajo completado ha dado lugar a la publicación de 6 artículos en revistas internacionales de alto índice de impacto y al envío de un artículo adicional para su evaluación. De manera complementaria, ha participado en otras colaboraciones como resultado de la transferencia de uno de los métodos desarrollados en esta investigación y que le han permitido publicar otros 3 artículos más.

Los principales avances conseguidos con el desarrollo de la investigación recogida en esta Memoria se deducen del título de la Tesis y son los siguientes:

1. El desarrollo y aplicación de metodologías innovadoras para la determinación de componentes minoritarios en aceite de oliva virgen y virgen extra para evaluar su potencial saludable y su asociación a la calidad.
2. La caracterización por primera vez del valor saludable de acuerdo con el Reglamento Europeo 432/2012 de un número importante de muestras de aceite de oliva virgen procedentes de productores españoles
3. La propuesta por primera vez de la determinación de glicerofosfolípidos como estrategia para evaluar la calidad del aceite de oliva y poder diferenciar entre diferentes categorías.
4. La monitorización de componentes bioactivos durante el proceso de extracción de aceite de oliva virgen extra a partir del análisis de las diferentes fases implicadas.
5. La propuesta de una estrategia de análisis para establecer la fijación de dietas en función de la fuente de grasa ingerida, haciendo especial énfasis sobre el aceite de oliva.
6. La presentación oral o en cartel de 3 comunicaciones en congresos nacionales e internacionales.
7. La realización de una estancia de 3 meses en la Universidad Nacional de Singapur como requisito para la mención de Doctora Internacional.

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 Inmaculada Criado Navarro.

Córdoba, 31 de mayo de 2021

Firma de los directores

Fdo.: Feliciano Priego Capote

Fdo.: Carlos Augusto Ledesma Escobar

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

TÍTULO DE LA TESIS: Desarrollo de métodos para evaluar el potencial saludable del aceite de oliva virgen y estudiar la asociación entre calidad y compuestos minoritarios

DOCTORANDA: INMACULADA CRIADO NAVARRO

PUBLICACIÓN	FI*	DECIL/CUARTIL
Determination of glycerophospholipids in vegetable edible oils: Proof of concept to discriminate olive oil categories. **Food chem.** 299, (2019) 125136	6.306	D1 6/139 Food Science & Technology
Profiling analysis of phospholipid fatty acids in serum as a complement to the comprehensive fatty acids method. **Journal of Chromatography A** 1619, (2020)460965	4.049	Q1 14/86 Chemistry, Analytical
Evaluating the variability in the phenolic concentration of extra virgin olive oil according to the Commission Regulation (EU) 432/2012 health claim. **J. Agric. Food Chem.**68 (34), (2020) 9070-9080	4.192	Q1 29/139 Food Science & Technology D1 4/58 Agriculture, Multidisciplinary

The decrease in the health benefits of extra virgin olive oil during storage is conditioned by the initial phenolic profile. **Food Chem.** 336, (2021)127730	6.306	D1 6/139 Food Science & Technology
Influence of fruit destoning on bioactive compounds of virgin olive oil. **LWT,** (2021) 111354	4.006	Q1 28/139 Food Science & Technology D1 4/58 Agriculture, Multidisciplinary
Alteration of the phenolic fraction of extra virgin olive oil subjected to frying conditions. Accepted in **ACS Food Sci. Technol.**		Not indexed
Monitoring the Transference of Bioactive Compounds in the Process for Extraction of Extra Virgin Olive Oil. Sent to **J. Agric. Food Chem.**	4.192	Q1 29/139 Food Science & Technology D1 4/58 Agriculture, Multidisciplinary

Acaba una exigente, pero gratificante etapa, en la que considero y consideraré mi casa: la Universidad de Córdoba. Y como no puede ser de otra manera, agradecer en estas líneas a compañeros, directores, familia y amigos el apoyo humano y académico recibido a lo largo de estos años; haciéndoles partícipes de esta Tesis Doctoral, pues el logro no es solo mío sino también vuestro.

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A todos los miembros del departamento de Química Analítica de la Universidad de Córdoba, ya sea personal técnico y administrativo, como profesores y becarios. Agradecer a los compañeros del grupo FQM-227, a los que continúan, y a los que han estado a lo largo de mi periodo en el laboratorio, porque además de la compañía en las horas de trabajo y descanso, cada uno de ellos me ha ayudado de alguna manera: Azahara, Ana, Marisi, Ángela, etc. Agradecerle de manera especial a Antonio, por ser el primero en enseñarme con mucha paciencia y comprensión, y ya como técnico, por seguir solucionando problemas. Gracias por contagiarme, junto con Mayte, ilusión y optimismo. A Mónica, que en un momento aclara todas las dudas. Gracias por tu ayuda y por aconsejarnos e interesarte siempre porque todo vaya bien en el grupo. Asun, gracias por compartir tu gran experiencia, por tu cariño, y por estar ahí para ayudarme en todo momento. María del Mar, gracias por tu apoyo

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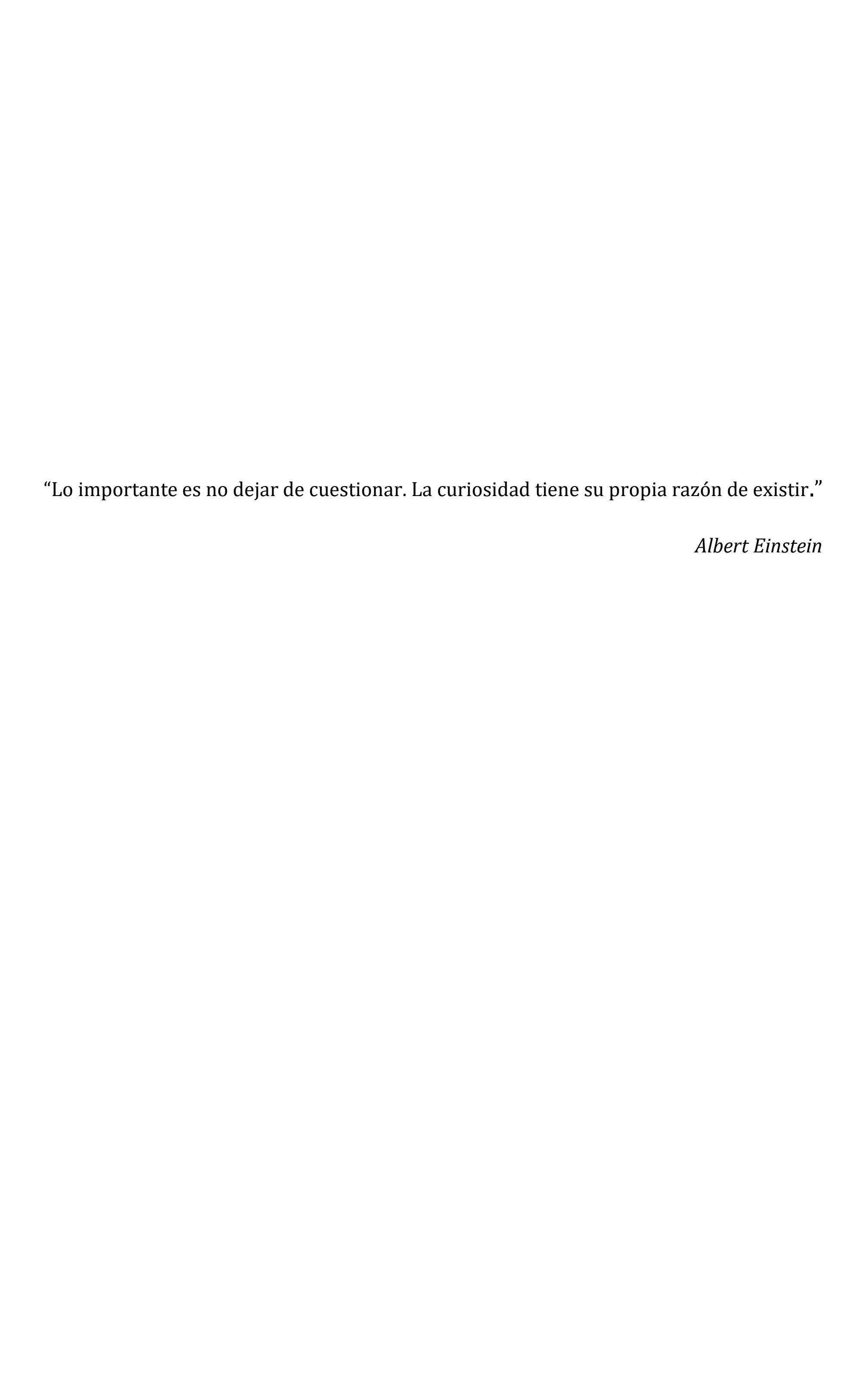
A todos, MUCHÍSIMAS GRACIAS



"Lo importante es no dejar de cuestionar. La curiosidad tiene su propia razón de existir."

Albert Einstein

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OBJETIVOS

El ***objetivo básico*** de la investigación recogida en esta Memoria de Tesis fue determinar familias de compuestos minoritarios presentes en aceite de oliva virgen (AOV) y virgen extra (AOVE) y estudiar su variabilidad en función de diferentes factores para reforzar la competitividad de ambos productos a partir de dos pilares fundamentales: la calidad y el valor saludable. El logro de este objetivo básico ha conducido a un mayor conocimiento del AOV(E) (abreviatura utilizada para referirse al AOV y, sobre todo, al AOVE por ser la referencia de máxima calidad) a partir de la aplicación de métodos analíticos capaces de proporcionar un mayor nivel de información, lo que constituye un hito clave para la resolución de problemas biológicos.

Este objetivo se dividió en cuatro ***objetivos generales*** de acuerdo con los diferentes bloques planteados en esta investigación:

- Evaluar la influencia de diferentes factores en el contenido fenólico del AOV(E) y, por tanto, sobre su valor saludable atendiendo a la declaración recogida en el Reglamento Europeo 432/2012.
- Caracterizar la fracción de glicerofosfolípidos en diferentes categorías de aceite de oliva y en otros aceites vegetales refinados aprovechando la combinación entre sensibilidad y selectividad del analizador de triple cuadrupolo (QqQ).
- Estudiar el comportamiento de dos familias de componentes bioactivos del AOV(E) (fenoles y triterpenos) durante el proceso de extracción de este a partir del análisis de las diferentes fases implicadas: masas sólidas, aceite y residuos.
- Proponer una estrategia que permita evaluar la adherencia de dietas con aceite de oliva como componente a partir de la determinación de ácidos grasos enlazados a triglicéridos y glicerofosfolípidos.

Cada objetivo general ha dado lugar a varios ***objetivos concretos***:

(i) Determinar el contenido de compuestos fenólicos en AOV(E)s obtenidos de productores durante dos campañas para conocer la

influencia de determinados factores agronómicos y geográficos sobre dicha familia de compuestos (Capítulo 1). Este estudio fue aplicado con la finalidad de conocer el valor saludable de AOV(E)s producidos en el área Mediterránea.

(ii) Interpretar la caída en contenido fenólico del AOV(E) en los 12 primeros meses desde su producción y evaluar la influencia del perfil fenólico inicial sobre la pérdida de valor saludable asociado a dicha familia de compuestos (Capítulo 2).

(iii) Estudiar el comportamiento del perfil fenólico inicial sobre la degradación de esta familia de compuestos en AOV(E)s sometidos a fritura a 180 °C durante 90 min. Para abordar este objetivo se seleccionaron cuatro AOVEs monovarietales con diferente perfil fenólico inicial (Capítulo 3).

(iv) Establecer una propuesta para la caracterización de glicerofosfolípidos en aceites vegetales basada en extracción en fase sólida (SPE) y cromatografía líquida con detección por espectrometría de masas en tándem (LC–MS/MS) (Capítulo 4). El método se aplicó a un conjunto de aceites vegetales refinados y a diferentes categorías de aceite de oliva para comparar el perfil cualitativo y cuantitativo de dicha fracción y como una posible aproximación para discriminar entre categorías de aceite de oliva atendiendo a calidad.

(v) Monitorizar dos familias de componentes bioactivos (fenoles y terpenos) en fases oleosas obtenidas en distintas etapas del proceso de producción de AOVE para dos variedades representativas en cuanto a perfil fenólico, 'Arbequina' y 'Picual'. También se pretendió abordar el efecto del deshuesado del fruto utilizando como referencia el perfil bioactivo de las fases oleosas obtenidas de fruto intacto (Capítulo 5).

(vi) Monitorizar dos familias de componentes bioactivos (fenoles y terpenos) en agua residual, aceite, y fases sólidas obtenidas durante el proceso de producción de AOVE para dos variedades

representativas en cuanto a perfil fenólico, 'Arbequina' y 'Picual', y su comparativa frente al contenido en el AOVE final (Capítulo 6).

(vii) Proponer una estrategia de análisis de adherencia de dieta basada en consumo de aceite de oliva a partir de la determinación de ácidos grasos enlazados a triglicéridos y a glicerofosfolípidos con el fin de obtener dos visiones complementarias para evaluar dicha adherencia (Capítulo 7).

La formación de la futura doctora, que es el ***objetivo final*** de una Tesis Doctoral, también ha incluido los complementos correspondientes para la especialidad de Química Analítica. Además, se completaron los pasos necesarios para cumplir con los requisitos para lograr la mención del Doctorado Internacional. Paralelamente a las tareas indicadas anteriormente y a la investigación en la parte principal de la Memoria, se ha buscado una formación más amplia de la estudiante de doctorado mediante el desarrollo de otras actividades resumidas a continuación como anexos:

(i) Anexo I: Tres artículos publicados como resultado de la colaboración con grupos del Centro Nacional de Epidemiología del Instituto de Salud Carlos III.

(ii) Anexo II: Comunicaciones orales en congresos nacionales e internacionales.

OBJECTIVES

The ***basic objective*** of the research that constitutes this PhD Book was to determinate minor families of compounds present in virgin olive oil (VOO) and extra virgin olive oil (EVOO), and to study their variability associated to different factors with the aim to reinforce the competitiveness of both products from the perspective of two fundamental pillars: quality and health value. The achievement of this basic objective has led to a greater knowledge of (E)VOO (abbreviation used to refer to VOO and, particularly, to EVOO as the highest quality reference) through the application of analytical methods capable of providing a higher level of information, which is a key for solving biological problems.

This objective was divided into four ***general objectives*** according to the different sections proposed in this study:

- To evaluate the influence of different factors on the phenolic content of (E)VOO and, therefore, on its healthy benefits, according to the Commission Regulation (EU) 432/2012 Health Claim.
- To characterize the glycerophospholipid fraction in different categories of olive oil, and in other refined vegetable oils, using the combination of sensitivity and selectivity offered by the triple quadrupole (QqQ) system.
- To study the behaviour of two families of bioactive compounds of EVOO (phenols and triterpenes) during the extraction process through the analysis of different phases involved: pastes, oil and residues.
- To propose a strategy that allows evaluating the adherence of diets with olive oil as a component by the determination of fatty acids linked to triglycerides and glycerophospholipids.

Each general objective has resulted in several ***concrete objectives***:

(i) To determine the phenolic content in (E)VOOs obtained from producers during two agronomic seasons to identify the influence of certain agronomic and geographical factors on this family of compounds (Chapter 1). This study was carried out to

know the health benefits of (E)VOOs produced in the Mediterranean area.

(ii) To interpret the decrease in phenolic content of (E)VOO after 12 months of its production and evaluate the loss of the health benefits conditioned by the initial phenolic profile (Chapter 2).

(iii) To study the influence of the initial phenolic profile on the degradation of these compounds in (E)VOOs subjected to frying at 180 °C for 90 min. To address this objective, four monovarietal EVOOs with different initial phenolic profiles were selected (Chapter 3).

(iv) To propose a method to characterize glycerophospholipids in vegetable oils based on solid phase extraction (SPE) and liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) (Chapter 4). The method was applied to refined vegetable oils, and to different categories of olive oil to compare the qualitative and quantitative profile of the mentioned fraction, and as a proof of concept to discriminate between olive oil categories according to quality.

(v) To monitor two families of bioactive compounds (phenols and terpenes) in oily phases obtained in different steps of the EVOO production process for two representative cultivars in terms of phenolic profile, 'Arbequina' and 'Picual', after fruit destoning. For this purpose, the bioactive profile of oily phases obtained with intact fruits was used as reference (Chapter 5).

(vi) To monitor two families of bioactive compounds (phenols and terpenes) in wastewater, oil and pastes obtained during the EVOO production process for two representative cultivars in terms of phenolic profile, 'Arbequina' and 'Picual', and their comparison with the content in the final EVOO (Chapter 6).

(vii) To propose a strategy for the analysis of diet adherence based on olive oil consumption through the determination of fatty acids

linked to triglycerides and glycerophospholipids to obtain two complementary views of mentioned adherence (Chapter 7).

The formation of the future PhD, which is the ***final objective*** of a Doctoral Thesis, has also included training complements (Analytical Chemistry Specialization), in which the PhD student completed the mandatory subjects. Also, the necessary steps to fulfil the requirements to achieve the International Doctorate mention were developed. In parallel to the above-mentioned tasks and to the research in the main part of this Book, a wider formation of the PhD student has been sought by development of other activities summarized below as annexes:

(i) Annex I: Three research articles that resulted from the collaboration with groups of the National Centre for Epidemiology, Carlos III Institute of Health

(ii) Annex II: Oral communications in national or international conferences.

INTRODUCTION

INTRODUCTION

1. Olive oil

Agriculture in Mediterranean countries is dominated by the cultivation of the olive tree, whose flagship products are olive oil (OO) and table olives. Currently, more than 11 million hectares of olives are grown in more than 67 countries worldwide according to the International Olive Council (IOC) [1]. The majority of this surface (97%) is localized in the Mediterranean countries. However, new intensive orchards have been planted in regions such as Australia, North and South America [2].

According to the IOC standard [3], oils obtained from the olive tree fruits are classified in VOO, refined olive oil (ROO), OO, and olive pomace oil (Figure 1). VOO is obtained solely by mechanical or other physical means without application of thermal conditions that lead to alterations in the oil composition. Additionally, any treatment in the extraction protocol other than washing, decantation, centrifugation or filtration must be discarded. VOO can be split into the following three categories that can be consumed directly:

(i) EVOO, which has a free acidity, expressed as oleic acid, of not more than 0.8 g per 100 g, and the other physico-chemical and organoleptical characteristics of which correspond to those fixed for this category in this standard.

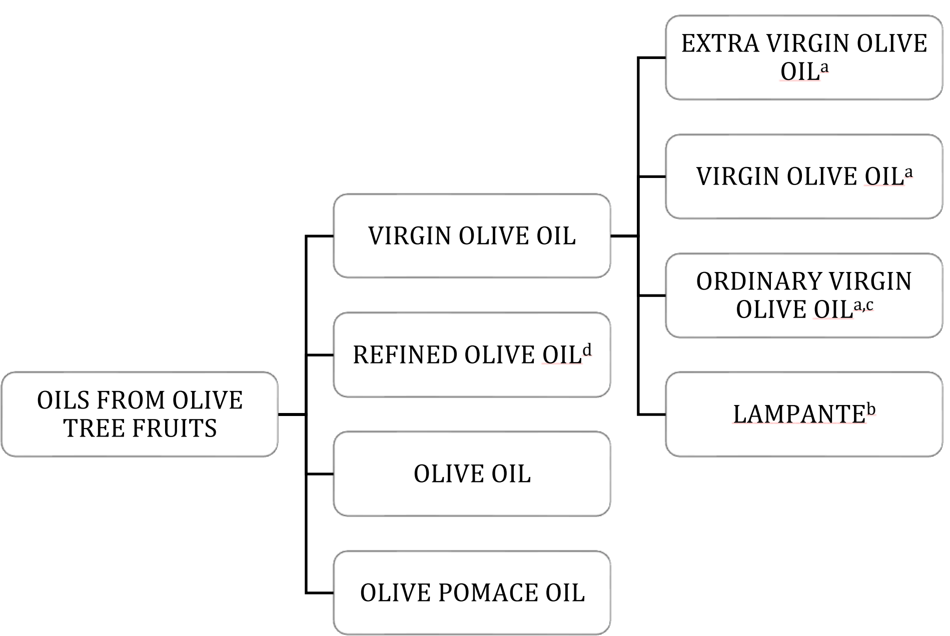
(ii) VOO, which has a free acidity, expressed as oleic acid, of not more than 2 g per 100 g and the other characteristics of which correspond to those fixed for this category in this standard.

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

Another class of olive oil that can be consumed is ROO. The ROO is obtained

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

ROO can be mixed with VOOs for consumption. This category, named olive oil, has a free acidity expressed as oleic acid of not more than 1 g per 100 g, and its other physico–chemical and organoleptic characteristics correspond to those fixed for this category in this standard [3].



[a] VOOs fit for consumption as they are.
[b] VOOs that must undergo processing prior to consumption.
[c] This product may only be sold directly to the consumer if permitted in the country of retail sale. If not permitted, the designation of this product shall comply with the legal provisions of the country concerned.
[d] This product may only be sold directly to the consumer if permitted in the country of retail sale.

Figure 1. Classification of the oils obtained from the fruit of the olive tree (*Olea europea* L.)

Olive oils must conform with different rules and standards depending on where they are traded. Three of the most important standards are those specified by the European Union, the IOC, and the Codex Alimentarius [4]. Table 1 reports the quality criteria for the oils according to the IOC COI/T.15/NC No 3/Rev. 15 November 2019 [3]. This table only includes those categories that are suitable for consumption. The limits established for each criterion and designation include the precision values of the attendant recommended method.

The past decade has seen a rise in interest in the Mediterranean Diet . Thus, the mentioned expansion is largely due to the fact that VOO and, particularly, EVOO, constitutes a supporting pillar for the health and nutritional benefits of the Mediterranean Diet [5]. (E)VOO has a particular fragrant flavor as a consequence of being obtained from olives by only physical means under conditions (particularly thermal) that do not lead to alterations in the oil [6].

The olive fruit is well-known for its high monounsaturated-fatty acids content (MUFAs), especially oleic acid; and, a phenolic profile specific of olive oil as secoiridoids, the main phenolic group, that are only found in *Olea europea* L. plant [7]. Other particularities of the olive fruit are its low sugar content (2-5%) and the high amount of oil accumulated during maturation (14–30% oil content) [8]. The final quality of the oil depends largely on the cultivar and ripening stage, and on the extration technique [9, 10].

2. Olive oil chemical composition and quality

The olive oil chemical composition is clustered into saponifiable and unsaponifiable fraction (Figure 2). The saponifiable fraction is made up primarily of triacylglycerols (TAGs), mono- (MAGs) and diacylglycerols (DAGs), phospholipids (PLs), waxes, and free fatty acids (FFAs). On the other hand, the unsaponifiable fraction is formed by an ample and heterogeneous group of minor compounds mainly constituted by tocopherols, hydrocarbons, sterols, aliphatic alcohols, carotenoids, chlorophylls, phenolic compounds, among others [11].

Table 1. Principal quality criteria for virgin olive oil categories, refined olive oil and olive oil according to the IOC

	EVOO	VOO	Ordinary-VOO	ROO	OO (ROO+VOOs)
1. Organoleptic characteristics					
Odour and taste				acceptable	good
Median of defect	Me = 0.0	0.0 < Me ≤ 3.5	3.5 < Me ≤ 6.0 [a]		
Median of the fruity attribute	Me > 0.0	Me > 0.0			
Colour				Light yellow	Light, yellow to green
Aspect at 20 °C for 24 hours				Limpid	Limpid
2. Free acidity (% m/m expressed in oleic acid)	≤ 0.80	≤ 2.0	≤ 3.3	≤ 0.30	≤ 1.00
3. Peroxide value (in milleq. Peroxide oxygen per kg/oil)	≤ 20.0	≤ 20.0	≤ 20.0	≤ 5.0	≤ 15.0
4. Absorbency in ultra-violet ($K^{1\%}_{1cm}$)					
270 nm (cyclohexane)/268 nm (iso-octane)	≤ 0.22	≤ 0.25	≤ 0.30	≤ 1.25	≤ 1.15
Δ K	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.16	≤ 0.15
232 nm [b]	≤ 2.50 [c]	≤ 2.60 [c]			
5. Moisture and volatile matter (% m/m)	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.1	≤ 0.1
6. Insoluble impurities in light petroleum (% m/m)	≤ 0.10	≤ 0.10	≤ 0.10	≤ 0.05	≤ 0.05
7. Flash point	-	-	-	-	-
8. Trace metal (mg/kg)					
Iron	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0	≤ 3.0
Copper	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1	≤ 0.1
9. Fatty acid ethyl ester (mg/kg)	≤ 35				
10. Phenols content					

[a] Or when the median of the defect is less than or equal to 3.5 and the median of the fruity attribute is equal to 0.0.
[b] This determination is solely for application by commercial partners on an optional basis.
[c] Commercial partners in the country of retail sale may require compliance with these limits when the oil is made available to the end consumer.

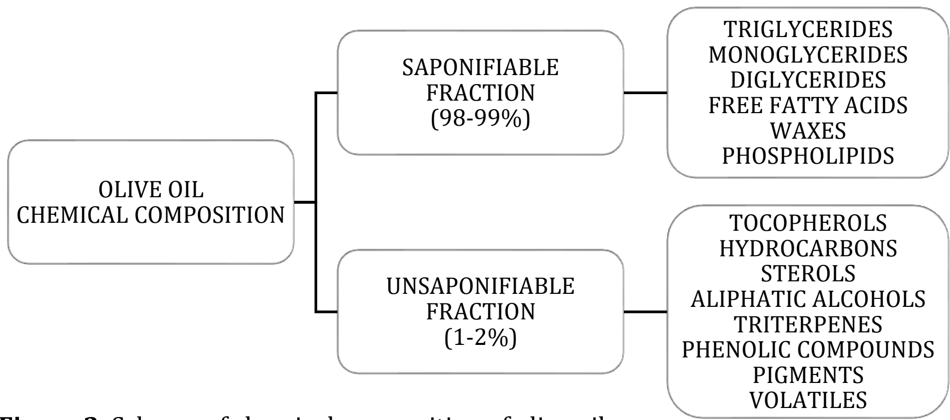


Figure 2. Scheme of chemical composition of olive oil

- *Saponifiable fraction*

This fraction represents approximately 98% of the total oil weight. The TAGs characterize almost entirely the saponifiable fraction (98–99% of total fats), while DAGs and MAGs are present with a concentration below 1% of total fats, respectively. TAGs are constituted by most of the identified fatty acids, although only six of them are major compounds: palmitic acid (6.30–20.93%), palmitoleic acid (0.32–3.52%), stearic acid (0.32–5.33%), oleic acid (55.23– 86.64%), linoleic acid (2.7–20.24%), and linolenic acid (0.11–1.52%) [12]. The wide range of TAGs and fatty acids is largely dependent on the cultivar, and to a certain extent, on the geographical origin. DAGs are major components of the polar fraction in VOOs (1–3%) and they are mainly found as 1,2- and 1,3-isomers. 1,2-DAGs are attributed to incomplete biosynthesis of TAGs (Kennedy pathway), whereas 1,3-DAGs are formed by enzymatic or chemical hydrolysis of TAGs [13]. MAGs are present in lower content (≤0.25%) and their major constituents are glycerol oleate, linoleate, and palmitate. During OO storage, the amount of 1,2-DAG decreases while that of 1,3-DAG increases. Thus, the ratio between these isomers has been suggested to be useful as indicator of VOO freshness, and together with the level of pyropheophytins, as hypothetical markers of the presence of deodorized olive oil in VOO. FFAs are markers of the processing of unhealthy olives or of a poor handling during olive processing, while acidity is a basic criterion for olive oil quality classification [6].

Waxes represent a further class of minor saponifiable fraction of olive fruit. They form the external hydrophobic layer produced by plants as a barrier against the biotic and abiotic environmental stresses. The waxes are characterized by homologous series of very-long-chain aliphatics, i.e., fatty acids, aldehydes, alcohols, ketones, alkanes, and alkyl esters [14]. Waxes are transferred into the oil in a small quantity if a mechanical extraction is applied and in a high quantity if a solvent (*n*-hexane) extraction is performed. Therefore, the concentration of waxes and wax esters may be an indicator for detection of fraudulent mixtures [15].

PLs, being the major components of biological membranes both in plant and

animal tissues, constitute the most important class of polar lipids. They are present in vegetable edible oils by their transfer to the oil phase during the extraction process. In olive oils, their content ranges between 21 and 124 mg kg^{-1} of oil [16]. PLs seem to exert a synergistic role on the stability of oils since they allow regeneration of other antioxidants such as phenols or tocopherols. On the other hand, excessive residual concentration of PLs could impact negatively on the quality of oil during refining [17].

- *Unsaponifiable fraction*

This fraction represents less than 2% of the oil weight and differentiates (E)VOOs from the other categories, due to it is partially or totally removed after thermal and/or physical–chemical treatments applied in the refining process. This set of minor compounds plays an important role in the quality and purity analyses, in the studies of authentication and validation and, more recently, in olive oil traceability and health [18].

Tocopherols are constituted by 6-OH-chromane ring and a lateral chain of 16 C atoms. Tocopherols exist in forms *α*, *β*, *γ*, and *δ*, according to the position of the methyl groups [19]. Olive oil is a reliable source of α-tocopherol, a molecule with vitamin E activity. The concentrations of tocopherols found in olive oil range approximately from 10 to 350 mg kg^{-1}. They contribute to the stability of olive oil, have a key biological role as antioxidants, and preserve oil quality during storage. Nevertheless, the presence of these compounds is not exclusive to olive oil. Other refined oils, such as sunflower oil, are characterized by relatively high concentration of tocopherols [20, 21].

Aliphatic alcohols consist of a linear chain containing a hydroxyl group, usually at the terminal position. The concentration of aliphatic alcohols in olive oils may not surpass the legal limit of 350 mg kg^{-1}, the most abundant being hexacosanol (C_{26}), octacosanol (C_{28}) and tetracosanol (C_{24}); while tricosanol (C_{23}), pentacosanol (C_{25}) and heptacosanol (C_{27}) may be present at trace levels [22].

In the unsaponifiable fraction of olive oil there are also triterpenic alcohols and other pentacyclic triterpenes. The main triterpenes found in (E)VOO are

oleanolic acid, maslinic acid, uvaol, and erythrodiol. These two hydroxyl pentacyclic triterpene acids (oleanolic and maslinic acid) and these two dialcohols (uvaol and erythrodiol) are differentiated according to the function present at the C-17 position. Maslinic acid has two vicinal hydroxyl groups at the C-2 and C-3 positions, besides the carboxyl radical. Uvaol and erythrodiol possess two hydroxyl groups in remote positions and are different regarding the methyl group location. In (E)VOOs the concentration of triterpenes oscillates between 8.9 and 112 mg kg^{-1} [23]. It is recognized that triterpene concentration varies depending on the type of cultivation and the handling of olive oil. In fact, triterpenes are present at higher concentrations in olive pomace oil than in VOOs [24].

Sterols are organic compounds with a molecular nucleus of 17 carbon atoms and a characteristic three-dimensional arrangement of four rings. Sterols, also known as plant sterols or phytosterols, are among the most abundant compounds of the unsaponifiable fraction (0.1-0.2%), with a known wide range of biological activities. Therefore, they constitute one of the most studied classes of this fraction, derivative from hydroxylated polycyclic isopentenoids [25]. The main olive oil sterols are β-sitosterol (75–90% of sterols fraction), Δ-5-avenasterol and campesterol, which after saponification might be present within the range 1000–2500 mg kg^{-1} oil [26]. The composition of phytosterols in oils may vary according to agronomic and climatic conditions, the quality of the fruits, extraction and refining procedures, and storage conditions [27]. Sterols with specific structures inhibit oxidative deterioration of oils acting as potential anti-polymerization agents for frying oils [28]. Compositional analysis of the sterol fraction in olive oil can be used to assess the quality of the oil, and the absence of other vegetal oils [29].

Hydrocarbons are formed by homologous series of linear compounds that are mainly saturated chains of 15–33 C atoms, although small amounts of ramified isomers are also present [30]. Squalene is a natural polyunsaturated triterpene, and the major hydrocarbon found in olive oil making up more than 90% of this fraction. Squalene is essential for the biosynthesis of steroids and triterpenes, and at the same time, constitutes an intermediate in the biosynthesis of phytosterols [31]. Squalene directly influences olive oil stability, and its chain-breaking ability

contributes to regenerate α-tocopherol. While VOOs contain squalene around 400 mg kg^{-1} oil, in refined olive oil the content is close to 3000 g kg^{-1}. Other edible vegetal oils do not surpass 240 g squalene kg^{-1} oil. Thus, this compound may be indicator of quality and origin [32].

The colour of olive oil is due to two types of natural pigments, chlorophylls, and carotenoids. Chlorophyll pigments account for the greenness of the oils, while carotenoids account for their yellowness [33]. The concentration of pigments typically ranges from a few mg kg^{-1} to approximately 100 mg kg^{-1}. They can be associated with the age, storage conditions, and to the authenticity and quality of the olive oil [34].

Volatile compounds found in olive oil can be grouped into alcohols, aldehydes, esters, ketones, sulfuric compounds and terpenes [35]. These compounds are synthesized through different pathways, with some of them activated during the fruit ripening, and others, during and after the extraction of the olive oil by several enzymes such as lipoxygenase and alcohol dehydrogenase, and by oxidation reactions, mainly during storage [36]. Volatiles are the main compounds responsible for the "green" and fruity desirable aroma attributes of VOOs. On the contrary, the low-quality olive oils have complex profiles composed of many volatiles responsible for off-flavours such as rancid, mustiness, fusty and muddy sediment [37]. As they are molecules strongly dependent on the olive oil sensory profiles, they are considered as relevant quality markers. During the last years, several studies have been focused on the development of robust analytical methods for evaluating the quali-quantitative profiles of volatile compounds in olive oils. In these studies, low number of volatile compounds were selected as relevant markers of the sensory defects and determined using less expensive instruments such as solid-phase microextraction followed by gas-chromatography with flame ionized detection (SPME–GC–FID) [38-40].

Finally, phenolic compounds are one of the most relevant chemical families found in olive oils. They are important minor constituents linked both to the flavour, and to its keeping properties. Many studies deal not only with the nutritional effects

of phenols, but also with the agronomic factors that influence their presence in the fruit and olive oil, the mechanism that contribute to a longer shelf life and stability, and the importance of the processing conditions [18, 41, 42]. Most phenolic compounds identified and quantified in olive oil belong to five different classes: (i) flavonoids (luteolin and apigenin); (ii) secoiridoids (aglycone derivatives of oleuropein and ligstroside); (iii) simple phenols (hydroxytyrosol, tyrosol); (iv) phenolic acids (especially derivatives of benzoic and cinnamic acids); and (v) lignans (pinoresinol and acetoxypinoresinol). Figure 3 shows the chemical structure representing these main classes. The phenolic content depends on different factors such as maturation index, genetics, geographical origin, pedoclimatic conditions, agronomical, and technological factors. VOOs may contain between 100 and 800 mg kg^{-1} total phenols, while refined oils have a concentration of less than 10 mg kg^{-1} [43]. Within the great variability of phenolic groups, the role of secoiridoids as conjugated forms of hydroxytyrosol and tyrosol is worth noting. This group of compounds, which are specific of the *Oleaceae* family, includes oleuropein and ligstroside aglycone isomers and the decarboxymethylated dialdehyde forms of oleuropein and ligstroside aglycones, which are more frequently referred to as oleacein (3,4-DHPEA-EDA) and oleocanthal (p-HPEA-EDA), respectively [44].

R = OH Luteolin
R = H Apigenin
Flavonoids

R = OH Oleuropein aglycone
R = H Ligstroside aglycone
Secoiridoids

R = OH Hydroxytyrosol
R = H Tyrosol
Simple Phenols

Caffeic acid
Phenolic acids

R = OH 1-Acetoxypinoresinol
R = $OCOCH_3$ 1-Hydroxypinoresinol
Lignans

Figure 3. Chemical structures representing the main families of phenolic compounds identified in olive oils.

3. Health benefits of (E)VOO

Being the Mediterranean Diet multi-factor in nature, it is difficult to single out the precise health contribution of its components. However, the case of (E)VOO is different because research on this is advanced and allows some evidence-based claims. In January 2018, the IOC convened several worldwide experts to discuss and summarize the available data on the effects of olive oil consumption on human health. A synthesis of the main reported findings were published [45]. Although different regions in the Mediterranean basin have their own diets there are common characteristics, most of which stem from the fact that olive oil occupies a central position in all of them [46].

Lipid oxidation has been recognized as the major problem affecting edible oils, as it is the cause of important deteriorative changes in their chemical, sensory, and nutritional properties. Autoxidation and photooxygenation, which are due to the presence of oxygen in air, are inevitable [47]. Fatty acids are the major olive oil constituents and, unlike other edible oils, (E)VOO contains high concentrations of MUFAs, particularly oleic acid, and low concentrations of saturated fatty acids (SFAs). Therefore, olive oil has fewer targets for reactive oxygen species, making it more stable than other edible oils and less likely to undergo peroxidation. As well, (E)VOO is considered to be resistant to oxidative degradation due to the presence of natural antioxidant minor components such as α-tocopherol and phenolic compounds.[8].

The human health benefits of minor olive oil components are primarily due to phenols, which demonstrate a broad spectrum of anti-inflammatory, antioxidant, antibacterial and anti-apoptotic effects. Table 2 summarizes the main (E)VOO phenolic compounds and their beneficial function [48]. Dietary intake of olive oil phenols has been estimated to be around 9 mg. The ingestion of hydroxytyrosol as oleuropein aglycones is probably the highest, given that conjugated forms are broken down in the gastrointestinal tract into hydroxytyrosol and elenolic acid [49].

- *Anti-inflammatory properties*

The benefits of (E)VOO are detectable only after a few weeks of its regular

consumption. Specifically, after 12 weeks of consuming (E)VOO, an increase of the anti-inflammatory effect of High Density Lipoprotein (HDL) was reported, alongside with a reduction of the anti-atherogenic activity in healthy subjects [50]. The regular consumption of (E)VOO from subjects with a high cardiovascular risk leads to the reduction of C-reactive protein, interleukin-6, as well as other pro-inflammatory interleukin levels [51, 52]. According to Yubero-Serrano *et al*. [53], the inflamatory reponses of phenolic compounds are accompanied by a downregulation of the expression of pro-inflammatory genes, low levels of pro-inflammatory proteins as well as a lower total plasma/serum concentration of pro-inflammatory markers in both chronic and postprandial levels. Hydroxytyrosol has the ability to inhibit the expression of cell adhesion molecules (VCAM-1 and ICAM-1) in human umbilical vein endothelial cells stimulated by lipopolysaccharides or cytokines. Furthermore, hydroxytyrosol reduces the inflammatory process during angiogenesis in endothelial cells in combination with oleuropein [54].

- *Antioxidant properties*

Antioxidant effects are considered basic and crucial benefits for health despite great variation in antioxidant potency among phenolic compounds. Most health benefits related to (E)VOO involve antioxidant defense systems, including the ageing process [55], cancer [56, 57], chronic liver injury [58], acrylamide toxicity [59], oxidative DNA damage [60], and colitis [61]. According to de la Torre-Carbot *et al*., (E)VOO consumption protects low density lipoproteins (LDLs) from being oxidized and turning to ox-LDLs due to the binding capability of phenolic compounds to LDL particles [62]. Hydroxytyrosol is believed to be capable of reducing the oxidative status when in the presence of hydrogen peroxide. This phenol, in combination with hydrogen peroxide, protects humans against oxidization by increasing the antioxidant activity and expression of both antioxidant glutathione peroxidase and reductase [63].

- *Regulation of the endothelial function*

Diet can affect the vascular endothelium function by exerting their effects in the vascular vasomotor capacity or influencing cellular regulators. These

dysfunctions lead to the production of an imbalance in the redox system due to the increase of oxidative stress and a decrease in nitric oxide availability caused at the vascular site [74]. Hydroxytyrosol has been found to be responsible for the high levels of nitric oxide in endothelial cultures [53]. In 2017, a research conducted by Lockyer *et al*. attributed the decrease of systolic and diastolic blood pressure of healthy subjects to the consumption of oleuropein extracts [69].

- *Lipids and lipase inhibition*

Several studies have reported a correlation between phenol concentrations in (E)VOO and a favorable plasma lipid response [69, 75, 76]. Buchholz and Melzig investigated potential mechanisms with anti-hyperlipidaemic effects caused by (E)VOO phenolic compounds. These potential mechanisms probably inhibit pancreatic lipases in the small intestine, thus causing the delay of postprandial lipaemia [77].

- *Neurodegenerative diseases*

Inflammation and oxidative stress are both associated with the pathophysiology of many degenerative diseases [78]. Mediterranean Diet leads to the decreased risk of pre-dementia [64]. Oleuropein aglycone showed an anti-amyloid effect, resulting in protection against the cytotoxic effects of amyloid aggregates [79, 80]. This may have a neuroprotective action in diseases such as Alzheimer's, characterized by amyloid deposition and autophagy impairment, contributing to a decrease in aggregated protein and to a reduction in cognitive impairment in *in vivo* models [81].

- *Ageing*

The proteostasis network is responsible for maintaining the proteome quality control [82]. In Nikou *et al*. study, selected polyphenol extracts from EVOOs, with different oleacein and oleocanthal levels, as well as isolated oleacein and oleocanthal in pure state were evaluated for their cytoprotective potential. Both compounds were found to activate ageing promoting cytoprotective pathways while suppressing the oxidative stress [83].

Table 2. (E)VOO phenolic compounds and beneficial functions

(E)VOO phenolic compound	Function	Mechanism	Year	Reference
Oleocanthal	Potent antioxidant		2018	[64]
	Non-steroidal anti-inflammatory agent		2018	[64]
	Neuroprotectant	Alters the structure and function of neurotoxins β-amyloid and Tau	2018	[64]
	Human breast, prostate, and colon cancer cell inhibitor	Inhibition of cancer cells proliferation, migration, and invasion	2015	[65]
	Inhibitor of multiple myeloma	Inhibition of macrophage inflammatory protein-1α	2015	[65]
	Responsible for the apoptotic and necrotic death of cancer cells	Altering the lysosomal membrane permeabilization	2015; 2003	[65,66]
	Reduction of inflammation	Inhibition of cyclooxygenase-1 and cyclooxygenase-2 activity	2015	[67]
Hydroxytyrosol	Inhibitor of protein expression in human umbical vein endothelial cells stimulated by lipopolysaccharides or cytokines	Inhibition of expression of cell adhesion molecules VCAM-1 and ICAM-1	2012	[54]
	Increase of antioxidant activity and reduction of oxidative status	Increase of expression of the antioxidant glutathione peroxidase and reductase in the presence of hydrogen peroxide	2014	[63]
	Decrease of inflammation and of oxidative stress	Increase of the nitric oxide production in endothelial cells	2011;2018	[53,68]
Hydroxytyrosol and oleuropein	Reduction of inflammation in endothelial cells during angiogenesis		2012	[54]
Oleuropein	Decrease of both systolic and diastolic blood pressure		2017	[69]
Oleuropein aglycone	Inhibition of amyloid diseases	Remodel and inactivate toxic amyloid oligomers	2011	[70]
	Protection against Aβ42 aggregation and plaque formation in tissue	Interference in the aggregates' binding to the cells' membrane	2013; 2018	[71-73]

4. Health claims and lack of standardized methods

Despite all health benefits mentioned above, that information does not reach the customer properly. Health claims represent a rarely used legal tool that could be helpful in designing comprehensive labelling to increase consumers' knowledge about the product quality and their willingness to pay. The aim of the Regulations is to "enhance the consumers' ability to make informed and meaningful choices" providing them with messages that are "clear, accurate and based on evidence accepted by the whole scientific community" through claims that appear on "labeling, presentation or marketing in the EU" [84]. Among the list of claims approved by European Food Safety Authority (EFSA), five claims are applicable to (E)VOO. Three out of them are authorized as functional health claims related to olive oil polyphenols, oleic acid, and vitamin E [85-87]; while others are authorized as a reduction of disease risk claim, which relate to unsaturated fatty acids [88] and lowering blood LDL-cholesterol in healthy and hypercholesterolaemic caused by plant sterols and stanols [89].

The claim of oleic acid is also valid for the other olive oil categories, such as olive oil and pomace olive oil, but also to other vegetable oils such as high-oleic sunflower oil as well as to other types of food. The claim may be used only for food where at least 70% of the fatty acids present in the product derive from unsaturated fat, under the condition that unsaturated fat provides more than 20% of energy of the product. The only claim related to the reduction of disease risk is based on monounsaturated and/or polyunsaturated fatty acid contents. Therefore, this claim is generic and does not allow any differentiation within the olive oil categories. On the other hand, the claim of phenols is applicable only to (E)VOOs because any type of refining process removes these molecules. This health claim included in the (EU) 432/2012 Commission Regulation stated: "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress." The claim may be used only for olive oil that contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of oil [85]. It is worth noting that no more than 10% of bottled oils available in the market have a suitable phenolic content for the application of the health claim [90]. The claim concerning the phenols can

reduce consumer's information asymmetry about the product and can create value in the olive growing sector, favouring better coordination in the supply chain between farmers, millers, packers, and distributors [91]. In addition, a few studies have found that some consumer's segments are willing to buy and pay a higher price for (E)VOOs with health claims [92, 93]. However, several problems may have hindered the claim implementation due to lack of clarity in terminology, and mostly, due to the absence of a official analytical protocol for the determination of the bioactive compounds behind the claim. The bioactive phenolic compounds of (E)VOO are mainly the simple phenols hydroxytyrosol, and tyrosol in their free form or linked to aldehydic forms of decarboxylmethyl elenolic acid (glycosylated or not), some phenolic acids and lignans [84].

Beyond concerns for ambiguous interpretation of determining bioactive compounds, there is a lack of a standardized analytical methods that allow quantitative determination of unequivocally identified individual phenolic compounds belonging to the group of hydroxytyrosol/tyrosol and its derivatives [4]. The two most frequently used methods for determination of phenols are the Folin–Ciocalteu method [94] and the method proposed by the IOC (IOC/T.20/Doc No. 29) [95]. Both methods are based on a relative quantitation with gallic/caffeic acid and tyrosol, respectively. Although they are widely extended in the olive oil sector, they are not suited to determine the phenolic content in (E)VOO according to the health claim.

One alternative is the application of nuclear magnetic resonance (NMR) based methods. NMR is a selective technique preferentially used for qualitative applications, but also it has proved applicability in quantitative methods. This technique enables the idea to detect and eventually quantify chemicals inside specific mixtures without troublesome chemical treatments or separation devices, and with simplicity of the measurement procedures, and instrumental stability. Magiatis *et al.* have widely proved the possibilities of this technique in the quantitative determination of phenols in VOOs [96,97]. The limitation of this technique concerns the sensitivity, and the instrumentation seems extremely sophisticated for quality control purposes [98]. A proposal is the application of the

hydrolysis-based method that quantifies the total tyrosol and hydroxytyrosol released from conjugated forms, mainly secoiridoids [99]. This protocol does not enable to discriminate the conjugated derivatives and still, the hydrolysis part needs consensus among scientists. A method based on LC–MS/MS could be a satisfactory method to absolute quantification of phenols due this technique is frequently considered the gold standard for comparison of methods due to its high sensitivity and selectivity levels. In fact, a LC–MS/MS method was used by Olmo-García *et al*. to prove that the Folin–Ciocalteu and IOC methods generally underestimate the phenolic content of olive oil [100]. The LC–MS/MS method allows to evaluate the phenolic content of (E)VOOs provided by producers and to study the influence of different factors on the concentration of these compounds. According to Miho *et al*., oxidative stability depends on the relative phenolic profile and not on the total phenol concentration [101]. This aspect would explain that (E)VOOs with the same total phenolic content may have different oxidative stability.

5. (E)VOO production and its impact in minor components

As previosuly mentioned, (E)VOO is a natural juice obtained exclusively by mechanical and physical processes. Typical olive oil extraction processes are generally based on traditional pressing, the three-phase system or the most recently developed, two-phase centrifugal approach (Figure 4). The latter has been widely accepted for production of (E)VOOs [102]. The general steps of the production process include harvest, washing and crushing of olives, malaxation of olive paste, decantation/centrifugation, storage, and filtration. The qualitative and quantitative profile of bioactive compounds in (E)VOO is widely affected by many variables related to production processes, from the ripening stage of olive fruits to storage conditions [103]. A suitable choice and an appropriate use of the various mechanical device combinations for (E)VOO extraction allows to modulate the nutritional and sensory quality of the final product by either enhancing or inhibiting the activity of enzymes present in olive tissues. Crushing and malaxation are considered as the most critical steps during olive oil extraction since the most important changes occur during these steps [104]. However, qualitative and quantitative changes take place in olive oil bioactive compounds during storage and filtration. Consequently,

rigorous controls of all olive oil processes are recommended to produce high quality oil. Unfortunately, the production of (E)VOO is associated with the generation of large quantities of wastes [105].

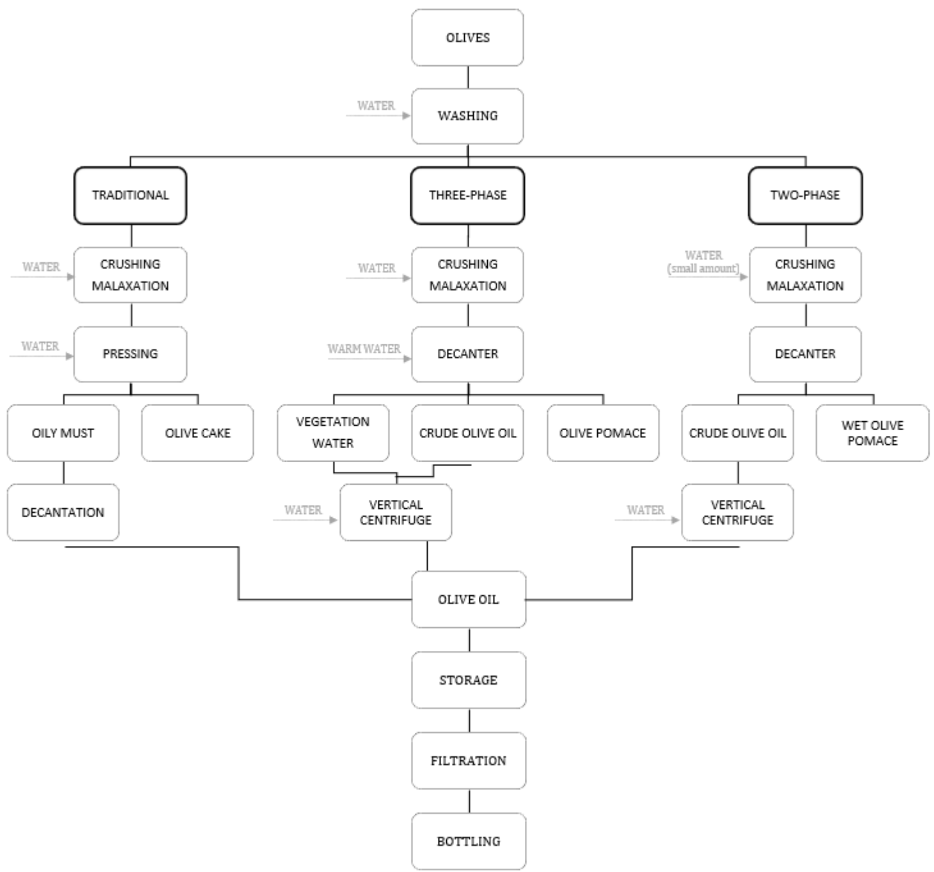


Figure 4. Scheme of olive oil extraction processes

- *Harvest*

The main phenolic compounds and derivatives, including hydroxytyrosol, ligstroside aglycone, oleuropein aglycone, acetoxy-pinoresinol, and elenolic acid, showed an increase in (E)VOO at the early stages of olive harvest, followed by a reduction of their concentrations at more advanced maturity stages. Consequently, early harvested fruit produces olive oil with high phenolic content and high

oxidative stability. However, harvesting too early might have unacceptable sensory oil attributes, such as excessive bitterness and pungency [106].

- *Crushing*

Crushing of olives is a physical process used to break the fruits tissues and release the oil drops contained in the vegetable cell vacuoles. After olive crushing, several enzymes involved in the generation and transformation of minor compounds can be activated such as β-glucosidases, esterases and oxidoreductases, which regulate the synthesis of secoiridoid derivatives. After crushing, both oleuropein and ligstroside, the main secoiridoids in the olives, are hydrolyzed into their corresponding aglycone forms. The hammer crusher is generally recognized as the strongest crushing technique, and generally produces more bitter oils. Hammer crushers usually possess fixed or mobile grids with different hole diameters to control the intensity of crushing depending on the maturity of the fruit, the variety, and the desired oil characteristics [107]. Stronger crushing conditions (i.e. smaller grid holes and higher rotation speed) are related to higher phenolic content, carotenoids, and chlorophyll pigments in the olive paste. This may be explained by the better breakage of the fruit tissue and by the increased activity of enzymes. However, lignans and volatile compounds indicate an opposite trend [108]. This can be beneficial in the case of very rich phenol olive cultivars as milder extraction conditions allow the volatile content to be increased and, at the same time, reduce the excessive bitterness related to excessive phenols. On the other hand, stronger crushing conditions should enhance the phenolic content of varieties characterized by a high volatile composition but low oxidative stability. The use of the different crushing conditions may be therefore very useful to modulate the (E)VOO content in minor compounds related to its overall quality [109].

- *Malaxation*

Malaxation consists of mixing olive paste to increase the percentage of free oil and help small oil droplets to coalesce and agglomerate, thus facilitating separation of the oil and water phases. The efficiency of malaxation depends on the rheological characteristics of the olive paste and the technological parameters of the

operation, such as temperature and time [110]. Regarding the phenolic composition, temperature, time, and the activity of several enzymes are involved in the evolution of these compounds during the malaxation step. Furthermore, increasing the temperature during the olive paste malaxation process increases the activity of oxidative enzymes such as polyphenoloxidase (PPO), peroxidase (POD), and lipoxygenase. These enzymatic activities explain the lineal increase of hydroxytyrosol and tyrosol obtained by degradation of complex phenolic compounds during malaxation. However, a few authors showed that phenolic concentration improved in the oil when malaxation temperature was increased. These conflicting results may be explained in term of O_2 concentration in the pastes during processing as the activity of PPO and POD is inhibited by a low O_2 concentration. Then, higher temperature improves solubility of phenols in the oil phase. The most important losses of different phenolic groups present in olive paste occur in the solid phase (wet pomace) and aqueous phase, by the low lipophilic behaviour of the phenolic structures that led to a low concentration in oil. However, in some (E)VOOs, a low phenolic concentration may improve their sensory quality [18, 102, 111].

A reduction of volatile compounds is correlated with malaxation. This may be due to their evaporation. Therefore, in terms of (E)VOO volatile content, a shorter malaxation time would appear to reduce the loss of these desirable aromatic compounds from the final product [109].

- *Decantation/Centrifugation*

Centrifugation is a separation of the olive oil fraction from the vegetable solid material and vegetation water. In these steps, minor compounds are partitioned in the different phases (oil, water, and solids) as a function of the affinity towards each phase, which is related to the relative polarities of the compounds, and phases ratio. In general, the content of bioactive compounds in the final olive oil is 1–2% of the available pool of these compounds in the fruit. The rest is lost with wastewater and the pomace [112].

Centrifugation step may be carried out using the combination of two

different systems: horizontal centrifugation (three- and two-phase decanter) and vertical centrifugation. Horizontal centrifugation using three-phase decanter requires the addition of warm water, while the two-phase decanter consist of "no-water" centrifugation for separating the oily phase from malaxed pastes without requiring adding warm water. This decanter has the advantage of recovering more complex hydrophilic phenolic compounds and preserving them more efficiently in (E)VOO than by the three-phase method. Concerning vertical centrifugation, this system is used to separate the oily must obtained from horizontal centrifugation. In vertical centrifugation, the addition of water can result in decrease of (E)VOO phenols owing to their hydrophilic nature in relation to the partition coefficient (Kp). Hence, the presence of simple phenols (tyrosol and hydroxytyrosol), with very low Kp in comparison to other phenols such as secoiridoids, decreases in oil [113, 114].

- *Storage*

In the Mediterranean area, olive oil is generally produced from September to February and stored in the mill until filtration and commercialization. During storage, hydrolysis, oxidation, hydration, and loss of the carboxylic group could involve degradation of the compounds. These reactions depend on storage conditions including time, temperature, oxygen availability, light exposure, and industrial or commercial containers. The decrease of quality-related (E)VOO parameters begins during the first month of storage and changes depending on further storage time. After storage for 9 months an increment of the peroxide values and a decrease of the total phenol content, and oxidative stability of olive oil, have been detected. Higher storage temperatures can initiate degradation reactions and facilitate the production of volatile compounds through the formation and decomposition of different compounds [115, 116]. The IOC practice guideline for the storage claims: "The temperature of the storage room should be kept between 13 and 25 °C and to store the oil in stainless-steel tanks, saturating the headspace with inert gases (e.g., nitrogen or argon under a controlled atmosphere), preferably insufflate from the bottom of the containers. If the stainless-steel tanks and connection pipes are maintained under a weak inert gas pressure, as far as possible,

from oil storage to bottling, there is a greater guarantee of reducing the oil's exposure to oxygen" [117]. Regarding commercial container type, (E)VOO stored at Tetra-Brik® container seems to preserve the best characteristics [118].

- *Filtration*

It is a special important final step to remove suspended solids and moisture. Filtration is recommended because moisture reduction improves the quality of (E)VOO. The higher polar phase content in unfiltered olive oils may increase alteration of (E)VOO, although only after several months of storage, especially at an inappropriate temperature, mainly affecting free acidity, sensory attributes, and the formation of simple phenols due to the hydrolysis rate of secoiridoid derivatives [119].

Regarding to minor compounds, waxes, volatiles, and pigments losses have been observed. In any case, it was reported that α-tocopherol content, lignans and flavonoids were unaffected by filtration [120-123]. From the point of view of the phenolic profile, the effects of this step are controversial. It seems that the total phenolic content is reduced by filtration. It is assumed that most phenolic compounds, having amphiphilic characteristics, are located around water droplets on olive oil. Through filtration, moisture is eliminated, thus, water content is decreased together with a proportion of the phenolic compounds. Regarding to secoiridoid aglycones, all these compounds decrease in concentration during storage due to the hydrolysis. It has been observed an increase in hydroxytyrosol and tyrosol content for unfiltered (E)VOO than for filtered ones. In this manner, oil filtration could reduce the rate of hydrolysis of secoiridoid aglycones [124].

- *Destoning*

The destoner is a mechanical system allowing crushing of the fruit flesh excluding the stone. The crushing of stones carries a loss of mechanical energy due to its conversion into thermal energy, causing degradation and oxidation phenomena. Likewise, it is worth mentioning the management of by-products with a lower environmental impact in destoned olive oil production. Nevertheless, the interest of this added step is focused on the quality improvement [125].

Concerning minor compounds, the passage of pigments from fruit to the oil is negatively influenced by destoning. The effect on tocopherols and carotenoids is not clear as a serious influence of cultivars was observed. However, destoned oils seem to contain a higher fraction of pleasant C_5 and C_6 volatile compounds. The increase seems to be due to the removal of the seeds containing 13-hydroperoxides metabolizing enzymes other than hydroperoxidelyase, able to lower the C_6 unsaturated aldehydes concentration [126, 127]. Regarding phenol content, the literature about the advantage of destoned process is controversial. Some authors did not find significant differences [128], while most have observed an increase in the total phenolic content. These data could be explained considering that most of the fruit oxidative enzymes, mainly located in the endocarp, such as PPO and POD, are removed, protecting the phenols from oxidation. However, it seems that destoning effects on phenolic compounds are different according to the cultivar [129-131].

6. Cooking influence in (E)VOO

Olive oil is a basic food for the people of the countries surrounding the Mediterranean Sea, but its use is now expanding to other parts of the world due to its unique flavor, high content of healthy MUFAs and the presence of biologically important minor constituents. As previosuly mentioned, (E)VOO has a remarkable oxidative stability. If properly stored, it can retain its characteristics for 18 months or more. This resistance to the development of rancidity, combined with a variety of flavors and distinct features, offers the opportunity for many culinary applications, many of which demand no or very mild processing (addition to salads, marinades, sauces, dressings, dips). Also, (E)VOO properties make it a good option for filling medium of canned food. It showed the highest protection against the thermal oxidation of n-3 fatty acids induced during sterilization. However, the health outcomes of a Mediterranean Diet are reportedly difficult to reproduce in non-Mediterranean populations, possibly because of different cooking practices [132, 133].

It is consensual that (E)VOO qualities are completely boosted when

consumed without being previously subjected to any thermal treatment. Otherwise, when it is used as the cooking base, thermal effects occur and compositional changes are expected. Whether or not the bioactive components reach the consumer will depend on the culinary practice carried out and on the hardness of this in terms of time and temperature. Frying, roasting, microwave and boiling are the typical thermal processes used for cooking. Each practice has particular characteristics regarding temperature and confecction time. In comparison to other vegetable oils, (E)VOO has a much lower rate of alteration during uses that require high temperature, which is a direct consequence of its high MUFA profile. Even on a direct association with other vegetable oils with equivalent oleic acid amounts, (E)VOO reveals an improved stability. This enhancement is mostly attributed to the antioxidant capacity of the phenolic compounds in combination with vitamin E, providing a balanced protection under thermal stress [134]. The conditions (temperature, time, cooking process and food presence), as well as the initial oil composition, will determine the degradation rate, and the time taken to consume the antioxidant pool. The temperature achieved during cooking is a determinant factor for most vegetable oils, and particularly, for VOOs. Indeed, the higher acidity of VOOs, due to the absence of refining, reduces their upper thermal limits, because of the lower boiling point of the FFA released. Still, when processed under normal cooking conditions, olive oil performance is comparable or better than other vegetable oils [135]. Nevertheless, under microwave heating, the temperatures achieved are uncontrolled and, all vegetable oils are rapidly degraded [136]. Under water boiling conditions, hydrolysis and leaching of phenolic compounds into the water phase are significant, but the heating time and the presence of food constitute the main contributor to their effective degradation. Therefore, when possible, olive oil should be added more closely to the final cooking process [135].

Frying is one of the most popular methods for preparation of foods worldwide. Deep-frying, in which the food is totally immersed in hot oil, is the most common frying method in domestic food preparation, and particularly, in restaurants and in the food industry. Fried products have unique organoleptic and sensorial properties including flavour, texture, and appearance, which turn them

largely enjoyed by consumers [137]. However, deep-frying is the most aggressive culinary technique for the oil, due to the high temperature and prolonged exposition time. The oil sustains some physical changes such as the colour darkens, the viscosity increases and smoke appears. Moreover, the hydrolysis, oxidation, and polymerization of oil components are common chemical reactions in frying oil and produce volatile or non-volatile compounds [138, 139]. A great concern regarding new formed compounds under thermal stress, including oxidized fatty acids and sterols or triacylglycerols polymers, and their possible impact on human health is rising. The intensity of these reactions is highly dependent on the type and quality of the oil used. Lignans were found to be very stable but there were significant losses of hydroxytyrosol derivatives (the main class of antioxidants) and α-tocopherol. It was observed that olive oil lost its radical scavenging activity in a shorter heating time relative to other vegetable oils much richer in tocopherols, but it reached the level of 25% total polar content (rejection point for a heated fat) after prolonged heating; all the other oils reached this upper limit in shorter periods. Monounsaturated oils show unique properties in frying, permitting the frequent replenishment of these oils to extend considerably their shelf life. Also, the most appropriate frying oil should be low in free fatty acids and polar compounds. With these premises, (E)VOO is a very interesting option when looking for a frying oil [140-142].

7. Metabolomics: study and quality improvement of the (E)VOO

Recent developments and applications of modern instrumental analytical techniques have attracted increasing attention from the research community as reliable and fast strategies to ensure (E)VOO quality and authenticity. However, research on (E)VOO are not restricted solely to its quality and authenticity issues, challenges cover all the aspects from the farm to the table [4, 143]. This is an arduous task as (E)VOO contains many components with broad ranges of concentrations, molecular properties, and polarities. The scientific community has given lately great attention to the development and validation of efficient modern analytical techniques which allow the rapid determination of olive oil metabolic content. It has been suggested that metabolomics may provide the most "functional"

information of the omics technologies. The metabolome represents the final "omic" level in a biological system, and metabolites represent functional entities. Thus, metabolites have a clear function in the life of the biological system and are also contextual reflecting the surrounding environment [144]. The field of metabolomics developed over the last two decades when a novel array of analytical methodologies and technologies were introduced. Recently, classical methodologies have been superseded by advanced analytical strategies. These novel analytical methods are widely used in contemporary research in food science and nutrition. In this direction, a new sub-field has been recently introduced, known as "Foodomics" [145]. A typical food metabolomics process is: (i) food samples are collected, and metabolites are extracted during sample preparation; (ii) the prepared samples are analyzed by the appropriate analytical technique; (iii) the raw output data are processed before statistical analysis; and (iv) interpretation of the data to find information that is biologically important. Furthermore, clinical studies are essential to ascertain the conditions and optimize ingestion schemes food compounds. *In vivo* human studies that provide evidence about the health properties of (E)VOO are promising [146].

MS-based metabolomics analyses have been broadly categorized into two main approaches, targeted and untargeted metabolomics. Targeted methods aim towards the determination of a particular group of metabolites of interest. In general, the main aim is the identification and quantification of as many metabolites within the group. More recently, efforts have been put on studies that focus on the development of semi-quantitative methods for a large number of metabolites [147, 148]. Untargeted metabolomics studies point at the simultaneous holistic measurement of as many metabolites as possible. An important issue in untargeted metabolomics research is to achieve global profiling coverage across a heterogeneous chemical landscape. It should also be noted that untargeted methods result in the collection of large volumes of data and, therefore, there is a need for multivariate statistical evaluation of acquired data. Discriminant Analysis and its variants are more appropriate for metabolic profiling, while Principal Component Analysis (PCA) is the statistical method preferred for fingerprinting [149, 150]. For

(E)VOO analysis, factors such as the number of samples, possible noise, the number of variables, the objective of the analysis, and mainly, the metabolomic approach, must be taken into consideration.

Metabolomic analysis uses different analytical platforms. These comprise NMR spectroscopy, direct infusion mass spectrometry (DI–MS), gas chromatography coupled to mass spectrometry (GC–MS), two-dimensional GC coupled to MS (GCxGC–MS), LC–MS, two-dimensional LC coupled to MS (LCxLC–MS), and capillary electrophoresis coupled to MS (CE–MS). These approaches allow the simultaneous analysis of a wide range of metabolites present in (E)VOO samples. All these techniques present distinct advantages and disadvantages. The best option of a specific strategy relies heavily on the objectives of the study and is generally a trade-off among sensitivity, selectivity, and speed [151, 152]. Over the last decade, metabolomics approaches seem to increasingly adopt LC–MS technology owing to its high sensitivity, wide availability, and metabolome coverage. Concerning LC–MS–based (E)VOO analysis, recent papers investigate some of the most crucial issues. Their application can be divided into three categories: (i) detection of adulteration with vegetable oils or any olive oil quality upgrade, and authentication of (E)VOO; (ii) characterization of (E)VOO bioactive compounds, and; (iii) (E)VOO safety: detection of pesticide residue metabolites [153].

In an increasingly globalized world, the "new consumers" buy the olive oil consciously and not because they are used to it as the traditional consumers in the Mediterranean region. In this sense, (E)VOO metabolomic studies can provide a useful tool for food testing laboratories, food processors, food chain suppliers, and plant breeders.

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MATERIALS AND METHODS

This section of the Thesis Book describes the different analytical tools and equipment used in the experimental part of the Thesis, which are described in more detail in the subsequent chapters.

1. Samples

The global objective of the Thesis research was to develop analytical strategies for improving the detection and identification by Ms in targeted and untargeted metabolomic analysis applied to olive oil samples. VOO samples used in Chapters 1, 2, 5, and 6 were provided directly by Spanish producers from different locations of the Mediterranean area. Samples used in Chapters 1 and 2 were part of the Aristoil Project, an European project of the Interreg-Med call. On the other hand, samples collected for Chapters 5 and 6 were provided by the Nutradaf Project, granted by the Ministerio de Ciencia e Innovación and CDTI. Oils from different producers were purchased in local supermarkets for Chapters 3 and 4. Human serum samples from volunteers were used in Chapter 7.

2. Sample preparation

The majority of the research in the Thesis was based on the analysis of bioactive compounds isolated by liquid-liquid extraction from the oil samples. In general terms, sample preparation consisted of solvent extration, separation of extracts by centrifugation; filtration if required; and dilution prior injection into the system.

For the extraction and preconcentration of phospholipids, conventional SPE cartidges, packaged with zirconia coated silica, were necessary, as detailed in Chapters 4 and 7. In these chapters, the influence of the SPE cartridges on sample preparation is discussed.

3. Separation and detection systems

The methods developed in the experimental part of this Doctoral Thesis have been based on a chromatographic separation (using LC or GC) and subsequent detection by MS.

For targeted analysis a Thermo Scientific UltiMate 3000 series LC system coupled to a Thermo Scientific triple quadrupole mass detector (QqQ) TSQ Quantum™ Access MAX detector (Waltham, MA, USA) was used in Chapters 1,2, and 3 for analysis of phenolic compounds. The LC system was furnished with a Mediterranea C18 (3 µm particle size, 5 × 0.46 cm i.d.) analytical column and a C18 guard column from Teknokroma (Madrid, Spain), using as mobile phases water and methanol. The QqQ detector was equipped with a electrospray ionization (ESI) probe for spraying the chromatographic eluate. Chromeleon™ software (version 6.80) was used for controlling the LC system, TSQ Tune software (version 1.2.1) was used to control the detector parameters and, finally, Thermo Xcalibur™ software (version 3.0.63) was used for methods and worklists creation.

A 1200 Series Agilent (Palo Alto, CA, USA) LC system furnished with a Poroshell 120 EC-C8 (2.7 µm particle size, 4.6×100mm i.d.) analytical column from Agilent. A 120 EC-C8 (2.7 µm particle size, 4.6×5mm i.d.) guard column, was used in Chapter 4. Water and methanol/2-propanol were the mobile phases.The LC system was coupled to a 6460 Agilent triple QqQ furnished with an Agilent Jet Stream Technology ESI. Agilent MassHunter Workstation (V-B.05) was the software for data acquisition, qualitative and quantitative analysis.

A GC (Agilent 7890B) coupled to a mass spectrometer (Agilent 5977A), using a SP TM 2560 fused silica column (100 m ×0.25 mm, 0.25 µm film thickness) from Supelco (Bellefonte, PA, USA) was used in Chapter 7 for fatty acids (FAs) analysis.

For untargeted analysis in Chapters 5 and 6 an Agilent 1200 series LC (Palo Alto, CA, USA) furnished with an Inertsil ODS-2 C18 analytical column (250 × 4.6 mm i. d., 5 µm particle) from GL Science (Tokyo, Japan) was used. Water and acetronitrile/2-propanol were the mobile phases. The chromatograph was coupled through an ESI source to a 6540 quadrupole–time-of-flight detector (QTOF MS/MS; Agilent Technologies, Santa Clara, CA) for detection. Agilent MassHunter Workstation LC–QTOF acquisition software (version B.06) was used to control the instrument and acquire the data.

4. Data processing

For targeted analysis, in Chapters 1, 2, and 3 a quantitative method was used. MS detection was performed by MS/MS in multiple reaction monitoring (MRM) mode for selective transitions from the precursor to product ions for each analyte. Calibration curves were prepared by using refined sunflower oil spiked with multistandard phenolic solutions at concentration ranges from limit of quantitation (LOQ) values to 20 μg g^{-1}. Tracefinder software (version 3.2), from Thermo Scientific, was used for data acquisition and qualitative and quantitative analysis.

In Chapter 4, a semi-quantitative method for analysis of glycerophospholipids (GPLs) was developed. MassHunter Workstation software was used to process all data obtained by LC–QqQ in different work modes. A data set containing the peak area and retention time (RT) of each GPL was obtained.

In Chapter 7, Qualitative Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to process the data obtained by GC–MS. This software allowed generating a data set containing the peak area and RT of each metabolite in all samples. Treatment of raw data files started by deconvolution of potential molecular features (MFs) by the algorithm included in the software. Quantitation was carried out by integrating the peak area for each considered FA. The relative amount of each fatty acid was expressed as percentage of total peak area considering all FAs.

Untargeted analysis was performed in Chapters 5 and 6. MassHunter Workstation software (version B8.00 Profinder; Agilent Technologies, Santa Clara, CA) was used to process the data obtained by LC–QTOF in auto-MS/MS mode. Treatment of the raw data file starts by extraction of potential MFs with the suited algorithm included in the software. Once the signal alignment has been done, the obtained chromatographic peaks were integrated to obtain a clean matrix, which was exported as a CSV file, to accomplish both the identification of the MFs and the corresponding statistical analyses. Once all MFs were extracted and aligned, the software MassHunter Qualitative v7.0 was used for the targeted extraction of

MS/MS information associated with the monitored MFs in the whole set of analyses.

5. Identification of metabolites

Tentative identification of metabolites was supported on MS/MS information that was searched in the METLIN MS/MS (http://metlin.scripps.edu), MassBank MS/MS (http://www.massbank.jp), and ReSpect MS/MS (http://spectra.psc.riken.jp) databases. Additionally, some compounds were confirmed by both MS/MS information and retention time, using commercially available standards. For identification of metabolites from GC–MS data, the NIST Mass Spectral Search Program v.11.0 (NIST, Washington, DC, USA) was used for spectra comparison. Tentative identification was reported when the correlation between experimental and database spectra was above 0.85 in normal search mode.

6. Data pretreatment and statistical analysis

Datasets including the peak area for all metabolites or potential metabolites detected in all samples were then processed for statistical analysis. In most studies analysis of variance (ANOVA) was performed to evaluate the significant differences (p-value <0.05) with Tukey's test followed by post hoc analysis. According to the objective of the study different statistical tools were used. The main softwares used are described below:

- Statgraphics Centurion XVI (version 16.1) allows performing different parametric and non-parametric statistical analysis.
- MetaboAnalyst is a comprehensive platform that enables high-throughput analysis for both targeted and untargeted metabolomic data (http://www.metaboanalyst.ca).

EXPERIMENTA PART

SECTION I

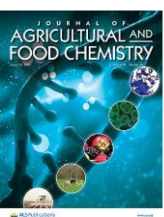
Determination of minor families in virgin olive oil for evaluation of quality and health benefits

Section I of this PhD Book is devoted to a methodological update for characterization of two minor families of compounds found in olive oil and, particularly, in (E)VOO. These two minor families are GPLs and phenolic compounds and their determination can be of interest to increase the commercial value of (E)VOO. Chapters 1–3 were dedicated to the characterization of the phenolic fraction of (E)VOO. The interest in this family of compounds has increased in the last decade due to its contribution to organoleptic features such as bitterness and pungency, its role on the (E)VOO stability due to the antioxidant capability, and finally, the health benefits associated to the daily consumption of (E)VOO with a relatively high phenolic content. This last aspect has been the focus in Section I by virtue of the health claim included in the European Commision (EU) 432/2012, specifically attributed to (E)VOO phenols. With this premise, Chapter 1 was aimed at the characterization of the phenolic fraction in (E)VOO samples from producers of the Mediterranean area obtained in two agronomic seasons. Due to the antioxidant properties associated to (E)VOO phenols, in Chapter 2 it was studied the decay of phenolic concentration 12 months after production, and this was explained by the initial phenolic profile. Finally, in Chapter 3 it was evaluated the decrease of phenolic content after subjecting monocultivar EVOOs to frying at 180 °C for 90 min. Similarly, alteration of the phenolic fraction during heating depended on its composition before the process.

GPLs, mainly, constitute vegetal cellular membranes and are partially transferred to olive oil during extraction. This polar lipid fraction has been scarcely studied despite it could be associated to quality of the final product. In Chapter 4, an analytical method is presented for characterization of GPLs in vegetable oils by combination of selectivity and sensitivity attained by SPE and LC–MS/MS. A novel aspect of this research was the elucidation of the GPLs profile by discrimination of sub-families and fatty acids identification. As a proof-of-concept, the method was applied to compare the GPLs profile in different categories of olive oil, but also in other vegetable refined oils. Results seem to point out that the characterization of this lipid fraction can aid to discriminate among olive oil categories by quality criteria.

CHAPTER 1

Evaluating the variability in the phenolic concentration of extra virgin olive oil according to the Commission Regulation (EU) 432/2012 health claim



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Evaluating the Variability in the Phenolic Concentration of Extra Virgin Olive Oil According to the Commission Regulation (EU) 432/2012 Health Claim

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Evaluating the Variability in the Phenolic Concentration of Extra Virgin Olive Oil According to the Commission Regulation (EU) 432/2012 Health Claim

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Abstract

The health benefits of extra virgin olive oil (EVOO) are associated to its fatty acids profile (with predominance of oleic acid) and to the minor components that include phenols, among others. Phenols are responsible for the only health claim of olive oil reported in the Commission Regulation (EU) 432/2012. Here, we have applied a LC–MS/MS method to determine the most abundant phenols included in the health claim (with special emphasis on secoiridoids) in 1239 EVOO samples produced in two consecutive agronomical seasons. The predominant cultivars in Spain ("Picual", "Arbequina", "Hojiblanca" and "Cornicabra") were evaluated. We also studied the influence of harvesting date and orchards location on the EVOO phenolic concentration. A great variability in phenolic content, from 1 to 2850 mg/kg, was found in these EVOOs and not all of them (4.6 and 23.1% in the two seasons) reported a concentration above 250 mg/kg to certify the health claim.

Keywords: Phenols; Extra-virgin olive oil; Health claim; LC–MS/MS; Cultivar; Healthy index.

1. Introduction

The consumption of olive oil is one of the fundamental pillars supporting the Mediterranean Diet. The beneficial effects associated to the frequently intake of olive oil have been widely described and recognized. The PREDIMED project revealed a considerably lower risk of cardiovascular diseases for individuals who consume olive oil. In fact, they recommended the preference for extra virgin olive oil (EVOO) [1-3].

The health benefits of (E)VOO (abbreviation used to refer to virgin olive oil and, particularly, to extra-virgin olive oil) are attributed to its composition. This includes major components (approximately 98% of the total oil weight), mainly triglycerides and other fatty acid (FA) derivatives, marked by a predominance of monounsaturated FAs, especially oleic acid (between 55 and 83% of the total FAs content) [4]. This monounsaturated profile allows olive oil to make use of the health claim included in the Commission Regulation (EU) 432/2012, attributable to foods with high content in unsaturated FAs [5]. In fact, the FAs profile is indicated in the nutritional information template that appears on the label of any commercial container of olive oil. Complementarily, olive oil contains minor components pertaining to different chemical families such as phenols, tocopherols, phytosterols, volatile compounds, terpenes or hydrocarbons, among others [6-8]. This fraction differentiates (E)VOOs from lower quality olive oils, and it is partially or totally removed in thermal and/or physical–chemical treatments applied in the refining process. Minor components also contribute to the health benefits of (E)VOOs. Thus, the Commission Regulation (EU) 432/2012 recognizes three health claims about minor components present in (E)VOO. These are vitamin E, mainly as α-tocopherol, phytosterols and phenols [5].

Vitamin E is the only minor component of olive oil included in the nutritional information template. Regularly, vitamin E concentration in (E)VOO is around 200 mg/kg [9,10]. According to the 12 mg recommended daily intake, it would be necessary to consume an amount above the daily intake recommended by the PREDIMED reports (between 40 and 50 g) [1]. Additionally, sunflower oil may

contain between 2.5 and 3.5 times more vitamin E than (E)VOO, despite sunflower oil is refined prior to consumption [6].

Concerning phytosterols, its concentration in (E)VOO does not exceed 2000 mg/kg, which implies a consumption of 2.0 mg/g of oil [11,12]. The beneficial effect is obtained with a minimum of 0.8 g daily intake of phytosterols and phytostanols. With these premises, it would be necessary a consumption of 400 g of oil per day. Due to its vegetable origin, (E)VOO is a source of phytosterols, but it does not represent the main dietary contribution of these compounds [13].

The Commission Regulation (EU) 432/2012 health claim for phenols is specific of olive oil since secoiridoids, the main phenolic family, are only found in plants of the *Oleaceae* family [14,15]. The health claim is attributable to olive oils providing a minimum amount of 5 mg of hydroxytyrosol, tyrosol and derivatives per 20 g of product [5]. Among the derivatives, it is worth mentioning oleuropein and ligstroside aglycone isomers, oleocanthal and oleacein. With this daily consumption, blood lipids are protected from oxidative stress, which is a key mechanism in the development of pathologies such as cardiovascular diseases [16-18]. Furthermore, the European Food Safety Authority (EFSA) pointed out other biological activities of olive oil phenols such as their anti-inflammatory properties, and their contribution to maintaining suitable cholesterol levels, normal blood pressure, respiratory health, normal gastrointestinal tract function and immune system strengthening [19].

Taking 20 g of olive oil intake per day as a reference, this means a concentration of phenols in oil above 250 mg/kg. However, the main limitation is found in the lack of reference analytical methods with capability to respond to the health claim. The two most frequently used methods for quantitative analysis of phenols in olive oil are the Folin–Ciocalteu method [20] and the method proposed by the International Olive Council (IOC) (IOC/T.20/Doc No. 29) [21]. Both methods are based on a relative quantitation. Gallic or caffeic acid are used as standards to determine a calibration curve in the Folin–Ciocalteu method [22]. Important limitations of this method are that any oxidizable specie interferes in the final result

and, there are critical differences in the oxidation kinetics between the standards and the main phenols found in olive oil [23]. Concerning the IOC method, this is based on a liquid–liquid extraction to isolate phenols in a hydroalcoholic phase that is analyzed by liquid chromatography with photometric detection. In this case, relative quantitation is supported on tyrosol as a reference, which leads to underestimation of the absolute phenolic content [24]. Low chromatographic resolution for separation of secoiridoid derivatives is an additional limitation, which significantly struggles the recognition of the chromatographic peaks and, consequently, the identification [25].

Although both methods are widely extended in the olive oil sector, they are not suited to determine the phenolic content in (E)VOO according to the health claim. A proposal is the application of the hydrolysis-based method that quantifies the total tyrosol and hydroxytyrosol released from conjugated forms, mainly secoiridoids [26]. This protocol is based on a hydrolysis reaction for 2 h at 80 ºC and does not enable to discriminate the conjugated derivatives. Karkoula *et al*. developed a quantitative method based on NMR for determination of major secoiridoid derivatives in olive oil and they analyzed 363 commercial Greek and Californian samples [27]. Miho *et al.* developed a method based on LC–MS/MS for quantitative determination of phenols in olive oil. This method was applied to evaluate the cultivar influence in the phenolic profile of VOO [28]. The same authors proved the association between the phenolic profile of VOO and its oxidative stability by using the Rancimat method as a reference test [29]. Later, a similar method also based on LC–MS/MS analysis was used by Olmo-García et al. in order to prove that the Folin–Ciocalteu and IOC methods generally underestimate the phenolic content of olive oil [30].

LC–MS/MS is considered as a gold-standard technique for quantitative analysis due to its high sensitivity and selectivity levels. According to these facts, one important step was to evaluate the phenolic content of EVOOs provided by producers and to study the influence of different factors on the concentration of these compounds. Currently, the commercial classification of olive oil categories identifies EVOO as the two most valuable product. The health properties of EVOO in

terms of phenolic content may be a factor to be considered as a future discriminating marker according to the Commission Regulation (EU) 432/2012. In this research, we have analyzed a total of 1239 Spanish EVOO samples obtained directly from producers in two consecutive agronomical seasons. An LC–MS/MS method developed previously was applied to estimate the phenolic content according to the health claim [28,31]. In addition, we have studied the influence of different variability factors such as the cultivar, the harvesting period, and the location of olive orchards (considering the height above the sea level, a.s.l.) on the EVOO phenolic profile.

2. Materials and methods

2.1 Reagents and standards

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

Hydroxytyrosol was purchased in Extrasynthese (Genay, France) while secoiridoid derivatives oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycone and ligstroside aglycone (both as monoaldehyde closed isomers) were provided by Prof. P. Magiatis (University of Athens, Greece). Hydroxytyrosol standard was prepared in methanol while those for secoiridoids were prepared in acetonitrile to preserve their stability and avoid undesired conversion to acetal and hemiacetal derivatives [31]. To simplify the nomenclature, oleuropein and ligstroside aglycones were used to name the monoaldehyde closed isomers, while oleomissional and oleokoronal were used to name the monoaldehyde open isomers of oleuropein and ligstroside aglycones, respectively.

Syringaldehyde from Sigma-Aldrich (St. Louis, MO, USA) was used as internal standard to control the LC–MS/MS performance during the analysis of all samples.

2.2. Samples

A set of 1239 Spanish olive oil samples were analyzed in this research. Samples were provided directly by Spanish producers from different locations of the Mediterranean area under the frame of the Aristoil project (granted by the Interreg-MED program). Oils were produced in two consecutive agronomical seasons, 690 and 549 samples in 2017/2018 and 2018/2019, respectively. All samples were classified after production as EVOOs by recognized entities or laboratories. Supplementary Tables 1 and 2 shows detailed information about the analyzed samples.

2.3. Sample preparation

GPLs were extracted from oil samples using 30 mg HybridSPE® phospholipid technology cartridges from Supelco. The protocol recommended by the manufacturer was adapted to the analysis of non-polar samples as vegetable oils. A 1-g aliquot of each oil was mixed with 500 µL of $CHCl_3$ for 30 s to prepare the loading solution. Vacuum was applied to the SPE manifold to favor the pass of the loading solution through the SPE sorbent cartridge, which was previously conditioned with 1 mL of MeOH acidified with 0.1% formic acid (v/v). Then, the cartridge was washed three times with 500 µL of 2-propanol. GPLs were eluted in alkaline medium using 1 mL of MeOH with 5% (v/v) ammonium hydroxide.

The eluted fraction was evaporated, and the residue was reconstituted with 50 µL of MeOH with 0.1% (v/v) formic acid prior to injection into the LC–QqQ MS/MS system. Three replicates per olive oil were analyzed.

2.4. Apparatus and instruments

A vortex shaker from IKA (Wilmington, NC, USA) and a centrifuge supplied by Ortoalresa (Madrid, Spain) were used for sample preparation. Chromatographic separation was carried out with a Thermo Scientific UltiMate 3000 series LC system furnished with a Mediterranea C18 (3 µm particle size, 5 × 0.46 cm i.d.) analytical column and a C18 guard column from Teknokroma (Madrid, Spain). The LC system was coupled to a Thermo Scientific QqQ TSQ detector equipped with an electrospray

ionization source (ESI). Chromeleon™ (version 6.80) and TSQ Tune (version 1.2.1) were used to control the LC–MS/MS system. Xcalibur™ (version 3.0.63) was used for creation of methods and worklists.

2.5. Sample preparation

Phenolic compounds were isolated by liquid–liquid extraction by following a previously published protocol [28]. A 0.5 g aliquot of oil was vortexed with 250 µL of *n*-hexane for 30 s. Then, 2 mL of 80:20 (v/v) methanol–water with the internal standard (1 µg/mL) was added and shaken for 2 min, and the hydroalcoholic phase was separated by centrifugation for 8 min at 900g. The resulting phenolic extract (top layer) and a dilution (1:10 or 1:20 v/v) were injected into the LC–QqQ MS/MS. Three replicates per sample were analyzed.

2.6. LC–MS/MS analysis of phenolic compounds

Analyses were performed by reversed-phase liquid chromatography followed by MS/MS detection with ionization in negative mode. The chromatographic mobile phases were deionized water (phase A) and MeOH (phase B), both solutions acidified with 0.1% (v/v) formic acid. The LC pump was programmed with a flow rate of 0.4 mL/min with the following elution gradient: 50% phase B was kept as initial mobile phase for 0.5 min; then, from 0.5 to 2 min, mobile phase B was from 50 to 80%; and from min 2 to 5, mobile phase B was from 80 to 100% [28]. This last composition was maintained for 1.5 min. A post-time of 4 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 10 µL. The autosampler was kept at 5 °C to increase sample stability and the column compartment was kept at 30 °C.

MS detection was performed by MS/MS in multiple reaction monitoring (MRM) mode for selective transitions from precursor to product ion for each analyte. The MRM parameters for the analysis of target phenols are listed in Supplementary Table 3 and Supplementary Figure 1 shows the MRM chromatograms obtained by analysis of an EVOO sample. The ionization parameters were set as follows: ionization probe, position B; spray voltage, 2750 V; sheath gas pressure, 25 arbitrary units; auxiliary gas, 10 psi; ion transfer capillary temperature,

300 °C; and FWHM for Q1 and Q3, 0.7. The scan time and width were 0.1 s and 0.5 *m/z*.

2.7. Quantitative analysis of phenols and estimation of the healthy index

Calibration curves were prepared by using refined sunflower oil spiked with multistandard phenolic solutions at concentration ranges from LOQ values to 20 µg/g (Supplementary Table 3). Spiked aliquots were analyzed with the complete protocol in triplicate to obtain the calibration models. Oleomissional and oleokoronal were quantified by using the calibration models prepared with the monoaldehyde closed isomers.

The healthy index was estimated according to the Commission Regulation (EU) 432/2012 and the EFSA opinion, which consider a 20 g daily intake of olive oil. Thus, the healthy index refers to the amount of phenols expressed in mg consumed with this daily intake. A positive healthy index is considered above 5.0 mg/20 g of oil.

2.8. Data processing and statistical analysis

Tracefinder™ software (version 3.2), from Thermo Scientific, was used for data acquisition, qualitative and quantitative analysis. The significant differences (p-value of < 0.05) were studied by ANOVA with Tukey's test followed by post hoc analysis. Statgraphics Centurion XVI.I version 16.1.18 (Warrenton, VA, EE.UU.) was used for statistical analysis.

3. Results and Discussion

3.1. Phenolic content in EVOOs produced in two consecutive agronomical seasons

One of the main objectives of this research was to characterize the phenolic content of EVOOs according to the Commission Regulation (EU) 432/2012 health claim. For this reason, samples produced in two consecutive agronomical seasons, 2017/2018 and 2018/2019, were analyzed to obtain an overview of the phenolic content of EVOOs produced in Spain in this period. The total number of samples was 1239, all of them classified by official laboratories as EVOOs. Samples were

distributed in 690 and 549 for the 2017/2018 and 2018/2019 seasons, respectively. Table 1 shows the average concentration of phenols in EVOO samples analyzed in both seasons, the standard deviation, and minimum and maximum detected concentrations. The total phenolic content and the healthy index were estimated by considering hydroxytyrosol, oleacein, oleocanthal, and the isomers of oleuropein aglycone and ligstroside aglycone. Despite the used method allowed differentiating between two isomers of oleuropein aglycone (the monoaldehyde closed form and oleomissional) and ligstroside aglycone (the monoaldehyde closed form and oleokoronal), the concentration of both isomers in each case were summed up and listed as oleuropein aglycone and ligstroside aglycone.

Table 1. Concentrations (mean, minimum and maximum) of phenolic compounds detected in the EVOO samples analyzed in the two seasons. The healthy index variation is also listed.

Season	**17/18 (*n*=690)**		**18/19 (*n*=549)**		**17/18**	**18/19**	**17/18**	**18/19**
Compound	**Mean**	**SD**	**Mean**	**SD**	**Max**		**Min**	
Hydroxytyrosol, mg/kg	1.8	1.5	2.8	2.3	14.2	18.8	<LOQ	<LOQ
Oleacein, mg/kg	343	208	85.8	66.1	1479	627	33.3	<LOQ
Oleocanthal, mg/kg	55.9	45.2	109	78.5	270	627	1.9	<LOQ
Oleuropein aglycone, mg/kg	273	151	171	130	931	943	<LOQ	<LOQ
Ligstroside aglycone, mg/kg	152	127	185	168	835	1120	<LOQ	<LOQ
Total phenols, mg/kg	826	409	553	381	2850	2604	51.9	1
Healthy index, mg/20g VOO	16.5	8.2	11.1	7.6	57	52.1	1	0.02

Concerning simple phenols, we only determined quantitatively hydroxytyrosol. The concentration of this phenol in the two seasons did not surpass 15 mg/kg, and most samples gave levels below 10 mg/kg (98.9% of 1239 EVOOs). Tyrosol was not considered for estimation of the healthy index in this research since this phenol was not detected in many samples and the maximum detected concentration was around 5 mg/kg.

The healthy index is the parameter that allows defining the EVOO value based on the health claim included in the Commission Regulation (EU) 432/2012. As Table 1 shows, the results for both seasons were characterized by a great

variability in the phenolic levels (Supplementary Figure 2). In the 2017/2018 season, the total phenolic concentration ranged from 51.9 to 2850 mg/kg, while in the following season, the range was from 1 to 2604 mg/kg. The average phenolic content was higher in the first season, 826 versus 553 mg/kg, which could be mainly explained by climatological conditions found in 2018/2019, with an intense drought period. Nevertheless, these results were above the cut-off value set for the health claim (250 mg/kg). Thus, the mean total phenolic content was 3.3 and 2.2 times the cut-off value in 2017/2018 and 2018/2019 seasons, respectively. According to these results, the mean healthy index was 16.5 and 11.1 mg/20 g of VOO for the 2017/2018 and 2018/2019 seasons, respectively, which are clearly above the 5.0 mg/20 g of VOO included in the health claim declaration. The analysis of the content of the different samples in both seasons allowed detecting that 95.4 and 76.9% of the samples had a concentration above 250 mg/kg. Therefore, a high proportion of analyzed samples reported a phenolic concentration to take benefits from the health claim with the recommended daily consumption. For a concentration cut-off of 500 mg/kg, the proportion of samples fulfilling the health claim was reduced to 76.7 and 51.6%, respectively. These results support that the adoption of appropriate agronomic, hygienic, and technological practices to obtain EVOO largely guarantees a product with benefits in accordance with the health claim.

Concerning individual phenols, in 2017/2018 season, oleacein and oleuropein aglycone were the two most concentrated phenols with average values of 343 and 273 mg/kg, respectively. Both phenols are hydroxytyrosol derivatives, while oleocanthal and ligstroside aglycone are tyrosol derivatives. In 2018/2019 season, the most concentrated phenols were oleuropein aglycone and ligstroside aglycone with average levels of 171 and 185 mg/kg, respectively. These two phenols are mainly formed during malaxation by hydrolysis of oleuropein and ligstroside by β-glucosidase action, while methylesterases are also involved in the production of oleacein and oleocanthal [32]. Oleacein decreased considerably its concentration in the 2018/2019 season from 343 to 85.8 mg/kg. On the other hand, oleocanthal increased its concentration from 55.9 to 109 mg/kg. The two aglycones also experienced a contrary effect since oleuropein aglycone passed from 273 to 171

mg/kg, while ligstroside aglycone varied from 152 to 185 mg/kg as average values. In contrast to the first season, in 2018/2019 EVOOs were characterized by a higher concentration of tyrosol derivatives.

This relative change in the phenolic profile was also observed in the maximum levels found for each phenol. Thus, only oleuropein and ligstroside aglycone reported a similar maximum concentration in the two consecutive seasons, above 800 mg/kg. A contrary effect was observed for oleacein and oleocanthal. Thus, oleocanthal maximum level was obtained in 2018/2019 season, 627 mg/kg, while oleacein maximum level was found in the previous season, reaching 14 mg/kg.

3.2. Cultivar influence in the phenolic profile of EVOO

One of the main factors contributing to the variability of phenols in EVOO is the cultivar [28]. In this research, we have evaluated the variability in the phenolic concentration of EVOOs corresponding to the most widespread cultivars in Spain. These were “Picual”, “Hojiblanca”, “Arbequina”, and “Cornicabra”. Hydroxytyrosol was not considered due to its low concentration in EVOOs as compared to secoiridoid derivatives. Table 2 shows the mean, maximum and minimum concentrations detected for each phenol in the four types of monocultivar EVOOs in the two seasons. Figure 1 highlights the significant differences among monocultivar EVOOs by ANOVA with Tukey’s test (p-value of < 0.05) followed by post hoc test. Picual EVOOs were characterized by a clear predominance of oleuropein and ligstroside aglycones in the two seasons. On the other hand, Arbequina EVOOs stood out for a totally different profile with a significant relative predominance of oleocanthal and oleacein. This result is in agreement with previous studies despite they were restricted only to one agronomical season [28, 33-35]. Less clear was the phenolic profile for Cornicabra and Hojiblanca EVOOs due to the high variability in concentration. Thus, Cornicabra samples were dominated by a high concentration of oleacein and oleocanthal, but a contrary effect was observed in the two seasons. Thus, the first season Cornicabra EVOOs were characterized by a high concentration of oleacein, while oleocanthal was identified as the most concentrated phenol in the

second season. This change was also observed in the other three cultivars, Picual, Arbequina, and Hojiblanca although the effect was less relevant. Hojiblanca EVOOs were more balanced in phenolic concentration. Thus, the first season samples reported a high concentration of oleacein and oleuropein aglycone, hydroxytyrosol derivatives, while the second season oils were more concentrated in oleuropein and ligstroside aglycones. Nevertheless, differences were not significant as compared to the other three cultivars. From the point of view of individual phenols, the aglycone forms were found at the highest concentration in Picual EVOOs in the two seasons, while Hojiblanca and Cornicabra were the oils reporting the highest levels of oleacein and oleocanthal in 2017/2018 and 2018/2019.

From the health claim perspective, Picual and Hojiblanca EVOOs reported the maximum health index in the 2017/2018 season with average values of 17.2 and 16.4 mg/20 g of oil, followed by "Cornicabra" EVOOs with 13.7 mg/20 g. On the other hand, Arbequina EVOOs reached a mean level of 6.2 mg/20 g of oil. In the next agronomical season, Picual and Cornicabra EVOOs were characterized by the greater healthy index with 14.2 and 13.0 mg/20 g of oil, while in this season Hojiblanca and Arbequina EVOOs provided 7.3 and 5.0 mg/20 g of oil. With these premises, Arbequina EVOOs have a lower probability to take benefits from the health claim than the other three cultivars. Similar results have been obtained in other studies by different research groups [34,35]. In fact, only 56.8 and 31.4% of the Arbequina samples reported a healthy index above 5 mg/20 g of oil in 2017/2018 and 2018/2019 seasons, respectively. On the other hand, 99.2, 97.7 and 100% of the Picual, Hojiblanca, and Cornicabra EVOOs surpassed the preset healthy index in 2017/2018 and 91.9, 59.7 and 91.7%, respectively, did it in 2018/2019. These results support the genotype predisposition of olive cultivars to produce EVOOs with a particular phenolic profile and with more possibilities to fulfill the health claim [28].

3.3. Influence of the harvesting period on the phenolic profile

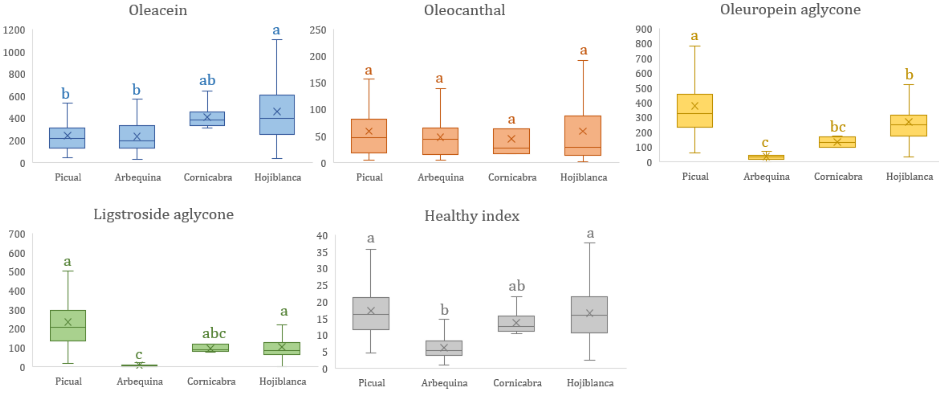
The influence of fruit ripening on the phenolic content of EVOO has been evaluated in studies dealing with limited number of samples [36,37]. In this study,

Table 2. Concentrations (mean, minimum and maximum) of phenolic compounds detected in the monocultivar EVOOs analyzed in the two seasons. The healthy index variation as a function of the cultivar is also listed.

Cultivar 17/18	Picual (n=239)				Arbequina (n=44)				Cornicabra (n=6)				Hojiblanca (n=300)			
Compound	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**
Oleacein, mg/kg	225	124	826	42.8	225	130	506	33.3	410	118	642	314	421	208	1086	38.5
Oleocanthal, mg/kg	52.9	36.3	155	4.6	44.1	31.6	138	4.4	44.3	47.5	140	16.2	51.9	47.7	201	1.9
Oleuropein aglycone, mg/kg	348	160	931	59.4	33.1	20.3	106	<LOQ	134	37.9	177	98.7	246	106	884	31.2
Ligstroside aglycone, mg/kg	236	143	835	19.6	6.8	8.7	31.6	<LOQ	96.2	18.4	119	75.2	102	72.1	718	<LOQ
Total phenols, mg/kg	861	381	2212	234	309	170	740	51.8	685	201	1076	516	821	380	2595	118
Healthy index, mg/20g VOO	17.2	7.6	44.2	4.7	6.2	3.4	14.8	1.0	13.7	4.0	21.5	10.3	16.4	7.6	51.9	2.4

Cultivar 18/19	Picual (n=260)				Arbequina (n=35)				Cornicabra (n=11)				Hojiblanca (n=159)			
Compound	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**
Oleacein, mg/kg	89.9	68.3	627	<LOQ	82.5	83.0	443	<LOQ	131	67.9	226	46.6	75.0	53.5	354	<LOQ
Oleocanthal, mg/kg	119	72.5	627	12.0	88.9	81.0	448	<LOQ	238	121	469	54.0	80.8	57.1	356	<LOQ
Oleuropein aglycone, mg/kg	233	135	943	21.9	44.1	74.1	378	<LOQ	120	68.2	234	38.9	115	89.9	692	<LOQ
Ligstroside aglycone, mg/kg	270	174	1120	2.3	32.4	63.5	274	<LOQ	160	111	350	23.0	93.9	96.9	705	<LOQ
Total phenols, mg/kg	711	384	2603	88.1	248	278	1403	2.9	649	302	1021	245	365	271	2090	1.8
Healthy index, mg/20g VOO	14.2	7.7	52.1	1.8	5.0	5.6	28.1	1.0	13.0	6.0	20.4	4.9	7.3	5.4	41.8	0.04

(A)



(B)

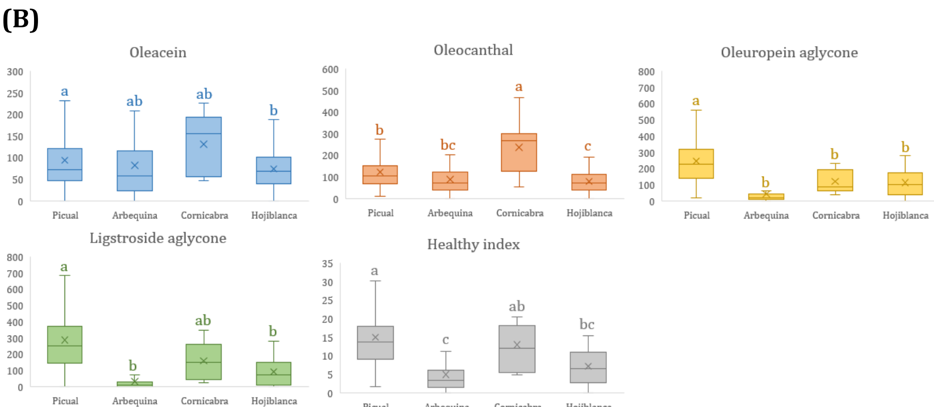


Figure 1. Variability in the concentration (expressed in mg/kg) of the main phenols detected in monocultivar EVOOs and the healthy index (mg/20 g of oil) in 17/18 **(A)** and 18/19 **(B)** seasons. Significance was evaluated by ANOVA with Tukey's test (p-value of < 0.05).

it was not possible to obtain the information of ripening index for samples because they were directly provided by producers from different geographical areas. For this reason, samples were grouped by harvesting date in five groups to balance them with a representative number of samples (between 55 and 196 samples, Supplementary Table 4). The groups were approximately 1 month periods except for the second group, which fit with the maximum production phase and, for this reason, was a 2-weeks period. Comparing the distribution of both periods, there is a difference of two weeks in the assignation of groups for both seasons due to

harvesting was postponed in general terms in the 2018/2019 season. The results presented for each secoiridoid derivative, total phenols, and healthy index for the different harvesting periods and season are presented in Table 3. Figure 2 highlights the significant differences in phenolic content detected in EVOOs according to the harvesting date by ANOVA (p-value of < 0.05). The two seasons led to same results interpretation. For all phenols and the healthy index, maximum mean levels were attained from the first to the third periods, which covered from November to January. These results are partially in agreement with previous studies dealing with the influence of ripening index, which concluded that maximum phenolic concentration is obtained in early ripening EVOOs [8,38,39], produced in October and November, and then, phenolic content is considerably decreased. It is worth mentioning that previous studies were carried out with samples produced under the same extraction conditions, which means that the only variable is the ripening index or harvesting period. However, in our research, samples were provided by producers that obtained their EVOOs under a great variability in terms of extraction conditions with multiple factors affecting the final results.

Here, the hypothesis to explain this trend is associated to the conditions of the extraction process. Typically, early harvesting EVOOs are produced at low temperature (below 25 °C) and under short extraction times to preserve the integrity of volatile compounds. On the other hand, EVOOs produced with ripened fruits or advanced harvesting are obtained by slight increasing of the temperature (below 30 °C) and lengthening the extraction time (above 60 min malaxation time) to increase the extraction efficiency. These conditions favor the enzymatic formation of secoiridoid derivatives and the enrichment of phenols in advanced harvesting EVOOs [40]. Nevertheless, an important result is that the maximum phenolic levels and also the maximum healthy index values were found in EVOOs produced at the first period, particularly, from November to the beginning of December and, therefore, corresponded to early harvesting EVOOs.

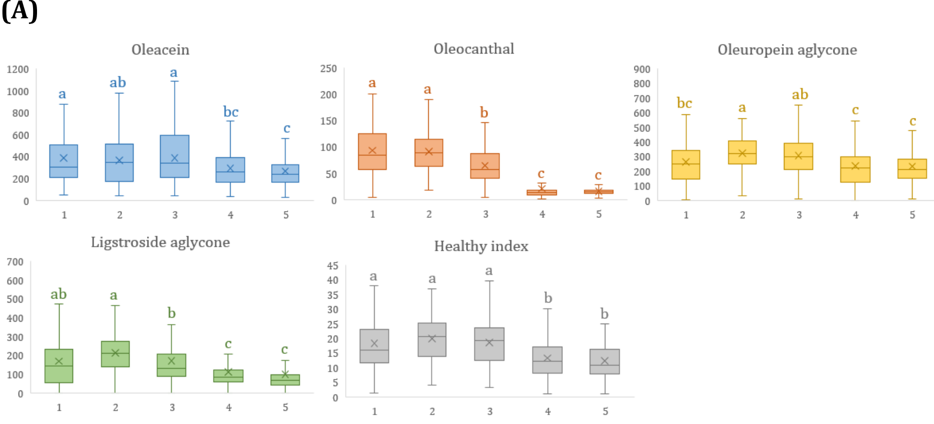
Table 3. Concentrations (mean, minimum and maximum) of phenolic compounds detected in the EVOOs according to the harvesting date in the two seasons. The healthy index variation as a function of the harvesting period is also listed.*

Date 17/18	1 (07/11-06/12)				2 (07/12-22/12)				3 (23/12-26/01)				4 (27/02-22/02)				5 (23/02-26/03)			
Compound	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**
Oleacein, mg/kg	377	238	1479	52.7	367	219	977	47.1	389	220	1086	42.8	296	174	970	37.1	268	143	716	33.3
Oleocanthal, mg/kg	92.6	48.4	270	4.4	91.1	37.5	190	18.3	65.2	32.3	201	4.6	21.5	23.7	152	1.9	16.1	6.0	37.8	3.8
Oleuropein aglycone, mg/kg	258	169	884	5.9	325	120	714	32.0	308	155	865	13.8	238	137	931	<LOQ	235	151	818	11.0
Ligstroside aglycone, mg/kg	167	147	718	<LOQ	214	111	654	1.1	170	144	835	<LOQ	113	86.2	550	<LOQ	99.8	110	607	<LOQ
Total phenols, mg/kg	895	494	2848	73.9	997	358	1849	204	933	398	2064	165	668	319	2212	51.8	618	323	1779	54.7
Healthy index, mg/20g VOO	17.9	9.9	57.0	1.5	19.9	7.2	37.0	4.1	18.7	8.0	41.3	3.3	13.4	6.4	44.2	1.0	12.4	6.5	35.6	1.1

Date 18/19	1 (19/11-19/12)				2 (20/12-04/01)				3 (05/01-05/02)				4 (06/02-09/03)				5 (10/03-10/04)			
Compound	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**	**Mean**	**SD**	**Max**	**Min**
Oleacein, mg/kg	98	106	627	<LOQ	102	50.7	290	23.9	93.2	47.4	226	<LOQ	56.8	37.2	194	<LOQ	64.6	61.2	225	<LOQ
Oleocanthal, mg/kg	115	116	627	<LOQ	121	57.1	510	18.7	114	62.0	368	27.6	91.0	68.6	469	3.8	88.3	83.6	335	<LOQ
Oleuropein aglycone, mg/kg	174	190	943	<LOQ	221	88	478	17.7	224	110	480	8.2	82.5	64.4	307	1.6	107	97.0	331	<LOQ
Ligstroside aglycone, mg/kg	205	214	1120	1.3	244	141	862	1.7	240	160	712	<LOQ	75.2	100	572	<LOQ	110	130	510	<LOQ
Total phenols, mg/kg	592	576	2603	2.9	689	255	1526	70.9	672	287	1431	72.4	306	225	1145	8.2	370	320	939	1.0
Healthy index, mg/20g VOO	11.8	11.5	52.1	0.06	13.8	5.1	30.5	1.4	13.4	5.7	28.6	1.4	6.1	4.5	22.9	0.2	7.4	6.4	18.8	0.02

*EVOO samples were distributed in five groups according to the harvesting date. Harvesting periods are detailed as day/month of the season.

(A)



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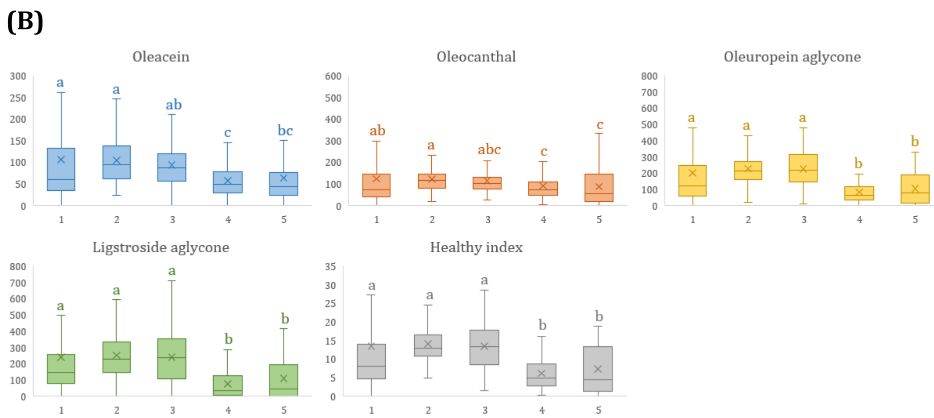

Figure 2. Variability in the concentration (expressed in mg/kg) of the main phenols detected in monocultivar EVOOs and the healthy index (mg/20 g of oil) according to the harvesting date **(Supplementary Table 4)** in 17/18 **(A)** and 18/19 **(B)** seasons. Significance was evaluated by ANOVA with Tukey's test (p-value of < 0.05).

3.4. Influence of the orchards location on the phenolic profile

The EVOOs analyzed in this research were produced in cultivation areas that can be classified according to the height a.s.l. in three representative groups: <300 m, between 300 and 600 m, and >600 m a.s.l. The influence of this parameter on the chemical composition of EVOO has been poorly investigated, and most of studies has been targeted at the fatty acids profile. Thus, it is well-known that the relative concentration of oleic acid is increased in EVOO obtained in areas at

elevated height a.s.l. [41]. Regarding the phenolic fraction, few studies have been dedicated to this association, and all of them were carried out with monocultivar EVOOs [42-44]. The hypothesis in this context is that EVOOs produced in cultivation areas located at elevated height a.s.l. would be characterized by a higher phenolic content than those obtained in cultivation areas at low height a.s.l. This hypothesis would be supported on the dependence between stress conditions of olive trees and the height a.s.l. of cultivation areas. This dependence is theoretically explained because at elevated height a.s.l., there is a predominance of (i) traditional cultivation systems, (ii) cultivations located in mountain areas, and, finally, (iii) non-irrigated cultivations. These three factors are associated to an increase of plants stress. Artajo *et al.* concluded that water status of the trees affected the phenol synthesis in the olive fruit and, therefore, in olive oil [45].

The influence of the orchards location on the phenolic profile of EVOOs was independently evaluated at the two agronomical seasons to find a consistency pattern. Figure 3 highlights the significant differences in phenolic content detected in EVOOs according to the height a.s.l. by ANOVA (p-value of < 0.05). In the 2017/2018 season, the ANOVA test only reported significant differences in the concentration of oleuropein aglycone isomers. Particularly, the mean concentration of these compounds found for the three groups were: 223 mg/kg in EVOOs produced at height a.s.l. below 300 m, 250 mg/kg in EVOOs produced between 300 and 600 m, and 330 mg/kg in EVOOs obtained above 600 m (Table 4). The post-hoc analysis allowed detecting differences in the concentration of this phenol in EVOOs obtained at heights a.s.l. below 300 m and above 600 m. No significant effects were observed for the rest of phenolic compounds and the healthy index. The trends found for aglycone isomers was reflected in the healthy index that reported values of 13.5, 16.4, and 18.0 mg/20 g of EVOO for the three groups.

The 2018/2019 season did not report the same effect observed in the previous season. Thus, the highest levels for secoiridoid derivatives were detected both at heights a.s.l. below 300 m and above 600 m. As previously mentioned, this season was characterized by a drought period, which could explain this variation. It is worth mentioning the important limitations of this study. On the one hand, the

influence of factors such as the cultivar, geographical area, ripening of fruits, agronomical practices, etc., on the phenolic content of EVOOs determines the great variability ranges found in terms of concentration. On the other hand, the geographical diversity is one other factor to be considered. EVOO samples were classified in this study in three groups as a function of the height a.s.l. However, it is possible to find cultivations subjected to high stress conditions in areas at non-elevated height a.s.l. but also the contrary situation, cultivations with reduced stress at elevated height a.s.l. This is a preliminary study developed with data from two agronomical seasons, which should be extended to obtain more representative results.

A general conclusion derived from this research is the influence of different factors on the phenolic content of EVOOs. This influence can be critical to discriminate EVOOs according to the health claim due to not all EVOOs contain phenols at concentration above 250 mg/kg. In this research, 4.6 and 23.1% of the analyzed EVOOs reported concentrations below this cutoff in the 2017/2018 and 2018/2019 agronomical seasons. A derived conclusion is associated to clinical and nutritional studies based on intervention diets with olive oil as main fat source. These studies should evaluate the phenolic content of administered olive oils due to the incidence of numerous factors on the composition of these minor components of virgin olive oil. A characterization of the phenolic fraction would enable to standardize these studies in terms of phenolic composition.

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Abbreviations Used

EVOO, extra virgin olive oil; EFSA, European Food Safety Authority; FAs, fatty acids; IOC, International Olive Council; a.s.l., above sea level; MeOH, methanol; 3,4-DHPEA-

EDA, oleacein; p-HPEA-EDA, oleocanthal; ESI, electrospray ionization source; MRM, multiple reaction monitoring

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Evaluating the Variability in the Phenolic Concentration of Extra Virgin Olive Oil According to the Commission Regulation (EU) 432/2012 Health Claim

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BACK CLOSE WINDOW

Supplementary Table 1. Production month, cultivar, region and height above sea level information of the samples analyzed in 2017/2018 season.

Sample code	Production month	Cultivar	Region	Height a.s.l.
1718_1	October	N.D.	Andalusia	300-600 m
1718_2	November	N.D.	Andalusia	300-600 m
1718_3	November	Arbequina	Andalusia	300-600 m
1718_4	November	Arbequina	Andalusia	300-600 m
1718_5	November	Hojiblanca	Andalusia	300-600 m
1718_6	November	Hojiblanca	Andalusia	300-600 m
1718_7	November	Picual	Andalusia	300-600 m
1718_8	November	Arbequina	Andalusia	<300m
1718_9	November	Picual	Andalusia	>600 m
1718_10	November	Arbequina	Andalusia	<300m
1718_11	November	Hojiblanca	Andalusia	300-600 m
1718_12	November	Hojiblanca	Andalusia	300-600 m
1718_13	November	Hojiblanca	Andalusia	300-600 m
1718_14	November	N.D.	Andalusia	300-600 m
1718_15	November	Arbequina	Andalusia	300-600 m
1718_16	November	N.D.	Andalusia	>600 m
1718_17	November	N.D.	Andalusia	300-600 m
1718_18	November	Arbequina	Andalusia	>600 m
1718_19	November	Hojiblanca	Andalusia	300-600 m
1718_20	November	Hojiblanca	Andalusia	300-600 m
1718_21	November	N.D.	Andalusia	300-600 m
1718_22	November	Hojiblanca	Andalusia	300-600 m
1718_23	November	Arbequina	Andalusia	300-600 m
1718_24	November	Hojiblanca	Andalusia	300-600 m
1718_25	November	Picual	Andalusia	300-600 m
1718_26	November	Arbequina	Andalusia	300-600 m
1718_27	November	Arbequina	Andalusia	<300m
1718_28	November	Picual	Andalusia	>600 m
1718_29	November	Picual	Andalusia	>600 m
1718_30	November	Hojiblanca	Andalusia	300-600 m
1718_31	November	N.D.	Andalusia	>600 m
1718_32	November	N.D.	Andalusia	300-600 m
1718_33	November	Picual	Andalusia	>600 m
1718_34	November	Hojiblanca	Andalusia	300-600 m
1718_35	November	Hojiblanca	Andalusia	300-600 m
1718_36	November	Hojiblanca	Andalusia	300-600 m
1718_37	November	Hojiblanca	Andalusia	300-600 m
1718_38	November	Hojiblanca	Andalusia	300-600 m
1718_39	November	Picual	Andalusia	300-600 m
1718_40	November	Arbequina	Andalusia	300-600 m
1718_41	November	N.D.	Andalusia	>600 m
1718_42	November	N.D.	Andalusia	>600 m
1718_43	November	N.D.	Andalusia	300-600 m
1718_44	November	N.D.	Andalusia	300-600 m
1718_45	November	Picual	Andalusia	300-600 m
1718_46	November	Picual	Andalusia	>600 m

1718_47	November	Arbequina	Andalusia	>600 m
1718_48	November	Arbequina	Andalusia	>600 m
1718_49	November	Arbequina	Andalusia	>600 m
1718_50	November	Hojiblanca	Andalusia	300-600 m
1718_51	November	N.D.	Andalusia	300-600 m
1718_52	November	N.D.	Andalusia	300-600 m
1718_53	November	Hojiblanca	Andalusia	300-600 m
1718_54	November	Hojiblanca	Andalusia	300-600 m
1718_55	November	Hojiblanca	Andalusia	300-600 m
1718_56	November	Picual	Andalusia	300-600 m
1718_57	November	Picual	Andalusia	300-600 m
1718_58	November	Picual	Andalusia	<300m
1718_59	November	Picual	Andalusia	<300m
1718_60	November	N.D.	Andalusia	300-600 m
1718_61	December	Arbequina	Andalusia	300-600 m
1718_62	December	Arbequina	Andalusia	300-600 m
1718_63	December	Hojiblanca	Andalusia	300-600 m
1718_64	December	Hojiblanca	Andalusia	>600 m
1718_65	December	N.D.	Andalusia	300-600 m
1718_66	December	Picual	Andalusia	300-600 m
1718_67	December	Picual	Andalusia	<300m
1718_68	December	Picual	Andalusia	<300m
1718_69	December	Picual	Andalusia	<300m
1718_70	December	N.D.	Andalusia	<300m
1718_71	December	Picual	Andalusia	<300m
1718_72	December	Picual	Andalusia	<300m
1718_73	December	Picual	Andalusia	<300m
1718_74	December	Hojiblanca	Andalusia	300-600 m
1718_75	December	Picual	Andalusia	300-600 m
1718_76	December	Picual	Andalusia	300-600 m
1718_77	December	Hojiblanca	Andalusia	300-600 m
1718_78	December	Hojiblanca	Andalusia	300-600 m
1718_79	December	Hojiblanca	Andalusia	300-600 m
1718_80	December	N.D.	Andalusia	300-600 m
1718_81	December	N.D.	Andalusia	300-600 m
1718_82	December	N.D.	Andalusia	300-600 m
1718_83	December	N.D.	Andalusia	300-600 m
1718_84	December	Picual	Andalusia	<300m
1718_85	December	Picual	Andalusia	>600 m
1718_86	December	Picual	Andalusia	>600 m
1718_87	December	N.D.	Andalusia	300-600 m
1718_88	December	N.D.	Andalusia	300-600 m
1718_89	December	Picual	Andalusia	300-600 m
1718_90	December	Picual	Andalusia	300-600 m
1718_91	December	Picual	Andalusia	>600 m
1718_92	December	Picual	Andalusia	>600 m
1718_93	December	Picual	Andalusia	>600 m
1718_94	December	N.D.	Andalusia	<300m
1718_95	December	N.D.	Andalusia	300-600 m

1718_96	December	N.D.	Andalusia	300-600 m
1718_97	December	Hojiblanca	Andalusia	300-600 m
1718_98	December	Hojiblanca	Andalusia	>600 m
1718_99	December	Hojiblanca	Andalusia	>600 m
1718_100	December	N.D.	Andalusia	300-600 m
1718_101	December	N.D.	Andalusia	300-600 m
1718_102	December	N.D.	Andalusia	300-600 m
1718_103	December	N.D.	Andalusia	300-600 m
1718_104	December	N.D.	Andalusia	300-600 m
1718_105	December	Arbequina	Andalusia	300-600 m
1718_106	December	Hojiblanca	Andalusia	>600 m
1718_107	December	Hojiblanca	Andalusia	300-600 m
1718_108	December	Hojiblanca	Andalusia	300-600 m
1718_109	December	Hojiblanca	Andalusia	300-600 m
1718_110	December	N.D.	Andalusia	300-600 m
1718_111	December	N.D.	Andalusia	300-600 m
1718_112	December	Picual	Andalusia	>600 m
1718_113	December	Picual	Andalusia	>600 m
1718_114	December	Hojiblanca	Andalusia	>600 m
1718_115	December	Hojiblanca	Andalusia	>600 m
1718_116	December	N.D.	Andalusia	<300m
1718_117	December	Picual	Andalusia	300-600 m
1718_118	December	Hojiblanca	Andalusia	300-600 m
1718_119	December	Hojiblanca	Andalusia	300-600 m
1718_120	December	Hojiblanca	Andalusia	>600 m
1718_121	December	Picual	Andalusia	300-600 m
1718_122	December	Hojiblanca	Andalusia	300-600 m
1718_123	December	Hojiblanca	Andalusia	300-600 m
1718_124	December	N.D.	Andalusia	300-600 m
1718_125	December	Picual	Andalusia	<300m
1718_126	December	Picual	Andalusia	<300m
1718_127	December	Picual	Andalusia	<300m
1718_128	December	Picual	Andalusia	<300m
1718_129	December	Picual	Andalusia	300-600 m
1718_130	December	Picual	Andalusia	300-600 m
1718_131	December	Picual	Andalusia	300-600 m
1718_132	December	N.D.	Andalusia	<300m
1718_133	December	N.D.	Andalusia	<300m
1718_134	December	N.D.	Andalusia	<300m
1718_135	December	N.D.	Andalusia	<300m
1718_136	December	N.D.	Andalusia	<300m
1718_137	December	Arbequina	Andalusia	<300m
1718_138	December	Picual	Andalusia	>600 m
1718_139	December	Picual	Andalusia	>600 m
1718_140	December	Picual	Andalusia	>600 m
1718_141	December	Picual	Andalusia	>600 m
1718_142	December	Picual	Andalusia	>600 m
1718_143	December	Picual	Andalusia	300-600 m
1718_144	December	Hojiblanca	Andalusia	300-600 m

1718_145	December	N.D.	Andalusia	300-600 m
1718_146	December	Hojiblanca	Andalusia	300-600 m
1718_147	December	Hojiblanca	Andalusia	300-600 m
1718_148	December	Picual	Andalusia	300-600 m
1718_149	December	Picual	Andalusia	300-600 m
1718_150	December	Picual	Andalusia	300-600 m
1718_151	December	N.D.	Andalusia	>600 m
1718_152	December	Hojiblanca	Andalusia	>600 m
1718_153	December	Picual	Andalusia	300-600 m
1718_154	December	N.D.	Andalusia	>600 m
1718_155	December	Picual	Andalusia	<300m
1718_156	December	Picual	Andalusia	300-600 m
1718_157	December	Picual	Andalusia	300-600 m
1718_158	December	Picual	Andalusia	300-600 m
1718_159	December	Picual	Andalusia	300-600 m
1718_160	December	Picual	Andalusia	300-600 m
1718_161	December	Picual	Andalusia	300-600 m
1718_162	December	N.D.	Andalusia	300-600 m
1718_163	December	Hojiblanca	Andalusia	300-600 m
1718_164	December	N.D.	Andalusia	300-600 m
1718_165	December	Picual	Andalusia	300-600 m
1718_166	December	Picual	Andalusia	300-600 m
1718_167	December	Picual	Andalusia	300-600 m
1718_168	December	Picual	Andalusia	300-600 m
1718_169	December	Picual	Andalusia	300-600 m
1718_170	December	Picual	Andalusia	300-600 m
1718_171	December	Picual	Andalusia	300-600 m
1718_172	December	Picual	Andalusia	300-600 m
1718_173	December	N.D.	Andalusia	<300m
1718_174	December	N.D.	Andalusia	<300m
1718_175	December	N.D.	Andalusia	<300m
1718_176	December	N.D.	Andalusia	<300m
1718_177	December	Arbequina	Andalusia	<300m
1718_178	December	Picual	Andalusia	>600 m
1718_179	December	Picual	Andalusia	>600 m
1718_180	December	Hojiblanca	Andalusia	300-600 m
1718_181	December	Hojiblanca	Andalusia	300-600 m
1718_182	December	Hojiblanca	Andalusia	300-600 m
1718_183	December	Hojiblanca	Andalusia	300-600 m
1718_184	December	N.D.	Andalusia	300-600 m
1718_185	December	Hojiblanca	Andalusia	300-600 m
1718_186	December	Hojiblanca	Andalusia	>600 m
1718_187	December	Hojiblanca	Andalusia	>600 m
1718_188	December	N.D.	Andalusia	300-600 m
1718_189	December	N.D.	Andalusia	300-600 m
1718_190	December	N.D.	Andalusia	300-600 m
1718_191	December	N.D.	Andalusia	>600 m
1718_192	December	N.D.	Andalusia	>600 m
1718_193	December	Hojiblanca	Andalusia	300-600 m

1718_194	December	Hojiblanca	Andalusia	300-600 m
1718_195	December	N.D.	Andalusia	>600 m
1718_196	December	Hojiblanca	Andalusia	>600 m
1718_197	December	N.D.	Andalusia	300-600 m
1718_198	December	N.D.	Andalusia	300-600 m
1718_199	December	Hojiblanca	Andalusia	>600 m
1718_200	December	Hojiblanca	Andalusia	300-600 m
1718_201	December	Hojiblanca	Andalusia	300-600 m
1718_202	December	Hojiblanca	Andalusia	300-600 m
1718_203	December	Hojiblanca	Andalusia	300-600 m
1718_204	December	Arbequina	Andalusia	300-600 m
1718_205	December	Picual	Andalusia	>600 m
1718_206	December	N.D.	Andalusia	300-600 m
1718_207	December	N.D.	Andalusia	300-600 m
1718_208	December	N.D.	Andalusia	300-600 m
1718_209	December	N.D.	Andalusia	300-600 m
1718_210	December	Hojiblanca	Andalusia	>600 m
1718_211	December	Hojiblanca	Andalusia	300-600 m
1718_212	December	N.D.	Andalusia	300-600 m
1718_213	December	Hojiblanca	Andalusia	300-600 m
1718_214	December	Hojiblanca	Andalusia	>600 m
1718_215	December	N.D.	Andalusia	300-600 m
1718_216	December	Picual	Andalusia	<300m
1718_217	December	Picual	Andalusia	300-600 m
1718_218	December	Picual	Andalusia	300-600 m
1718_219	December	Picual	Andalusia	300-600 m
1718_220	December	Picual	Andalusia	300-600 m
1718_221	December	Picual	Andalusia	>600 m
1718_222	December	Hojiblanca	Andalusia	300-600 m
1718_223	December	Hojiblanca	Andalusia	300-600 m
1718_224	December	Picual	Andalusia	>600 m
1718_225	December	Picual	Andalusia	>600 m
1718_226	December	Hojiblanca	Andalusia	300-600 m
1718_227	December	N.D.	Andalusia	300-600 m
1718_228	December	Hojiblanca	Andalusia	>600 m
1718_229	December	N.D.	Andalusia	300-600 m
1718_230	December	Picual	Andalusia	>600 m
1718_231	December	Picual	Andalusia	300-600 m
1718_232	December	Hojiblanca	Andalusia	>600 m
1718_233	December	Hojiblanca	Andalusia	>600 m
1718_234	December	Hojiblanca	Andalusia	>600 m
1718_235	December	Picual	Andalusia	>600 m
1718_236	December	Hojiblanca	Andalusia	>600 m
1718_237	December	N.D.	Andalusia	300-600 m
1718_238	December	N.D.	Andalusia	300-600 m
1718_239	December	Picual	Andalusia	>600 m
1718_240	December	Picual	Andalusia	>600 m
1718_241	December	Picual	Andalusia	>600 m
1718_242	December	Picual	Andalusia	>600 m

1718_243	December	Picual	Andalusia	>600 m
1718_244	December	Picual	Andalusia	>600 m
1718_245	December	Picual	Andalusia	>600 m
1718_246	December	Hojiblanca	Andalusia	300-600 m
1718_247	December	Hojiblanca	Andalusia	300-600 m
1718_248	December	Picual	Andalusia	>600 m
1718_249	December	Picual	Andalusia	>600 m
1718_250	December	Hojiblanca	Andalusia	>600 m
1718_251	December	Picual	Andalusia	<300m
1718_252	January	Picual	Andalusia	300-600 m
1718_253	January	Picual	Andalusia	300-600 m
1718_254	January	Picual	Andalusia	300-600 m
1718_255	January	Picual	Andalusia	300-600 m
1718_256	January	Picual	Andalusia	>600 m
1718_257	January	Picual	Andalusia	300-600 m
1718_258	January	Picual	Andalusia	>600 m
1718_259	January	Picual	Andalusia	>600 m
1718_260	January	Picual	Andalusia	>600 m
1718_261	January	Picual	Andalusia	>600 m
1718_262	January	Picual	Andalusia	<300m
1718_263	January	Picual	Andalusia	>600 m
1718_264	January	Picual	Andalusia	<300m
1718_265	January	Picual	Andalusia	<300m
1718_266	January	Picual	Andalusia	<300m
1718_267	January	Hojiblanca	Castilla-La Mancha	300-600 m
1718_268	January	Picual	Andalusia	>600 m
1718_269	January	Picual	Andalusia	300-600 m
1718_270	January	Hojiblanca	Andalusia	300-600 m
1718_271	January	Cornicabra	Andalusia	>600 m
1718_272	January	Picual	Andalusia	>600 m
1718_273	January	Hojiblanca	Andalusia	300-600 m
1718_274	January	Picual	Andalusia	>600 m
1718_275	January	Picual	Andalusia	>600 m
1718_276	January	Picual	Andalusia	>600 m
1718_277	January	Arbequina	Andalusia	>600 m
1718_278	January	Hojiblanca	Andalusia	300-600 m
1718_279	January	Hojiblanca	Andalusia	>600 m
1718_280	January	N.D.	Andalusia	300-600 m
1718_281	January	Arbequina	Andalusia	300-600 m
1718_282	January	Picual	Andalusia	<300m
1718_283	January	Hojiblanca	Andalusia	>600 m
1718_284	January	Hojiblanca	Andalusia	>600 m
1718_285	January	N.D.	Andalusia	>600 m
1718_286	January	Picual	Andalusia	<300m
1718_287	January	Picual	Andalusia	>600 m
1718_288	January	Picual	Andalusia	>600 m
1718_289	January	Picual	Andalusia	300-600 m
1718_290	January	Hojiblanca	Andalusia	>600 m
1718_291	January	Hojiblanca	Andalusia	300-600 m

1718_292	January	Picual	Andalusia	>600 m
1718_293	January	Picual	Andalusia	300-600 m
1718_294	January	Hojiblanca	Andalusia	300-600 m
1718_295	January	Hojiblanca	Andalusia	300-600 m
1718_296	January	Hojiblanca	Andalusia	300-600 m
1718_297	January	Hojiblanca	Andalusia	300-600 m
1718_298	January	Picual	Andalusia	300-600 m
1718_299	January	Picual	Andalusia	>600 m
1718_300	January	Picual	Andalusia	>600 m
1718_301	January	Picual	Andalusia	>600 m
1718_302	January	N.D.	Andalusia	300-600 m
1718_303	January	Picual	Andalusia	>600 m
1718_304	January	Picual	Andalusia	<300m
1718_305	January	Arbequina	Andalusia	<300m
1718_306	January	Arbequina	Andalusia	<300m
1718_307	January	Hojiblanca	Andalusia	>600 m
1718_308	January	Hojiblanca	Andalusia	300-600 m
1718_309	January	Picual	Andalusia	>600 m
1718_310	January	Picual	Andalusia	<300m
1718_311	January	Picual	Andalusia	>600 m
1718_312	January	Hojiblanca	Andalusia	300-600 m
1718_313	January	Picual	Andalusia	>600 m
1718_314	January	Picual	Andalusia	>600 m
1718_315	January	Picual	Andalusia	>600 m
1718_316	January	Hojiblanca	Andalusia	300-600 m
1718_317	January	Hojiblanca	Andalusia	>600 m
1718_318	January	N.D.	Andalusia	300-600 m
1718_319	January	Picual	Andalusia	<300m
1718_320	January	Picual	Andalusia	<300m
1718_321	January	Hojiblanca	Andalusia	300-600 m
1718_322	January	Hojiblanca	Andalusia	300-600 m
1718_323	January	Hojiblanca	Andalusia	300-600 m
1718_324	January	Arbequina	Andalusia	<300m
1718_325	January	Arbequina	Andalusia	<300m
1718_326	January	Picual	Andalusia	<300m
1718_327	January	Arbequina	Andalusia	<300m
1718_328	January	Picual	Andalusia	300-600 m
1718_329	January	Hojiblanca	Andalusia	300-600 m
1718_330	January	Hojiblanca	Andalusia	300-600 m
1718_331	January	Hojiblanca	Andalusia	>600 m
1718_332	January	Hojiblanca	Andalusia	>600 m
1718_333	January	Hojiblanca	Andalusia	300-600 m
1718_334	January	Hojiblanca	Andalusia	300-600 m
1718_335	January	N.D.	Andalusia	<300m
1718_336	January	N.D.	Andalusia	<300m
1718_337	January	Arbequina	Andalusia	<300m
1718_338	January	Arbequina	Andalusia	<300m
1718_339	January	Arbequina	Andalusia	<300m
1718_340	January	Picual	Andalusia	<300m

1718_341	January	Hojiblanca	Andalusia	300-600 m
1718_342	January	Hojiblanca	Andalusia	300-600 m
1718_343	January	Hojiblanca	Andalusia	300-600 m
1718_344	January	Hojiblanca	Andalusia	300-600 m
1718_345	January	N.D.	Andalusia	300-600 m
1718_346	January	Picual	Andalusia	>600 m
1718_347	January	Hojiblanca	Andalusia	>600 m
1718_348	January	Hojiblanca	Andalusia	300-600 m
1718_349	January	Hojiblanca	Andalusia	300-600 m
1718_350	January	Hojiblanca	Andalusia	300-600 m
1718_351	January	Hojiblanca	Andalusia	300-600 m
1718_352	January	Picual	Andalusia	>600 m
1718_353	January	Picual	Andalusia	>600 m
1718_354	January	Picual	Andalusia	>600 m
1718_355	January	Picual	Andalusia	<300m
1718_356	January	Hojiblanca	Andalusia	300-600 m
1718_357	January	Hojiblanca	Andalusia	300-600 m
1718_358	January	Hojiblanca	Andalusia	300-600 m
1718_359	January	Hojiblanca	Andalusia	300-600 m
1718_360	January	Hojiblanca	Andalusia	300-600 m
1718_361	January	N.D.	Andalusia	300-600 m
1718_362	January	N.D.	Andalusia	300-600 m
1718_363	January	Hojiblanca	Andalusia	>600 m
1718_364	January	Hojiblanca	Andalusia	300-600 m
1718_365	January	Picual	Andalusia	300-600 m
1718_366	January	Picual	Andalusia	300-600 m
1718_367	January	Picual	Andalusia	300-600 m
1718_368	January	Picual	Andalusia	300-600 m
1718_369	January	Picual	Andalusia	>600 m
1718_370	January	Hojiblanca	Andalusia	300-600 m
1718_371	January	Hojiblanca	Andalusia	>600 m
1718_372	January	Picual	Andalusia	<300m
1718_373	January	Picual	Andalusia	<300m
1718_374	January	Picual	Andalusia	<300m
1718_375	January	Picual	Andalusia	<300m
1718_376	January	Hojiblanca	Andalusia	300-600 m
1718_377	January	Hojiblanca	Andalusia	300-600 m
1718_378	January	Hojiblanca	Andalusia	300-600 m
1718_379	January	Hojiblanca	Andalusia	300-600 m
1718_380	January	Hojiblanca	Andalusia	300-600 m
1718_381	January	Hojiblanca	Andalusia	300-600 m
1718_382	January	N.D.	Andalusia	300-600 m
1718_383	January	Hojiblanca	Andalusia	>600 m
1718_384	January	Hojiblanca	Andalusia	300-600 m
1718_385	January	Hojiblanca	Andalusia	300-600 m
1718_386	January	Picual	Andalusia	>600 m
1718_387	January	N.D.	Andalusia	<300m
1718_388	January	N.D.	Andalusia	<300m
1718_389	January	N.D.	Andalusia	<300m

1718_390	January	N.D.	Andalusia	<300m
1718_391	January	Picual	Andalusia	<300m
1718_392	January	Picual	Andalusia	<300m
1718_393	January	Picual	Andalusia	<300m
1718_394	January	Arbequina	Andalusia	<300m
1718_395	January	Arbequina	Andalusia	<300m
1718_396	January	Picual	Andalusia	300-600 m
1718_397	January	Hojiblanca	Andalusia	300-600 m
1718_398	January	Hojiblanca	Andalusia	>600 m
1718_399	January	N.D.	Andalusia	300-600 m
1718_400	January	Hojiblanca	Andalusia	300-600 m
1718_401	January	Hojiblanca	Andalusia	300-600 m
1718_402	January	Picual	Andalusia	300-600 m
1718_403	January	Hojiblanca	Andalusia	300-600 m
1718_404	January	Picual	Andalusia	>600 m
1718_405	January	Picual	Andalusia	>600 m
1718_406	January	Picual	Andalusia	>600 m
1718_407	January	Picual	Andalusia	>600 m
1718_408	January	Picual	Andalusia	>600 m
1718_409	January	Picual	Andalusia	<300m
1718_410	January	Picual	Andalusia	<300m
1718_411	January	Arbequina	Andalusia	300-600 m
1718_412	January	Hojiblanca	Andalusia	>600 m
1718_413	January	Hojiblanca	Andalusia	>600 m
1718_414	January	Hojiblanca	Andalusia	300-600 m
1718_415	January	Hojiblanca	Andalusia	>600 m
1718_416	January	Picual	Andalusia	>600 m
1718_417	January	Picual	Andalusia	>600 m
1718_418	January	Picual	Andalusia	>600 m
1718_419	January	Picual	Andalusia	>600 m
1718_420	January	Hojiblanca	Andalusia	300-600 m
1718_421	January	Hojiblanca	Andalusia	300-600 m
1718_422	January	Picual	Andalusia	300-600 m
1718_423	January	Picual	Andalusia	>600 m
1718_424	January	Hojiblanca	Andalusia	300-600 m
1718_425	January	Hojiblanca	Andalusia	300-600 m
1718_426	January	Hojiblanca	Andalusia	300-600 m
1718_427	January	Hojiblanca	Andalusia	>600 m
1718_428	January	N.D.	Andalusia	300-600 m
1718_429	January	N.D.	Andalusia	300-600 m
1718_430	January	Picual	Andalusia	>600 m
1718_431	January	Hojiblanca	Andalusia	300-600 m
1718_432	January	Hojiblanca	Andalusia	300-600 m
1718_433	January	Hojiblanca	Andalusia	>600 m
1718_434	January	Hojiblanca	Andalusia	>600 m
1718_435	January	Hojiblanca	Andalusia	>600 m
1718_436	January	Picual	Andalusia	>600 m
1718_437	January	Picual	Andalusia	<300m
1718_438	January	Hojiblanca	Andalusia	>600 m

1718_439	January	N.D.	Andalusia	300-600 m
1718_440	January	Hojiblanca	Andalusia	>600 m
1718_441	January	Picual	Andalusia	>600 m
1718_442	January	Picual	Andalusia	<300m
1718_443	February	Hojiblanca	Andalusia	300-600 m
1718_444	February	Picual	Andalusia	300-600 m
1718_445	February	N.D.	Andalusia	300-600 m
1718_446	February	N.D.	Andalusia	300-600 m
1718_447	February	N.D.	Andalusia	300-600 m
1718_448	February	Hojiblanca	Andalusia	>600 m
1718_449	February	Picual	Andalusia	300-600 m
1718_450	February	Hojiblanca	Andalusia	>600 m
1718_451	February	Hojiblanca	Andalusia	>600 m
1718_452	February	Hojiblanca	Andalusia	>600 m
1718_453	February	Picual	Andalusia	300-600 m
1718_454	February	N.D.	Andalusia	<300m
1718_455	February	Hojiblanca	Andalusia	300-600 m
1718_456	February	Picual	Andalusia	>600 m
1718_457	February	Hojiblanca	Andalusia	300-600 m
1718_458	February	Hojiblanca	Andalusia	300-600 m
1718_459	February	N.D.	Andalusia	300-600 m
1718_460	February	Picual	Andalusia	300-600 m
1718_461	February	Hojiblanca	Andalusia	300-600 m
1718_462	February	Hojiblanca	Andalusia	300-600 m
1718_463	February	Hojiblanca	Andalusia	300-600 m
1718_464	February	Hojiblanca	Andalusia	300-600 m
1718_465	February	Hojiblanca	Andalusia	300-600 m
1718_466	February	Hojiblanca	Andalusia	300-600 m
1718_467	February	Hojiblanca	Andalusia	300-600 m
1718_468	February	Hojiblanca	Andalusia	300-600 m
1718_469	February	Picual	Andalusia	<300m
1718_470	February	Picual	Andalusia	<300m
1718_471	February	Picual	Andalusia	<300m
1718_472	February	Arbequina	Andalusia	<300m
1718_473	February	Picual	Andalusia	>600 m
1718_474	February	Picual	Andalusia	>600 m
1718_475	February	Picual	Andalusia	>600 m
1718_476	February	N.D.	Andalusia	300-600 m
1718_477	February	N.D.	Andalusia	300-600 m
1718_478	February	Hojiblanca	Andalusia	>600 m
1718_479	February	Hojiblanca	Andalusia	>600 m
1718_480	February	Hojiblanca	Andalusia	>600 m
1718_481	February	Hojiblanca	Andalusia	300-600 m
1718_482	February	Hojiblanca	Andalusia	>600 m
1718_483	February	Hojiblanca	Andalusia	>600 m
1718_484	February	Picual	Andalusia	>600 m
1718_485	February	Picual	Andalusia	>600 m
1718_486	February	Hojiblanca	Andalusia	300-600 m
1718_487	February	Hojiblanca	Andalusia	300-600 m

1718_488	February	Hojiblanca	Andalusia	300-600 m
1718_489	February	Hojiblanca	Andalusia	>600 m
1718_490	February	Arbequina	Andalusia	>600 m
1718_491	February	Hojiblanca	Andalusia	300-600 m
1718_492	February	Hojiblanca	Andalusia	>600 m
1718_493	February	Hojiblanca	Andalusia	300-600 m
1718_494	February	N.D.	Andalusia	300-600 m
1718_495	February	N.D.	Andalusia	300-600 m
1718_496	February	Hojiblanca	Andalusia	300-600 m
1718_497	February	Hojiblanca	Andalusia	300-600 m
1718_498	February	Hojiblanca	Andalusia	>600 m
1718_499	February	Hojiblanca	Andalusia	300-600 m
1718_500	February	Hojiblanca	Andalusia	300-600 m
1718_501	February	Hojiblanca	Andalusia	300-600 m
1718_502	February	Hojiblanca	Andalusia	300-600 m
1718_503	February	Hojiblanca	Andalusia	>600 m
1718_504	February	Hojiblanca	Castilla-La Mancha	>600 m
1718_505	February	Hojiblanca	Castilla-La Mancha	>600 m
1718_506	February	Cornicabra	Andalusia	>600 m
1718_507	February	Cornicabra	Andalusia	>600 m
1718_508	February	Cornicabra	Castilla-La Mancha	>600 m
1718_509	February	Hojiblanca	Andalusia	300-600 m
1718_510	February	Picual	Andalusia	300-600 m
1718_511	February	Picual	Andalusia	300-600 m
1718_512	February	Hojiblanca	Andalusia	300-600 m
1718_513	February	Hojiblanca	Andalusia	300-600 m
1718_514	February	Hojiblanca	Andalusia	300-600 m
1718_515	February	Hojiblanca	Andalusia	300-600 m
1718_516	February	Hojiblanca	Andalusia	300-600 m
1718_517	February	Hojiblanca	Andalusia	>600 m
1718_518	February	Hojiblanca	Andalusia	>600 m
1718_519	February	N.D.	Andalusia	300-600 m
1718_520	February	Hojiblanca	Andalusia	>600 m
1718_521	February	Hojiblanca	Andalusia	300-600 m
1718_522	February	Hojiblanca	Andalusia	300-600 m
1718_523	February	Hojiblanca	Andalusia	300-600 m
1718_524	February	Hojiblanca	Andalusia	>600 m
1718_525	February	Hojiblanca	Andalusia	>600 m
1718_526	February	Hojiblanca	Andalusia	>600 m
1718_527	February	Hojiblanca	Andalusia	>600 m
1718_528	February	Hojiblanca	Andalusia	>600 m
1718_529	February	Picual	Andalusia	>600 m
1718_530	February	Picual	Andalusia	>600 m
1718_531	February	Picual	Andalusia	>600 m
1718_532	February	Picual	Andalusia	>600 m
1718_533	February	Picual	Andalusia	>600 m
1718_534	February	Picual	Andalusia	>600 m
1718_535	February	Hojiblanca	Andalusia	>600 m
1718_536	February	Picual	Andalusia	<300m

1718_537	February	Picual	Andalusia	<300m
1718_538	February	Picual	Andalusia	<300m
1718_539	February	Picual	Andalusia	<300m
1718_540	February	Picual	Andalusia	<300m
1718_541	February	Picual	Andalusia	<300m
1718_542	February	Picual	Andalusia	<300m
1718_543	February	Picual	Andalusia	<300m
1718_544	February	Picual	Andalusia	>600 m
1718_545	February	Picual	Andalusia	<300m
1718_546	February	Picual	Andalusia	<300m
1718_547	February	Picual	Andalusia	<300m
1718_548	February	Hojiblanca	Andalusia	>600 m
1718_549	February	Hojiblanca	Andalusia	>600 m
1718_550	February	N.D.	Andalusia	300-600 m
1718_551	February	Hojiblanca	Andalusia	>600 m
1718_552	February	Hojiblanca	Andalusia	300-600 m
1718_553	February	Hojiblanca	Andalusia	300-600 m
1718_554	February	Hojiblanca	Andalusia	300-600 m
1718_555	February	Hojiblanca	Andalusia	300-600 m
1718_556	February	Hojiblanca	Castilla-La Mancha	300-600 m
1718_557	February	Hojiblanca	Castilla-La Mancha	300-600 m
1718_558	February	Hojiblanca	Andalusia	>600 m
1718_559	February	Hojiblanca	Andalusia	300-600 m
1718_560	February	Hojiblanca	Andalusia	300-600 m
1718_561	February	Hojiblanca	Andalusia	300-600 m
1718_562	February	Hojiblanca	Andalusia	300-600 m
1718_563	February	Hojiblanca	Andalusia	300-600 m
1718_564	February	Arbequina	Andalusia	<300m
1718_565	February	N.D.	Andalusia	<300m
1718_566	February	Arbequina	Andalusia	<300m
1718_567	February	Arbequina	Andalusia	<300m
1718_568	February	Hojiblanca	Andalusia	300-600 m
1718_569	February	Hojiblanca	Andalusia	300-600 m
1718_570	February	Hojiblanca	Andalusia	>600 m
1718_571	February	Hojiblanca	Andalusia	>600 m
1718_572	February	Hojiblanca	Andalusia	>600 m
1718_573	February	Picual	Andalusia	300-600 m
1718_574	February	Picual	Andalusia	300-600 m
1718_575	February	Hojiblanca	Andalusia	300-600 m
1718_576	February	Picual	Andalusia	300-600 m
1718_577	February	Picual	Andalusia	>600 m
1718_578	February	Hojiblanca	Andalusia	300-600 m
1718_579	February	Hojiblanca	Andalusia	300-600 m
1718_580	February	Hojiblanca	Andalusia	300-600 m
1718_581	February	Hojiblanca	Andalusia	300-600 m
1718_582	February	Hojiblanca	Andalusia	>600 m
1718_583	February	Hojiblanca	Andalusia	>600 m
1718_584	February	Hojiblanca	Andalusia	>600 m
1718_585	February	Picual	Andalusia	300-600 m

1718_586	February	Hojiblanca	Andalusia	>600 m
1718_587	February	Hojiblanca	Andalusia	>600 m
1718_588	February	Picual	Andalusia	>600 m
1718_589	February	Picual	Andalusia	>600 m
1718_590	February	Hojiblanca	Andalusia	300-600 m
1718_591	February	Picual	Andalusia	>600 m
1718_592	February	Picual	Andalusia	>600 m
1718_593	February	Hojiblanca	Andalusia	300-600 m
1718_594	February	Hojiblanca	Andalusia	>600 m
1718_595	February	Hojiblanca	Andalusia	>600 m
1718_596	February	Hojiblanca	Andalusia	>600 m
1718_597	February	Picual	Andalusia	<300m
1718_598	February	Hojiblanca	Andalusia	300-600 m
1718_599	February	Hojiblanca	Andalusia	>600 m
1718_600	February	Hojiblanca	Andalusia	300-600 m
1718_601	February	Picual	Andalusia	>600 m
1718_602	February	Hojiblanca	Andalusia	300-600 m
1718_603	February	Picual	Andalusia	300-600 m
1718_604	February	Hojiblanca	Andalusia	300-600 m
1718_605	February	Picual	Andalusia	<300m
1718_606	February	Picual	Andalusia	<300m
1718_607	February	Picual	Andalusia	300-600 m
1718_608	February	Hojiblanca	Andalusia	>600 m
1718_609	February	Hojiblanca	Andalusia	300-600 m
1718_610	February	Hojiblanca	Andalusia	300-600 m
1718_611	February	Hojiblanca	Andalusia	300-600 m
1718_612	February	N.D.	Andalusia	>600 m
1718_613	February	Hojiblanca	Andalusia	>600 m
1718_614	February	Hojiblanca	Andalusia	>600 m
1718_615	February	Hojiblanca	Andalusia	>600 m
1718_616	February	Hojiblanca	Andalusia	>600 m
1718_617	February	Hojiblanca	Andalusia	>600 m
1718_618	February	Hojiblanca	Andalusia	>600 m
1718_619	February	Arbequina	Andalusia	<300m
1718_620	February	Arbequina	Andalusia	<300m
1718_621	February	Picual	Andalusia	<300m
1718_622	February	Arbequina	Andalusia	<300m
1718_623	February	Hojiblanca	Andalusia	>600 m
1718_624	February	Hojiblanca	Andalusia	300-600 m
1718_625	February	Picual	Andalusia	>600 m
1718_626	February	Picual	Andalusia	>600 m
1718_627	February	Picual	Andalusia	>600 m
1718_628	February	Hojiblanca	Andalusia	300-600 m
1718_629	February	Hojiblanca	Andalusia	300-600 m
1718_630	February	Hojiblanca	Andalusia	300-600 m
1718_631	February	Hojiblanca	Andalusia	300-600 m
1718_632	February	Hojiblanca	Andalusia	300-600 m
1718_633	February	Hojiblanca	Andalusia	300-600 m
1718_634	February	Hojiblanca	Andalusia	300-600 m

1718_635	February	Hojiblanca	Andalusia	300-600 m
1718_636	February	Hojiblanca	Andalusia	300-600 m
1718_637	February	Hojiblanca	Andalusia	300-600 m
1718_638	February	Hojiblanca	Andalusia	300-600 m
1718_639	February	Hojiblanca	Andalusia	300-600 m
1718_640	February	Hojiblanca	Andalusia	300-600 m
1718_641	February	Hojiblanca	Andalusia	300-600 m
1718_642	February	Hojiblanca	Andalusia	300-600 m
1718_643	February	Hojiblanca	Andalusia	>600 m
1718_644	February	Hojiblanca	Andalusia	>600 m
1718_645	February	Hojiblanca	Andalusia	>600 m
1718_646	February	Hojiblanca	Andalusia	300-600 m
1718_647	February	Hojiblanca	Andalusia	300-600 m
1718_648	February	Hojiblanca	Andalusia	>600 m
1718_649	February	Hojiblanca	Andalusia	>600 m
1718_650	February	Hojiblanca	Andalusia	>600 m
1718_651	February	Picual	Andalusia	>600 m
1718_652	February	Picual	Andalusia	>600 m
1718_653	February	Cornicabra	Castilla-La Mancha	>600 m
1718_654	February	Cornicabra	Castilla-La Mancha	>600 m
1718_655	February	Picual	Andalusia	300-600 m
1718_656	February	Picual	Andalusia	300-600 m
1718_657	February	Picual	Andalusia	300-600 m
1718_658	March	Hojiblanca	Andalusia	>600 m
1718_659	March	Hojiblanca	Andalusia	>600 m
1718_660	March	Hojiblanca	Andalusia	>600 m
1718_661	March	Hojiblanca	Andalusia	300-600 m
1718_662	March	N.D.	Andalusia	300-600 m
1718_663	March	Picual	Andalusia	<300m
1718_664	March	Picual	Andalusia	>600 m
1718_665	March	Picual	Andalusia	>600 m
1718_666	March	N.D.	Andalusia	300-600 m
1718_667	March	Hojiblanca	Andalusia	>600 m
1718_668	March	Hojiblanca	Andalusia	300-600 m
1718_669	March	Hojiblanca	Andalusia	>600 m
1718_670	March	Hojiblanca	Andalusia	300-600 m
1718_671	March	Picual	Andalusia	>600 m
1718_672	March	Picual	Andalusia	>600 m
1718_673	March	Picual	Andalusia	>600 m
1718_674	March	Arbequina	Castilla-La Mancha	>600 m
1718_675	March	Hojiblanca	Andalusia	300-600 m
1718_676	March	Hojiblanca	Andalusia	300-600 m
1718_677	March	Hojiblanca	Andalusia	300-600 m
1718_678	March	Arbequina	Andalusia	<300m
1718_679	March	Picual	Andalusia	>600 m
1718_680	March	Hojiblanca	Andalusia	300-600 m
1718_681	March	Arbequina	Andalusia	<300m
1718_682	March	Arbequina	Andalusia	<300m
1718_683	March	Picual	Andalusia	<300m

1718_684	March	Hojiblanca	Andalusia	>600 m
1718_685	March	Picual	Andalusia	<300m
1718_686	March	Hojiblanca	Andalusia	300-600 m
1718_687	March	Hojiblanca	Andalusia	300-600 m
1718_688	March	Hojiblanca	Andalusia	>600 m
1718_689	March	Hojiblanca	Andalusia	>600 m
1718_690	March	Hojiblanca	Andalusia	300-600 m

N.D.: non-defined.

Supplementary Table 2. Production Month, Cultivar, Region and Height Above Sea Level Information of the Samples Analyzed in 2018/2019 Season

Sample code	Production month	Cultivar	Region	Heigh a.s.l.
1819_1	November	N.D.	Andalusia	<300m
1819_2	November	N.D.	Andalusia	>600 m
1819_3	November	N.D.	Andalusia	300-600 m
1819_4	December	Picual	Andalusia	300-600 m
1819_5	December	Picual	Andalusia	<300m
1819_6	December	Arbequina	Andalusia	>600 m
1819_7	December	Arbequina	Andalusia	<300m
1819_8	December	Hojiblanca	Andalusia	300-600 m
1819_9	December	N.D.	Andalusia	<300m
1819_10	December	N.D.	Andalusia	>600 m
1819_11	December	N.D.	Andalusia	>600 m
1819_12	December	Picual	Andalusia	300-600 m
1819_13	December	Picual	Andalusia	300-600 m
1819_14	December	Picual	Andalusia	>600 m
1819_15	December	Picual	Andalusia	300-600 m
1819_16	December	Arbequina	Andalusia	<300m
1819_17	December	Hojiblanca	Andalusia	300-600 m
1819_18	December	Hojiblanca	Andalusia	>600 m
1819_19	December	Hojiblanca	Andalusia	300-600 m
1819_20	December	Hojiblanca	Andalusia	>600 m
1819_21	December	N.D.	Andalusia	300-600 m
1819_22	December	N.D.	Andalusia	300-600 m
1819_23	December	Picual	Andalusia	<300m
1819_24	December	Picual	Andalusia	>600 m
1819_25	December	Picual	Andalusia	>600 m
1819_26	December	Picual	Andalusia	>600 m
1819_27	December	Arbequina	Andalusia	<300m
1819_28	December	N.D.	Andalusia	300-600 m
1819_29	December	Picual	Andalusia	<300m
1819_30	December	Picual	Andalusia	300-600 m
1819_31	December	Picual	Andalusia	300-600 m
1819_32	December	Picual	Andalusia	>600 m
1819_33	December	Hojiblanca	Andalusia	300-600 m
1819_34	December	Picual	Andalusia	>600 m
1819_35	December	Picual	Andalusia	>600 m
1819_36	December	Picual	Andalusia	>600 m
1819_37	December	Picual	Andalusia	>600 m

1819_38	December	Picual	Andalusia	>600 m
1819_39	December	Picual	Andalusia	>600 m
1819_40	December	Picual	Andalusia	>600 m
1819_41	December	Picual	Andalusia	>600 m
1819_42	December	Picual	Andalusia	300-600 m
1819_43	December	Picual	Andalusia	<300m
1819_44	December	Picual	Andalusia	>600 m
1819_45	December	Picual	Andalusia	>600 m
1819_46	December	Arbequina	Andalusia	>600 m
1819_47	December	Picual	Andalusia	<300m
1819_48	December	Picual	Andalusia	>600 m
1819_49	December	Picual	Andalusia	<300m
1819_50	December	Picual	Andalusia	<300m
1819_51	December	Picual	Andalusia	300-600 m
1819_52	December	Picual	Andalusia	<300m
1819_53	December	Picual	Andalusia	<300m
1819_54	December	Arbequina	Castilla-La Mancha	<300m
1819_55	December	Picual	Andalusia	>600 m
1819_56	December	Picual	Andalusia	300-600 m
1819_57	December	Picual	Andalusia	>600 m
1819_58	December	Picual	Andalusia	>600 m
1819_59	December	Picual	Andalusia	300-600 m
1819_60	December	Picual	Andalusia	<300m
1819_61	December	Picual	Andalusia	300-600 m
1819_62	December	Arbequina	Andalusia	300-600 m
1819_63	December	Arbequina	Andalusia	<300m
1819_64	December	Arbequina	Andalusia	<300m
1819_65	December	Arbequina	Andalusia	>600 m
1819_66	December	Arbequina	Andalusia	>600 m
1819_67	December	Arbequina	Castilla-La Mancha	<300m
1819_68	December	Arbequina	Andalusia	>600 m
1819_69	December	Hojiblanca	Andalusia	>600 m
1819_70	December	Hojiblanca	Andalusia	300-600 m
1819_71	December	Hojiblanca	Andalusia	300-600 m
1819_72	December	Hojiblanca	Andalusia	300-600 m
1819_73	December	Hojiblanca	Andalusia	300-600 m
1819_74	December	Hojiblanca	Andalusia	>600 m
1819_75	December	Hojiblanca	Andalusia	>600 m
1819_76	December	N.D.	Andalusia	300-600 m
1819_77	December	N.D.	Andalusia	300-600 m
1819_78	December	N.D.	Andalusia	<300m
1819_79	December	Picual	Andalusia	<300m
1819_80	December	Picual	Andalusia	<300m
1819_81	December	Picual	Andalusia	>600 m
1819_82	December	Picual	Andalusia	<300m
1819_83	December	Picual	Andalusia	<300m
1819_84	December	Arbequina	Andalusia	<300m
1819_85	December	Arbequina	Andalusia	<300m
1819_86	December	Arbequina	Andalusia	<300m

1819_87	December	Hojiblanca	Andalusia	300-600 m
1819_88	December	N.D.	Andalusia	300-600 m
1819_89	December	Picual	Andalusia	>600 m
1819_90	December	Picual	Andalusia	>600 m
1819_91	December	Picual	Andalusia	>600 m
1819_92	December	Picual	Andalusia	>600 m
1819_93	December	Picual	Andalusia	>600 m
1819_94	December	Picual	Andalusia	>600 m
1819_95	December	Picual	Andalusia	>600 m
1819_96	December	Picual	Andalusia	>600 m
1819_97	December	Picual	Andalusia	>600 m
1819_98	December	Picual	Andalusia	>600 m
1819_99	December	Picual	Andalusia	>600 m
1819_100	December	Picual	Andalusia	>600 m
1819_101	December	Hojiblanca	Andalusia	300-600 m
1819_102	December	Hojiblanca	Andalusia	300-600 m
1819_103	December	N.D.	Andalusia	300-600 m
1819_104	December	Hojiblanca	Andalusia	300-600 m
1819_105	December	N.D.	Andalusia	300-600 m
1819_106	December	Picual	Andalusia	>600 m
1819_107	December	Picual	Andalusia	>600 m
1819_108	December	Picual	Andalusia	<300m
1819_109	December	Hojiblanca	Andalusia	300-600 m
1819_110	December	N.D.	Andalusia	<300m
1819_111	December	N.D.	Andalusia	>600 m
1819_112	December	Picual	Andalusia	300-600 m
1819_113	December	Hojiblanca	Andalusia	>600 m
1819_114	December	N.D.	Andalusia	300-600 m
1819_115	December	N.D.	Andalusia	300-600 m
1819_116	December	N.D.	Andalusia	300-600 m
1819_117	December	Picual	Andalusia	>600 m
1819_118	December	Picual	Andalusia	<300m
1819_119	December	Picual	Andalusia	>600 m
1819_120	December	Picual	Andalusia	<300m
1819_121	December	N.D.	Andalusia	300-600 m
1819_122	December	Picual	Andalusia	>600 m
1819_123	December	Hojiblanca	Andalusia	>600 m
1819_124	December	Picual	Andalusia	>600 m
1819_125	December	Picual	Andalusia	300-600 m
1819_126	December	Picual	Andalusia	>600 m
1819_127	December	Picual	Andalusia	>600 m
1819_128	December	Picual	Andalusia	>600 m
1819_129	December	Picual	Andalusia	>600 m
1819_130	December	Picual	Andalusia	>600 m
1819_131	December	Picual	Andalusia	>600 m
1819_132	December	Picual	Andalusia	300-600 m
1819_133	December	Picual	Andalusia	>600 m
1819_134	December	Picual	Andalusia	>600 m
1819_135	December	Hojiblanca	Andalusia	300-600 m

1819_136	December	Picual	Andalusia	300-600 m
1819_137	December	Picual	Andalusia	300-600 m
1819_138	December	Picual	Andalusia	<300m
1819_139	December	Picual	Andalusia	300-600 m
1819_140	December	Picual	Andalusia	300-600 m
1819_141	December	Picual	Andalusia	>600 m
1819_142	December	Picual	Andalusia	>600 m
1819_143	December	Picual	Andalusia	300-600 m
1819_144	December	Picual	Andalusia	300-600 m
1819_145	December	Hojiblanca	Andalusia	300-600 m
1819_146	December	Hojiblanca	Andalusia	>600 m
1819_147	December	Hojiblanca	Andalusia	300-600 m
1819_148	December	Hojiblanca	Andalusia	>600 m
1819_149	December	N.D.	Andalusia	300-600 m
1819_150	December	N.D.	Andalusia	300-600 m
1819_151	December	N.D.	Andalusia	300-600 m
1819_152	December	Picual	Andalusia	>600 m
1819_153	December	Picual	Andalusia	<300m
1819_154	December	Picual	Andalusia	>600 m
1819_155	December	Picual	Andalusia	>600 m
1819_156	December	Picual	Andalusia	<300m
1819_157	December	Picual	Andalusia	>600 m
1819_158	December	Picual	Andalusia	>600 m
1819_159	December	Picual	Andalusia	>600 m
1819_160	December	Picual	Andalusia	>600 m
1819_161	December	Arbequina	Andalusia	<300m
1819_162	December	Arbequina	Castilla-La Mancha	<300m
1819_163	December	Arbequina	Castilla-La Mancha	<300m
1819_164	December	Hojiblanca	Andalusia	>600 m
1819_165	December	Hojiblanca	Andalusia	300-600 m
1819_166	December	Hojiblanca	Andalusia	300-600 m
1819_167	December	Hojiblanca	Andalusia	300-600 m
1819_168	December	Hojiblanca	Andalusia	>600 m
1819_169	December	Hojiblanca	Andalusia	>600 m
1819_170	December	N.D.	Andalusia	<300m
1819_171	December	Picual	Andalusia	300-600 m
1819_172	December	Picual	Andalusia	300-600 m
1819_173	December	Picual	Andalusia	300-600 m
1819_174	December	Picual	Andalusia	300-600 m
1819_175	December	Picual	Andalusia	>600 m
1819_176	December	Picual	Andalusia	>600 m
1819_177	December	Picual	Andalusia	300-600 m
1819_178	December	Picual	Andalusia	>600 m
1819_179	December	Hojiblanca	Andalusia	>600 m
1819_180	December	Hojiblanca	Andalusia	300-600 m
1819_181	December	Hojiblanca	Andalusia	>600 m
1819_182	December	Hojiblanca	Andalusia	300-600 m
1819_183	December	Hojiblanca	Andalusia	>600 m
1819_184	December	Hojiblanca	Andalusia	300-600 m

1819_185	December	N.D.	Andalusia	300-600 m
1819_186	December	N.D.	Andalusia	>600 m
1819_187	December	Picual	Andalusia	<300m
1819_188	December	Picual	Andalusia	300-600 m
1819_189	December	Hojiblanca	Andalusia	300-600 m
1819_190	December	Hojiblanca	Andalusia	>600 m
1819_191	December	Picual	Andalusia	>600 m
1819_192	December	Picual	Andalusia	>600 m
1819_193	December	Picual	Andalusia	>600 m
1819_194	December	Picual	Andalusia	>600 m
1819_195	December	Picual	Andalusia	300-600 m
1819_196	December	Picual	Andalusia	>600 m
1819_197	December	Picual	Andalusia	>600 m
1819_198	December	Picual	Andalusia	>600 m
1819_199	December	Hojiblanca	Andalusia	>600 m
1819_200	December	N.D.	Andalusia	>600 m
1819_201	December	N.D.	Andalusia	>600 m
1819_202	December	N.D.	Andalusia	>600 m
1819_203	December	N.D.	Andalusia	>600 m
1819_204	December	Picual	Andalusia	>600 m
1819_205	December	Picual	Andalusia	>600 m
1819_206	December	Picual	Andalusia	>600 m
1819_207	December	Picual	Andalusia	>600 m
1819_208	December	Picual	Andalusia	>600 m
1819_209	December	Picual	Andalusia	>600 m
1819_210	December	Picual	Andalusia	>600 m
1819_211	December	Picual	Andalusia	300-600 m
1819_212	December	Picual	Andalusia	>600 m
1819_213	December	Picual	Andalusia	>600 m
1819_214	December	Picual	Andalusia	>600 m
1819_215	December	Picual	Andalusia	>600 m
1819_216	December	Picual	Andalusia	300-600 m
1819_217	December	Picual	Andalusia	300-600 m
1819_218	December	Picual	Andalusia	>600 m
1819_219	December	Picual	Andalusia	>600 m
1819_220	December	Picual	Andalusia	>600 m
1819_221	December	Picual	Andalusia	>600 m
1819_222	December	Picual	Andalusia	>600 m
1819_223	December	Picual	Andalusia	300-600 m
1819_224	December	Picual	Andalusia	300-600 m
1819_225	December	Picual	Andalusia	<300m
1819_226	December	Picual	Andalusia	300-600 m
1819_227	December	Picual	Andalusia	>600 m
1819_228	December	Hojiblanca	Andalusia	>600 m
1819_229	December	Hojiblanca	Andalusia	>600 m
1819_230	December	Hojiblanca	Andalusia	>600 m
1819_231	December	Hojiblanca	Andalusia	>600 m
1819_232	December	Hojiblanca	Andalusia	>600 m
1819_233	December	Hojiblanca	Andalusia	>600 m

1819_234	December	Hojiblanca	Andalusia	300-600 m
1819_235	December	Hojiblanca	Andalusia	300-600 m
1819_236	December	Hojiblanca	Andalusia	300-600 m
1819_237	December	N.D.	Andalusia	<300m
1819_238	December	N.D.	Andalusia	<300m
1819_239	December	N.D.	Andalusia	300-600 m
1819_240	January	Picual	Andalusia	<300m
1819_241	January	Picual	Andalusia	>600 m
1819_242	January	Picual	Andalusia	<300m
1819_243	January	Arbequina	Andalusia	<300m
1819_244	January	Hojiblanca	Andalusia	300-600 m
1819_245	January	Hojiblanca	Andalusia	300-600 m
1819_246	January	Hojiblanca	Andalusia	300-600 m
1819_247	January	N.D.	Andalusia	>600 m
1819_248	January	Picual	Andalusia	300-600 m
1819_249	January	Hojiblanca	Andalusia	>600 m
1819_250	January	Hojiblanca	Andalusia	300-600 m
1819_251	January	Hojiblanca	Andalusia	>600 m
1819_252	January	Hojiblanca	Andalusia	>600 m
1819_253	January	N.D.	Andalusia	<300m
1819_254	January	N.D.	Andalusia	300-600 m
1819_255	January	N.D.	Andalusia	300-600 m
1819_256	January	Picual	Andalusia	>600 m
1819_257	January	Picual	Andalusia	>600 m
1819_258	January	Picual	Andalusia	>600 m
1819_259	January	Picual	Andalusia	300-600 m
1819_260	January	Hojiblanca	Andalusia	>600 m
1819_261	January	N.D.	Andalusia	>600 m
1819_262	January	N.D.	Andalusia	<300m
1819_263	January	Picual	Andalusia	>600 m
1819_264	January	Picual	Andalusia	>600 m
1819_265	January	Picual	Andalusia	>600 m
1819_266	January	Picual	Andalusia	>600 m
1819_267	January	N.D.	Andalusia	300-600 m
1819_268	January	Picual	Andalusia	>600 m
1819_269	January	Picual	Andalusia	>600 m
1819_270	January	Hojiblanca	Andalusia	>600 m
1819_271	January	Hojiblanca	Andalusia	300-600 m
1819_272	January	Hojiblanca	Andalusia	>600 m
1819_273	January	Hojiblanca	Andalusia	300-600 m
1819_274	January	Hojiblanca	Andalusia	300-600 m
1819_275	January	Hojiblanca	Andalusia	300-600 m
1819_276	January	Hojiblanca	Andalusia	300-600 m
1819_277	January	N.D.	Andalusia	300-600 m
1819_278	January	Picual	Andalusia	300-600 m
1819_279	January	Picual	Andalusia	300-600 m
1819_280	January	Arbequina	Andalusia	<300m
1819_281	January	N.D.	Andalusia	>600 m
1819_282	January	N.D.	Andalusia	>600 m

1819_283	January	Picual	Andalusia	>600 m
1819_284	January	Picual	Andalusia	<300m
1819_285	January	Picual	Andalusia	<300m
1819_286	January	Picual	Andalusia	<300m
1819_287	January	Arbequina	Andalusia	>600 m
1819_288	January	Picual	Andalusia	>600 m
1819_289	January	Picual	Andalusia	300-600 m
1819_290	January	Picual	Andalusia	>600 m
1819_291	January	Picual	Andalusia	>600 m
1819_292	January	Picual	Andalusia	>600 m
1819_293	January	Picual	Andalusia	300-600 m
1819_294	January	Picual	Andalusia	<300m
1819_295	January	Picual	Andalusia	<300m
1819_296	January	Picual	Andalusia	<300m
1819_297	January	Picual	Andalusia	<300m
1819_298	January	Arbequina	Andalusia	<300m
1819_299	January	Picual	Andalusia	>600 m
1819_300	January	Picual	Andalusia	>600 m
1819_301	January	Picual	Andalusia	>600 m
1819_302	January	N.D.	Andalusia	300-600 m
1819_303	January	Picual	Andalusia	>600 m
1819_304	January	Picual	Andalusia	300-600 m
1819_305	January	Hojiblanca	Andalusia	300-600 m
1819_306	January	Hojiblanca	Andalusia	>600 m
1819_307	January	Cornicabra	Castilla-La Mancha	>600 m
1819_308	January	Cornicabra	Castilla-La Mancha	>600 m
1819_309	January	N.D.	Andalusia	300-600 m
1819_310	January	N.D.	Andalusia	300-600 m
1819_311	January	Picual	Andalusia	300-600 m
1819_312	January	Picual	Andalusia	300-600 m
1819_313	January	Picual	Andalusia	300-600 m
1819_314	January	Picual	Andalusia	300-600 m
1819_315	January	Picual	Andalusia	>600 m
1819_316	January	Picual	Andalusia	>600 m
1819_317	January	Picual	Andalusia	>600 m
1819_318	January	Picual	Andalusia	>600 m
1819_319	January	Picual	Andalusia	>600 m
1819_320	January	Picual	Andalusia	>600 m
1819_321	January	Picual	Andalusia	>600 m
1819_322	January	Picual	Andalusia	>600 m
1819_323	January	Picual	Andalusia	>600 m
1819_324	January	Hojiblanca	Andalusia	>600 m
1819_325	January	N.D.	Andalusia	>600 m
1819_326	January	Picual	Andalusia	>600 m
1819_327	January	Picual	Andalusia	>600 m
1819_328	January	Arbequina	Andalusia	>600 m
1819_329	January	Hojiblanca	Andalusia	>600 m
1819_330	January	Picual	Andalusia	>600 m
1819_331	January	Hojiblanca	Andalusia	>600 m

1819_332	January	Hojiblanca	Andalusia	300-600 m
1819_333	January	Cornicabra	Castilla-La Mancha	>600 m
1819_334	January	Hojiblanca	Andalusia	>600 m
1819_335	January	N.D.	Castilla-La Mancha	>600 m
1819_336	January	N.D.	Castilla-La Mancha	>600 m
1819_337	January	Picual	Andalusia	300-600 m
1819_338	January	Picual	Andalusia	300-600 m
1819_339	January	Hojiblanca	Andalusia	300-600 m
1819_340	January	Hojiblanca	Andalusia	>600 m
1819_341	January	Picual	Andalusia	300-600 m
1819_342	January	Picual	Andalusia	300-600 m
1819_343	January	Picual	Andalusia	300-600 m
1819_344	January	Cornicabra	Castilla-La Mancha	>600 m
1819_345	January	N.D.	Andalusia	300-600 m
1819_346	January	N.D.	Andalusia	>600 m
1819_347	January	Arbequina	Andalusia	300-600 m
1819_348	January	Hojiblanca	Andalusia	>600 m
1819_349	January	Hojiblanca	Andalusia	>600 m
1819_350	January	Hojiblanca	Andalusia	300-600 m
1819_351	January	Hojiblanca	Andalusia	300-600 m
1819_352	January	Hojiblanca	Andalusia	300-600 m
1819_353	January	Hojiblanca	Andalusia	300-600 m
1819_354	January	N.D.	Andalusia	300-600 m
1819_355	January	Picual	Andalusia	>600 m
1819_356	January	Picual	Andalusia	>600 m
1819_357	January	Picual	Andalusia	>600 m
1819_358	January	Picual	Andalusia	300-600 m
1819_359	January	Picual	Andalusia	>600 m
1819_360	January	Picual	Andalusia	>600 m
1819_361	January	Picual	Andalusia	300-600 m
1819_362	January	Picual	Andalusia	<300m
1819_363	January	Picual	Andalusia	<300m
1819_364	January	Arbequina	Andalusia	<300m
1819_365	January	Hojiblanca	Andalusia	300-600 m
1819_366	January	Hojiblanca	Andalusia	>600 m
1819_367	January	Hojiblanca	Andalusia	>600 m
1819_368	January	Hojiblanca	Andalusia	>600 m
1819_369	January	Hojiblanca	Andalusia	300-600 m
1819_370	February	Arbequina	Andalusia	300-600 m
1819_371	February	Arbequina	Castilla-La Mancha	<300m
1819_372	February	Hojiblanca	Andalusia	300-600 m
1819_373	February	Picual	Andalusia	>600 m
1819_374	February	Picual	Andalusia	>600 m
1819_375	February	Picual	Andalusia	>600 m
1819_376	February	Arbequina	Castilla-La Mancha	<300m
1819_377	February	Hojiblanca	Andalusia	300-600 m
1819_378	February	Hojiblanca	Andalusia	300-600 m
1819_379	February	Hojiblanca	Andalusia	300-600 m
1819_380	February	Hojiblanca	Andalusia	>600 m

1819_381	February	Hojiblanca	Andalusia	>600 m
1819_382	February	Hojiblanca	Andalusia	300-600 m
1819_383	February	N.D.	Andalusia	300-600 m
1819_384	February	Arbequina	Andalusia	>600 m
1819_385	February	N.D.	Andalusia	300-600 m
1819_386	February	Picual	Andalusia	300-600 m
1819_387	February	Picual	Andalusia	300-600 m
1819_388	February	Picual	Andalusia	300-600 m
1819_389	February	Picual	Andalusia	>600 m
1819_390	February	Picual	Andalusia	>600 m
1819_391	February	Picual	Andalusia	300-600 m
1819_392	February	Picual	Andalusia	>600 m
1819_393	February	Hojiblanca	Andalusia	>600 m
1819_394	February	Hojiblanca	Andalusia	300-600 m
1819_395	February	Hojiblanca	Andalusia	300-600 m
1819_396	February	Hojiblanca	Andalusia	300-600 m
1819_397	February	Hojiblanca	Andalusia	300-600 m
1819_398	February	Hojiblanca	Andalusia	300-600 m
1819_399	February	Picual	Andalusia	300-600 m
1819_400	February	Picual	Andalusia	<300m
1819_401	February	Picual	Andalusia	<300m
1819_402	February	Picual	Andalusia	<300m
1819_403	February	Cornicabra	Castilla-La Mancha	>600 m
1819_404	February	Cornicabra	Castilla-La Mancha	>600 m
1819_405	February	Cornicabra	Castilla-La Mancha	>600 m
1819_406	February	Cornicabra	Castilla-La Mancha	>600 m
1819_407	February	Cornicabra	Castilla-La Mancha	>600 m
1819_408	February	Hojiblanca	Andalusia	300-600 m
1819_409	February	Hojiblanca	Andalusia	300-600 m
1819_410	February	Hojiblanca	Andalusia	300-600 m
1819_411	February	Hojiblanca	Andalusia	300-600 m
1819_412	February	Hojiblanca	Andalusia	300-600 m
1819_413	February	N.D.	Andalusia	>600 m
1819_414	February	N.D.	Andalusia	300-600 m
1819_415	February	N.D.	Andalusia	300-600 m
1819_416	February	N.D.	Andalusia	>600 m
1819_417	February	Picual	Andalusia	300-600 m
1819_418	February	Hojiblanca	Andalusia	300-600 m
1819_419	February	Picual	Andalusia	<300m
1819_420	February	Picual	Andalusia	<300m
1819_421	February	Picual	Andalusia	<300m
1819_422	February	Picual	Andalusia	>600 m
1819_423	February	Picual	Andalusia	300-600 m
1819_424	February	Picual	Andalusia	>600 m
1819_425	February	Picual	Andalusia	<300m
1819_426	February	Picual	Andalusia	>600 m
1819_427	February	Picual	Andalusia	300-600 m
1819_428	February	Picual	Andalusia	>600 m
1819_429	February	Picual	Andalusia	>600 m

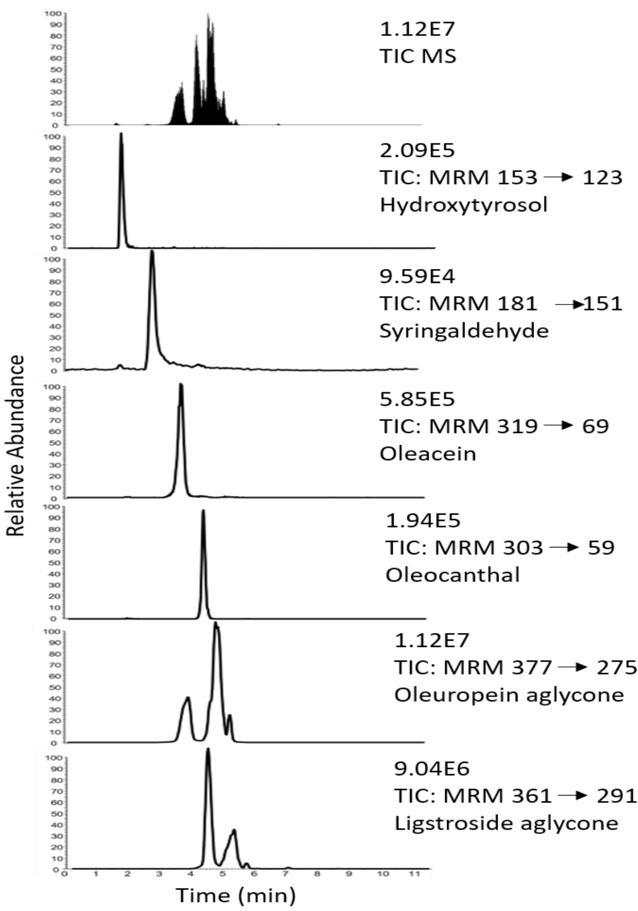
1819_430	February	Picual	Andalusia	300-600 m
1819_431	February	Picual	Andalusia	>600 m
1819_432	February	Arbequina	Andalusia	<300m
1819_433	February	Hojiblanca	Andalusia	300-600 m
1819_434	February	Hojiblanca	Andalusia	300-600 m
1819_435	February	N.D.	Andalusia	300-600 m
1819_436	February	Picual	Andalusia	300-600 m
1819_437	February	Picual	Andalusia	300-600 m
1819_438	February	Cornicabra	Castilla-La Mancha	>600 m
1819_439	February	Hojiblanca	Andalusia	>600 m
1819_440	February	Hojiblanca	Andalusia	>600 m
1819_441	February	Hojiblanca	Andalusia	>600 m
1819_442	February	N.D.	Andalusia	>600 m
1819_443	February	N.D.	Andalusia	300-600 m
1819_444	February	Picual	Andalusia	>600 m
1819_445	February	Hojiblanca	Andalusia	300-600 m
1819_446	February	Hojiblanca	Andalusia	300-600 m
1819_447	February	Hojiblanca	Andalusia	>600 m
1819_448	February	Hojiblanca	Andalusia	>600 m
1819_449	February	Hojiblanca	Andalusia	>600 m
1819_450	February	Hojiblanca	Andalusia	300-600 m
1819_451	February	Hojiblanca	Andalusia	300-600 m
1819_452	February	Hojiblanca	Andalusia	300-600 m
1819_453	February	N.D.	Andalusia	>600 m
1819_454	February	N.D.	Andalusia	>600 m
1819_455	February	N.D.	Andalusia	>600 m
1819_456	February	Picual	Andalusia	<300m
1819_457	February	Picual	Andalusia	<300m
1819_458	February	Picual	Andalusia	<300m
1819_459	February	Picual	Andalusia	<300m
1819_460	February	Arbequina	Andalusia	<300m
1819_461	February	Hojiblanca	Andalusia	>600 m
1819_462	February	Hojiblanca	Andalusia	>600 m
1819_463	February	Hojiblanca	Andalusia	>600 m
1819_464	February	Hojiblanca	Andalusia	300-600 m
1819_465	February	Hojiblanca	Andalusia	300-600 m
1819_466	February	Hojiblanca	Andalusia	300-600 m
1819_467	February	Hojiblanca	Andalusia	300-600 m
1819_468	February	N.D.	Andalusia	300-600 m
1819_469	February	Hojiblanca	Andalusia	300-600 m
1819_470	March	Cornicabra	Castilla-La Mancha	>600 m
1819_471	March	Hojiblanca	Andalusia	300-600 m
1819_472	March	N.D.	Castilla-La Mancha	>600 m
1819_473	March	Picual	Andalusia	>600 m
1819_474	March	Hojiblanca	Andalusia	>600 m
1819_475	March	Hojiblanca	Andalusia	>600 m
1819_476	March	Hojiblanca	Andalusia	300-600 m
1819_477	March	Hojiblanca	Andalusia	300-600 m
1819_478	March	Hojiblanca	Andalusia	>600 m

1819_479	March	N.D.	Andalusia	300-600 m
1819_480	March	N.D.	Andalusia	300-600 m
1819_481	March	Picual	Andalusia	300-600 m
1819_482	March	Picual	Andalusia	300-600 m
1819_483	March	Picual	Andalusia	300-600 m
1819_484	March	Hojiblanca	Andalusia	300-600 m
1819_485	March	Hojiblanca	Andalusia	>600 m
1819_486	March	Hojiblanca	Andalusia	>600 m
1819_487	March	Hojiblanca	Andalusia	300-600 m
1819_488	March	Hojiblanca	Andalusia	300-600 m
1819_489	March	Hojiblanca	Andalusia	>600 m
1819_490	March	Arbequina	Andalusia	>600 m
1819_491	March	Arbequina	Andalusia	>600 m
1819_492	March	Hojiblanca	Andalusia	300-600 m
1819_493	March	N.D.	Andalusia	300-600 m
1819_494	March	Picual	Andalusia	300-600 m
1819_495	March	Picual	Andalusia	>600 m
1819_496	March	Picual	Andalusia	300-600 m
1819_497	March	Arbequina	Andalusia	300-600 m
1819_498	March	Hojiblanca	Andalusia	>600 m
1819_499	March	Hojiblanca	Andalusia	>600 m
1819_500	March	Hojiblanca	Andalusia	>600 m
1819_501	March	N.D.	Andalusia	300-600 m
1819_502	March	N.D.	Andalusia	300-600 m
1819_503	March	Picual	Andalusia	>600 m
1819_504	March	Picual	Andalusia	>600 m
1819_505	March	Picual	Andalusia	>600 m
1819_506	March	Picual	Andalusia	>600 m
1819_507	March	Picual	Andalusia	>600 m
1819_508	March	Picual	Andalusia	<300m
1819_509	March	Picual	Andalusia	<300m
1819_510	March	Picual	Andalusia	>600 m
1819_511	March	N.D.	Andalusia	300-600 m
1819_512	March	Picual	Andalusia	>600 m
1819_513	March	Picual	Andalusia	>600 m
1819_514	March	Hojiblanca	Andalusia	>600 m
1819_515	March	Hojiblanca	Andalusia	>600 m
1819_516	March	Hojiblanca	Andalusia	>600 m
1819_517	March	N.D.	Andalusia	300-600 m
1819_518	March	N.D.	Castilla-La Mancha	>600 m
1819_519	March	Hojiblanca	Andalusia	>600 m
1819_520	March	Hojiblanca	Andalusia	>600 m
1819_521	March	Hojiblanca	Andalusia	300-600 m
1819_522	March	Picual	Andalusia	300-600 m
1819_523	March	Hojiblanca	Andalusia	300-600 m
1819_524	March	Hojiblanca	Andalusia	300-600 m
1819_525	March	Hojiblanca	Andalusia	300-600 m
1819_526	March	Hojiblanca	Andalusia	300-600 m
1819_527	March	Picual	Andalusia	>600 m

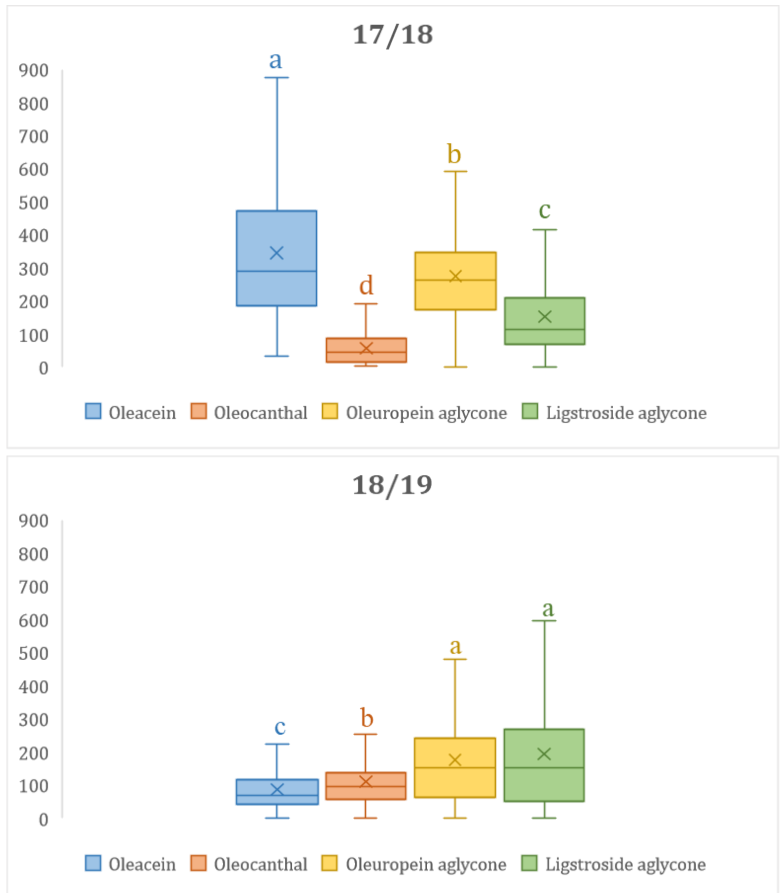
1819_528	March	Hojiblanca	Andalusia	>600 m
1819_529	March	Hojiblanca	Andalusia	>600 m
1819_530	March	Hojiblanca	Andalusia	300-600 m
1819_531	March	Hojiblanca	Andalusia	300-600 m
1819_532	March	Hojiblanca	Andalusia	>600 m
1819_533	March	Hojiblanca	Andalusia	300-600 m
1819_534	March	Picual	Andalusia	>600 m
1819_535	March	Picual	Andalusia	>600 m
1819_536	March	Hojiblanca	Andalusia	300-600 m
1819_537	March	N.D.	Andalusia	>600 m
1819_538	March	Hojiblanca	Andalusia	>600 m
1819_539	March	N.D.	Andalusia	>600 m
1819_540	March	N.D.	Andalusia	>600 m
1819_541	March	N.D.	Andalusia	>600 m
1819_542	March	N.D.	Andalusia	>600 m
1819_543	March	N.D.	Andalusia	>600 m
1819_544	March	N.D.	Andalusia	>600 m
1819_545	March	N.D.	Andalusia	>600 m
1819_546	March	Picual	Andalusia	>600 m
1819_547	April	Picual	Andalusia	>600 m
1819_548	April	N.D.	Andalusia	<300m
1819_549	April	Hojiblanca	Andalusia	>600 m

Supplementary Table 3. MRM parameters and limits of quantitation for determination of phenolic compounds in olive oil.

Compound	RT (min)	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (eV)	Tube Lens (V)	LOQ (mg/kg)
Hydroxytyrosol	1.7	153	123	17	54	0.1
Syringaldehyde (IS)	2.8	181	151	22	68	-
Oleacein	3.7	319	69	39	62	0.5
Oleomissional	3.9	377	275	16	103	0.25
Oleocanthal	4.5	303	59	17	56	0.5
Oleokoronal	4.6	361	291	17	88	0.25
Oleuropein aglycone	5.1	377	275	16	103	0.25
Ligstroside aglycone	5.3	361	291	17	88	0.25



Supplementary Figure 1. MRM chromatograms obtained by LC-MS/MS analysis of an EVOO sample.



Supplementary Figure 2. Variability in the concentration of the main phenolic compounds in samples analyzed in the two seasons. Letters indicate significant differences according to anova with Tukey's test (p-value of < 0.05).

Supplementary Table 4. Distribution of EVOO samples in five groups according to the harvesting date in the two consecutive seasons.

Season	17/18		18/19	
Group	**Date**	***n***	**Date**	***n***
1	07/11-06/12	124	19/11-19/12	105
2	07/12-22/12	105	20/12-04/01	157
3	23/12-26/01	186	05/01-05/02	111
4	27/02-22/02	196	06/02-09/03	121
5	23/02-26/03	79	10/03-10/04	55

CHAPTER 2

The decrease in the health benefits of extra virgin olive oil during storage is conditioned by the initial phenolic profile

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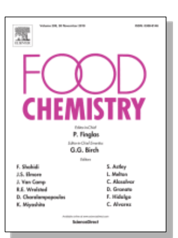

The decrease in the health benefits of extra virgin olive oil during storage is conditioned by the initial phenolic profile

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The decrease in the health benefits of extra virgin olive oil during storage is conditioned by the initial phenolic profile

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Abstract

Phenols are responsible for the only health claim of virgin olive oil (VOO) recognized by the European Commission (EU) 432/2012 and the European Food Safety Authority. In this research, we studied the decrease in the phenolic content of 160 extra VOOs (EVOOs) after 12 months storage in darkness at 20 °C. Phenolic concentration was decreased 42.0 ± 24.3% after this period and this reduction strongly depended on the initial phenolic profile. Hence, EVOOs with predominance in oleacein and oleocanthal experienced a larger decrease in phenolic content than oils enriched in other phenols. Complementarily, hydroxytyrosol and oleocanthalic acid increased significantly in aged EVOOs, which allowed their discrimination from recently produced EVOOs. These changes are explained by degradation of main secoiridoids during storage due to their antioxidant properties. Hydroxytyrosol and oleocanthalic acid can be considered markers of olive oil ageing, although they can also provide information about quality or stability.

Keywords: Phenols; Extra-Virgin Olive Oil; LC–MS/MS; Health Claim; Storage; Secoiridoids; Hydroxytyrosol; Oleocanthalic Acid.

1. Introduction

The health benefits of virgin olive oil (VOO) are attributed to its characteristic composition that can be divided into two main fractions. Major or saponifiable fraction (~98% of the total content) consists of fatty acids derivatives such as triglycerides, diglycerides, monoglycerides, phospholipids, and sterol esters, being oleic acid the predominant fatty acid (between 55 and 83% of the total fatty acids content). On the other hand, the minor or unsaponifiable fraction (~2%) contains different chemical families such as phenols, tocopherols, phytosterols, volatile compounds, terpenes and hydrocarbons, among others. These compounds are partially or totally removed when low quality oil, named *lampante* olive oil, is subjected to thermal, physical and/or chemical refining treatments. For this reason, the minor fraction is mostly present in VOO and, especially, in extra virgin olive oil (EVOO) [1].

Phenolic compounds are highlighted in the minor fraction of (E)VOO (abbreviation used to refer to virgin olive oil and, particularly, to the extra-virgin category), with special attention to secoiridoids because of their high concentration. These compounds are mainly produced during crushing and malaxation of the olive paste and they are formed by conversion of oleuropein and ligstroside, which are accumulated in the fruit during ripening [2]. This conversion is based on an enzymatic process involving two classes of endogenous enzymes, β-glucosidases and methylesterases. β-glucosidases hydrolyze oleuropein and ligstroside to generate the corresponding aglycone isomers. The closed monoaldehyde forms are mainly detected in (E)VOO. Nevertheless, it is frequent to detect the open monoaldehyde forms named as oleokoronal and oleomissional [3]. The combined action of β-glucosidases and methylesterases results in oleocanthal and oleacein by additional decarboxymethylation (Supplementary Figure 1) [4].

The importance of phenols in the (E)VOO is mainly attributed to three aspects. Firstly, several authors have found a strong relationship between particular organoleptic properties such as pungency and bitterness, and the content of phenolic compounds [5-7]. Secondly, phenolic fraction allows (E)VOOs to make use

of the health claim included in the European Commission (EU) 432/2012, “phenols protect blood lipids against oxidative stress”, which is one of the main mechanisms involved in the development of several pathologies such as cardiovascular diseases [8]. However, the health claim is only attributable to olive oils providing a minimum amount of 5 mg of hydroxytyrosol, tyrosol and derivatives with a daily consumption of 20 g of product, in concentration terms above 250 mg/kg [9]. This amount corresponds to the consumption recommended by the European Food Safety Authority (EFSA) to follow a healthy diet with a balanced fat content [10]. Additionally, phenols have other health benefits recognized by the EFSA such as their anti-inflammatory properties, the contribution to maintaining the suitable concentration of cholesterol, normal blood pressure, respiratory health, normal gastrointestinal tract function and immune system strengthening [8,10]. A third reason that justifies the interest of (E)VOO phenols is their antioxidant properties. Most studies indicate that phenolic concentration is positively related to oxidative stability of (E)VOOs [1,11]. According to Miho *et al.*, oxidative stability depends on the relative phenolic profile and not on the total phenol concentration. These authors defined the “*f* factor” as the ratio between the concentration of oleuropein and ligstroside aglycone isomers and the concentration of oleocanthal and oleacein. Thus, a high *f* value is typical of (E)VOOs with high oxidative stability, while a low *f* refers to less stable (E)VOOs. It means that (E)VOOs with a high content of oleuropein and ligstroside aglycone isomers tend to be more stable than those with a lower content in these phenols [12]. This aspect would explain that (E)VOOs with the same total phenolic content may have different oxidative stability.

Phenolic compounds are influenced by many factors, among them, the genotype or cultivar [13]. Miho *et al.* showed that cultivars have a genetic predisposition to provide (E)VOOs with a certain phenolic composition [14]. In this study, the authors identified groups of cultivars with tendency to give EVOOs rich in aglycone isomers or in oleocanthal and oleacein. On the other hand, agronomic factors such as the fruit ripening, irrigation practices or the presence of certain pathologies, together with technological conditions such as temperature and malaxation time or added amount of water, also influence significantly the phenolic content of (E)VOO [15,16].

However, the (E)VOO composition changes during the best before date period recommended for 18 months [17]. This alteration strongly depends on variables such as storage time, filtration, type of container or exposure to light and high temperature [18]. In this research, we aimed to study the decrease of phenolic content in EVOOs stored in darkness at room temperature (20 °C) for 12 months to evaluate its incidence on the health benefits. Additionally, we have analyzed the influence of the initial phenolic content, in absolute and relative terms, on the decrease of EVOO phenolic content after storage. The hypothesis is that the decrease in the concentration of total phenols in EVOO after storage for 12 months is conditioned by its initial phenolic profile.

2. Materials and methods

2.1. Samples

A set of 160 EVOO samples produced in the 2018/2019 crop season were selected in this research. Samples were directly provided by Spanish producers from different locations of the Mediterranean area under the frame of the Aristoil project (granted by the Interreg-MED program). One aliquot of each sample was analyzed just after production in the 2018/2019 season and a second aliquot was stored at room temperature (20 °C) in darkness for 12 months. After this period, the second aliquots were analyzed following the same methodology.

2.2. Reagents and standards

MS-grade methanol (MeOH) from Fischer Scientific (Hampton, NH, USA) and *n*-hexane from Scharlab (Barcelona, Spain) were used for sample preparation. MS-grade formic acid, also from Fischer Scientific, was used as ionization agent. Mobile phases and the hydroalcoholic mixture used as extractant were prepared with MeOH and deionized water (18 MΩ • cm) supplied by a Milli-Q purification system from Millipore (Bedford, MA, USA).

Hydroxytyrosol was purchased in Extrasynthese (Genay, France) while secoiridoid derivatives oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycone and ligstroside aglycone (both as monoaldehyde closed

isomers), and oleocanthalic acid were provided by Prof. P. Magiatis (University of Athens, Greece). Hydroxytyrosol standard was prepared in MeOH while secoiridoid standards were prepared in acetonitrile to preserve their stability and avoid undesired conversion to acetal and hemiacetal derivatives [19]. To simplify the nomenclature, oleuropein and ligstroside aglycones were used to name the monoaldehyde closed isomers, while oleomissional and oleokoronal were used to name the monoaldehyde open isomers of oleuropein and ligstroside aglycones, respectively. Syringaldehyde from Sigma-Aldrich (St. Louis, MO, USA) was used as internal standard (IS) to control the LC–MS/MS performance during the analysis of all samples.

2.3. Apparatus and instruments

A vortex shaker from IKA (Wilmington, NC, USA) and a centrifuge supplied by Ortoalresa (Madrid, Spain) were used for sample preparation. A Thermo Scientific UltiMate 3000 series LC system coupled to a Thermo Scientific QqQ TSQ Quantum™ Access MAX detector (Waltham, MA, USA) was used for LC–MS/MS analysis. The QqQ detector was equipped with an electrospray ionization (ESI) source. Chromeleon™ (version 6.80) and TSQ Tune (version 1.2.1) were used to control the LC–MS/MS system. Xcalibur™ (version 3.0.63) was used for creation of methods and worklists and Tracefinder™ (version 3.2) was the software for data acquisition, qualitative and quantitative analysis.

2.4. Sample preparation

Phenolic compounds were isolated by liquid–liquid extraction by following a previously published protocol [14]. A 0.5 g aliquot of oil was vortexed with 250 µL of *n*-hexane for 30 s. Then, 2 mL of 80:20 (v/v) MeOH:water with the IS (1 µg/mL) was added and shaken for 2 min, and the hydroalcoholic phase was separated by centrifugation for 8 min at 900g. The resulting phenolic extract (top layer) and a dilution (1:10 v/v) were injected into the LC–QqQ MS/MS. Three replicates per sample were analyzed.

2.5. LC–MS/MS analysis of phenolic compounds

Analyses were performed by reversed-phase liquid chromatography followed by MS/MS detection with ionization in negative mode. Chromatographic separation was performed by using a Mediterranea C_{18} column (3 μm particle size, 5 × 0.46 cm i.d.), which was thermostated at 30 °C and protected with a C_{18} precolumn (3 μm particle size × 0.46 cm i.d.) from Teknokroma (Madrid, Spain). The chromatographic mobile phases were deionized water (phase A) and MeOH (phase B), both containing 0.1% (v/v) formic acid as ionization agent. The LC pump was programmed with a flow rate of 0.4 mL/min with the following elution gradient: 50% phase B was kept as initial mobile phase for 0.5 min; then, from 0.5 to 2 min, mobile phase B was from 50 to 80%; and from min 2 to 5, mobile phase B was from 80 to 100%. This last composition was kept for 1.5 min. A post-time of 4 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 10 μL and the autosampler was kept at 4 °C.

MS detection was performed by MS/MS in multiple reaction monitoring (MRM) mode for selective transitions from precursor to product ions for each analyte. The MRM parameters for the analysis of target phenols are listed in Supplementary Table 1. The ionization parameters were set as follows: ionization probe, position B; spray voltage, 2750 V; sheath gas pressure, 25 arbitrary units; auxiliary gas, 10 psi; ion transfer capillary temperature, 300 °C; and FWHM for Q1 and Q3, 0.7. The scan time and width were 0.1 s and 0.5 *m/z*.

2.6. Quantitative analysis of phenolic compounds and estimation of the healthy index

Calibration models were prepared by using refined sunflower oil spiked with multistandard phenolic solutions at five concentration levels (1 to 20 μg/g). Spiked aliquots were analyzed with the complete protocol, in triplicate, to obtain the calibration models (Supplementary Table 2). Oleomissional and oleokoronal were quantified by using the calibration models prepared with the corresponding monoaldehyde closed isomers.

The healthy index was estimated according to the Commission Regulation (EU) 432/2012 and the EFSA opinion, which recommend a 20 g daily intake of olive

oil. Thus, the healthy index refers to the amount of phenols expressed in mg consumed with this daily intake. A positive healthy index is considered above 5 mg/20 g of oil, which is equivalent to an absolute concentration of 250 mg/kg. The *f* factor was calculated as the ratio between the sum of aglycone isomers of oleuropein and ligstroside and that of oleocanthal and oleacein. Complementarily, the *h* factor was calculated as the ratio between the sum of concentrations of hydroxytyrosol and tyrosol secoiridoid derivatives.

2.7. Statistical analysis

The Kolmogorov-Smirnov test (p-value of <0.05) was applied to reject the hypothesis of normality for all evaluated variables. For this reason, the Wilcoxon test was performed to detect significant alterations in the concentration of phenolic compounds after 12 months (p-value of <0.01). Subsequently, Pearson or Spearman analysis was carried out (p-value of <0.01 and R>0.45) to evaluate correlations between variables. A paired t-test was applied to detect significant changes in the concentration of hydroxytyrosol and oleocanthalic acid after storage. Finally, univariate receiver operating characteristic curve (ROC) analysis was performed to evaluate the ability of hydroxytyrosol and oleocanthalic acid to classify oils according to the crop season. Statgraphics Centurion XVI.I version 16.1.18 (Warrenton, VA, EE.UU.) and Metaboanalyst (version 4.0, www.metaboanalyst.ca) were used for data analysis [20].

3. Results and Discussion

3.1. Phenolic profiles of the analyzed EVOOs

Phenolic compounds analyzed in the set of samples were those that mainly contribute to the health benefits recognized by the EFSA claim and included in the European Commission 432/2012. These were hydroxytyrosol, oleocanthal, oleacein, oleuropein aglycone, oleomissional, ligstroside aglycone and oleokoronal. Additionally, we measured the concentration of oleocanthalic acid, a derivative of oleocanthal produced by oxidation. Tyrosol was monitored but was finally excluded in this research. Tyrosol concentration in the complete set of VOOs did not surpass 5 mg/kg and numerous samples reported a concentration below quantitation limits.

In addition, tyrosol determination was affected by a high variability and significant effects were not found. Table 1 lists the results obtained by quantitative determination of the target phenols in the EVOOs analyzed in the 2018/2019 crop season, when they were produced. These results prove a huge variability in the concentration of all monitored phenols. This variability is associated to the genotype (cultivar), agronomical (ripening, practices, etc.) and technological factors (malaxation time and temperature). This variability is crucial to evaluate the decrease in the concentration of phenols according to the initial phenolic profile. The most concentrated phenols were the secoiridoid derivatives while hydroxytyrosol did not surpass 10 mg/kg. Maximum levels detected for secoiridoid derivatives ranged from 387 mg/kg for oleomissional to 974 mg/kg for oleokoronal. It is worth noting that EVOOs with minimum levels of these derivatives were also detected (from unquantifiable levels to 8.2 mg/kg). We also estimated the variability of the phenolic profiles by determination of the *f* and *h* factors. The *f* factor is a direct indicator of the transformation of precursor biophenols, oleuropein and ligstroside, to the aglycone forms or to oleocanthal and oleacein. This factor was from 0 to 6.3, with an average level of 0.75. Thus, the set of EVOOs included samples with different phenolic profiles as reported by Miho *et al.*, which were grouped in those with predominance of oleocanthal and oleacein (*f* factor lower than 0.67), those enriched in the aglycone isomers of oleuropein and ligstroside (*f* factor higher than 1.5), and those without a quantitative predominance in secoiridoid derivatives (*f* factor between 0.67 and 1.5). Complementarily, the *h* factor is an indicator of the prevalence of hydroxytyrosol or tyrosol secoiridoid derivatives in the phenolic profile. The *h* factor ranged from 0.19 to 10.7 with a mean value of 1.04.

The analyzed EVOOs were characterized by their health benefits according to the phenolic content. The mean healthy index was 17.0 mg/20 g of oil, which triplicates the limit level established by the EFSA at 5 mg/20 g of oil or, in phenolic concentration terms, 250 mg/kg. The percentage of EVOO samples with phenolic concentration above this limit was 83.2%.

Concerning oleocanthalic acid, this was detected within a reduced range of concentrations, from 0 to 3.7 mg/kg, and the mean content was 0.46 mg/kg.

Therefore, the formation of oleocanthalic acid by oleocanthal oxidation is minimum in recently produced EVOOs, which agrees with the results reported by Tsolakou *et al.* [21]. Complementarily, Table 1 also shows the results provided by analysis of the same set of EVOOs after storage for 12 months at 20 °C in darkness. The comparison between results clearly reveals a decrease in the content of all secoiridoid derivatives. On the contrary, we observed an increase in the levels of hydroxytyrosol and oleocanthalic acid. Hydroxytyrosol mean concentration was 5.9 mg/kg with a maximum detected level of 15.4 mg/kg, whereas oleocanthalic acid reported an average concentration of 4.4 mg/kg with a maximum detected level of 14.2 mg/kg. These results point out a degradation effect and hydroxytyrosol and oleocanthalic acid would be considered degradation markers.

The phenol content of the EVOOs decreased after storage for 12 months despite they were stored under optimal conservation conditions. This substantial degradation is also manifested in the healthy index that was decreased up to 7.7 mg/20 g of oil with a maximum found value of 20.2 mg/20 g of oil. After 12 months, the percentage of samples with average content above 250 mg/kg was reduced to 67.2%, which highlights a relevant decrease in the health benefits of analyzed EVOOs after storage for 12 months.

3.2. Evaluation of the phenolic decrease in EVOO

The total phenolic content in EVOO samples experienced a mean decrease of 42.0 ± 24.3%. This result agrees with other previous studies that have estimated a decrease of phenolic concentration in EVOO from 40 to 70% after storage for 12 or 24 months [22]. Table 1 lists the concentration decreases experienced by the secoiridoid derivatives in the set of analyzed EVOOs. This decrease ranged between 41.3% for oleuropein aglycone and 62% for oleocanthal. Oleokoronal and oleomissional, both monoaldehyde open forms, were decreased at higher level than the corresponding monoaldehyde closed forms, ligstroside aglycone and oleuropein aglycone, respectively. If the comparison is carried out between tyrosol and hydroxytyrosol conjugated compounds, tyrosol derivatives, namely, oleocanthal, oleokoronal and ligstroside aglycone, experienced a higher concentration decrease

than their corresponding hydroxytyrosol derivatives, namely, oleacein, oleomissional and oleuropein aglycone.

Concerning the increase in concentration measured for hydroxytyrosol and oleocanthalic acid, this is explained by degradation of secoiridoid derivatives. The increase in the concentration of hydroxytyrosol is identified as a marker of antioxidant effect due to the degradation of hydroxytyrosol conjugated secoiridoids, particularly, oleacein, oleomissional and oleuropein aglycone. Oleacein and oleuropein aglycone are recognized by their antioxidant properties [23,24] and, this protective effect over EVOO oxidation induces an increase in the concentration of hydroxytyrosol. Nevertheless, despite the high levels of secoiridoid derivatives and the decrease in their levels during storage, the concentration increase of hydroxytyrosol was not of the same magnitude, which means that there is not a direct conversion. On the other hand, oleocanthalic acid, initially undetectable or detected at sub-mg/kg levels, was detectable in all stored EVOOs with a mean concentration of several mg/kg units. Tsolakou *et al.* identified oleocanthalic acid as an ageing marker in EVOOs due to the oxidation of oleocanthal to the corresponding oleocanthalic acid [21].

Table 1. Concentration (mean, minimum and maximum) of phenolic compounds and healthy index in the EVOOs analyzed in the 2018/2019 crop season and after storage for 12 months. The *f* and *h* factors provide information about the variability of phenolic profiles. The concentration decrease (expressed in percentage) for individual phenols and healthy index experienced in EVOOs after storage is also included.

	2018/2019 crop season			After storage for 12 months			
Compound (mg/kg)	**Mean±SD[a]**	**Maximum**	**Minimum**	**Mean±SD[a]**	**Maximum**	**Minimum**	**% Degradation±SD[a]**
Hydroxytyrosol	2.6 ± 1.9	8.9	0	5.9 ± 3.1	15.4	0.15	-
Oleacein	120 ± 110	627	4.4	49.2 ± 28.8	172	4.8	48.8 ± 23.6
Oleocanthal	154 ± 125	627	7.9	46.8 ± 27.6	192	11.1	62.0 ± 16.6
Oleomissional	67.9 ± 81.1	387	0	34 ± 28.1	122	0	43.9 ± 29.9
Oleocanthalic acid	0.46 ± 0.73	3.7	0	4.4 ± 2.4	14.2	0.25	-
Oleokoronal	181 ± 189	974	0	74.8 ± 68.5	316	0.19	51.8 ± 19.9
Oleuropein aglycone	191 ± 171	819	8.2	106 ± 67	271	5	41.3 ± 28.5
Ligstroside aglycone	133 ± 142	908	0	66.2 ± 56.9	274	2.6	46.3 ± 24.9
Healthy index (mg/20g)	17 ± 14.5	78.6	1.9	7.7 ± 4.4	20.2	0.71	42.0 ± 24.3
***f* factor**	0.75 ± 0.96	6.3	0				
***h* factor**	1.04 ± 1.09	10.7	0.19				

[a]SD: Standard deviation.

3.3. Association of the initial phenolic profile of EVOO and the decrease of concentration during storage

EVOO samples were distributed in two balanced groups according to the degradation of phenolic compounds after 12 months storage. The cut-off value was set at 35% expressed as total phenolic degradation, which divided samples in a group with reduced decrease (22.5 ± 9.9%) and a second group with high degradation up to 72.7% (average decrease 60.6 ± 18.7%). A Wilcoxon test (adjusted FDR *p*-value of <0.01) was applied to the initial concentrations of phenols as well as to other related parameters such as the *f* and *h* factors (Supplementary Table 3). The two groups of EVOOs were characterized by significantly different concentrations of oleacein and oleocanthal (*p*-value of <0.0001), while no discrimination was observed for the aglycone isomers. In fact, oleacein and oleocanthal were twice more concentrated in EVOOs with high phenolic degradation as compared with the low degradation group (Figure 1). This result can be explained by the chemical structure and reactivity of secoiridoid derivatives. Oleacein and oleocanthal, with two aldehydic functional groups in an open configuration, possess larger reactivity than monoaldehyde oleuropein aglycone isomers, mainly detected in a closed configuration. Nevertheless, the *f* factor did not result significantly different in the two groups of EVOOs. The significant effects were also absent for the *h* factor, which means that there is not a predominant degradation of hydroxytyrosol or tyrosol secoiridoid derivatives.

We studied the association between the concentrations of oleacein and oleocanthal and the degradation of total phenols in EVOO by Spearman correlation analysis. The concentration of both phenols in EVOOs was correlated with the decrease in the total phenolic content after 12 months storage (Figure 2). A second-degree polynomial function seems to regulate the association between the concentration of oleocanthal (R = 0.4876, *p*-value of <0.0001) and oleacein (R = 0.5160, *p*-value of <0.0001) and the degradation of health benefits according to the phenolic content. The correlation plots reveal that EVOOs with concentrations of oleacein and oleocanthal above 200 mg/kg were characterized by a large degradation of total phenolic compounds in absolute terms. These results point out

that EVOOs with high content in oleocanthal and oleacein tend to lose their phenolic concentration and, therefore, their health benefits associated to these compounds after storage for 12 months.

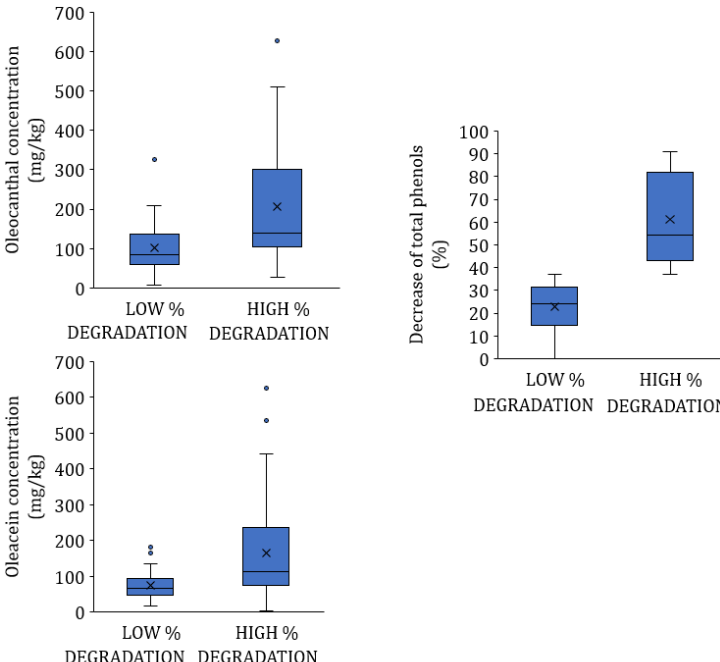


Figure 1. Box-and-whisker plots that represent the significant differences in the concentration of oleacein and oleocanthal measured in the 2018/2019 crop season in EVOOs that experienced a high or low degradation of total phenols after storage for 12 months.

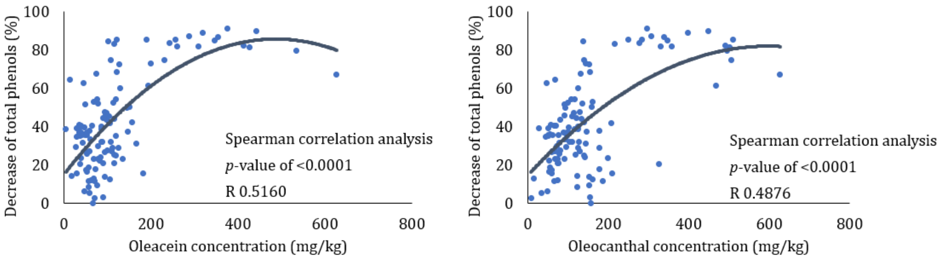


Figure 2. Correlation plot between the concentration of oleacein and oleocanthal measured in EVOOs in the 2018/2019 crop season versus the decrease in total phenolic content of EVOOs stored for 12 months.

3.4 Markers for detection of EVOO ageing

Two phenolic compounds, hydroxytyrosol and oleocanthalic acid, increased their concentrations after storage for 12 months in contrast to secoiridoid derivatives. Nevertheless, this change did not quantitatively correspond to that experienced by secoiridoids, which means that other intermediates or final products are also formed. In this section, we studied the change in the concentration of these two phenols after storage by a paired t-test (p-value of <0.01) due to the concentration variability detected in the two time points. Both hydroxytyrosol and oleocanthalic acid increased their concentration significantly in EVOOs after storage for 12 months (p-value of <0.0001). As previously indicated, oleocanthalic acid has previously been proposed as a marker of EVOO ageing [21] formed by oxidation of oleocanthal. We confirmed this result in our study since oleocanthalic acid changed from a mean concentration of 0.46 to 4.4 mg/kg after 12 months storage. In fact, we found a linear correlation between oleocanthal levels in recently produced EVOOs and the increase in the concentration of oleocanthalic acid after storage (Supplementary Figure 2, R = 0.7335, p-value of <0.0001). Similarly, the mean concentration of hydroxytyrosol increased from 2.6 to 5.9 mg/kg after 12 months storage due to the degradation of hydroxytyrosol conjugated secoiridoids and their antioxidant properties (Supplementary Figure 1).

The high significance level found for both phenols is explained because they are typically detected at low concentration levels in recently produced EVOOs. With these premises, these alterations can be suitable markers to classify oils according to the crop season or to detect blends with aged EVOOs. To evaluate this classification performance, we carried out a univariate ROC analysis for both phenols to discriminate recently produced EVOOs and after storage for 12 months. Figure 3 shows the individual ROC curves for hydroxytyrosol and oleocanthalic acid, which were characterized by a high classification capability. Thus, the area under the curve (AUC) for oleocanthalic acid and hydroxytyrosol were 0.986 (0.972–0.995 confidence interval at 95%) and 0.824 (0.766–0.874 CI 95%), respectively. The box-and-whisker plots comparing concentration levels in the two situations reveal the high significance of these changes. As the increase in hydroxytyrosol and

oleocanthalic acid seems to be promoted by different pathways, we prepared a ROC model with the response of the two phenols (Figure 4). The AUC for this model was 0.966 (0.931–0.992 CI 95%) and the confusion matrix obtained by cross-validation reported 92.2 and 94.4% for the discrimination of recently produced EVOOs and after storage for 12 months.

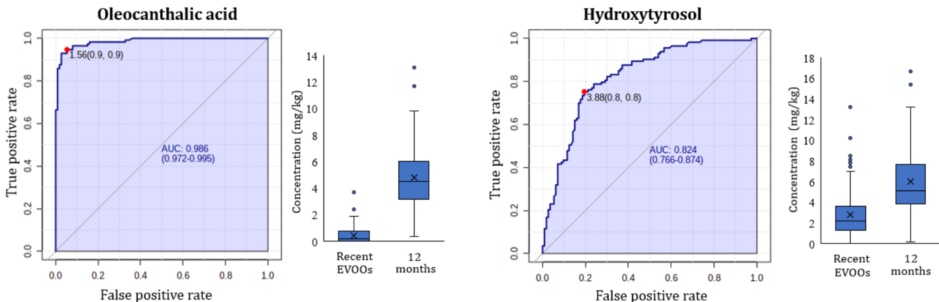
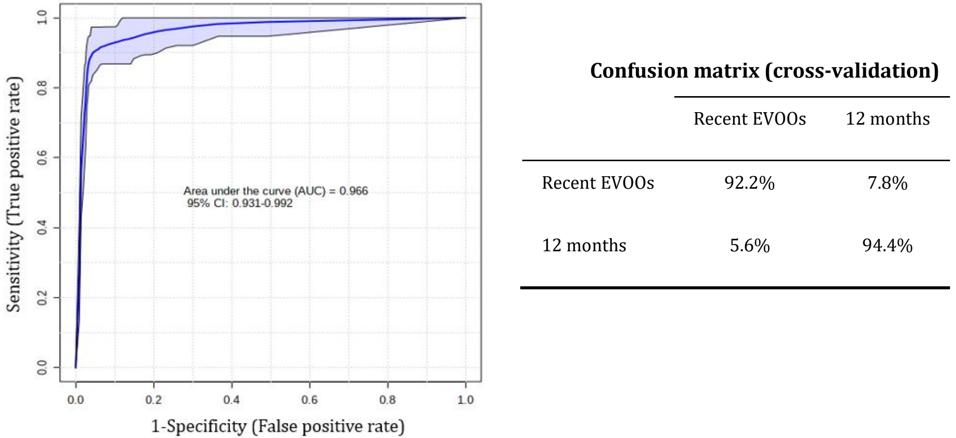


Figure 3. ROC curves that evaluate the capability of oleocanthalic acid and hydroxytyrosol to discriminate recently produced EVOOs and those stored for 12 months. Box-and-whisker plots comparing the concentration of both phenols in the two groups of EVOOs are also shown.



Confusion matrix (cross-validation)

	Recent EVOOs	12 months
Recent EVOOs	92.2%	7.8%
12 months	5.6%	94.4%

Figure 4. ROC curve that evaluates the combined capability of oleocanthalic acid and hydroxytyrosol to discriminate recently produced EVOOs and those stored for 12 months. The confusion matrix provided by cross-validation is also reported.

4. Conclusions

The degradation level of the phenolic content in 160 EVOOs stored in darkness at room temperature (20 °C) for 12 months was evaluated. The health benefits of EVOOs estimated by the total phenolic content were decreased 42.0 ± 24.3% after this storage period. The decrease in the concentration of total phenols in EVOOs was conditioned by the predominance of certain phenols such as oleacein and oleocanthal. Thus, EVOOs with oleacein and oleocanthal above 200 mg/kg were characterized by a large reduction of the total phenolic concentration.

On the other hand, levels of hydroxytyrosol and oleocanthalic acid were significantly increased after storage. These changes, associated to the degradation of main secoiridoids, allowed discriminating recently produced EVOOs from those stored for one year (0.966 AUC). These two phenols can be proposed as reliable markers to detect aged EVOOs and blends prepared with aged EVOOs.

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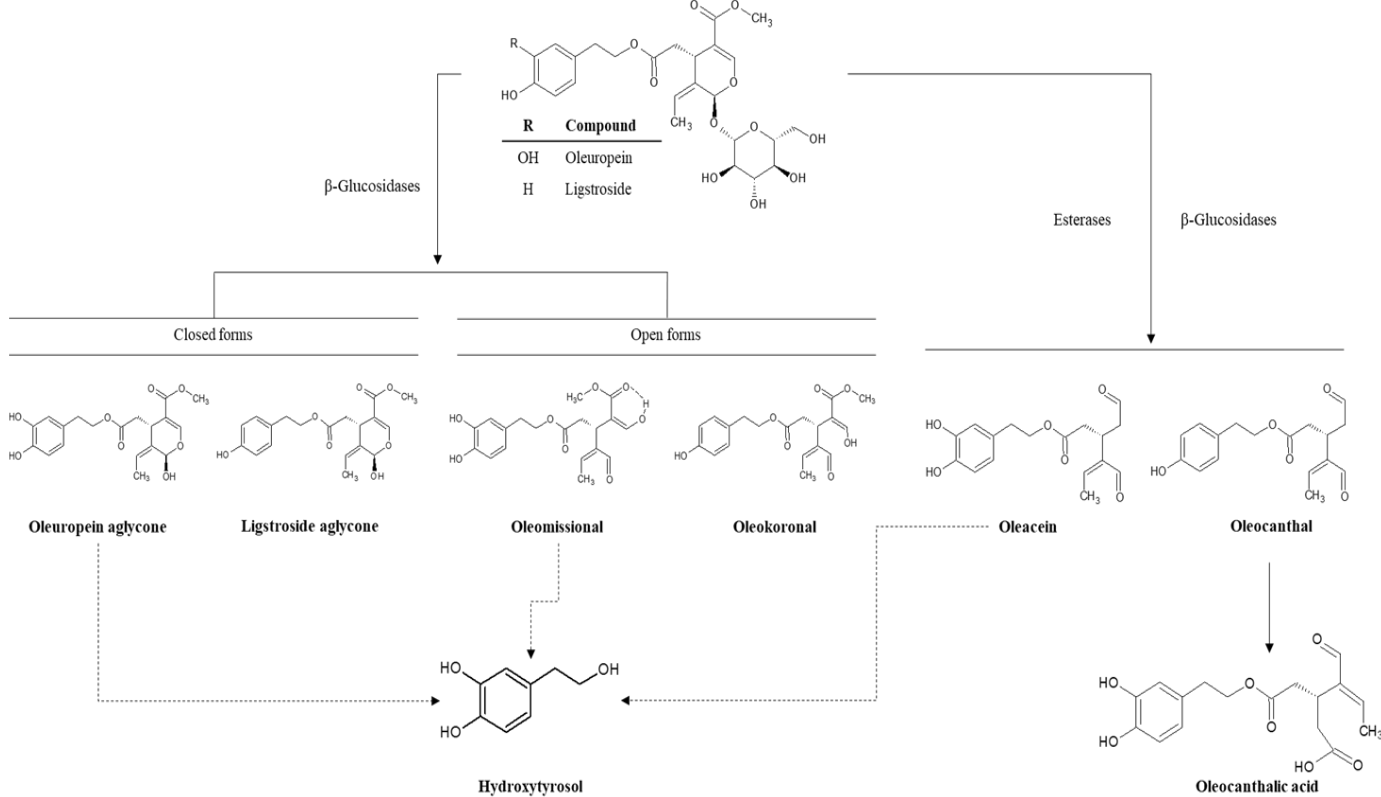


Supplementary Figure 1. Pathways for synthesis of main secoiridoid phenols.

Supplementary Table 1. LC–MS/MS parameters for determination of phenolic compounds in olive oil.

Compound	RT (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Tube lens (V)
Hydroxytyrosol	1.7	153	123	17	54
Syringealdehyde (IS)	2.8	181	151	22	68
Oleacein	3.7	319	69	39	62
Oleomissional	3.9	377	275	16	103
Oleocanthal	4.5	303	59	17	56
Oleokoronal	4.6	361	291	17	88
Oleocanthalic acid	4.7	319	200	17	103
Oleuropein aglycone	5.1	377	275	16	103
Ligstroside aglycone	5.1	361	291	17	88

[a]Retention Time

Supplementary Table 2. Calibration models prepared for quantitative analysis of target phenols.

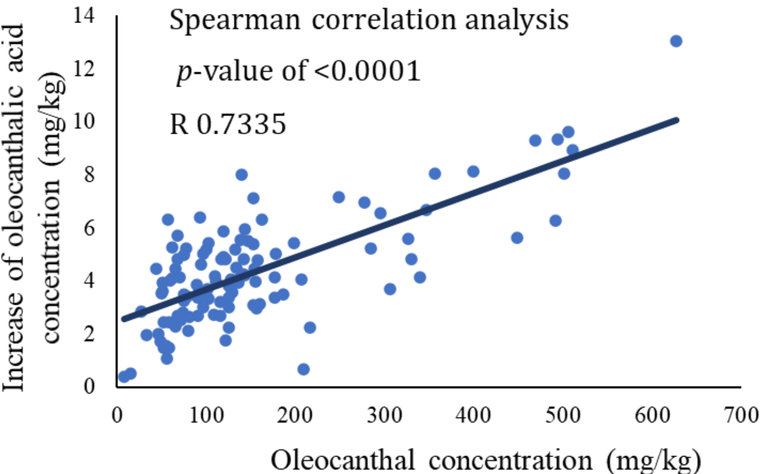
Compound	Calibration model	R^{2a}	Calibration range
Hydroxytyrosol	y = 65496 × ± 1912 + 38081 ± 19685	0.9932	1-20 mg/kg
Oleacein	y = 2661 × ± 42 –1739 ± 432	0.9980	1-20 mg/kg
Oleomissional	y = 3848 × ± 135 + 2418 ± 1386	0.9903	1-20 mg/kg
Oleocanthal	y = 262214 × ± 5473 + 138927 ± 56350	0.9965	1-20 mg/kg
Oleocanthalic acid	y = 116839 × ± 1773 + 46877 ± 18259	0.9982	1-20 mg/kg
Oleokoronal	y= 89717 × ± 2641 + 10403 ± 27195	0.9931	1-20 mg/kg
Oleuropein aglycone	y = 262214 × ± 5473 + 138927 ± 56350	0.9965	1-20 mg/kg
Ligstroside aglycone	y= 89717 × ± 2641 + 10403 ± 27195	0.9931	1-20 mg/kg

[a]Regression coefficient

Supplementary Table 3. Wilcoxon test (*p*-value of < 0.01) to detect significant differences in the initial phenolic profile of EVOOs according to the degradation of the total phenolic content after 12 months storage.

Compound (mg/kg)	*p*-value	-log10(p)	FDR[a]
Degradation of total phenols	5.22E-20	19.282	1.04E-18
Oleocanthal	1.22E-06	49.144	6.09E-5
Oleacein	5.72E-05	42.424	2.29E-04

[a]False discovery rate



Supplementary Figure 2. Correlation plot between the concentration of oleocanthal measured in EVOOs in the 2018/2019 crop season *versus* the increase of oleocanthalic acid detected in EVOOs stored for 12 months.

CHAPTER 3

Alteration of the phenolic fraction of extra virgin olive oil subjected to frying conditions

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Alteration of the phenolic fraction of extra virgin olive oil subjected to frying conditions

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Alteration of the phenolic fraction of extra virgin olive oil subjected to frying conditions.

Inmaculada Criado-Navarro, María Asunción López-Bascón, Carlos Augusto Ledesma-Escobar, Feliciano Priego-Capote

Abstract

Deep-frying in extra-virgin olive oil (EVOO) is an appreciated culinary practice that confers unique organoleptic features to fried food. However, high temperature (above 180 °C) contributes to deteriorate the health benefits of EVOO by accelerating degradation reactions. In this research, we measured the concentration of main phenolic compounds contributing to the health claim included in the European Commission (EU) 432/2012 in EVOOs subjected to frying for 90 min at 180ºC. For this purpose, four monocultivar EVOOs ('Arbequina', 'Cornicabra', 'Hojiblanca' and 'Picual') with different phenolic profile were selected to study the impact of frying on phenols. A significant decay in the phenolic content was observed for all EVOOs during frying process. In addition, we detected changes in the phenolic profile by conversion of open forms of oleuropein and ligstroside aglycones (oleomissional and oleokoronal) to oleacein and oleocanthal in the first frying cycles.

Keywords: Phenols; Virgin olive oil; Frying; Antioxidant; Health claim; Oleocanthal; Oleuropein aglycone.

Chemical compounds studied in this article: Hydroxytyrosol (PubChem CID: 82755); Oleacein (PubChem CID: 18684078); Oleocanthal (PubChem CID: 11652416); Oleuropein aglycone (PubChem CID: 56842347); Ligstroside aglycone (PubChem CID: 71718370); Luteolin (PubChem CID: 5280445); Apigenin (PubChem CID: 5280443); Oleocanthalic acid.

1. Introduction

The benefits of the Mediterranean diet are well-known [1, 2]. The PREDIMED study concluded that the Mediterranean diet supplemented with extra virgin olive oil (EVOO) reduces the risk of cardiovascular events in high-risk population [3]. The health benefits of (E)VOO (abbreviation used to refer to virgin olive oil and, particularly, to the extra-virgin category) are attributed to its balanced composition. This is divided into a major fraction (approximately ~98% of the total content) formed by fatty acid derivatives with predominant concentration of monounsaturated fatty acids (MUFAs), especially oleic acid (between 55 and 83% of the total fatty acids content), and a minor fraction that contains chemical families with recognized bioactivity such as phenols, phytosterols, tocopherols (vitamin E) and terpenes [4].

Phenolic compounds stand out in the minor fraction with special attention to secoiridoids because of their relative high concentration. These compounds are mainly produced during fruits ripening by conversion of oleuropein and ligstroside, but also during crushing and malaxation of the olive paste [5, 6]. The importance of phenols in (E)VOOs is mainly explained by three reasons. Firstly, several authors have found a strong relationship between particular organoleptic attributes such as pungency and bitterness, and the phenolic content [7-9]. Secondly, phenols are responsible for the only health claim included in the European Commission (EU) 432/2012, that is specific of olive oil. This, is attributable to olive oils providing a minimum mass of 5 mg of hydroxytyrosol, tyrosol and derivatives with a daily consumption of 20 g of product (in concentration units above 250 mg/kg) [10]. Thirdly, (E)VOO phenols are characterized by their antioxidant properties. According to Miho *et al.*, oxidative stability depends on the relative phenolic profile and not on the total concentration. This study explained that (E)VOOs with the same total phenolic content may have different oxidative stabilities [11].

As (E)VOO is consumed without any refining, it retains minor components, being distinguishable from other edible oils. [12].

The culinary use of (E)VOO is supported on its resistance to rancidity combined with a variety of flavours and distinct features. Many of these culinary practices demand no or mild processing conditions (addition to salads, marinades, sauces, dressings, dips), but there are also many applications based on the preparation of fried and baked or grilled foods [13,14].

It is consensual that (E)VOO quality is completely boosted when consumed directly, without any previous thermal treatment. Otherwise, when it is used as the cooking base, thermal effects promote compositional changes. Whether or not the bioactive components reach the consumer will depend on the culinary practice carried out in terms of time and temperature. The oil resistance depends on the antioxidant capacity of the phenolic compounds in combination with vitamin E, providing a balanced protection under thermal stress [15]. Under microwave heating, the temperature achieved are uncontrolled, and all vegetable oils are rapidly degraded. Under water boiling conditions, hydrolysis and leaching of phenols into the water phase is significant, but the heating time and the presence of food constitute the main contributor to their effective degradation [16,17]. Deep-frying, in which the food is totally immersed in hot oil, is the most common frying method in domestic food preparation, and particularly, in restaurants and food industry. Fried products have unique organoleptic properties, including flavour, texture, and appearance, which turn them largely enjoyed by consumers [18]. However, deep-frying is the most aggressive culinary technique for the oil, due to the high temperature and prolonged exposition time [19,20]. The oil sustains some physical changes such as the colour darkens, the viscosity increases, and smoke appears. Moreover, hydrolysis, oxidation, and polymerization of oil components are common chemical reactions in frying oil and produce volatile or non-volatile compounds. A great concern regarding new formed compounds under thermal stress, including oxidized fatty acids and sterols or triacylglycerols polymers, and their possible impact on human health is rising [21-23]. The intensity of these reactions is highly dependent on the type and quality of the oil used. Oils with a monounsaturated fatty acids profile show unique properties for frying, permitting the frequent replenishment of these oils to extend considerably their shelf life [24].

The most appropriate frying oil should be low in free fatty acids and polar compounds [25]. In addition, the presence of phenolic compounds could effectively inhibit thermo-oxidative degradation of frying oil as well as the formation of toxic thermo-oxidative degradation compounds such as acrylamide and heterocyclic amines [26,27]. With these premises, EVOO is a very interesting option when looking for a frying oil considering its composition rich in antioxidants (phenols and tocopherols) and its balanced fat profile. Understanding the role of phenolic compounds in improving the performance of frying oil contributes to the extension of its fry-life and quality maintenance of both oil and fried food. Some studies have evaluated the potential of adding phenolic extracts to vegetables oils to limit the negative effects of frying [28,29]. Nevertheless, the influence of frying process on the EVOO phenols has not been deeply studied. In this research, we have studied the changes in the absolute and relative phenolic content of EVOO following domestic deep-frying conditions. For this purpose, we selected four monocultivar EVOOs characterized by different phenolic profiles to correlate them with degradation patterns occurring after frying.

2. Materials and methods

2.1 Samples

A set of 4 monocultivar EVOOs produced in the 2019/2020 crop season were used in this research. Purposely, 'Hojiblanca', 'Cornicabra', 'Arbequina' and 'Picual' were the Spanish cultivars selected due to their different phenolic profile. The frying experiments were carried out four months after the production of EVOOs. Regulatory parameters were previously monitored to ensure the extra-virgin category (free acidity < 0.2% expressed as oleic acid; peroxide value < 10.0 meq O_2/kg; extinction indices K270 < 0.15 and K232 < 1.80).

2.2. Reagents and standards

MS-grade methanol (MeOH) and formic acid from Fischer Scientific (Hampton, NH, USA) and *n*-hexane from Scharlab (Barcelona, Spain) were used for analysis. Deionized water (18 MΩ • cm) was supplied by a Milli-Q purification system Millipore (Bedford, MA, USA).

Hydroxytyrosol and flavonoids apigenin and luteolin were purchased in Extrasynthese (Genay, France) and Sigma-Aldrich (Steinheim, Germany), respectively. Oleacein (3,4-DHPEA-EDA), oleocanthal (p-HPEA-EDA), oleuropein aglycone and ligstroside aglycone (both as monoaldehyde closed isomers), and oleocanthalic acid were provided by Prof. P. Magiatis (University of Athens, Greece).

Commercial standard solutions were prepared in MeOH while secoiridoid standards were prepared in acetonitrile to avoid undesired conversion to acetal and hemiacetal derivatives [30]. Syringaldehyde from Sigma-Aldrich (St. Louis, MO, USA) was used as internal standard (IS).

2.3. Apparatus and instruments

A 1-L domestic fryer with programmable temperature was used for deep-frying. A UltiMate 3000 series LC system coupled to a QqQ TSQ Quantum™ Access MAX detector (Thermo Scientific, Waltham, MA, USA) equipped with an electrospray ionization (ESI) source was used for phenolic analysis. The QqQ detector was equipped with an electrospray ionization (ESI) source.

2.4. Frying protocol

The fryer was filled with 1 kg of each EVOO heated to 180 ± 1 °C according to the recommendations reported by the Food and Agriculture Organization/World Health Organization (FAO/WHO). [31]. A total of 18 batches containing 20 g of pre-fried potatoes were fried for 5 min. Between consecutive batches the system was equilibrated for 1 min and temperature was periodically controlled to standardize the process. Oil aliquots (1.0 g) were sampled in triplicate at 10, 30, 45, 60, and 90 min of the frying process. EVOOs were also analyzed before heating to be used as reference. Thus, 18 aliquots of each EVOO were analyzed.

2.5. LC–MS/MS analysis of phenolic compounds

Phenolic compounds were analyzed by following a previously published protocol [32–34]. MS detection was performed in multiple reaction monitoring (MRM) mode. The MRM parameters for the analysis of target phenols are listed in Supplementary Table 1.

Calibration models were obtained by using refined sunflower oil aliquots spiked with multistandard phenolic solutions at variable concentration levels (1 to 20 μg/g, Supplementary Table 2). Oleomissional and oleokoronal, the monoaldehyde open isomers of oleuropein and ligstroside aglycone, were quantified by using the calibration models prepared with the corresponding monoaldehyde closed isomers.

The healthy index, estimated according to the Commission Regulation (EU) 432/2012, refers to the mass of phenols expressed in mg consumed with 20 g of oil daily intake. The *f* factor was calculated as the ratio between the sum of aglycone isomers of oleuropein and ligstroside and that of oleocanthal and oleacein. Complementarily, the *h* factor was calculated as the ratio between the sum of concentrations of hydroxytyrosol and tyrosol secoiridoid derivatives [11,33].

2.6. Statistical analysis

Analysis of variance (ANOVA) and multiple range test (Tukey, HSD, $p \geq 0.05$) were used to evaluate differences among samples using Statgraphics Centurion XVIII software (Statgraphics Technologies, Inc. 2018, USA).

3. Results and Discussion

3.1. Phenolic profile of monocultivar EVOOs

The phenolic profile of the four monocultivar EVOOs was measured in absolute terms. All monitored phenols except for the two flavonoids and oleocanthalic acid were used for estimation of the healthy index according to the EFSA declaration and Commission Regulation (EU) 432/2012 health claim. Table 1 shows the concentrations for individual phenols in EVOOs as well as the healthy index. Hydroxytyrosol concentration was at similar level in the four monocultivar EVOOs, from 4.0 mg/kg in 'Picual' to 6.6 mg/kg in 'Cornicabra'. The highest concentration of oleacein and oleocanthal was found in 'Arbequina' EVOO (153 and 130 mg/kg, respectively) followed by 'Hojiblanca' (71.0 and 91.3 mg/kg), 'Cornicabra', (25.4 and 41.0 mg/kg) and finally, 'Picual' (25.4 and 16.9 mg/kg). On the other hand, the highest level of oleuropein aglycone and its isomer,

oleomissional, was detected in ‘Cornicabra’ (281 and 234 mg/kg, respectively), followed by ‘Hojiblanca’ (200 and 144 mg/kg), ‘Picual’ (134 and 118 mg/kg) and ‘Arbequina’ (60.4 and 8.8 mg/kg). Similarly, the same trend was observed for ligstroside aglycone and its isomer oleokoronal. Thus, these two compounds were found at higher concentration in ‘Cornicabra’ (481 and 728 mg/kg) as compared to ‘Hojiblanca’ (375 and 348 mg/kg), ‘Picual’ (90.5 and 146 mg/kg) and, finally, ‘Arbequina’ (16.2 and 11.7 mg/kg). These concentrations agreed with measured levels in EVOOs obtained from the same cultivars produced in two consecutive agronomical seasons in Spain and, therefore, can be considered representative for these monocultivar EVOOs [32,34,35].

The variability in phenolic concentration was also reported in relative terms. Thus, we evaluated the phenolic profile by estimating the *f* factor. This parameter oscillated from 0.34 for ‘Arbequina’, with a marked concentration in oleacein and oleocanthal, to 26.0 for ‘Cornicabra’, with a predominant concentration in aglycone isomers of oleuropein and ligstroside. ‘Picual’ and ‘Hojiblanca’ gave intermediate values, 11.7 and 6.6, respectively. The *h* factor also provides information about the phenolic profile of monocultivar EVOOs since it points out the predominance of hydroxytyrosol or tyrosol derivatives in the phenolic profile. This factor ranged from 0.44 and 0.52 for ‘Cornicabra’ and ‘Hojiblanca’, which means that tyrosol derivatives predominated in these two EVOOs, to 1.11 and 1.44 for ‘Picual’ and ‘Arbequina’, which were more enriched in hydroxytyrosol derivatives.

Concerning flavonoids, apigenin and luteolin were quite similarly concentrated in ‘Arbequina’ (1.0 and 7.6 mg/kg, respectively), ‘Picual’ (1.3 and 6.2 mg/kg) and ‘Hojiblanca’ (1.0 and 5.3 mg/kg), while ‘Cornicabra’ reported the lowest levels (0.4 and 2.9 mg/kg) of these phenols.

Finally, we also monitored oleocanthalic acid to evaluate the oxidation of oleocanthal as a measurement of EVOO deterioration [36]. Previous studies have identified oleocanthalic acid as an oxidation marker but also as an EVOO ageing marker [33]. In this research, this compound was not detected in ‘Cornicabra’, ‘Hojiblanca’ and ‘Picual’ EVOOs, while trace level (below 1 mg/kg) was detected in

'Arbequina', which could be explained by its high oleocanthal content. Therefore, the practical absence of oleocanthalic acid is a marker of freshness in the four EVOOs.

Table 1. Phenolic concentrations (mean value and standard deviation) measured in the four monocultivar EVOOs before frying.

Compound (mg/kg)	Arbequina	Cornicabra	Hojiblanca	Picual
Hydroxytyrosol	5.4 ± 0.1	6.6 ± 0.1	6.1 ± 0.1	4.0 ± 0.1
Oleacein	153 ± 4	25.4 ± 0.5	71.0 ± 1	25.4 ± 3
Oleocanthal	130 ± 4	41.0 ± 3	91.3 ± 8	16.9 ± 2
Oleuropein aglycone	60.4 ± 0.5	281 ± 3	200 ± 8	134 ± 0.2
Oleomissional	8.8 ± 0.4	234 ± 3	144 ± 4	118 ± 0.2
Ligstroside aglycone	16.2 ± 1	481 ± 22	375 ± 29	90.5 ± 6
Oleokoronal	11.7 ± 1	728 ± 18	348 ± 10	146 ± 5
Luteolin	7.6 ± 0.01	2.9 ± 0.18	5.3 ± 0.1	6.2 ± 0.1
Apigenin	1.00 ± 0.02	0.40 ± 0.03	1.00 ± 0.05	1.30 ± 0.02
Oleocanthalic acid	0.8 ± 0.05	0.0	0.0	0.0
Total phenolic content	395 ± 9	1800 ± 49	1241 ± 58	542 ± 6
Healthy index (mg/20 g)	7.7 ± 0.2	35.9 ± 1	24.7 ± 1	10.7 ± 0.1
***f* factor**	0.34 ± 0.02	26.0 ± 1	6.6 ± 0.4	11.7 ± 1
***h* factor**	1.44 ± 0.05	0.44 ± 0.01	0.52 ± 0.01	1.11 ± 0.05

3.2. Alteration in the phenolic profile of monocultivar EVOOs during frying

The frying process was carried out in 5-min cycles for 90 min. Figure 1A shows the phenolic concentration measured in aliquots sampled at 10, 30, 45, 60, and 90 min for each EVOO. The degradation in total phenolic content (including the concentration of all measured phenols except for oleocanthalic acid) after the complete frying process ranged from 54.7% in 'Arbequina' to 77.9% in 'Hojiblanca'. The result obtained with 'Hojiblanca' EVOO agrees with that reported by Lozano-Castellón *et al.*, who showed a decrease of 75% in the total phenol content after pan-frying at 170 °C [37]. 'Cornicabra' and 'Picual' EVOOs experienced a degradation in total phenolic content around 72.5%. These results mean that phenolic degradation is intense during frying considering that phenols and tocopherols represent the most active barrier against oxidation. It is worth mentioning that phenolic degradation was especially severe after 10 min frying in 'Picual', 'Hojiblanca' and 'Cornicabra' with rates of 45.5, 51.8 and 52.3%, respectively. On the other hand, degradation was more attenuated in relative terms in 'Arbequina' EVOO with a value

of 18.4% at the same time. The patterns observed for 'Arbequina' and 'Picual' EVOOs were previously described by Abenoza *et al.* and Allouche *et al.* [22,38], who reported a lower relative degradation of phenols in 'Arbequina' EVOO as compared to 'Picual' EVOO after the first frying cycles.

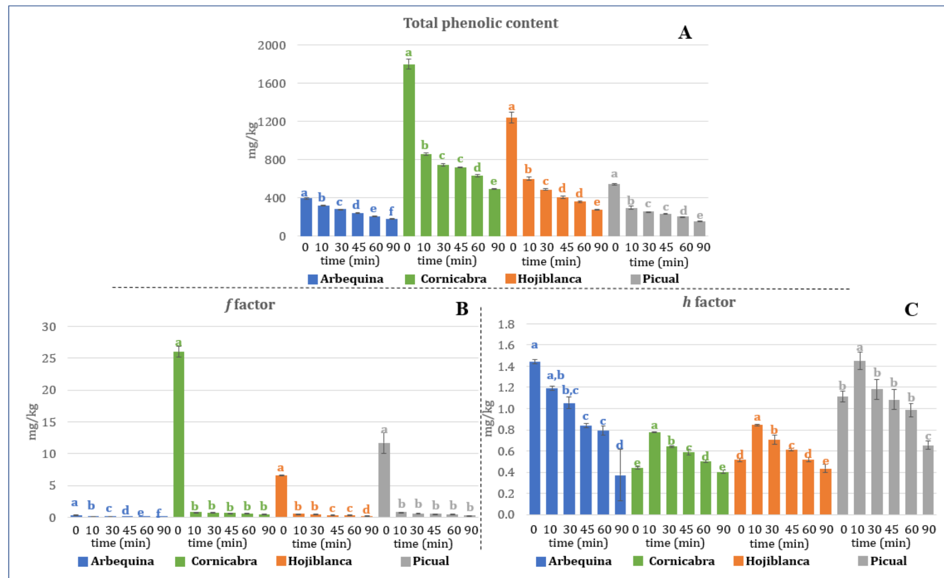

Figure 1. Variation in the total phenolic content (A), *f* factor (B) and *h* factor (C) in the four monocultivar EVOOs subjected to frying by analysis of aliquots sampled at 0, 10, 30, 45, 60, and 90 min.

We further evaluated the impact of frying on the phenolic profile of EVOOs. For this purpose, we studied the variations in the *f* and *h* factors. Thus, the *f* factor experienced a substantial decrease for all EVOOs. 'Cornicabra' EVOO passed from a f factor of 26.0 to 0.51, 'Picual' EVOO from 11.7 to 0.30, 'Hojiblanca' from 6.6 to 0.23 and 'Arbequina' from 0.34 to 0.004 (Figure 1B). These results pointed out that frying especially influenced the concentration of aglycone isomer forms of oleuropein and ligstroside attending to the *f* factor definition. On the other hand, the *h* factor was affected in the frying process as a function of the cultivar. Thus, in 'Arbequina' and 'Picual' EVOOs, more enriched in hydroxytyrosol derivatives, the *h* factor changed to 0.37 and 0.65, respectively. Therefore, phenolic degradation substantially affected to hydroxytyrosol derivatives. These results are consistent with the higher antioxidant capacity described for hydroxytyrosol and derivatives as compared to that of the tyrosol family [39,40]. On the other hand, in 'Cornicabra' and 'Hojiblanca'

EVOOs, the initial *h* factor was 0.44 and 0.52, respectively, and after the frying process this changed to 0.40 and 0.44, respectively (Figure 1C). Nevertheless, it seems that the phenolic profile especially changed in these two cultivars during the frying process. Thus, in 'Cornicabra' the *h* factor increased to 0.78 after 10 min frying, while in 'Hojiblanca' this parameter was increased up to 0.84 in the same time period. With these premises, there should be phenolic conversions during frying, which could promote other chemical reactions such as hydrolysis or oxidations accelerated by heating. Additionally, the higher stability of tyrosol derivatives means a lower protecting effect against oxidative reactions as compared to hydroxytyrosol derivatives [28].

3.3. Alteration of individual phenols in monocultivar EVOOs during frying

After overall evaluation of the frying effect on phenolic profiles of monocultivar EVOOs, in the next step we monitored the concentration changes experienced by main individual phenols (Figure 2), which agrees with other previous studies [40,41]. Special attention was paid in this study to secoiridoid derivatives such as oleacein, oleocanthal, and the aglycone isomers of oleuropein and ligstroside. A significant and progressive concentration decay was found for oleuropein aglycone and ligstroside aglycone in all monocultivar EVOOs. This decrease was particularly severe after 10 min frying. In absolute terms, the concentration decrease observed after the two first frying cycles was significant for 'Hojiblanca' and 'Cornicabra'. In fact, these two cultivars reported a decrement in ligstroside aglycone levels from 375 and 481 mg/kg up to 78.2 and 145 mg/kg, respectively. 'Picual' and 'Arbequina' reported a concentration decay for this phenol from 90.5 and 16.1 mg/kg to 22.3 and 5.3 mg/kg, respectively. In relative terms, all monocultivar EVOOs provided a similar performance after 10 min frying for ligstroside aglycone because its concentration was decreased in averaged terms 73.0 ± 5%. A common pattern was found for oleuropein aglycone, but the concentration decrease was lower for all cultivars (32.0 ± 17%). An opposite trend was obtained by Esposto et al. for 'Coratina' EVOO, where the ligstroside aglycone degradation was slightly lower than that found for oleuropein aglycone, which stands out the cultivar influence [28]. 'Arbequina' EVOO led to the highest

concentration decrease for oleuropein aglycone (53.8%) after 10 min. A drastic total degradation was observed for these two aglycone isomers after 90 min frying with average decrease levels of 89.5 ± 7% for ligstroside aglycone and 86.4 ± 9% for oleuropein aglycone. For both compounds, the highest degradation was measured in 'Arbequina' EVOO (around 95%) whereas the lowest degradation was found in 'Cornicabra' EVOO (around 78%).

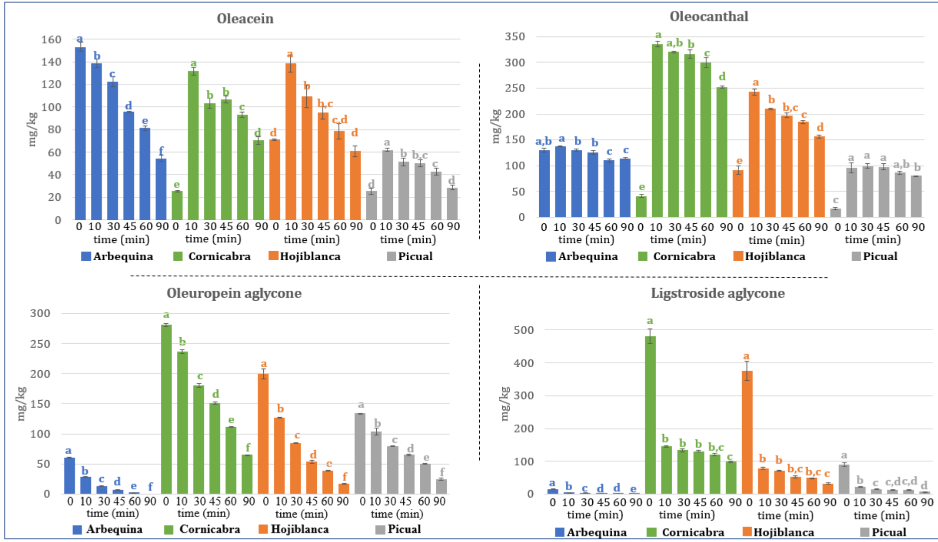

Figure 2. Variation in the concentration of the main secoiridoid derivatives in the four monocultivar EVOOs subjected to frying by analysis of aliquots sampled at 0, 10, 30, 45, 60, and 90 min.

Degradation rate was substantially fast for the other open isomers of the aglycone forms, oleomissional and oleokoronal. In fact, these two compounds were completely degraded after 10 min frying, even in 'Cornicabra' EVOO, where one of these compounds was found at concentration above 700 mg/kg (Supplementary Figure 1). This result can be associated to the concentration variations found for oleocanthal and oleacein after 10 min frying. Thus, 'Hojiblanca', 'Cornicabra' and 'Picual' EVOOs increased their oleocanthal concentration from 91.3, 41.0 and 16.9 mg/kg to 243, 335 and 95.7 mg/kg, respectively, which means increment of 151.7, 294 and 78.8 mg/kg, respectively. These variations fit the initial content of oleokoronal in these monocultivar EVOOs, being detected at the highest concentration in 'Cornicabra', followed by 'Hojiblanca' and 'Picual'. The analysis of

aliquots sampled during the frying process for these three EVOOs revealed a relatively small variation in the concentration of oleocanthal.

Oleacein reported the same pattern visualized for oleocanthal, observing an initial concentration increase and then, a lowering tendency until the end of the frying process as reported in other studies [17,38]. Thus, oleacein concentration significantly increased in 'Hojiblanca', 'Cornicabra' and 'Picual' EVOOs subjected to 10 min frying while no increase was observed in 'Arbequina'. Again, the highest increase was found in 'Cornicabra' followed by 'Hojiblanca' and 'Picual', which provided initial contents of oleomissional of 234, 144 and 118 mg/kg, respectively.

Conversion of the pair oleomissional/oleokoronal to oleacein/oleocanthal is based on a simple hydrolysis that would be enhanced by frying conditions in terms of temperature and humidity (Supplementary Figure 2). We calculated the conversion rate by plotting mmoles decreased of oleomissional/oleokoronal *versus* mmoles increased of oleacein/oleocanthal for the three monocultivar EVOOs. As Figure 3 shows, a high correlation was observed with a correlation coefficient (R) of 0.9952 (regression coefficient R^2 0.9905). This evaluation allowed estimating the average conversion rate that was 51.7 ± 9% after 10 min frying.

Oleacein and oleocanthal were partially degraded during the frying process. Nevertheless, degradation was not complete and after frying for 90 min oleacein was detected from 28.5 mg/kg in 'Picual' up to 70.4 in 'Cornicabra', and oleocanthal from 80.2 mg/kg in 'Picual' to 252 mg/kg in 'Cornicabra'. It is worth mentioning that the highest initial concentrations of oleacein and oleocanthal were detected in 'Arbequina' EVOO (153 and 130 mg/kg, respectively), but after 90 min frying both phenols were more concentrated in 'Hojiblanca' and 'Cornicabra' EVOOs (60.8 and 70.4 mg/kg for oleacein, 156 and 252 mg/kg for oleocanthal, respectively).

Flavonoids apigenin and luteolin decreased their concentration significantly during the frying process for 90 min. Allouche *et al*. pointed out a slower degradation for apigenin as compared to luteolin. This difference may be attributed to the structure, since their antioxidant activity is correlated with the number of phenolic hydroxyl groups in the molecule [38]. Nevertheless, the

percentage of decrement did not surpass 45% in any EVOO (Supplementary Figure 1). The same trend was observed for hydroxytyrosol. Despite hydroxytyrosol concentration increases in VOOs with ageing [28], theoretically by degradation of hydroxytyrosol derivatives, this effect was not found during frying in the selected 90 min period.

The opposite trend was found for oleocanthalic acid in all oils (Figure 4). This phenol, obtained by oxidation of oleocanthal, increased its concentration with frying time. In fact, oleocanthalic acid was almost not detected in the non-treated EVOOs, except for trace levels in 'Arbequina', but its concentration ranged from 11.9 mg/kg in 'Picual' up to 26.9 mg/kg in 'Hojiblanca' after frying for 90 min. In all cases, there was a progressive increase from the first frying cycles. Apart from being a marker of olive oil ageing, oleocanthalic acid can be proposed as a marker of temperature alteration and frying exposure.

3.4. Impact of the frying process on the healthy index of monocultivar EVOOs

The healthy properties associated to the presence of phenolic compounds in monocultivar EVOOs were influenced by the frying process. The four EVOOs were characterized by concentrations of hydroxytyrosol and tyrosol derivatives above 250 mg/kg before frying (Table 1). This concentration is equivalent to an intake of 5.0 mg of these phenols with a daily consumption of 20 g of oil. Particularly, this parameter ranged from 7.7 mg/20 g oil for 'Arbequina' to 35.9 mg/20 g oil for 'Cornicabra'. Intermediate values were obtained for 'Picual' and 'Hojiblanca' EVOOs, 10.7 and 24.7 mg/20 oil, respectively.

The effect of frying on the healthy index depended on the initial phenolic profile. Thus, after 90 min frying only 'Hojiblanca' and 'Cornicabra' EVOOs kept the phenolic content above the preset limit, and the healthy index was decreased up to 5.4 and 9.8 mg/20 g oil, respectively. On the other hand, the healthy benefits of 'Picual' and 'Arbequina' EVOOs were affected by frying process and, after 90 min, both oils reported phenolic contents below those established by the EFSA to recognize health effects associated to consumption (Figure 5). In relative terms, the three EVOOs with an initial phenolic content above 500 mg/kg (equivalent to a

healthy index above 10 mg/20 g oil) experienced a decrease of healthy index superior to 70%, while 'Arbequina' EVOO provided a decay above 50%.

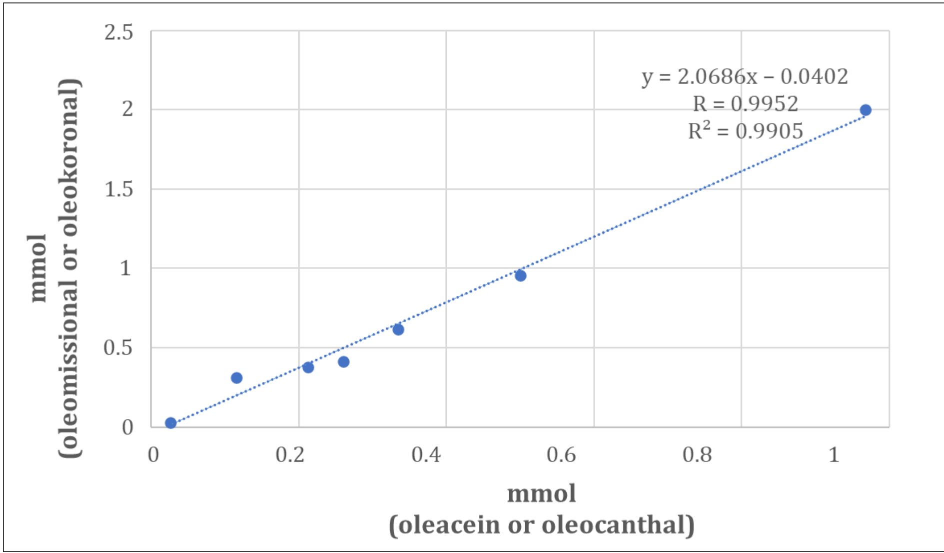

Figure 3. Correlation plot between the amount of oleomissional or oleokoronal decreased in EVOOs after frying for 10 min versus the amount of oleacein or oleocanthal increased in EVOOs in the same treatment period.

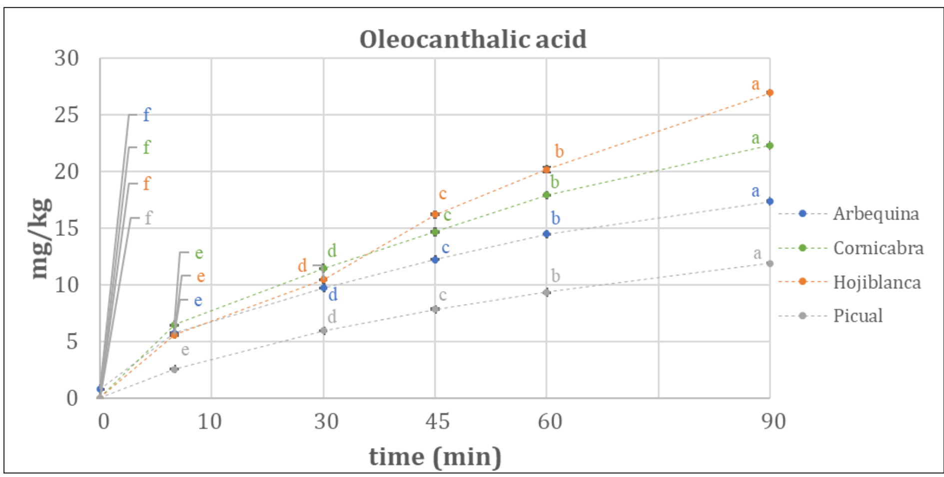

Figure 4. Variation in the concentration of oleocanthalic acid in the four monocultivar EVOOs subjected to frying by analysis of aliquots sampled at 0, 10, 30, 45, 60, and 90 min.

Previous studies pointed out that phenolic concentration can decrease around 42.0 ± 24% after 12 months [33]. Thus, a high initial phenolic concentration increases considerably the possibilities to take benefits from EVOO consumption. The same conclusion can be derived from this study. Frying is an aggressive cooking practice due to high temperature. Therefore, to preserve the healthy properties it is recommended to use EVOOs with high healthy index.

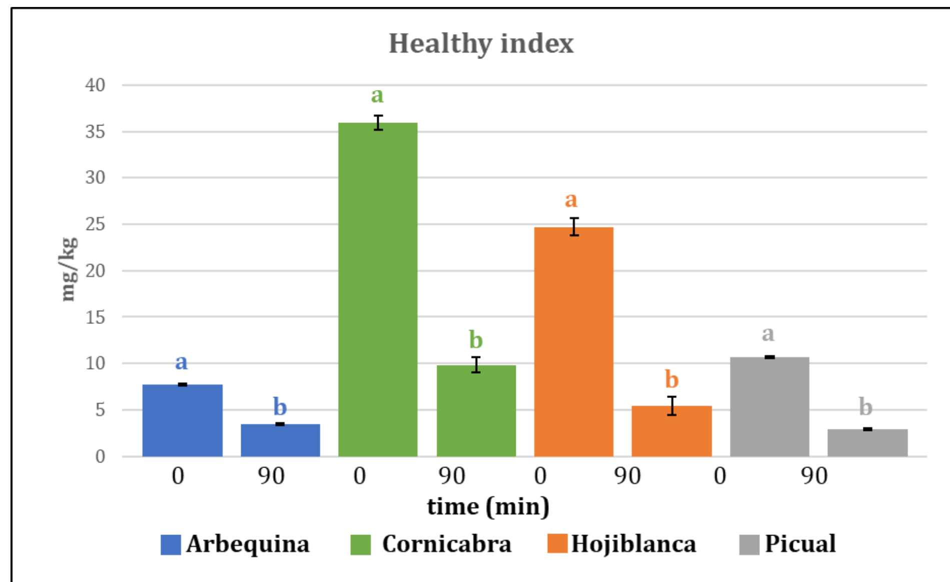

Figure 5. Change in the healthy index in the four EVOOs subjected to frying for 90 min as compared to EVOOs before heating.

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Abbreviations Used

(E)VOO, extra virgin olive oil; EU, European Union; MUFA, monounsaturated fatty acid; MeOH, methanol; IS, internal standard; LC, liquid

chromatography; MS/MS, quadrupole–time-of-flight detector; QqQ, triple quadrupole; ESI, electrospray ionization; FAO, food and agriculture organization; WHO, world health organization; MRM, multiple reaction monitoring; FWHM, full width at half maximum; EFSA, European Food Safety Authority; ANOVA, analysis of variance; HSD, honestly significant difference.

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Supplementary Table 1. LC–MS/MS parameters for determination of phenolic compounds in olive oil.

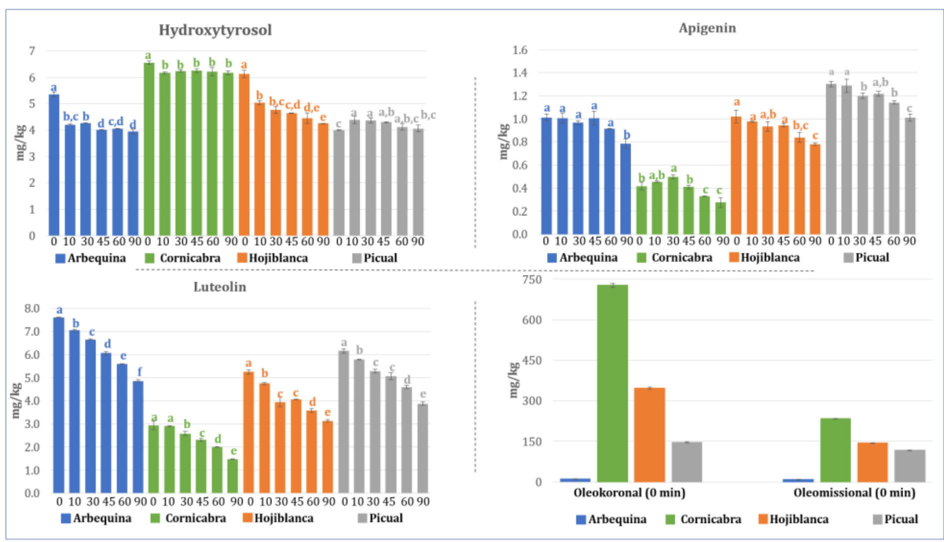
Compound	RT[a] (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Tube lens (V)
Hydroxytyrosol	1.7	153	123	17	54
Syringealdehyde (IS)	2.8	181	151	22	68
Oleacein	3.7	319	69	39	62
Oleomissional	3.9	377	275	16	103
Oleocanthal	4.5	303	59	17	56
Oleokoronal	4.6	361	291	17	88
Oleocanthalic acid	4.7	319	200	17	103
Oleuropein aglycone	5.1	377	275	16	103
Ligstroside aglycone	5.1	361	291	17	88
Luteolin	5.5	285	133	35	103
Apigenin	6	269	117	35	70

[a]Retention time

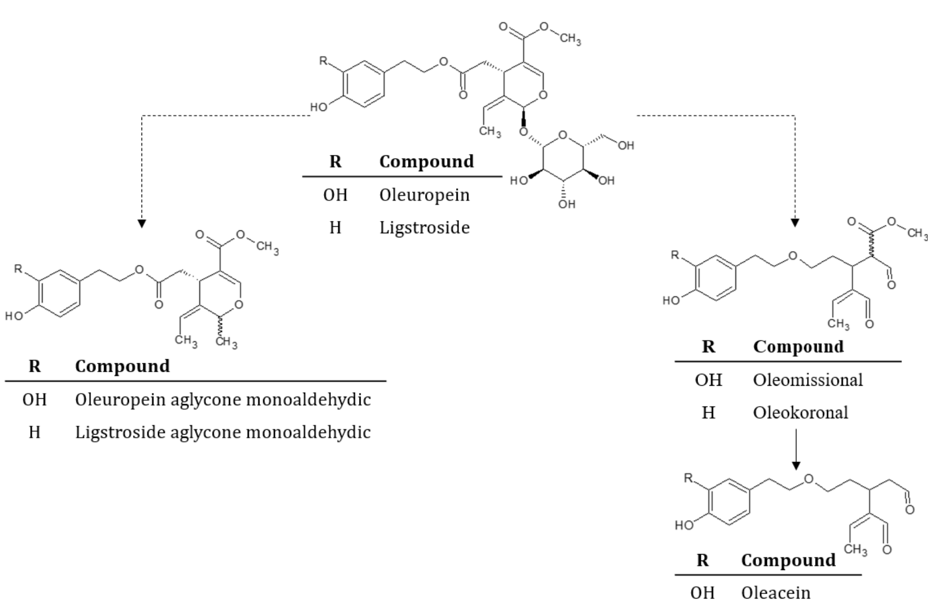
Supplementary Table 2. Calibration models prepared for quantitative analysis of target phenols.

Compound	Calibration model	R^{2a}	Calibration range (mg/kg)
Hydroxytyrosol	y = (66019 ± 1276) x + 16386 ± 13139	0.9970	1-20
Oleacein	y = (1762 ± 36) x– 1802 ± 368	0.9967	1-20
Oleocanthal	y = (2375 ± 83) x + 945 ± 850	0.9904	1-20
Oleocanthalic acid	y = (992116 ± 2062) x + 54197 ± 21226	0.9982	1-20
Oleuropein aglycone	y = (101303 ± 1926) x + 25928 ± 49832	0.9971	1-20
Ligstroside aglycone	y = (11064 ± 297) x + 3914 ± 3060	0.9943	1-20
Luteolin	y = (139025 ± 6430) x + 266806 ± 66407	0.9852	1-20
Apigenin	y= (89380 ± 3414) x + 135534 ± 35257	0.9899	1-20

[a]Determination coefficient



Supplementary Figure 1. Variation in the concentration of other phenols in the four monocultivar EVOOs subjected to frying by analysis of aliquots sampled at 0, 10, 30, 45, 60, and 90 min.



Supplementary Figure 2. Mechanism proposed for the formation of main secoiridoid derivatives in olive oil [6]. The main alteration detected in the initial frying cycles (10 min) was the quantitative conversion of oleomissional/oleokoronal to oleacein/oleocanthal.

CHAPTER 4

Determination of glycerophospholipids in vegetable edible oils: Proof of concept to discriminate olive oil categories

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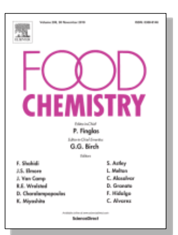

Determination of glycerophospholipids in vegetable edible oils: Proof of concept to discriminate olive oil categories

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Abstract

Glycerophospholipids (GPLs) constitute a chemical family within the saponifiable fraction of vegetable oils. GPLs have been scarcely studied in edible oils owing to the lack of sensitive and selective analytical methods. We have developed a method for identification, confirmation and relative quantitation of GPLs in vegetable oils. The method is based on solid-phase extraction (SPE) for isolation of GPLs and determination by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). As proof of concept, the approach has been applied to characterize GPLs in different olive oil categories, thus revealing compositional changes, which could be explained by factors such as the quality of fruits and the extraction process. Families such as glycerophosphatidic acids and phosphatidylglycerides are remarkable because of their capability to discriminate virgin olive oils from the rest of categories. These results open a door to additional studies targeted at the identification of olive oil quality by monitoring these lipids.

Keywords: Glycerophospholipids; Vegetable oils; Extra-virgin olive oil; Virgin olive oil; Refined olive oil; Mass spectrometry; Liquid chromatography.

1. Introduction

The saponifiable fraction of vegetable edible oils is formed by different chemical families, namely, triglycerides, diglycerides, monoglycerides, glycerophospholipids (GPLs), sterols of fatty acid esters, and free fatty acids [1,2]. This fraction is typically characterized by estimation of the fatty acids profile using a relative quantitation strategy [3,4]; therefore, no discrimination among the different families is carried out.

GPLs (also known as phospholipids —PLs), constitute a particular class of membrane components with a glycerol backbone, in which two hydroxyl groups are esterified by fatty acids, whereas the third hydroxyl group is phosphorylated. The phosphate group can be esterified by several polar molecules, particularly amino alcohols and alcohols such as ethanolamine, choline, inositol or glycerol, among others. GPLs play a key role both in the structure of the cell membranes and in various processes linked to the functioning of these membranes such as selective permeability, active transportation, and electrical conductivity [5,6].

GPLs are present in vegetable edible oils by their transfer to the oil phase during the extraction process. According to Meng *et al.*, GPLs concentration in vegetable oils ranges from 1 to 18 g/kg [7]. Despite their presence, the studies on characterization of GPLs in vegetable oils are scant. There are some evidences on the biological activity of GPLs such as the influence on the antioxidant capacity and oxidative stability [8-10] GPLs seem to exert a synergistic role on the oxidative stability of edible oils since they allow regeneration of other antioxidants such as phenols or tocopherols [11] by donation of hydrogen atoms from amino groups. Therefore, the main antioxidant effect has been observed for GPLs with amino alcohols as polar groups, particularly phosphatidylcholines (PCs), phosphatidylethanolamines (PEs) and phosphatidylserines (PS) [12]. On the other hand, excessive residual concentration of GPLs could impact negatively on the quality of oil during refining. Thus, GPLs could be responsible for several undesired processes such as oil losses due to emulsion formation during alkali treatment, decolouration during deodorization/steam distillation, and reduced smoke point of the end product [13].

GPLs are typically determined as total phosphorous content by using official methods such as the AOCS Ca 12-55, which is extensively used to determine total GPLs in oils and dietary supplements. This method is based on colorimetric analysis of phosphorous after acid digestion. The method is not specific for GPLs *per se*, although most phosphate present in vegetable oils is in form of GPLs. Furthermore, this method is based on a tedious and time-consuming procedure that requires between 5 and 9 h [13]. Hatzakis *et al.* developed in 2008 a method based on ^{31}P nuclear magnetic resonance (NMR) for quantitative determination of GPL families in olive oil [14]. Additionally, relative quantitation of fatty acids was also possible with this method. A first approach dealing with PLs profiling using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) was developed by Boukhchina *et al.* in 2004 [15]. The method was applied to determine GPLs in edible oils such as rapeseed, olive, almond and sunflower oils. The number of GPLs identified in the analyzed oils was small, probably owing to the lack of sensitivity of the proposed method, but it reported relative quantitation of the detected GPL families. Verardo *et al.* proposed a method based on solid-phase extraction (SPE) and LC–MS/MS for determination of GPLs in olive oil. They identified 13 GPLs and quantified in relative terms the total content of GPLs, which resulted in 8.25 mg/kg [16]. This content is close to that provided by Koidis & Boskou, who reported concentration levels from 21 to 124 mg/kg [9]. More recently, Alves *et al.* detected five classes of GPLs in olive oil samples by SPE and LC–MS/MS, which allowed the identification of 18 GPLs, mostly, PCs [17].

The objective of this research was to propose a method to characterize the GPL fraction in edible vegetable oils. The method is based on an SPE protocol for selective isolation of GPLs from edible oils with further determination by LC–MS/MS in multiple reaction monitoring mode (MRM). This combination is adopted to attain the high sensitivity and selectivity required to detect GPLs in the presence of more concentrated fractions as triglycerides. As proof of concept, the method has been applied to four commercial categories of olive oil for comparison of their GPL profiles. These categories have been extra virgin olive oil (EVOO), virgin olive oil (VOO), olive oil (OO) and pomace olive oil (POO). EVOO is obtained from olive fruits

collected under sanitary conditions that lead to the product with the highest quality (acidity below 0.8% and no organoleptic defects). The extraction conditions should also be controlled to avoid undesired alterations. VOO is obtained from olive fruits collected under good conditions, but the product is characterized by slight organoleptic defects as compared to EVOO owing to some deficiency in the fruits state or unsuited processing. OO is mainly prepared from lampante olive oil subjected to refining. Lampante olive oil is obtained either from low quality olive fruits or after an unsuited processing. This oil is refined and then mixed with a low proportion of EVOO or VOO to give color, odor and taste to the final OO product. Finally, POO is obtained from crude pomace oil subjected to refining to avoid undesired organoleptic features. By analogy to OO, the refined pomace oil is mixed at low concentration with EVOO or VOO to improve the final POO product [18]. The determination of GPLs in these samples has involved identification and confirmatory analysis of GPLs, and semiquantitative analysis to compare their GPL profiles.

2. Materials and methods

2.1. Reagents and samples

Mass-spectrometry grade methanol (MeOH) and formic acid from Scharlab (Barcelona, Spain) were used for sample treatment and also to prepare the chromatographic mobile phases. Acetonitrile (ACN), ammonia and HPLC grade chloroform were also from Scharlab and MS-grade 2-propanol was from Fischer (Madrid, Spain). Deionized water (18 MΩ • cm) from Milli-Q water purification system was used to prepare the chromatographic phases.

Five bottles of sunflower oil (SO) and high-oleic sunflower oil (HOSO) from different producers were purchased in local supermarkets. Additionally, four commercial categories of olive oil were studied in this research. They encompassed EVOO, VOO, OO (refined olive oil mixed with EVOO or VOO at proportions typically ranging from 5 to 15% v/v) and POO (refined pomace oil mixed with EVOO or VOO at proportions typically ranging from 5 to 15% v/v). Five producers were selected

for each category, which means a total number of 60 samples (4 categories × 5 producers × 3 replicates).

2.2. Apparatus and instruments

A vacuum manifold (Supelco, PA, USA) with disposable liners (Supelco, PA, USA) was used for SPE as strategy for isolation of GPLs from vegetable oils. A speed-vac ConcentratorPlus from Eppendorf Ibérica (Madrid, Spain) was used to evaporate the MeOH phase after SPE elution to concentrate the GPL fraction.

Chromatographic separation was carried out with a 1200 Series Agilent (Palo Alto, CA, USA) LC system furnished with a Poroshell 120 EC-C8 (2.7 µm particle size, 4.6×100 mm i.d.) analytical column from Agilent. A 120 EC-C8 (2.7 µm particle size, 4.6×5 mm i.d.) guard column, also from Agilent, was used to preserve the integrity of the analytical column. The LC system was coupled to a 6460 Agilent triple quadrupole mass detector (QqQ) furnished with an Agilent Jet Stream Technology electrospray ion source (ESI).

Agilent MassHunter Workstation (V-B.05) was the software for data acquisition, qualitative and quantitative analysis.

2.3. Sample preparation

GPLs were extracted from oil samples using 30 mg HybridSPE® phospholipid technology cartridges from Supelco. The protocol recommended by the manufacturer was adapted to the analysis of non-polar samples as vegetable oils. A 1-g aliquot of each oil was mixed with 500 µL of $CHCl_3$ for 30 s to prepare the loading solution. Vacuum was applied to the SPE manifold to favor the pass of the loading solution through the SPE sorbent cartridge, which was previously conditioned with 1 mL of MeOH acidified with 0.1% formic acid (v/v). Then, the cartridge was washed three times with 500 µL of 2-propanol. GPLs were eluted in alkaline medium using 1 mL of MeOH with 5% (v/v) ammonium hydroxide. The eluted fraction was evaporated, and the residue was reconstituted with 50 µL of MeOH with 0.1% (v/v) formic acid prior to injection into the LC–QqQ MS/MS system. Three replicates per olive oil were analyzed.

2.4. LC–MS/MS analysis of glycerophospholipids

The chromatographic mobile phases were water (phase A) and 90/10 (v/v) MeOH/2-propanol (phase B), both phases acidified with 0.1% (v/v) formic acid as ionization agent. The LC pump was programmed with a flow rate of 0.6 mL/min and the elution gradient was started at 60% phase B, which was kept as initial mobile phase for 5 min; then, phase B was linearly increased up to 100% from min 5 to 15 min, which was kept for 5 min. A post-run time of 5 min was set to equilibrate the initial conditions for the analysis of the next sample. The injection volume was 5 µL and the injector needle was washed between injections with ACN.

MS detection was performed by MS/MS using different acquisition modes —*viz.*, multiple reaction monitoring (MRM), precursor ion scanning (PIS) and neutral loss scanning (NLS). In all cases, the ESI parameters were set as follows: gas temperature, 325 °C; drying gas, nitrogen 10 L/min; nebulizer pressure, 50 psi; sheath gas temperature, 300 °C; sheath gas flow, 12 L/min; capillary voltage, 2000 V; and nozzle voltage, 1500 V.

2.5. Identification and confirmatory analysis of glycerophospholipids

Tentative identification of the different GPL families was carried out by LC–MS/MS analysis of the reconstituted eluate in PIS and NLS acquisition modes. For this purpose, representative product ions and neutral losses generated by fragmentation of the GPL families were monitored. Additionally, the identity of each GPL was confirmed by MRM by monitoring representative product ions corresponding to the fatty acids from each precursor.

2.6. Quantitative analysis of glycerophospholipids

Once the presence of different families of GPLs was confirmed in the edible oils, a quantitative analysis of each reconstituted eluate was planned by using MRM mode due to its higher sensitivity as compared to other acquisition methods.

Quantitative analysis of GPLs in relative terms was carried out by selecting the most sensitive transition for each compound. Quantitative results were

supported on the peak area for each GPL, which was obtained by triplicate analysis of each reconstituted eluate.

2.7. Data treatment

MetaboAnalyst (www.metaboanalyst.ca) (version 4.0) was used for statistical analysis, visualization and interpretation of results. Quantitative data were normalized by log transformation and scaled. Variability among groups of samples was checked by Tuckey post-hoc analysis of variance (ANOVA) with 95% confidence level. Principal Component Analysis (PCA) was tested to identify the main GPL families reporting significant differences between classes of edible oils.

3. Results and Discussion

3.1. Solid-phase extraction for isolation of glycerophospholipids

Isolation of GPLs from edible oils using SPE has been mainly carried out with different generic sorbent materials such as amino, diol or silica phases. Among them, Verardo *et al.* obtained recoveries close to 100% with a diol sorbent for different GPLs families [16]. In this research, we selected zirconia coated silica as SPE sorbent for isolation of GPLs due to its high selectivity. This sorbent allows setting a selective Lewis interaction between Zr atoms and the phosphate group, which is not produced with any other lipid family. Additionally, the cartridge possesses a hydrophobic membrane for partial retention of neutral lipids such as acylglycerides, the most concentrated family in edible oils. This sorbent has been used for characterization of GPLs in plasma and serum [19-21]. Also, zirconia coated silica has been used for isolation of phospholipids in food samples. One example is the application of this sorbent to samples collected in the butter production chain, but it offered lower extraction efficiency as compared to C8 and silica [22]. Alves *et al.* used the zirconia coated silica sorbent for determination of GPLs in EVOO and VOO samples [17]. However, the detection coverage was limited to 18 GPLs and experimental variability was not estimated.

The protocol recommended by the manufacturer was adapted for determination of GPLs in edible oils with some modifications. The oil sample was mixed with chloroform to reduce viscosity. As washing solvent, 2-propanol was

used to remove interferents due to the non-polar character of the sample matrix, mainly constituted by acylglycerides (~98%). The elution of GPLs is carried out by MeOH with 5% (v/v) ammonium hydroxide, which is the solution proposed by the manufacturer. The two most critical steps in the SPE process (the washing and elution steps) were evaluated for quantitative isolation of GPLs. For this purpose, several washing steps with 500 μL 2-propanol aliquots were tested to evaluate the influence of this step on the signal-to-background ratio. Figure 1.A shows the Total Ion Chromatograms (TICs) provided by LC–MS/MS analysis of the eluted fractions from an EVOO sample after 1, 2 and 3 washing steps. As can be seen, the background contribution is minimized after 3 washing cycles and the chromatographic signals of GPLs were enhanced. No significant improvement was found for the fourth washing step. On the other hand, the elution volume was tested by analysis of several 1-mL aliquots. The highest proportion of GPLs was eluted with 1 mL aliquot, as shows Figure 1.B, which reveals that the second and third aliquots eluted a minor fraction of GPLs. Quantitation of the total chromatographic signals corresponding to GPLs enabled to estimate that approximately 86% of GPLs were eluted in the first step, while 11 and 3% were eluted in the second and third steps, respectively. Thus, the optimum elution volume was set at 1 mL.

3.2. Identification of glycerophospholipids in edible oils

After SPE optimization, this sample preparation approach was tested in edible oils to confirm the presence of GPL families in them. For this purpose, a strategy was planned to identify GPLs in the extracts of analyzed oils by considering the known fragmentation pattern for each GPL family. Both positive and negative ionization modes were tested to enhance the identification. It is well-known that PCs are detected with high sensitivity in positive ionization mode, while other subclasses such as glycerophosphatidic acids (PAs) are preferentially detected in the negative mode [23,24]. The preference of one or other ionization mode is strongly associated to the polar head group. Furthermore, representative product ions generated after fragmentation of GPLs have been widely described [25]. In this research, an algorithm including main product ions and neutral losses detected after

fragmentation of GPL subclasses was designed to support the automated identification of these lipids (Table 1).

(A)

(B)

Figure 1. Total ion chromatograms (TICs) obtained by LC–MS/MS in the optimization of the SPE step for isolation of GPLs from vegetable oils. **(A)** Influence of the washing step (1, 2 and 3 cycles) with 2-propanol in the LC–MS/MS analysis of GPLs to compare the washing effect on background contribution. **(B)** Influence of the elution volume by analysis of three 1-mL eluted aliquots containing GPLs.

Table 1. Fragments and neutral loss used for identification of GPLs.

	PIS[1]		NLS[2]	MRM[3]
	Product ion (+)	Product ion (-)	Neutral loss	Product ion (-)
PC	184.1			
PE		196	141	
PG		227		
PA		153		
C16:0				255.2
C16:1				253.2
C18:0				283.2
C18:1				281.2
C18:2				279.2
C18:3				277.2

[1]Product ions (*m/z*) in positive and negative ionization modes were used for identification of GPLs by PIS methods.
[2]Neutral losses were tuned for identification of GPLs by NLS.
[3]Product ions (*m/z*) in negative ionization were used for confirmatory analysis of GPLs by MRM.

PCs are activated by collision induced dissociation (CID) in positive ionization to cleave the polar group. This fragmentation generated a product ion at *m/z* 184.1, which fits the phosphorylcholine moiety. By analogy, the identification of PEs was supported on the detection of the product ion at *m/z* 196.0, which also fits the glycerophosphorylethanolamine polar group with loss of a water molecule. Complementarily, PEs also provided a neutral loss of 141.0 mass units corresponding to the phosphorylethanolamine moiety, which can be used with confirmatory purposes. The extracted ion chromatograms by monitoring product ions at *m/z* 184.1 and 196.0 offered the profile of PCs and PEs in the analysis of EVOO samples (Supplementary Figure 1), which represent a fingerprint of both subfamilies. According to polarity, the chromatographic signals eluted in the elution range from 10 to 11.5 min corresponded to lysophosphatidylcholines (lysoPCs) and lysophosphatidylethanolamines (lysoPEs), while the peaks eluted from 11.7 to 15 min were ascribed to PCs and PEs. PCs, PAs and PEs were the three most abundant GPL subclasses in edible oils.

Other less abundant families are also characterized by selective CID fragmentation patterns. In this research, PAs were determined through a characteristic fragment formed in negative ionization at *m/z* 153.0, which corresponds to cyclic glycerophosphate. The fragmentation of phosphatidylglycerides (PGs) led to a product ion at *m/z* 227.0, also in negative ionization, corresponding to the glycerophosphate group. Other two families of GPLs, PSs and glycerophosphatidylinositols (PIs), were not detected in edible oils.

3.3. Confirmatory analysis of glycerophospholipids in edible oils

After the presence of GPL families in edible oils was verified, an MRM method was created with a list of tentative precursor ions corresponding to detected families. The list was prepared by considering the most abundant fatty acids in the targeted oils: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3). The fatty acids fragments were selected as product ions to confirm the identity of the chains. Thus, product ions at *m/z* 255.2, 253.2, 283.2, 281.2, 279.2 and 277.2 were tuned in Q3 for monitoring GPLs with C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3, respectively. Negative ionization was adopted to improve the sensitivity of the method by monitoring the fatty acid fragments that are only visible in this ionization mode. In the case of lysoPLs, the product ion corresponded to the unique fatty acid in the structure. On the other hand, the fatty acid that led to the highest sensitivity in MS/MS detection was selected for definition of MRM transitions for GPLs.

The resulting MRM method was applied to all edible oils selected for this study and the confirmed GPLs are listed in Table 2. Information about chromatographic retention time, precursor ion as well as the characteristic product ion supporting the identity confirmation of 41 GPLs are detailed. As can be seen, lysoPLs were eluted in the range from 10.2 to 12.1 min while GPLs eluted from 12.1 to 14.8 min. Two different families of lysoPLs (lysoPCs and lysoPEs) were detected in the analyzed oils. The detected lysoPCs were those with C16:0, C18:0, C18:1, C18:2 and C18:3 in their structure, while detected lysoPEs contained C18:0, C18:1 and C18:2. Concerning GPLs, several compounds were detected for the different

families, particularly, PCs, PEs, PGs and PAs. Thus, PAs were the most diverse family with 15 identified compounds, followed by PCs and PEs with 11 and 10 compounds, respectively, and PGs with 5 compounds. Table 3 shows the GPLs identified in the two sunflower oils, HOSO and SO, and in the four categories of olive oil.

A precision study was carried out to evaluate the methodological variability associated to the determination of GPLs in edible oils (n = 3 replicates). For this purpose, precision was calculated as relative standard deviation (RSD). Table 2 reports the obtained results for each detected GPL. As can be seen, the highest values of RSD were 17.3 and 16.5% for PCs and PEs, respectively, and 27.0 and 30.0% for PGs and PAs, respectively. These values are not unbalanced according to the low concentration of GPLs in edible oils and the sample preparation protocol, which involves an SPE purification step to reduce the interference caused by acylgycerides.

3.4. Comparison of glycerophospholipid profiles in the olive oil categories

Variability of the GPLs profile was assessed for the different olive oil categories included in this study: EVOO, VOO, OO and POO. The purpose of this comparison was to find compositional changes in the GPL fraction associated to the olive oil category. Categorization of EVOO, VOO and OO is basically supported on the presence of organoleptic defects and chemical tests, indicative of oil deterioration or alteration. These organoleptic and chemical anomalies are explained by two main reasons: the use of lower quality fruits to obtain VOO and lampante oils (main component of OO once it is refined) as compared to those for EVOO extraction, or an unsuited processing that deteriorates the oil composition. The hypothesis here is that these two reasons also change the GPL profile of olive oil by alteration of cell membranes. A particular case is that of POO, mainly formed by refined olive pomace oil, which is isolated from the solid residue of the first extraction process. Therefore, the second treatment of the paste should also lead to compositional changes in the GPL profile.

Table 2. Glycerophospholipids identified and confirmed in edible oils by using the method proposed here.

Compound	Formula	RT (min)	Precursor ion *m/z*		Product ion *m/z*	RSD (%)
			[M+FA]⁻	[M-H]⁻		
LysoPC(18:3)	$C_{26}H_{48}NO_7P$	10.2	562.3		277.2	17.3
LysoPC(18:2)	$C_{26}H_{50}NO_7P$	10.7	564.3		279.2	13.1
LysoPC(16:0)	$C_{24}H_{50}NO_7P$	11.1	540.3		255.2	14.4
LysoPC(18:1)	$C_{26}H_{52}NO_7P$	11.3	566.3		281.2	2.6
LysoPC(18:0)	$C_{26}H_{54}NO_7P$	11.8	568.4		283.2	16.6
LysoPE(18:2)	$C_{23}H_{44}NO_7P$	11		476.3	279.2	11.2
LysoPE(18:1)	$C_{23}H_{46}NO_7P$	11.6		478.3	281.2	6.5
LysoPE(18:0)	$C_{23}H_{48}NO_7P$	12.1		480.3	283.2	3.4
PA(16:0/16:1)[1]	$C_{35}H_{67}O_8P$	12.1		645.4	255.2	4.7
PA(16:0/16:1)[2]	$C_{35}H_{67}O_8P$	12.2		645.4	255.2	8.5
PA(16:1/18:1)	$C_{37}H_{67}O_8P$	12.3		671.5	281.2	7.5
PA(16:0/18:1)	$C_{37}H_{71}O_8P$	12.3		673.5	281.2/255.2	7.6
PA(16:0/18:0)	$C_{37}H_{73}O_8P$	12.7		675.5	283.3	4.3
PA(18:2/18:3)	$C_{39}H_{67}O_8P$	12.8		693.5	279.2	8.8
PA(18:2/18:2)	$C_{39}H_{69}O_8P$	12.9		695.5	279.2	23.8
PA(18:1/18:3)	$C_{39}H_{69}O_8P$	13.1		695.5	281.2	17.9
PA(18:1/18:2)[1]	$C_{39}H_{71}O_8P$	13.1		697.5	281.2	30
PA(18:1/18:2)[2]	$C_{39}H_{71}O_8P$	13.2		697.5	281.2	29.8
PA(18:0/18:3)	$C_{39}H_{71}O_8P$	13.2		697.5	283.3	5.8
PA(18:1/18:1)[1]	$C_{39}H_{73}O_8P$	13.2		699.5	281.2	25
PA(18:1/18:1)[2]	$C_{39}H_{73}O_8P$	13.3		699.5	281.2	3.8
PA(18:0/18:2)	$C_{39}H_{73}O_8P$	13.4		699.5	283.3	16.9
PA(18:0/18:0)	$C_{39}H_{77}O_8P$	13.6		703.5	283.3	21.4
PE(16:0/16:1)	$C_{37}H_{72}NO_8P$	12.5		688.5	255.2	2.4
PE(18:2/18:3)	$C_{41}H_{72}NO_8P$	13.7		736.5	279.2	3.4
PE(18:2/18:2)	$C_{41}H_{74}NO_8P$	13.8		738.5	279.2	6.3
PE(18:1/18:3)	$C_{41}H_{74}NO_8P$	13.9		738.5	281.2	9.7
PE(18:1/18:2)	$C_{41}H_{76}NO_8P$	14.1		740.5	281.2	16.5
PE(18:1/16:0)	$C_{39}H_{76}NO_8P$	14.2		716.5	281.2	9.6
PE(18:0/18:1)	$C_{41}H_{80}NO_8P$	14.7		744.5	281.2	7.9
PG(18:1/16:0)	$C_{40}H_{77}O_{10}P$	13.3		747.5	281.2/255.2	27
PG(16:0/18:0)	$C_{41}H_{79}O_{10}P$	13.5		749.5	255.2	22
PG(18:1/18:2)	$C_{42}H_{77}O_{10}P$	14.2		771.5	281.2/279.2	21.4
PG(18:1/18:1)	$C_{42}H_{79}O_{10}P$	14.5		773.5	281.2	16.4
PG(18:0/18:0)	$C_{42}H_{83}O_{10}P$	14.8		777.6	283.2	2.3
PC(18:2/18:2)	$C_{44}H_{80}NO_8P$	13.8	826.5		279.2	2.7
PC(18:1/18:3)	$C_{44}H_{80}NO_8P$	13.9	826.5		281.2	4.1
PC(18:1/18:2)	$C_{44}H_{82}NO_8P$	14.1	828.7		281.2	11.1
PC(16:1/18:1)	$C_{42}H_{80}NO_8P$	14.2	802.5		281.2	11.7
PC(16:0/18:1)	$C_{42}H_{82}NO_8P$	14.3	804.8		281.2	10.5
PC(18:1/18:1)	$C_{44}H_{84}NO_8P$	14.4	830.9		281.2	15

Superscript numbers mean isomer GPLs detected in edible oils.

Table 3. Glycerophospholipids identified and confirmed in the different types of edible oils.

Compound	EVOO	VOO	OO	POO	HOSO	SO
LysoPC(18:3)	X	X	X		X	X
LysoPC(18:2)	X	X	X		X	X
LysoPC(16:0)	X	X	X	X	X	X
LysoPC(18:1)	X	X	X		X	X
LysoPC(18:0)	X	X	X	X	X	X
LysoPE(18:2)	X	X			X	X
LysoPE(18:1)	X	X	X	X	X	X
LysoPE(18:0)				X		
PA(16:0/16:1)[1]		X	X	X	X	X
PA(16:0/16:1)[2]	X	X	X	X		X
PA(16:1/18:1)	X	X	X	X	X	X
PA(16:0/18:1)	X	X	X	X	X	X
PA(16:0/18:0)	X	X	X	X	X	X
PA(18:2/18:3)	X	X	X	X	X	X
PA(18:2/18:2)	X	X	X	X	X	X
PA(18:1/18:3)	X	X	X	X	X	X
PA(18:1/18:2)[1]	X	X	X	X	X	X
PA(18:1/18:2)[2]	X	X	X	X	X	X
PA(18:0/18:3)	X	X				
PA(18:1/18:1)[1]	X	X	X	X	X	X
PA(18:1/18:1)[2]	X	X	X	X	X	X
PA(18:0/18:2) PA(18:0/18:0)					X	X
PE(16:0/16:1)	X	X	X	X	X	X
PE(18:2/18:3)			X			X
PE(18:2/18:2)			X			X
PE(18:1/18:3)	X	X	X	X	X	X
PE(18:1/18:2)				X		
PE(18:1/16:0)	X	X	X	X		
PE(18:0/18:1)		X	X	X	X	
PG(18:1/16:0)				X		
PG(16:0/18:0)			X	X		
PG(18:1/18:2)				X		
PG(18:1/18:1)			X	X	X	X
PG(18:0/18:0)			X	X		
PC(18:2/18:2)	X	X			X	X
PC(18:1/18:3)	X	X				
PC(18:1/18:2)	X	X			X	X
PC(16:1/18:1)			X			
PC(16:0/18:1)			X	X	X	X
PC(18:1/18:1)		X				

Superscript numbers mean isomer GPLs detected in edible oils.

The Venn diagram in Figure 2 compares the GPL profiles provided by the four categories of olive oil. These Venn diagrams were built by considering only those GPLs identified in at least four out of five samples for each category (33 GPLs). The Venn diagram revealed that EVOO and VOO were characterized by a common GPL profile in qualitative terms, except for two compounds —PA(18:0/18:3) and PC(18:2/18:2)—, which were only identified in VOO. On the other hand, clear differences in GPLs composition were found in OO and PO *versus* EVOO and VOO. These differences particularly affected to PG, PA and PC families.

Principal component analysis (PCA) applied to all the samples clearly revealed a discrimination trend as a function of the olive oil category (Figure 2). Thus, three main groups corresponding to POO, OO and EVOO+VOO samples were perfectly distinguishable. Additionally, EVOO and VOO samples were not completely separated, but a certain differentiation was observed. The combination of PC1–PC2 components explained 71.6% of the total variability, which allowed concluding that the GPL profile depends on the olive oil category. The bi-plot PCA (Supplementary Figure 2) obtained by overlapping the scores and loadings plots allows detecting the contribution of the different GPL families in the separation virgin olive oils (EVOO and VOO) *versus* non-virgin oils (OO and POO). Some families explained the position of EVOO and VOO samples, while most GPLs contributed to discriminate OO and POO samples.

Statistical analysis was carried out by ANOVA with post-hoc test (95% confidence level) to compare the GPLs profile of the different categories of olive oil. Thus, 37 GPLs were found at significantly different concentration in EVOO, VOO, OO and POO. Figure 3 shows the list of GPLs detected at different concentrations (p-value < 0.05) in the analyzed oils. As can be seen, the main differences were found between virgin olive oils *versus* OO and POO, with special emphasis on PAs, PCs, PEs and PGs. LysoPLs allowed differentiation of POO from the other three categories. Also, PGs were found at significantly different concentration in virgin olive oils versus OO and POO. Concerning virgin olive oils, the main differences between EVOO and VOO were found in three PAs.

Qualitative analysis was complemented with semiquantitative analysis to compare the concentration levels of the GPL families in the four categories of olive oils, shown in Figure 4. As can be seen, a common trend was observed for lysoPCs and lysoPEs, which were found at the highest concentration in VOO and OO, followed by EVOO. POO samples reported the lowest concentration of both lysoPL families, with special emphasis on lysoPCs, which were practically not detected in POO samples. The low lysoPL levels can be explained by the olive pomace oil extraction process, which is based on a solid–liquid extraction with an organic solvent (generally *n*-hexane). As lysoPLs are more polar compounds as compared to disubstituted GPLs, they are not efficiently extracted in the non-polar solvent.

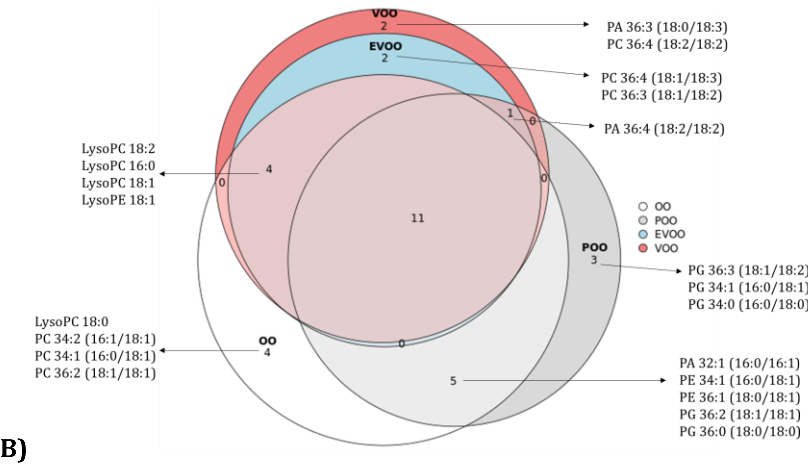
The levels of PCs in the four olive oil categories followed a similar pattern to that found in lysoPLs. Thus, EVOO and VOO showed a similar concentration of PCs, but lower than that measured in OO. Again, POO reported the lowest concentration of PCs, being this group of GPLs at low level in POO samples. PEs concentration levels were not similar to that observed for lysoPEs. Thus, PEs were more concentrated in EVOO, while VOO, OO and POO yielded lower concentration of this family of GPLs.

Among the different families of GPLs, PAs and PGs were highlighted by their discrimination capability. Thus, PAs were present at the lowest concentration in EVOO, followed by VOO; while refined oils, OO and POO, provided higher concentration of PAs. On the other hand, PGs clearly allowed discrimination between refined and non-refined olive oils. In fact, only few PGs were detected in EVOO and VOO samples. Supplementary Figure 3 shows the variability in the concentration of GPLs for the different samples evaluated within each category. Some GPL families provided higher variability for some categories of olive oil, particularly when these families were detected at low concentration. Nevertheless, the variability was not high in general.

4. Conclusions

The method developed for qualitative and quantitative analysis of GPLs in vegetable edible oils involves the combination of SPE, with high selectivity for

(A)



(B)

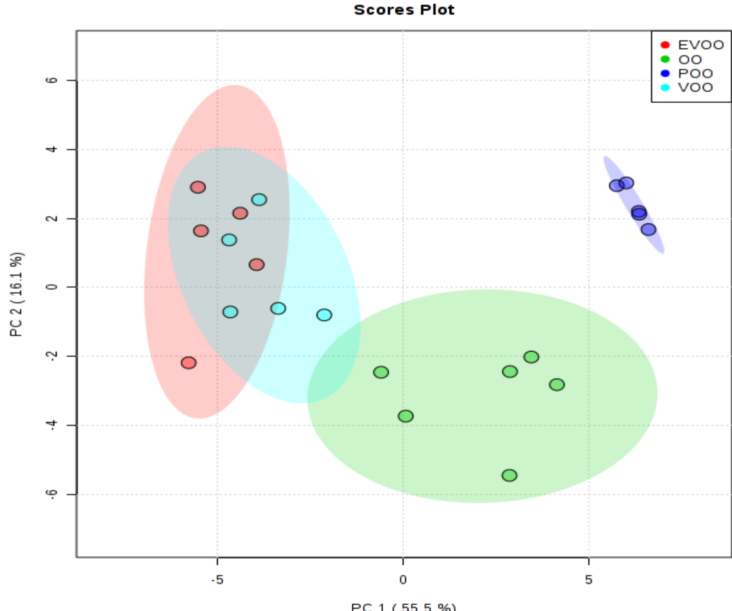


Figure 2. Comparative analysis of the GPLs composition in four categories of olive oil: EVOO (extra virgin olive oil), VOO (virgin olive oil), OO (olive oil) and POO (pomace olive oil). **(A)** Venn diagram built by considering only those GPLs that were identified in at least four out of five samples for each class (33 GPLs). **(B)** Principal components analysis (PCA) obtained with the complete data set showing the distribution of olive oil categories according to the GPL fraction.

EVOO-VOO	EVOO-OO	VOO-OO	EVOO-POO	VOO-POO	OO-POO	
						LysoPC(16:0)
						LysoPC(18:0)
						LysoPC(18:1)
						LysoPC(18:2)
						LysoPC(18:3)
						LysoPE(18:0)
						LysoPE(18:1)
						LysoPE(18:2)
						PA(16:0/16:1)^{1}
						PA(16:0/16:1)^{2}
						PA(16:0/18:0)
						PA(16:0/18:1)
						PA(16:1/18:1)
						PA(18:0/18:0)
						PA(18:1/18:1)
						PA(18:0/18:3)
						PA(18:1/18:2)
						PA(18:2/18:2)
						PA(18:1/18:3)
						PA(18:2/18:3)
						PE(16:0/16:1)
						PE(18:1/16:0)
						PE(18:0/18:1)
						PE(18:1/18:2)
						PE(18:1/18:3)
						PE(18:2/18:3)
						PG(16:0/18:0)
						PG(18:1/16:0)
						PG(18:0/18:0)
						PG(18:1/18:1)
						PG(18:1/18:2)
						PC(16:0/18:1)
						PC(16:1/18:1)
						PC(18:1/18:1)
						PC(18:1/18:2)
						PC(18:2/18:2)
						PC(18:1/18:3)

Figure 3. Discrimination pattern between pairs of olive oil categories based on GPLs found at significantly different concentration. Superscript numbers mean isomer GPLs detected in edible oils.

retention of GPLs, and LC–MS/MS analysis using different acquisition methods for identification, confirmatory analysis and relative quantitation of GPLs. The method has been applied to characterizing the GPLs fraction of four categories of olive oil defined in terms of quality: EVOO, VOO, OO and POO. The comparison of the GPL

profile in olive oils categories allowed identifying qualitative and quantitative compositional differences that would be correlated to the quality of olive fruits and processing factors used to obtain olive oil. Attending to these changes, the determination of the GPLs profile could be considered a promising marker to differentiate olive oil categories with quality purposes or to detect adulterations. Polar lipids have been scarcely studied in olive oils and, for this reason, their presence in olive oil could be associated to the quality of fruits and the production process. On the other hand, GPLs are not completely removed during the refining process and they could be target markers to monitor adulterations with low quality oils.

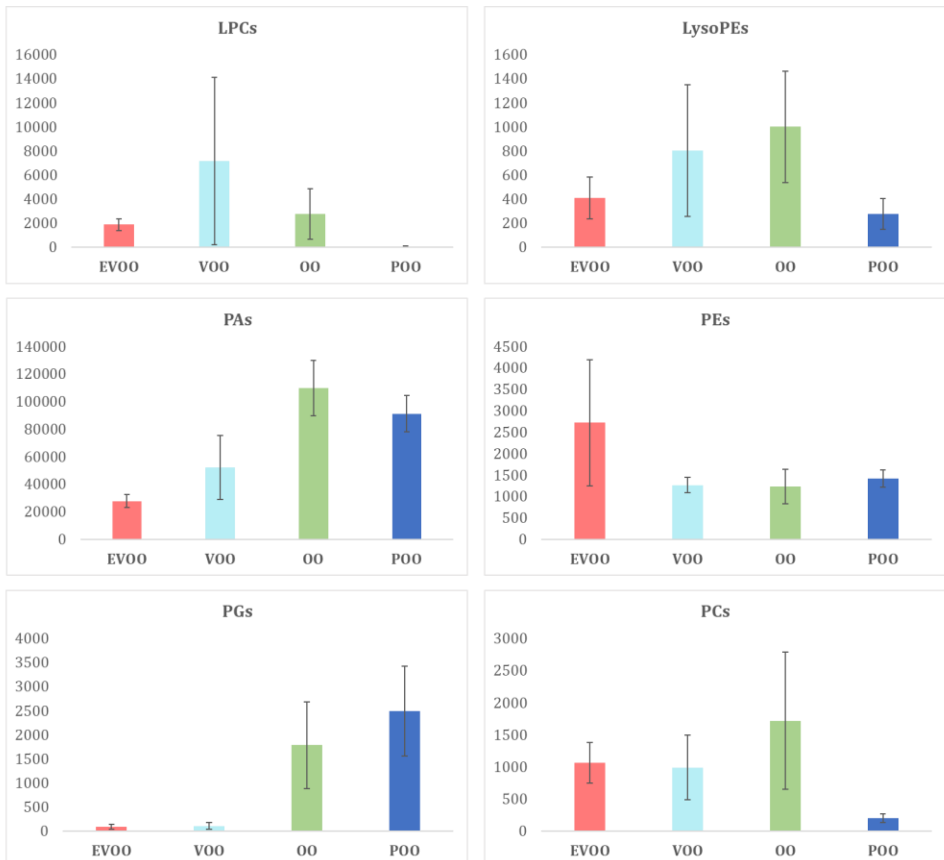


Figure 4. Mean relative concentration and standard deviation of the different GPL families found in each category of olive oil: lysophospahtildylcholines (LPCs), lysophosphatidylethanolamines (LPEs), glycerophosphatidic acids (PAs), glycerophosphatidylethanolamines (PEs), glycerophospatidylglycerols (PGs) and glycerophosphatidylcholines (PCs).

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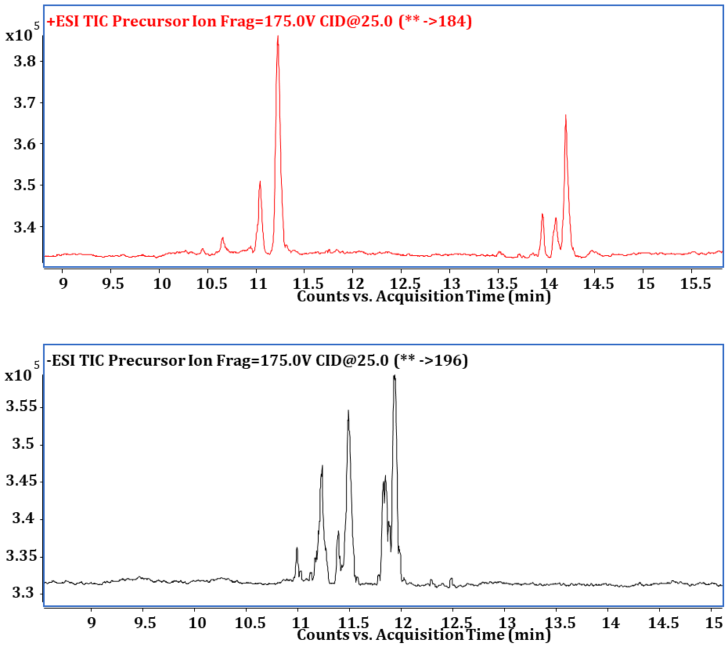
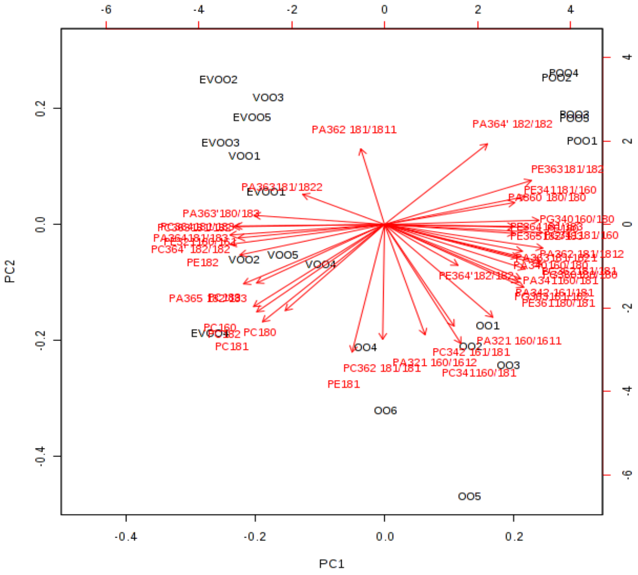
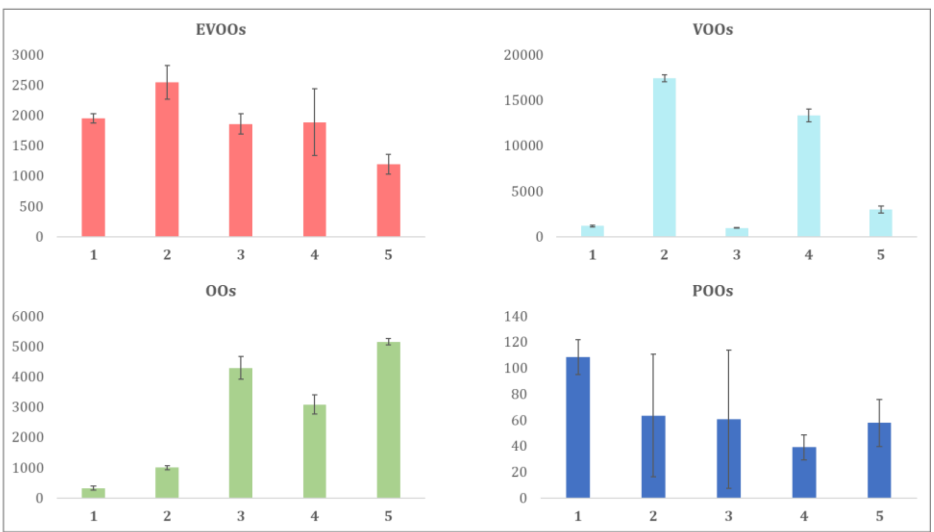


Supplementary Figure 1. Extracted ion chromatograms (EICs) obtained in precursor ion scanning by monitoring the product ions *m/z* 184.1 and 196.0, which correspond to PCs and PEs. The analyzed sample was extra virgin olive oil.

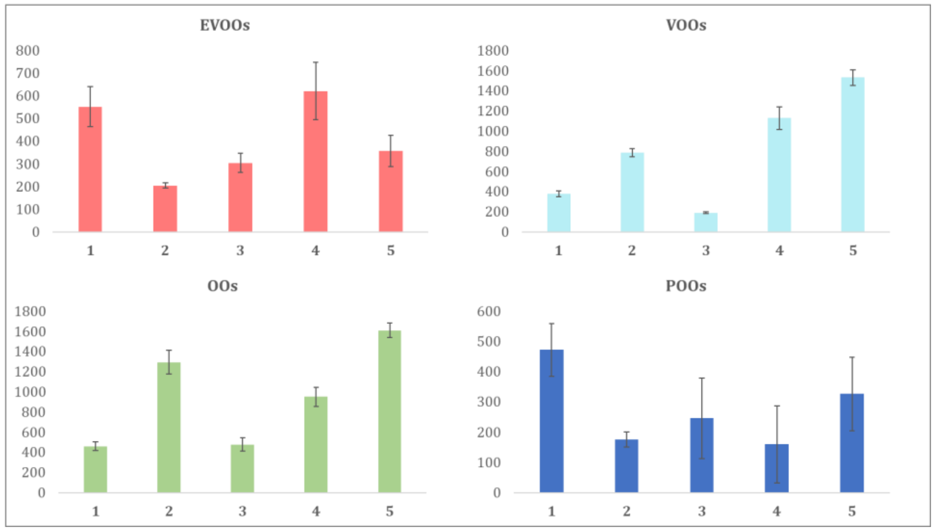


Supplementary Figure 2. Biplot principal component analysis that allows visual discrimination of olive oil categories and the main families of GPLs involved in discrimination.

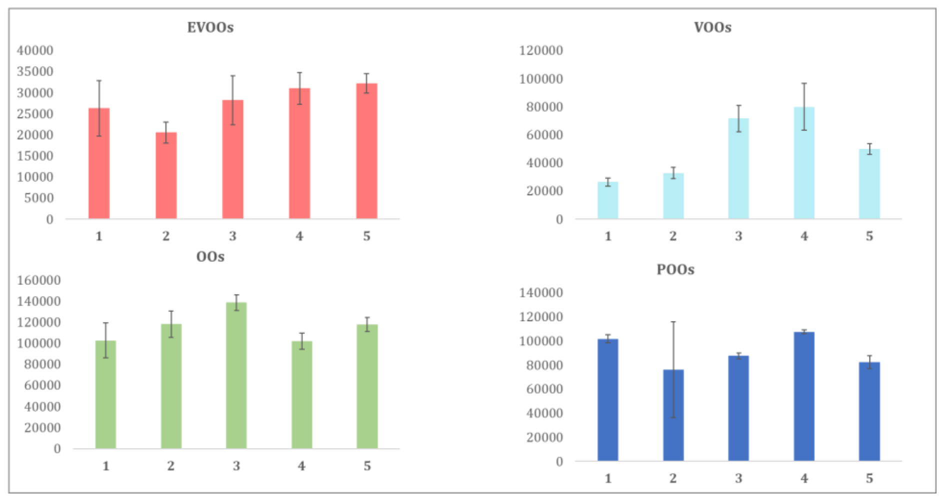
LysoPCs



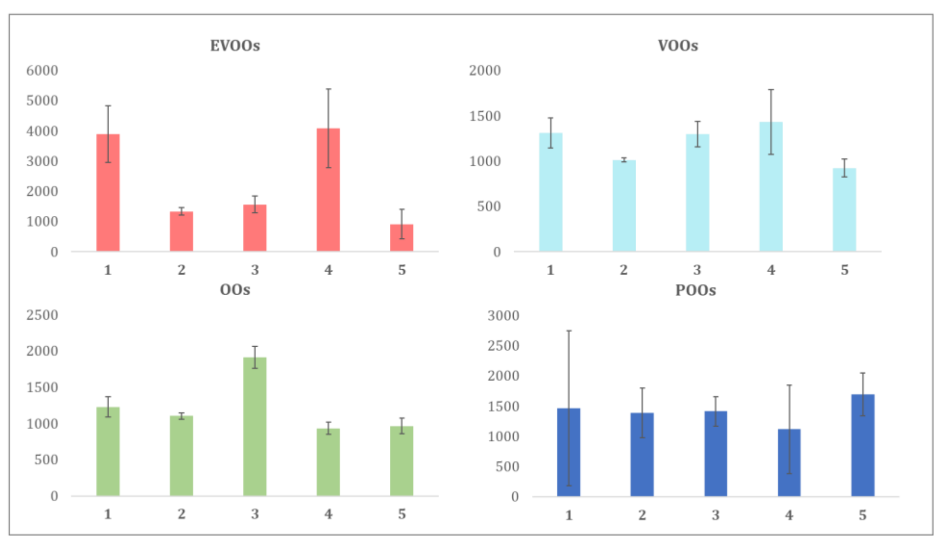
LysoPEs



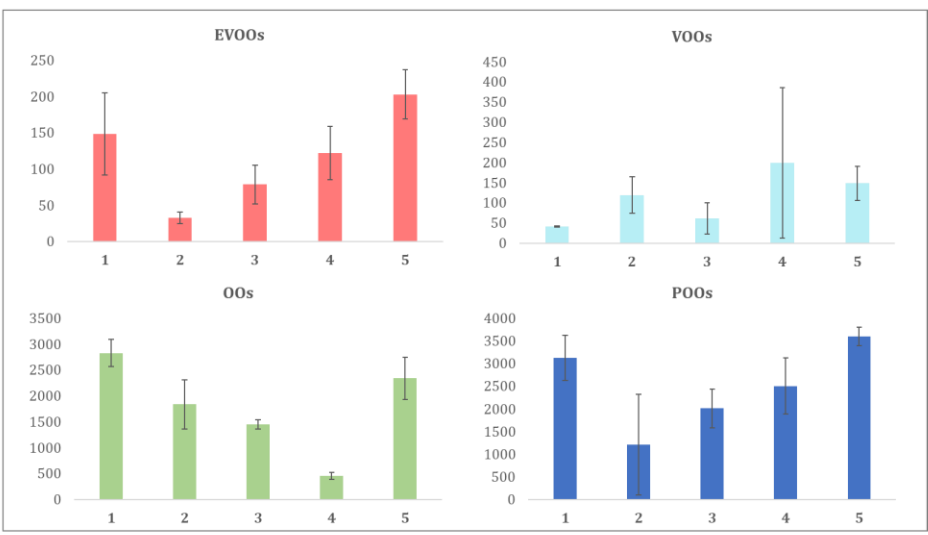
PAs



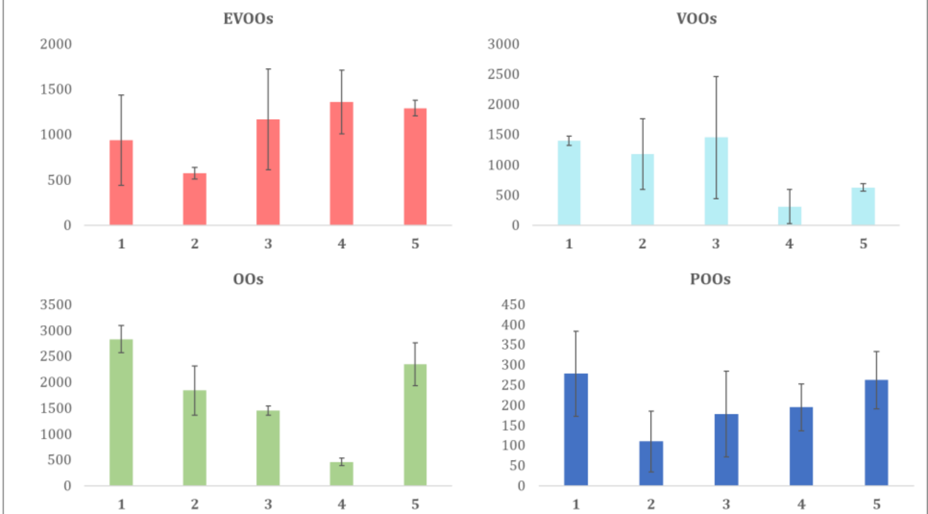
PEs



PGs



PCs



Supplementary Figure 3. Relative concentration of the different GPLs families found in the analyzed samples of each olive oil category: lysophospahtildylcholines (lysoPCs), lysophosphatidylethanolamines (lysoPEs), phosphatidic acids (PAs), phosphatidylethanolamines (PEs), phospatidylglycerols (PGs) and phosphatidylcholines (PCs).

SECTION II

Monitoring bioactive components during the extraction of extra virgin olive oil

Section II of this PhD Book is devoted to study the formation and partition of bioactive compounds in the different phases, both solids and liquids, involved in the process for extraction of EVOO. Two main families of bioactive compounds were monitored in this research, namely, phenolic compounds and terpenic acids. Phenolic compounds are valued because of their recognized health benefits, apart from their contribution to organoleptic features and antioxidant stability. Terpenic acids have been characterized at lesser extent, but they are also associated to relevant bioactive properties. Concerning the extraction process, the two-phase system, as the most extended approach, was adopted for this block because of its suitability to obtain EVOO enriched in minor components. According to the variability in the concentration of phenolic compounds identified in Section I, two different cultivars were selected in this research, 'Arbequina' and 'Picual'. With these premises, two research studies were developed to monitor these two bioactive families. Chapter 5 was focused on the determination of phenols and triterpenes in oily phases by comparing oil samples obtained after decantation, after vertical centrifugation and after filtration (final EVOO). In this chapter, the influence of fruit destoning was also considered to compare its effect on the profile of bioactive compounds in both monocultivar EVOOs.

On the other hand, Chapter 6 was intended to evaluate the formation and partition of phenols and triterpenes in intact fruits, pastes, final olive oil, wastewater and olive pomace (alperujo). The main aim here was to complement results obtained in the previous chapter to compare the distribution of bioactive compounds in the different phases along the extraction process.

It is worth mentioning that both studies were part of the Nutradaf research project granted by the CDTI FEDER-Interconecta Program in the framework of a collaboration involving private and public entities.

CHAPTER 5

Influence of fruit destoning on bioactive compounds of virgin olive oil

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Influence of Fruit Destoning on Bioactive Compounds of Virgin Olive Oil

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Influence of Fruit Destoning on Bioactive Compounds of Virgin Olive Oil

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Abstract

Fruit destoning before crushing is gaining interest due to this operation enhances the working capacity, reduces the waste generation, and improves the quality of virgin olive oil (VOO). Despite these benefits, the influence of olives destoning on the concentration of bioactive compounds in VOO has not been evaluated. In this research, we studied the changes occurring in two different families of bioactive compounds (phenols and terpenoids) in VOO obtained from two cultivars (‘Arbequina’ and ‘Picual’) after fruits destoning. For this purpose, we used as reference the bioactive profile of VOOs obtained with intact fruits. A cultivar significant effect was found in the concentration of the main phenols included in the health claim of the Commission Regulation (EU)432/2012. Thus, fruit destoning contributed to significantly decrease the health benefits of VOO in ‘Arbequina’ while no changes were observed in ‘Picual’. These variations affected particularly to the aglycone isomers of oleuropein and ligstroside.

Keywords: Destoning; bioactive compounds; virgin olive oil; health claim; cultivar.

Chemical compounds studied in this article: Hydroxytyrosol (PubChem CID: 82755); Oleacein (PubChem CID: 18684078); Oleocanthal (PubChem CID: 11652416); Oleuropein aglycone (PubChem CID: 56842347); Ligstroside aglycone (PubChem CID: 71718370); Luteolin (PubChem CID: 5280445); Apigenin (PubChem CID: 5280443); Maslinic acid (PubChem CID: 73659); Oleanolic acid (PubChem CID: 10494); Erythrodiol (PubChem CID: 101761)

1. Introduction

Virgin olive oil (VOO) is produced from fresh and healthy olive fruits (*Olea europaea* L.) exclusively by mechanical and physical processes, unlike other edible oils such as sunflower and soybean oils, which need refining before consumption [1]. Typical extraction processes for olive oil extraction are generally based on traditional pressing, the three-phase system, or the most recently developed two-phase centrifugal approach. The latter has been widely accepted for production of (E)VOOs (abbreviation used to refer to virgin olive oil and, particularly, to the extra-virgin category) [2]. The two-phase system consists of: (i) olive fruits collection; (ii) washing; (iii) crushing, which is a physical process to break the fruits tissues and release the oil drops contained in the vegetable cell vacuoles; (iv) malaxation, mixing olive paste to increase the percentage of free oil and help small oil droplets to coalesce and agglomerate, thus facilitating separation of the oil and water phases; (v) decantation, involving "no-water" horizontal centrifugation for separating the oily phase from malaxed pastes; (vi) vertical centrifugation, which is used to wash the oil; (vii) storage; and (viii) filtration, the final step to remove suspended solids and humidity (Supplementary Figure 1) [3-5]. This system is characterized by two inputs, olive paste and water, and two outputs with three by-products: pomace and wastewater–oil.

Phenolic compounds have been recognized to play a significant role in the health benefits of (E)VOO. According to the European Commission (EU)432/2012, "phenols protect blood lipids from oxidative stress", which is one of the main mechanisms involved in the development of pathologies such as cardiovascular diseases [6, 7]. Additionally, phenols are responsible for organoleptic attributes, pungency and bitterness, but also for the shelf life of the (E)VOO due to their antioxidant capabilities [8]. The phenolic fraction is highly influenced by the extraction process. Crushing and malaxation induce the activation of endogenous enzymes, such as β-glucosidases, esterases and oxidoreductases, which regulate the synthesis of secoiridoid derivatives. After crushing, both oleuropein and ligstroside, the main secoiridoids in the olives, are hydrolyzed into their corresponding aglycone forms [9]. Additionally, during malaxation, an oxidation of phenolic

compounds is catalyzed by polyphenoloxidases (PPO) and peroxidases (POD) leading to a loss of phenolic compounds [9-11]. In the decanter the malaxated paste is partitioned in the different phases (oil, water and solids) as a function of the affinity towards each phase, which is related to the relative polarities of the compounds and phases ratio [12, 13]. In vertical centrifugation, the addition of water can result in decrease of (E)VOO phenols owing to their hydrophilic nature in relation to the partition coefficient (*Kp*). Hence, the presence of simple phenols (tyrosol and hydroxytyrosol), with very low *Kp* in comparison to other phenols such as secoiridoids, decreases in oil [14, 15]. Finally, after the filtration, a reduction of the total phenolic content can occur. It is assumed that most phenolic compounds, having amphiphilic characteristics, are located around water droplets on olive oil. Through filtration, moisture is eliminated, thus, water content is decreased together with a proportion of the phenolic compounds [5, 16]. During storage, hydrolysis, oxidation, hydration, and loss of the carboxylic group could involve degradation of the phenolic compounds. These reactions depend on storage conditions including time, temperature, oxygen availability, light exposure and industrial or commercial containers [17-19].

Most of the studies on olive oil processing are focused on phenolic compounds, disregarding other minor compounds like triterpenes. In recent years, the number of studies focused on the bioactivity of VOO triterpenes is increasing. Antitumoral, anti-inflammatory, antioxidant, hepatoprotective, cardioprotective, and antimicrobial activities have been recently described [20, 21]. However, there is scant literature regarding their behaviour related to VOO processing conditions. Allouche *et al*. studied the influence of different parameters (sieve diameters of the hammer mill, malaxation temperature and time) on triterpenic content. The process was carried out under laboratory-scale conditions and, the triterpenic content was analyzed only in the extracted olive oils [22]. Thus, the changes that these compounds undergo during industrial processing were unknown.

Destoning before crushing in the process of olive oil extraction is gaining interest since it could enhance the working capacity. The crushing of pits carries a large loss of mechanical energy due to its conversion into thermal energy increasing

degradation and oxidation phenomena [23]. Likewise, it is worth mentioning the management of by-products with a lower environmental impact in destoned olive oil production. In fact, the stones represent about 25% of the total olive paste volume and their exclusion before the extraction significantly reduces the amount of solid waste processing [24, 25]. Nevertheless, the interest of this added step is focused on the quality improvement of the oil due to most of the fruit oxidative enzymes such as PPO and POD located in the endocarp are removed, protecting the phenolic and volatile composition [26, 27]. Despite most studies of destoning have been targeted at quality evaluation (sensory analysis, UV specific extinction coefficients K232 and K270, acidity and peroxide index), contradictory results are found in literature mainly related to genetic factors of different cultivars [28, 29]. It is generally accepted that peroxide value and the UV specific extinction coefficients are not substantially affected by destoning. These parameters are highly dependent on the cultivar, quality, and health status of the fruits Also, positive effects of destoning on acidity values and sensory notes have been described [26]. Studies of the destoning effect on the (E)VOO quality have been mainly carried out considering these parameters; however, the effect on the composition of phenols and terpenoids, which are determinant in both (E)VOO stability and health properties, has been underestimated.

In this study, we have evaluated the effect of destoning on two families of bioactive compounds found in VOOs from two traditional expanded Spanish cultivars, 'Arbequina' and 'Picual', which predominate in new plantations due to their suitable edaphoclimatic adaptation. These two cultivars have been selected due to their significant differences in terms of pulp-stone ratio and phenolic composition [30, 31]. Thus, oleuropein and ligstroside aglycones are the most abundant phenols in 'Picual' VOOs, while oleacein and oleocanthal predominate in the profile of 'Arbequina' VOOs [32, 33].

2. Materials and methods

2.1. Samples

'Arbequina' and 'Picual' olive fruits were collected at optimum ripening stage (2.0, yellowish-red color) according to the method proposed by the International Olive Oil Council [34] in two independent orchards located at Alcaudete and Torredonjimeno (Jaen, Spain), respectively. Fruits from each cultivar were processed in two batches by following the same workflow but in one of them fruits were destoned prior to crushing (Supplementary Figure 1). VOO samples were taken just after decantation (decanter oil), after vertical centrifugation (centrifuge oil) and as final product (final oil). Each process was replicated to obtain a total of 36 samples (2 cultivars × 2 processes × 3 types of samples × 3 replicates).

2.2. Reagents and standards

Ethanol, acetone, chloroform methanol and *n*-hexane used as solvents for sample preparation were from Scharlab (Barcelona, Spain). Formic acid, used as ionization agent in the chromatographic mobile phases, acetonitrile and 2-propanol were from Fisher Scientific (Madrid, Spain) with LC–MS grade. Deionized water (18 MΩ • cm) from a Mili-Q water purification system was used to prepare both the aqueous mobile phase and the extractant. Secoiridoid derivatives oleacein, oleocanthal, oleuropein aglycone and ligstroside aglycone were provided in pure forms by Prof. P. Magiatis (University of Athens, Greece). Hydroxytyrosol was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Apparatus and instruments

A vortex shaker from IKA (Wilmington, NC, USA) and a centrifuge supplied by Ortoalresa (Madrid, Spain) were used for sample preparation. Chromatographic separation was carried out by an Agilent 1200 series LC (Palo Alto, CA, USA) furnished with an Inertsil ODS-2 C18 analytical column (250 × 4.6 mm i.d., 5 μm particle) from GL Science (Tokyo, Japan). The chromatograph was coupled through an electrospray ionization (ESI) source to a 6540 quadrupole–time-of-flight detector (QTOF MS/MS; Agilent Technologies, Santa Clara, CA) for detection.

Quantitative determination of the main secoiridoids was carried out with an Agilent 1200 series LC furnished with a Mediterranea C18 (3 μm particle size, 5 × 0.46 cm i.d.) analytical column and a C18 guard column from Teknokroma

(Madrid, Spain). The LC system was coupled to a 6460 Agilent triple quadrupole mass detector (QqQ) equipped with an Agilent Jet Stream Technology ESI source.

2.4. Sample preparation

Phenolic compounds were isolated by liquid–liquid extraction from the three oil samples. An aliquot of 2 mL of each oil was mixed with 3 mL of 70:10:10:10 (v/v) ethanol-chloroform-acetone-water and shaken for 2 min. The phases were separated by centrifugation (8 min, 900 × *g*). The top layer was filtered by 0.2 µm nylon filter from Agilent prior the injection into the LC–QTOF MS/MS. Three replicates per sample were analyzed.

For the quantification of phenols, three 0.5-g aliquots of each oil were vortexed with 250 µL of *n*-hexane for 30 s. Then, 2 mL of 80:20 (v/v) methanol-water with the internal standard (syringaldehyde, 1 µg/mL) was added to each aliquot and shaken for 2 min, and the hydroalcoholic phase was separated by centrifugation for 8 min at 900 × *g*. A dilution of the resulting phenolic extracts (1:10 v/v) was injected into the LC–QqQ MS/MS.

2.5. LC–QTOF MS/MS analysis for characterization of oil samples

The chromatographic mobile phases were water (phase A) and 50:50 (v/v) acetonitrile-isopropanol (phase B), both solutions acidified with 0.1% (v/v) formic acid, at a constant flow rate of 0.75 mL/min. The LC pump was programmed with the following elution gradient: 20% to 45% B in 3 min, change from 45% to 70% B in 9 min, from 70% to 100% B in 3 min and constant at 100% B during 10 min (total time 25 min). After analysis, the column was equilibrated to the initial conditions for 5 min. The injection volume was 10 µL. The column compartment was thermostated at 30 °C.

The parameters of the ESI source operated in negative ionization mode were as follows: nebulizer gas at 40 psi, the flow rate and temperature of the N_2 as drying gas were 12 L min^{-1} and 325 °C, respectively. The capillary voltage was set at ± 3.5 kV, while the Q1, skimmer, and octapole voltages were fixed at 130, 65, and 750 V, respectively. Data were collected in centroid mode in the extended dynamic

range (2 GHz). Full scan was carried out at 6 spectra per second within the *m/z* range of 40–1200, with subsequent activation of the three most intense precursor ions (allowed charge: single or double) by MS/MS using a collision energy of 12 eV and 25 eV at 3 spectra per second within the *m/z* range 30–1200. An exclusion window of 0.75 min after the first spectrum was programmed to avoid repetitive fragmentation of the most intense precursor ions. To assure the desired mass accuracy of the recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in negative ion mode.

Relative quantitation was carried out using oleuropein as reference standard. For this purpose, a calibration curve was built by analysis of oleuropein solutions at concentrations ranging from 1 to 20 μg/g. Detected compounds were quantified in concentration units expressed as oleuropein (mg/kg, Supplementary Table 1).

2.6. Quantitative analysis of phenolic compounds and estimation of the healthy index

For LC–QqQ MS/MS analyses the chromatographic mobile phases were deionized water (phase A) and MeOH (phase B), both solutions acidified with 0.1% (v/v) formic acid. The LC pump was programmed with a flow rate of 0.4 mL/min with the following elution gradient: 50% phase B was kept as initial mobile phase for 0.5 min; then, from 0.5 to 2 min, mobile phase B was from 50 to 80%; and from min 2 to 5, mobile phase B was from 80 to 100%. This last composition was maintained for 1.5 min. A post-time of 4 min was set to equilibrate the initial conditions for the next analysis. The injection volume was 10 μL. The autosampler was kept at 5 °C to increase sample stability and the column compartment was kept at 30 °C.

MS detection was performed by MS/MS in multiple reaction monitoring (MRM) mode for selective transitions from precursor to product ion for each analyte. The MRM parameters for the analysis of target phenols are listed in Table 1. The ionization parameters were set as follows: ionization probe, position B; spray voltage, 2750 V; sheath gas pressure, 25 arbitrary units; auxiliary gas, 10 psi; ion

transfer capillary temperature, 300 °C; and FWHM for Q1 and Q3, 0.7. The scan time and width were 0.1 s and 0.5 *m/z*.

Calibration models were prepared by using refined sunflower oil spiked with the target phenols at five concentration levels (1 to 20 μg/g). Target phenols were hydroxytyrosol, oleacein, oleocanthal, oleuropein aglycone and ligstroside aglycone. Three spiked aliquots were analyzed for each concentration level analyzed in triplicate to obtain the calibration models (Supplementary Table 1).

The healthy index was estimated according to the Commission Regulation (EU) 432/2012 and the EFSA opinion, which recommend a 20 g daily intake of olive oil. Thus, the healthy index refers to the sum of phenolic concentration expressed in mg consumed with this daily intake. A positive healthy index is considered above 5 mg/20 g of oil, which is equivalent to an absolute concentration of 250 mg/kg.

2.7. Data processing and statistical analysis

MassHunter Workstation software (version B8.00 Profinder; Agilent Technologies, Santa Clara, CA) was used to process the data obtained by LC–QTOF in auto-MS/MS mode. Treatment of the raw data file starts by extraction of potential molecular features (MFs) with the suited algorithm included in the software. The recursive extraction algorithm considered all ions exceeding 5000 counts as cut-off. Additionally, the isotopic distribution to consider a molecular feature as valid should be defined by two or more ions (with a peak spacing tolerance of *m/z* 0.0025, plus 10.0 ppm in mass accuracy). Apart from $[M-H]^-$ ions, adducts formation in the negative ionization ($HCOO^-$, Cl^-) modes, as well as neutral loss by dehydration, were included, to identify features corresponding to the same potential metabolite. Thus, ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by their retention time (RT), intensity at the apex of the chromatographic peaks and accurate mass. Background contribution was removed by subtraction of MFs linked to the blank under identical operational conditions of the samples.

Table 1. List of identified compounds with the main parameters (formula, neutral mass, retention time) and the MRM parameters for targeted quantitative analysis in VOO.

Compound Name	Formula	Mass (g/mol)	RT (min)	MRM Parameters Precursor (m/z)	MRM Parameters Product (m/z)
Flavonoids					
Luteolin*	$C_{15}H_{10}O_6$	286.048	6.17		
Diosmetin*	$C_{16}H_{12}O_6$	300.064	6.73		
Apigenin*	$C_{15}H_{10}O_5$	270.053	7.05		
Secoiridoids					
Oleacein*	$C_{17}H_{20}O_6$	320.126	4.4	319	69
Hydroxylated form of-decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_7$	336.121	4.57		
Oleuropein*	$C_{25}H_{32}O_{13}$	540.183	4.64		
2-Methoxyoleuropein	$C_{26}H_{34}O_{14}$	570.191	4.89		
Ligstroside	$C_{25}H_{32}O_{12}$	524.152	5.2		
10-Hydroxyoleuropein aglycone	$C_{19}H_{22}O_9$	394.127	5.23		
Oleocanthal*	$C_{17}H_{20}O_5$	304.132	5.24	303	59
Demethyloleuropein aglycone	$C_{18}H_{20}O_8$	364.114	6.21		
Oleocanthalic acid	$C_{17}H_{20}O_6$	320.126	5.41		
Methyl oleuropein aglycone	$C_{20}H_{24}O_8$	392.148	5.65		
Oleuropein aglycone methyl ester	$C_{20}H_{26}O_9$	410.158	5.86		
Ligstroside aglycone*	$C_{19}H_{22}O_7$	362.137	-1	361	291
Oleuropein aglycone*	$C_{19}H_{22}O_8$	378.132	-2	377	275
Triterpenes					
Maslinic acid*	$C_{30}H_{48}O_4$	472.355	9.33		
Oleanolic acid*	$C_{30}H_{48}O_3$	456.361	11.67		
Erythrodiol*	$C_{30}H_{50}O_2$	442.381	11.84		
Simple phenols					
Hydroxytyrosol*	$C_8H_{10}O_3$	154.062	1.65	153	123
Tyrosol*	$C_8H_{10}O_2$	138.068	2.45		
Hydroxytyrosol acetate	$C_{10}H_{12}O_4$	196.074	4.15		
Tyrosol acetate	$C_8H_8O_3$	152.047	5.24		
Phenolic acids and derivatives					
Methyl salicylate	$C_8H_8O_3$	152.048	1.75		
3,4-Dimethoxybenzoic acid*	$C_9H_{10}O_4$	182.058	2.24		
p-Hydroxybenzoic acid*	$C_7H_6O_3$	138.032	2.34		
Caffeic acid*	$C_9H_8O_4$	180.042	3.08		
3,4-Dihydroxybenzaldehyde	$C_7H_6O_3$	138.032	3.14		
Coumaric acid*	$C_9H_8O_3$	164.048	4.33		
Ferulic acid*	$C_{10}H_{10}O_4$	194.058	5.1		
Phenylacetic acid	$C_8H_8O_2$	136.052	5.31		
Elenolic acid and derivative					
Dialdehydicform of decarboxymethyl-elenolic acid	$C_9H_{12}O_4$	184.073	2.38		
Elenolic acid	$C_{11}H_{14}O_6$	242.079	6.59		
Lignans					
1-Acetoxypinoresinol	$C_{22}H_{24}O_8$	416.149	4.96		
1-Hydroxypinoresinol	$C_{20}H_{22}O_7$	374.138	3.91		

[1]Ligstroside aglycone is the combination of three isomers with the following retention times: 6.2; 6.9 and 7.6 min.

[2]Oleuropein aglycone is the combination of five isomers with the following retention times: 4.5; 5.1; 5.8; 6.7 and 7.8 min.

*Identification confirmed by analytical standards.

Once the signal alignment has been done, the obtained chromatographic peaks were integrated to obtain a clean matrix, which was exported as a CSV file, to accomplish both the identification of the MFs and the corresponding statistical analyzes. Metaboanalyst v4.0 [35] was used for further processing and statistical analyzes. Normalization by logarithmic transformation (log2) and autoscaling were used as a pre-processing step. Statistical analysis included the ANOVA test applied to find the number of significant molecular entities ($p \leq 0.05$), and pairwise combinations (Tukey HSD), to identify significant differences in relative concentration of identified compounds, between samples belonging to different growth times.

Once all MFs were extracted and aligned, the software MassHunter Qualitative v7.0 was used for the targeted extraction of MS/MS information associated with the monitored MFs in the whole set of analyses. This information was used for tentative identification of metabolites by searching in the METLIN MS/MS (http://metlin.scripps.edu), MassBank MS/MS (http://www.massbank.jp), and ReSpect MS/MS (http://spectra.psc.riken.jp) databases. Additionally, some compounds were confirmed by both MS/MS information and retention time, using commercially available.

3. Results and Discussion

3.1. Identification of bioactive compounds in oil samples

After processing the experimental data obtained by LC–QTOF MS/MS, a total of 35 bioactive compounds (17 secoiridoids and derivatives, 8 phenolic acids, 3 flavonoids, 3 triterpenes, 2 lignans, and elenolic acid and a derivative) were tentatively identified in all analyzed oils (Supplementary Figure 2). Eighteen out of them were confirmed by injection of analytical standards. Table 1 summarizes the main parameters (formula, neutral mass, retention time, precursor ion and main fragments) for identification of these compounds.

Secoiridoids are characteristic of *Oleaceae* plants and constitute the main phenolic fraction of VOO. Oleuropein aglycone, ligstroside aglycone, oleacein and oleocanthal can represent around 90% of the total phenolic content of (E)VOOs [36].

Secoiridoids are produced from the secondary metabolism of terpenes, but the main olive secoiridoid glucosides, oleuropein and ligstroside, are scarcely found in (E)VOO [10].

Phenolic acids and flavonoids are widely distributed in the plant kingdom. Ferulic, vanillic, caffeic and *p*-coumaric acids were the most abundant phenolic acids in the analyzed VOOs. The two latter are precursors of apigenin and luteolin, which were the two main flavonoids identified in this study [37]. These two compounds are present in small amounts in EVOOs, frequently at concentrations below 2 mg/kg. Contrarywise, the concentration of lignans in (E)VOOs may be above 100 mg/kg and has been proposed as a cultivar marker [38].

The main triterpenes identified in VOO were oleanolic acid, maslinic acid and erythrodiol. Maslinic acid has two vicinal hydroxyl groups at C-2 and C-3 positions apart from the carboxyl radical. On the other hand, erythrodiol possesses two hydroxyl groups in remote positions and are different with regard to the methyl group location. In virgin olive oil, the concentration of triterpenes oscillates between 8.9 and 112 mg/kg [20]

3.2. Phenolic composition in 'Arbequina' and 'Picual' oils during the conventional extraction process

We analyzed changes occurring in the relative concentration of identified bioactive compounds in oils sampled at three different steps of the conventional process for VOO extraction. Samples were obtained at the output of the decanter (decanter oil), after vertical centrifugation (centrifuge oil), and the final oil. Changes were evaluated for the two cultivars, 'Arbequina' and 'Picual'.

The ANOVA and pairwise analysis (Tukey HSD, p-value < 0.05) revealed significant differences in the composition of bioactive compounds (phenols and terpenes) among the decanter oil, centrifuge oil and final oil for each cultivar. Figure 1 shows relative concentrations for each family of compounds expressed as mg/kg equivalents of oleuropein. A significant effect was found for the most concentrated phenolic family, secoiridoids, since their concentration in 'Picual' decreased in the final oil as compared to previous samples. On the other hand, the maximum content

of secoiridoids in 'Arbequina' was found in the final oil. A contrary pattern was found for the rest of phenolic families such as flavonoids, phenolic acids and derivatives, simple phenols and lignans. Thus, in 'Arbequina' these four families showed a trend decay in concentration along the extraction process, while in 'Picual' these groups of compounds were increased in the final oil as compared to the samples collected after decantation and centrifugation. It is worth mentioning that these compounds were detected in the 'Arbequina' decanter oil at higher concentration than in the 'Picual' decanter oil. However, the two cultivars led to final oils with quite similar levels of flavonoids, lignans and phenolic acids and derivatives (Figure 1). Simple phenols, including tyrosol, hydroxytyrosol and acetate esters were more concentrated in 'Arbequina' than in 'Picual' final oil.

Concerning triterpenes, the highest concentration in 'Arbequina' was found in the decanter and centrifuge oils, whereas they significantly decreased in the final oil. In 'Picual', the concentration of triterpenes increased gradually during the extraction process to achieve the maximum level in the final oil.

The biosynthesis of both secoiridoids and triterpenes is initiated with geranyl diphosphate (GPP) as common precursor (Supplementary Figure 3). However, their production is highly dependent on the olive cultivar, among other factors such as environmental factors [39, 40]. Generally, GPP can be converted into geraniol by geranyl diphosphate diphosphatase to produce secoiridoids. In parallel, GPP is converted to squalene, the immediate precursor of triterpenoids, in two steps starting by the conversion of GPP into farnesyl diphosphate by farnesyl diphosphate synthase and then into prescualene diphosphate by farnesyl diphosphate farnesyltransferase. Additionally, both hydroxytyrosol and tyrosol, namely simple phenols, are the final products in the degradation of secoiridoids. The high concentration of simple phenols and the low content of secoiridoids in 'Arbequina' oil, as compared to 'Picual', suggests a greater stability of 'Picual' during the conventional production process. Also, the high concentration of triterpenes in the decanter oils (almost three times superior in 'Arbequina') suggests a favoured conversion of GPP into triterpenes. Nevertheless, the concentration of these compounds in the final oil was similar in both cultivars.

In general terms, minor families of bioactive compounds such as flavonoids, simple phenols, lignans, phenolic acids and triterpenes in 'Arbequina' decreased their concentration from the decanter up to the final oil. On the other hand, secoiridoids increased slightly their overall concentration. In turn, 'Picual' final oil was more enriched in these minor families as compared to the decanter and centrifuge oils. Secoiridoids in 'Picual' samples showed a reduced variation along the production process. These results allow pointing out that bioactive compounds in VOO may experience concentration changes during the production process. These changes should be explored for each cultivar to obtain VOO with a desired profile of bioactive compounds in the final oil.

3.3. Effect of destoning on the VOO profile of bioactive compounds

The influence of fruit destoning on the concentration of bioactive compounds was compared separately in 'Arbequina' and 'Picual' VOOs during the extraction process. For this purpose, we only evaluated the composition of final oils. The most significant effect was observed in the concentration of secoiridoids in 'Arbequina' because they were seriously decreased when olive fruits were destoned prior extraction (Figure 2). Less significant was the increase in the concentration of secoiridoids found in 'Picual' VOO obtained from destoned fruits. With these premises, destoning of olive fruits seems to affect significantly to the metabolism of secoiridoids and, especially, to β-glucosidases and esterases, which are the two enzymes involved in the production of major secoiridoid derivatives found in VOO. An explanation to this effect was that destoning in 'Arbequina' fruits could reduce the activity of these enzymes.

The contrary effect was detected for flavonoids since their concentration was significantly increased in 'Arbequina' VOO obtained by the destoning process while in 'Picual' flavonoids were higher in VOO produced from intact fruits as compared to destoned fruits. For lignans, we observed the same effect than that found for flavonoids in 'Arbequina', but no significant changes were found in 'Picual'.

VOOs produced with 'Picual' and 'Arbequina' destoned fruits reported a lower concentration of phenolic acid and derivatives while no effect was described

for simple phenols. Finally, triterpenes also allowed differentiating between both cultivars when VOO was obtained from destoned fruits. Thus, in 'Arbequina' we found a decrease in the concentration of triterpenes when fruits were destoned while it was totally different in 'Picual', the concentration of triterpenes was increased when fruits were destoned.

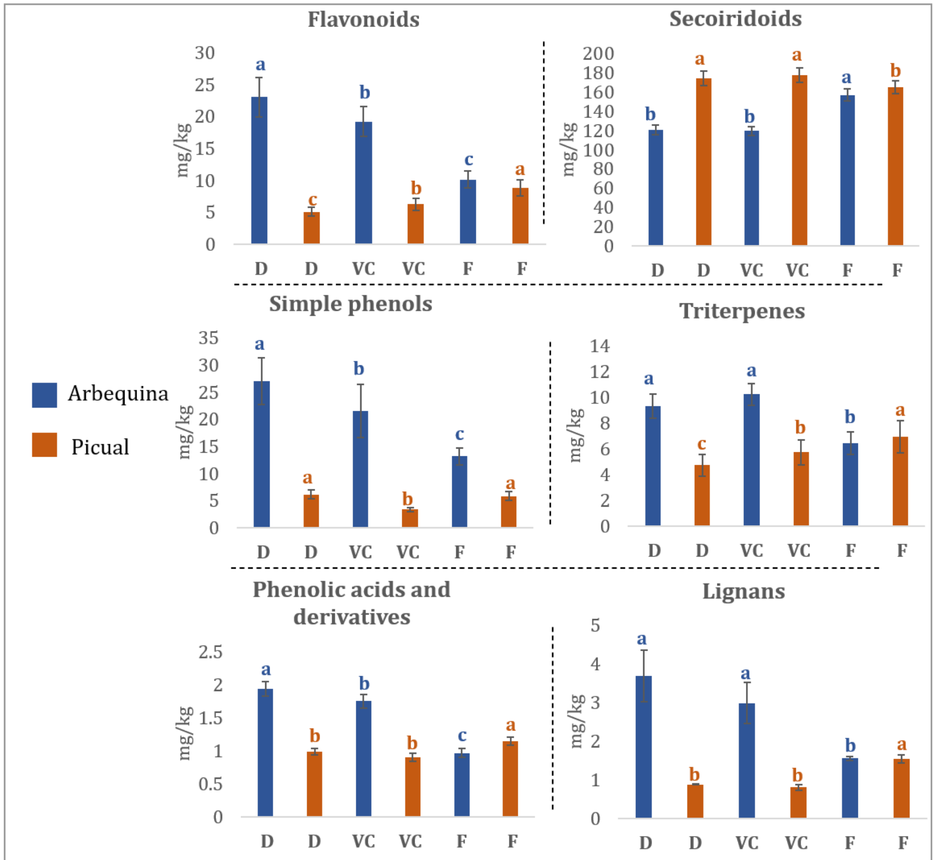

Figure 1. Relative concentrations for each family of compounds expressed as mg/kg equivalents of oleuropein in 'Arbequina' and 'Picual' VOOs (decanter **D**, vertical centrifuge **VC** and final **F** oils) obtained with the conventional process. Three replicates per sample were analyzed. Different letters for the same cultivar indicate significant changes in the concentration of bioactive compounds in oil samples obtained in the conventional process (Tukey HSD, p-value < 0.05).

3.4 Effect of the destoning on the heathy index of 'Arbequina' and 'Picual' VOOs

Phenolic compounds in VOO contribute to the health benefits recognized by the EFSA claim and included in the European Commission Regulation

(EU)432/2012 [41]. We evaluated the effect of fruit destoning on the healthy properties of the studied oils. For this purpose, we quantified the concentration of oleuropein aglycone, ligstroside aglycone, oleocanthal, oleacein and hydroxytyrosol in the final oils (Figure 3). In concordance with the results discussed above, we detected a non-significant variation in the healthy index of 'Picual' oil when fruits were previously destoned, from 14 to 15 mg/20 g. On the other hand, in 'Arbequina' the healthy index substantially decreased from 11 mg/20 g in the conventional process to 5 mg/20 g with destoned olives. These results confirm the undesirable effect of destoning in 'Arbequina' oils from the health perspective.

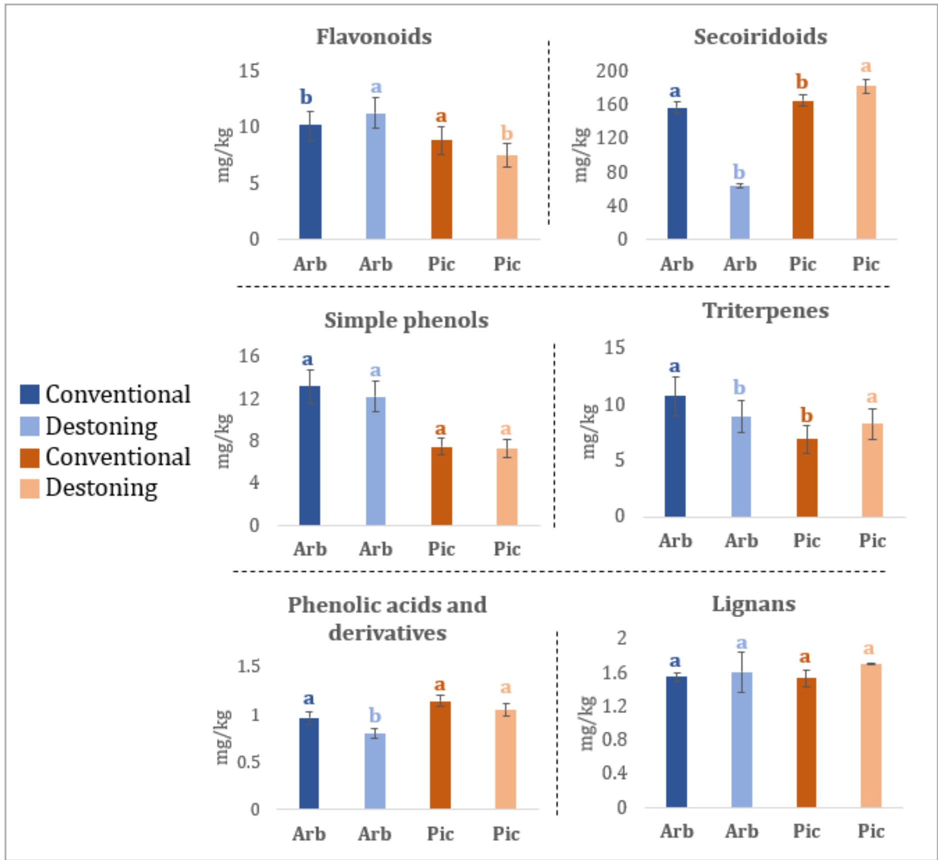

Figure 2. Relative concentrations for each family of compounds expressed as mg/kg equivalents of oleuropein in 'Arbequina' (Arb) and 'Picual' (Pic) final oils obtained with the conventional process (intact olive fruits) and the alternative process (with destoning of olive fruits). Three replicates per sample were analyzed. Different letters for the same cultivar indicate significant change in the concentration of bioactive compounds in oil samples obtained with the two processes (Tukey HSD, p-value < 0.05).

To understand the reduced phenolic content in ‘Arbequina’ oils obtained with destoned fruits, we compared levels of individual phenols in the final oils for both cultivars. Thus, no significant changes were found in the concentration of oleocanthal and oleacein in ‘Arbequina’, while these two phenols were increased in ‘Picual’ oil after fruits destoning. This process affected more significantly to the aglycone forms of oleuropein and ligstroside, which are produced by β-glucosidase hydrolysis of oleuropein and ligstroside precursors. As Figure 4 shows, the aglycone forms were slightly altered by comparing the final oils provided by the two processes, but in ‘Arbequina’ there was a substantial effect when fruits were destoned. Thus, the content of the aglycone forms of oleuropein and ligstroside were reduced approximately up to 25% of the content found in the ‘Arbequina’ (E)VOO obtained with the conventional process.

According to these results, destoning of olive fruits does not affect equally to ‘Arbequina’ and ‘Picual’ cultivars. Despite the benefits associated to this process in terms of working capacity, waste generation and VOO quality, fruits destoning reduced the health benefits of the final product in ‘Arbequina’ whereas increased slightly these properties in ‘Picual’.

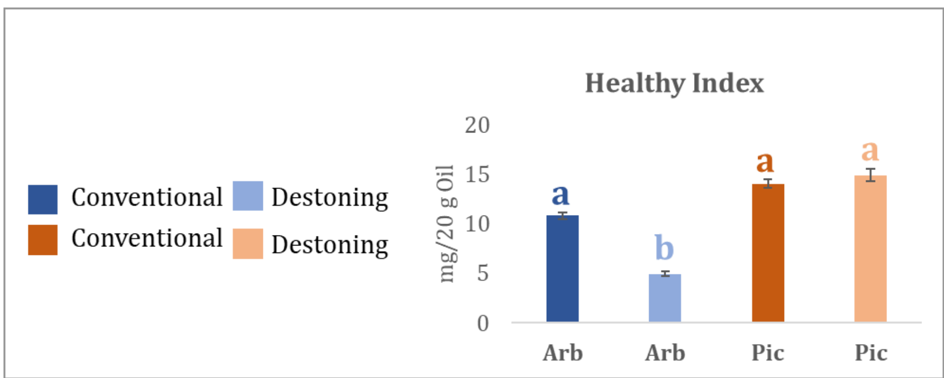

Figure 3. Healthy index expressed as mg/20 of oil measured for ‘Arbequina’ (Arb) and ‘Picual’ (Pic) final oils obtained with the conventional protocol (intact olive fruit) and that with a previous destoning of fruits. Three replicates per sample were analyzed. Different letters for the same cultivar indicate significant change in the healthy index of oil samples obtained with the two processes (Tukey HSD, p-value < 0.05).

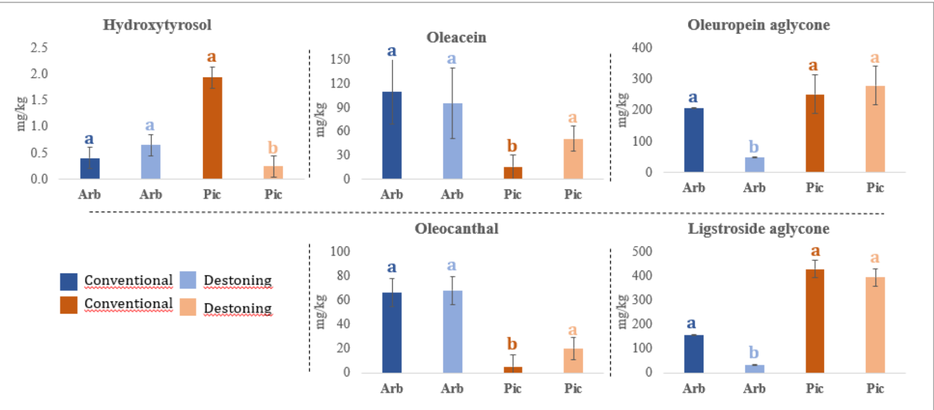

Figure 4. Phenolic concentrations expressed in mg/kg found in 'Arbequina' (Arb) and 'Picual' (Pic) VOOs obtained with the conventional process and with the alternative process with fruit destoning. Three replicates per sample were analyzed. Different letters for the same cultivar indicate significant change in the phenolic concentration of oil samples obtained with the two processes (Tukey HSD, *p*-value < 0.05).

4. Conclusions

Despite destoning have been demonstrated positive effects in the olive oil extraction process, in terms of reducing energy consumption and wastes, our results revealed a significant effect on the concentration of bioactive compounds in the final (E)VOO. This effect was different for 'Arbequina' and 'Picual' cultivars and specially affected to secoiridoid derivatives. Thus, these compounds were less concentrated in 'Arbequina' oil when fruits were previously destoned, while a slight increase was observed in the level of these compounds in 'Picual' oil obtained with destoned fruits as compared to the conventional process. This result had a significant effect on the healthy index associated to the claim that recognizes benefits of phenolic compounds.

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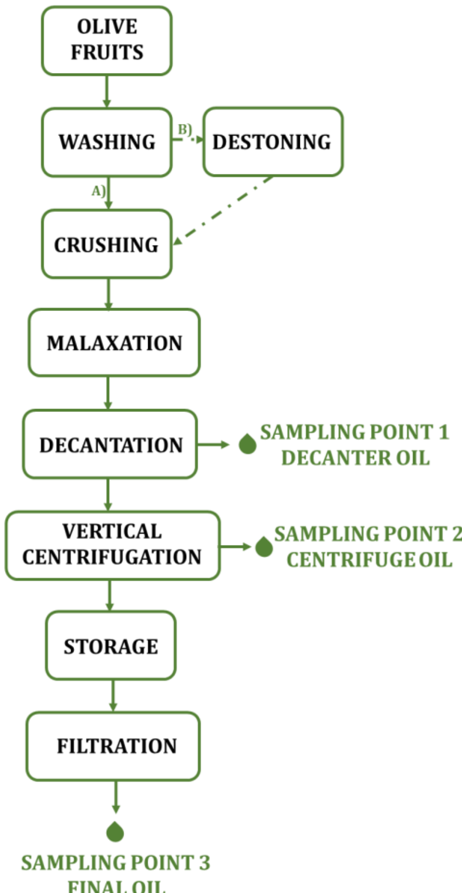


Supplementary Figure 1. Flow diagram of the industrial process for (E)VOO production. A) Conventional process. B) Alternative process with fruits destoning.

Supplementary Table 1. Calibration models prepared for quantitative analysis of target phenols.

Compound	Calibration model	R^{2c}	Calibration range
Oleuropein[a]	y = (2661 ± 42) x − 1739 ± 432	0.9895	1-20 mg/kg
Hydroxytyrosol[b]	y = (65496 ± 1912) x + 38081 ± 19685	0.9932	1-20 mg/kg
Oleacein[b]	y = (388021 ± 7310) x + 359347 ± 95883	0.998	1-20 mg/kg
Oleocanthal[b]	y = (262214 ± 5473) x + 138927 ± 56350	0.9965	1-20 mg/kg
Oleuropein aglycone[b]	y = (262214 ± 5473) x + 138927 ± 56350	0.9965	1-20 mg/kg
Ligstroside aglycone[b]	y= (89717 ± 2641) x + 10403 ± 27195	0.9931	1-20 mg/kg

[a]Calibration model obtained with the LC–QTOF system.
[b]Calibration models obtained with the LC–QqQ system.
[c]Determination coefficient.

R = OH Luteolin
R = H Apigenin
Flavonoids

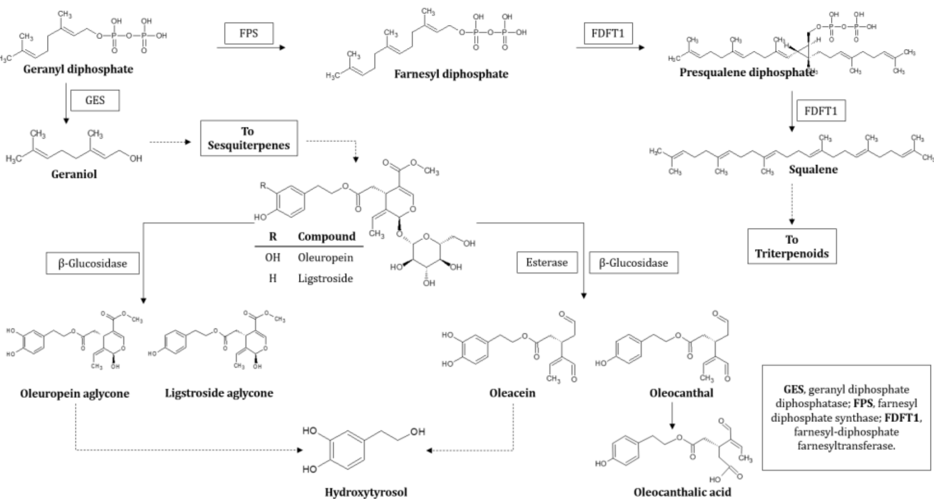
R = OH Oleuropein aglycone
R = H Ligstroside aglycone
Secoiridoids

R = OH Hydroxytyrosol
R = H Tyrosol
Simple Phenols

Caffeic acid
Phenolic acids

R = OH 1-Acetoxypinoresinol
R = OCOCH3 1-Hydroxypinoresinol
Lignans

Supplementary Figure 2. Chemical structures representing the main families of bioactive compounds identified in VOO: flavonoids, secoiridoids, triterpenes, simple phenols, phenolic acids, and derivatives, and lignans.



Supplementary Figure 3. Pathway involved in the formation of bioactive compounds.

CHAPTER 6

Monitoring the transference of bioactive compounds in the process for extraction of extra virgin olive oil

Journal of Agricultural and Food Chemistry

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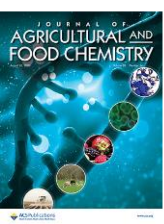

Monitoring the Transference of Bioactive Compounds in the Process for Extraction of Extra Virgin Olive Oil

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Monitoring the Transference of Bioactive Compounds in the Process for Extraction of Extra Virgin Olive Oil

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Abstract

The influence of the extraction process on the content of bioactive components in extra virgin olive oil (EVOO) is critical. Nevertheless, the behavior of individual compounds in terms of partition among the involved phases (pastes, wastewater and oil) has not been deeply evaluated. In this research, we aimed at monitoring individual bioactive compounds in different samples obtained in the production process, from fruits to the final oil. Two cultivars with recognized different profile in bioactive components, 'Arbequina' and 'Picual', were evaluated to obtain complementary results. Secoiridoids were predominantly transferred to EVOOs with different relative profiles for both cultivars. Main differences between the extraction process for 'Arbequina' and 'Picual' were found for major phenols, oleuropein aglycone, ligstroside aglycone, oleocanthal and oleacein. Lignans also reported a high transfer to EVOO while trace levels were detected in olive pomace (alperujo) and wastewater. Concerning hydroxytyrosol and its conjugated derivatives, they were partitioned among the three output phases according to polarity. Finally, flavonoids and triterpenes remained predominantly in the olive pomace phase, particularly, oleanolic and maslinic acid were low detected in EVOOs and wastewaters.

Keywords: Extra virgin olive oil; bioactive compounds; phenols; triterpenes; olive pomace; paste; wastewater.

1. Introduction

The past decade has been a rise in interest in the beneficial effects of the Mediterranean diet. This expansion is largely since extra virgin olive oil (EVOO) is one of the main pillars supporting the health and nutritional benefits associated to this diet. EVOO has a particular fragrant flavor because of the extraction from olives by only physical means under conditions (particularly thermal) that do not lead to alterations in the oil [1, 2]. The sensory properties and health characteristics of EVOO are linked to its chemical characteristics, particularly, to the presence of several minor compounds, which represents approximately 2% of the total weight. These compounds pertain to families such as tocopherols, hydrocarbons, sterols, aliphatic alcohols, carotenoids, chlorophylls, volatiles, triterpenoids, and phenolic compounds [3].

Phenolic compounds are one of the most relevant chemical families. According to the European Commission (EU) 432/2012, "phenols protect blood lipids from oxidative stress", which is one of the main mechanisms involved in the development of pathologies such as cardiovascular diseases [4, 5]. Additionally, phenols are responsible for organoleptic attributes, pungency, and bitterness, but also for the shelf life of the EVOO due to their antioxidant capabilities [6]. Most phenolic compounds identified and quantified in EVOO belong to five different classes: (i) flavonoids; (ii) secoiridoids; (iii) simple phenols; (iv) phenolic acids; and (v) lignans. Within this great chemical variability, it is worth noting the role of secoiridoids as conjugated forms of hydroxytyrosol and tyrosol. This group of compounds, which are specific to the Oleaceae family, includes oleuropein and ligstroside aglycone isomers and the decarboxymethylated dialdehyde forms of oleuropein and ligstroside aglycones, which are more frequently referred to as oleacein (3,4-DHPEA-EDA) and oleocanthal (p-HPEA-EDA), respectively [7].

Regarding to other important bioactive compounds, terpenes are not only the largest group of plant natural products, comprising at least 30.000 compounds, but also contain the widest assortment of structural types. Hundreds of different monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), and triterpenes (C30) carbon skeletons are known [8]. In recent years, the number of studies focused on

the bioactivity of EVOO triterpenes is increasing. Antitumoral, anti-inflammatory, antioxidant, hepatoprotective, cardioprotective, and antimicrobial activities have been recently described. Oleanolic acid, maslinic acid, uvaol and erythrodiol are the main triterpenes found in EVOO [9, 10].

Typical olive oil extraction processes are generally based on traditional pressing, the three-phase system, or the most extended two-phase centrifugal approach. The latter has been widely accepted for production of EVOOs [11]. The general steps of the production process include harvest, washing, and crushing of olives, malaxation of olive paste, decantation/centrifugation, storage, and filtration. The two-phase process is characterized by the "no-water" decanter (or horizontal centrifugation). The important advantages of this decanter are the reduction in the amount of wastewaters and the greater recovery of phenolic compounds, mainly due to their better solubility in water than in oily phase [12].

Bioactive compounds are strongly influenced by the operational conditions in the technological extraction process. The quality of EVOO depends on physical, chemical, and biochemical reactions which occur during extraction process, and during storage [13]. In this sense, crushing and malaxation are critical steps. Crushing of olives is a physical process used to break the tissues and release the oil drops contained in the vegetable cell vacuoles. After olive crushing, several enzymes involved in the generation and transformation of minor compounds can be activated such as β-glucosidases, esterases and oxidoreductases, which regulate the synthesis of secoiridoid derivatives. After crushing, both oleuropein and ligstroside, the main secoiridoids in the olives, are hydrolyzed into their corresponding aglycone forms [14, 15]. Malaxation consists of mixing olive paste to increase the percentage of free oil and help small oil droplets to coalesce and agglomerate. Increasing the temperature during the olive paste malaxation process enhances the activity of oxidative enzymes such as polyphenoloxidase, peroxidase, and lipoxygenase. These enzymatic activities explain the lineal increase of hydroxytyrosol and tyrosol obtained by degradation of complex phenolic compounds during malaxation [16, 17]. Centrifugation is a separation of the olive oil fraction from the vegetable solid material and vegetation water. In these steps, the minor compounds are partitioned

in the different phases (oil, water, and solids) as a function of the affinity towards each phase which is related to the relative polarities of the compounds, and phases ratio. In general, the number of bioactive compounds in the final olive oil is 1–2% of the available pool of these compounds in the fruit. The rest is lost with wastewater and pomace [18]. In vertical centrifugation, the addition of water can result in decrease of EVOO phenols owing to their hydrophilic nature [19]. Filtration is a special important final step to remove suspended solids and moisture. The higher polar phase content in unfiltered olive oils may augment alteration of EVOO, especially, at an inappropriate temperature, mainly affecting free acidity, sensory attributes, and the formation of simple phenols due to the hydrolysis rate of secoiridoid derivatives [20]. Most of the studies on olive oil processing are focused on the total content of phenolic compounds, disregarding other minor compounds like triterpenes.

Unfortunately, the production of EVOO is associated with the generation of large quantities of wastes. In the two-phase process, humid semi-solid pomace is obtained as by-product (wet olive pomace), which is a rich source of bioactive compounds. Therefore, there is a growing concern on finding effective ways to extract, isolate and use the bioactive compounds for added-value applications [21, 22]. In this sense, it is essential to know how the bioactive compounds are partitioned, which phase is the most enriched, and what would be the most suitable matrix to extract them. The objective of this study was to evaluate changes occurring in bioactive compounds during the two-phase extraction process. For this purpose, six types of samples were analyzed by LC–QTOF MS/MS: intact olives, crushing paste, malaxation paste, final oil, wastewater and olive pomace (alperujo). 'Picual' and 'Arbequina' were the two cultivars selected due to their different bioactive profiles in the final oil.

2. Materials and methods

2.1 Samples

'Arbequina' and 'Picual' olive fruits were collected at optimum ripening stage (2.0, yellowish-red color) according to the method proposed by the

International Olive Oil Council [23] in two independent orchards located at Alcaudete and Torredonjimeno (Jaen, Spain), respectively. Fruits from each cultivar were processed in two batches to obtain EVOO by following the same process. Six types of samples were taken during the olive extraction process (Supplementary Figure 1): intact olives, crushing paste, malaxation paste, final olive oil, wastewater and olive pomace. Three replicates of each sample were analyzed.

2.2. Reagents and standards

LC-grade ethanol, acetone, chloroform, and methanol used for sample preparation were from Scharlab (Barcelona, Spain). MS-grade formic acid, used as ionization agent, acetonitrile and 2-propanol, used for preparation of chromatographic mobile phases, were from Fisher Scientific (Madrid, Spain). Deionized water (18 MΩ • cm) was obtained from a Milli-Q water purification system. Oleuropein standard was purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Apparatus and instruments

A vortex shaker from IKA (Wilmington, NC, USA) and a centrifuge supplied by Ortoalresa (Madrid, Spain) were used for sample preparation. Chromatographic separation was carried out by an Agilent 1200 series LC (Palo Alto, CA, USA) furnished with an Inertsil ODS-2 C18 analytical column (250 × 4.6 mm i.d., 5 μm particle) from GL Science (Tokyo, Japan). The chromatograph was coupled through an electrospray ionization (ESI) source to a 6540 quadrupole–time-of-flight hybrid mass spectrometer (QTOF MS/MS; Agilent Technologies, Santa Clara, CA) for detection.

2.4. Sample preparation

Bioactive compounds were isolated from oil samples by liquid–liquid extraction. An aliquot of 2 mL of oil was mixed with 3 mL of 70:10:10:10 (v/v) ethanol-chloroform-acetone-water and shaken for 2 min. The phases were separated by centrifugation (8 min, 900 *g*). The top layer was filtered by 0.2 μm nylon filter, from Agilent, prior to injection into the LC–QTOF system. For solid

samples, these were previously homogenized with a mortar and liquid nitrogen. An aliquot of 2.5 g (olives, crushing paste, malaxation paste and olive pomace) was mixed with 10 mL of 70:10:10:10 (v/v) ethanol-chloroform-acetone-water and shaken for 1 hour. Then, extracts were filtrated with a 0.2 µm nylon filter prior to analysis. Wastewater samples were filtered and analyzed. Each sample was characterized in triplicate.

2.5. LC–QTOF MS/MS analysis

The chromatographic mobile phases were water (phase A) and 50:50 (v/v) acetonitrile/2-propanol (phase B), both solutions acidified with 0.1% (v/v) formic acid. Flow-rate was set at 0.6 mL/min. The injection volume was 10 µL. The LC pump was programmed with the following elution gradient: 20% to 45% B in 3 min, change from 45% to 70% B in 9 min, from 70% to 100% B in 3 min and constant at 100% B for 10 min (total time 25 min). After analysis, the column was equilibrated to the initial conditions for 5 min. The column compartment was kept at 30 °C.

The parameters of the ESI source, operating in negative ionization mode, were as follows: nebulizer gas, 40 psi; flow rate and temperature of the drying gas (N_2), 12 L min^{-1} and 325 °C; capillary voltage, ± 3.5 kV; Q1, skimmer, and octapole voltages, 130, 65, and 750 V, respectively. Data were collected in centroid mode in the extended dynamic range (2 GHz). Full scan was carried out at 6 spectra per second within the *m/z* range of 40–1200, with subsequent activation of the three most intense precursor ions (allowed charge: single or double) by MS/MS using collision energies of 12 and 25 eV at 3 spectra/s within the *m/z* range 30–1200. An exclusion window of 0.75 min after the first spectrum was programmed to avoid repetitive fragmentation of the most intense precursor ions. To assure the desired mass accuracy of the recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 112.9856 (trifluoroacetic acid anion) and *m/z* 1033.9881 (HP-921) in negative ion mode.

Relative quantitation was carried out using oleuropein as reference standard. For this purpose, a calibration model was prepared by analysis of oleuropein solutions at concentrations ranging from 1 to 20 µg/g (Supplementary

Table 1). Detected compounds were quantified in concentration units expressed as oleuropein (mg/kg).

2.6. Data processing and statistical analysis

MassHunter Workstation software (version B8.00 Profinder; Agilent Technologies, Santa Clara, CA) was used to process the data obtained by LC–QTOF in MS/MS mode. Treatment of the raw data file was initiated by extraction of potential molecular features (MFs) with the suited algorithm included in the software. The recursive extraction algorithm considered all ions exceeding 5000 counts as cut-off. Additionally, the isotopic distribution to consider a molecular feature as valid should be defined by two or more ions (with a peak spacing tolerance of *m/z* 0.0025, plus 10.0 ppm in mass accuracy). Apart from $[M-H]^-$ ions, adducts formation in the negative ionization mode ($HCOO^-$, Cl^-), as well as neutral loss by dehydration, were included to identify features corresponding to the same metabolite. Thus, ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as entities characterized by their retention time (RT), intensity at the apex of the chromatographic peaks and accurate mass. Background contribution was removed by subtraction of MFs linked to the blank under identical operational conditions of the samples.

Once the signal alignment was completed, the obtained chromatographic peaks were integrated to obtain a clean matrix, which was exported as a CSV file, to accomplish the identification of the MFs. Metaboanalyst v4.0 [24] was used for further processing and statistical analysis. Normalization by logarithmic transformation (log2) and autoscaling were used as a pre-processing step. Statistical analysis included the ANOVA test ($p \leq 0.05$) and pairwise combinations (Tukey HSD) to identify significant differences in relative concentration of identified compounds. Principal component analysis (PCA) was used to identify discrimination patterns among samples.

Once all MFs were extracted and aligned, the software MassHunter Qualitative v7.0 was used for the targeted extraction of MS/MS information

associated with the monitored MFs in the whole set of analyses. This information was used for tentative identification of metabolites by searching in the METLIN MS/MS (http://metlin.scripps.edu), MassBank MS/MS (http://www.massbank.jp), and ReSpect MS/MS (http://spectra.psc.riken.jp) databases. Additionally, some compounds were confirmed by both MS/MS information and retention time using standards.

3. Results and Discussion

3.1. Identification of bioactive compounds

A total of 40 compounds were tentatively identified in the complete set of samples, 15 out of them were confirmed by analytical standards. Table 1 shows the main parameters (formula, neutral mass, and retention time) of the identified compounds, which are grouped by chemical classes in flavonoids (7); iridoids (3); secoiridoids (17); triterpenes (2); simple phenols (4); phenolic acids and derivatives (5); and elenolic acid and derivative.

During oil extraction bioactive compounds are distributed in the oil, water, and solids as a function of affinity, which is related to the relative polarity of the compounds and phases ratio [25]. Supplementary Figure 2 shows the Base Peak Chromatograms (BPCs) for 'Picual' cultivar. Simple phenols and phenolic acids (between 1 and 4.3 min) were detected at high levels in wastewater, with low presence in the oil due to their hydrophilic nature. Thus, wastewater samples were abundant in hydroxytyrosol, hydroxytyrosol glucoside, tyrosol, *p*-hydroxybenzoic acid, coumaric acid and caffeic acid.

Flavonoids are widely distributed in the plant kingdom. Apigenin, luteolin and diosmetin were the main compounds identified in olive oils. On the other hand, conjugated forms such as luteolin-7-O-glucoside and rutin have only been reported in olive peel [11]. We only identified this compound in the extracts from solid samples (olives, crushing and malaxation paste, and olive pomace).

Secoiridoids are characteristic of *Oleaceae* plants and constitute the main phenolic fraction of VOOs. Oleuropein aglycone, ligstroside aglycone, oleacein and

oleocanthal can represent around 90% of the total phenolic content of EVOOs [3]. Due to the less polar properties of these compounds, they are found at higher concentration in oils as compared to wastewaters (Supplementary Figure 2). On the other hand, main secoiridoid glucosides, oleuropein and ligstroside, were found at trace concentrations in oils. Iridoids, precursor of secoiridoids, followed a common pattern to oleuropein and ligstroside, and they were practically undetected in oil samples. In turn, lignans, one other phenolic class, were mainly detected in olive oils.

Triterpenoids represent the major terpenic compounds in olive fruits. Triterpenic diols are replaced by triterpenic acids during ripening. Maslinic acid and oleanolic acid are the main triterpenoids found in fruits and they decreases during ripening [6]. Supplementary Figure 2 reveals that chromatographic signal of maslinic acid (16.7 min) is clearly lower in solid samples than those found in liquid samples (wastewater and oil).

3.2. Bioactive compounds in 'Picual' and 'Arbequina' samples

Changes occurring in the concentration of bioactive compounds were evaluated in the samples collected after EVOO extraction by unsupervised analysis. Scores plots reported by PCA (Figures 1) show a clear discrimination of samples in both 'Arbequina' and 'Picual' cultivars with a common pattern. Wastewater was clearly discriminated along component 1 (PC1), while the solid samples including olive, crushing and malaxation paste and olive pomace, were discriminated along component 2 (PC2). In fact, there was a sequential discrimination following the extraction process: olive fruits, crushing paste, malaxation paste and olive pomace. These groups can be explained by metabolic transformations occurring during the extraction process but also by partitioning in the involved phases. Heat maps illustrated in Figure 2 allow visualizing these effects for both cultivars. Concentration changes observed for main families of bioactive compounds can be monitored in the diagrams, which reveal a common performance for both cultivars. Thus, the concentration of iridoids was maximum in olive pomace and wastewater while minimum levels were found in olive oil, which is clearly attributed to the polar profile of this family. On the other hand, secoiridoids were decreased after

malaxation to reach minimum levels in olive pomace and wastewater, but the maximum concentration was found in EVOO.

Table 1. List of identified compounds with the main parameters (formula, neutral mass, and retention time).

Compound Name	Formula	Mass (g/mol)	RT (min)
Flavonoids			
Luteolin7-rutinoside	$C_{27}H_{30}O_{15}$	594.159	3.94
Rutin	$C_{27}H_{30}O_{16}$	610.155	4.15
Luteolin-7-O-glucoside	$C_{21}H_{20}O_{11}$	448.102	4.19
Apigenin7-O-neohesperidoside (Rhoifolin)	$C_{27}H_{30}O_{14}$	578.168	4.55
Luteolin*	$C_{15}H_{10}O_6$	286.048	6.14
Diosmetin*	$C_{16}H_{12}O_6$	300.064	6.66
Apigenin*	$C_{15}H_{10}O_5$	270.053	7.01
Iridoids			
10-Hydroxyloganin	$C_{17}H_{26}O_{11}$	406.147	2.07
Loganin +H2O	$C_{17}H_{28}O_{11}$	408.164	3.7
Secologanol (Loganin)	$C_{17}H_{26}O_{10}$	390.157	5.13
Secoiridoids			
Hydroxylated form of decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_7$	336.122	2.2
Oleoside 11-methyl ester	$C_{17}H_{24}O_{11}$	404.133	2.27
10-Hydroxyoleuropein	$C_{25}H_{32}O_{14}$	556.178	2.76
Oleuropein+4H	$C_{25}H_{36}O_{13}$	544.216	3.53
Nuzhenide	$C_{31}H_{42}O_{17}$	686.242	3.59
Verbacoside	$C_{29}H_{36}O_{15}$	624.206	3.87
Oleacein*	$C_{17}H_{20}O_6$	320.126	4.37
Oleuropein-Glucoside	$C_{31}H_{42}O_{18}$	702.239	4.4
10-Hydroxyoleuropein aglycone	$C_{19}H_{22}O_9$	394.128	4.61
Demethyloleuropein aglycone	$C_{18}H_{20}O_8$	364.116	4.61
GL3	$C_{48}H_{64}O_{27}$	1.072.364	4.75
Oleuropein*	$C_{25}H_{32}O_{13}$	540.187	4.78
Oleocanthal*	$C_{17}H_{20}O_5$	304.131	5.24
Ligstroside	$C_{25}H_{32}O_{12}$	524.189	5.72
Methy oleuropein aglycone	$C_{20}H_{24}O_8$	392.146	6.87
Ligstroside aglycone*	$C_{19}H_{22}O_7$	362.138	-1
Oleuropein aglycone*	$C_{19}H_{22}O_8$	378.131	-2
Triterpenes			
Maslinic acid*	$C_{30}H_{48}O_4$	472.355	14.22
Ursolic/Oleanolic acid*	$C_{30}H_{48}O_3$	456.361	16.66
Simple phenols			
Hydroxytyrosol glucoside	$C_{14}H_{20}O_8$	316.117	1.51
Hydroxytyrosol*	$C_8H_{10}O_3$	154.063	1.64
Tyrosol*	$C_8H_{10}O_2$	138.068	2.55
Hydroxytyrosol acetate	$C_{10}H_{12}O_4$	196.074	4.36
Phenolic acids and derivatives			
3-Caffeoylquinic acid (Chlorogenic acid)	$C_{16}H_{18}O_9$	354.091	1.05
4-Caffeoylquinic acid (Cryptochlorogenic acid)	$C_{16}H_{18}O_9$	354.096	1.82
p-Hydroxybenzoic acid*	$C_7H_6O_3$	138.032	3.04
Caffeic acid*	$C_9H_8O_4$	180.042	3.14
Coumaric acid*	$C_9H_8O_3$	164.048	4.33
Elenolic acid and derivative			
Elenoic acid glucoside	$C_{23}H_{34}O_{16}$	566.183	1.71
Dialdehydicform of decarboxymethyl elenolic acid	$C_9H_{12}O_4$	184.073	1.72
Lignans			
1-Acetoxypinoresinol	$C_{22}H_{24}O_8$	416.149	4.96
1-Hydroxypinoresinol	$C_{20}H_{22}O_7$	374.138	3.91

[1] Ligstroside aglycone is the combination of three isomers with the following retention times: 6.25;6.89 and 7.64.

[2] Oleuropein aglycone is the combination of five isomers with the following retention times: 4.47; 5.1; 5.79; 6.73 and 7.82.

*Identification confirmed by analytical standards.

Following with other phenolic families, flavonoids were preferentially found in solid samples while minimum levels were detected in wastewaters. Low concentrations of these phenols were also found in olive oils as compared to solid material. The contrary scenario was found for lignans since minimum concentrations were detected in fruits, solid pastes and wastewaters and maximum levels were found in olive oil.

Significant differences were found for simple phenols and phenolic acids. These two groups were at higher concentration in wastewater derived from 'Picual' olive oil extraction, while in 'Arbequina' simple phenols and phenolic acids were more concentrated in olive oil and crushing paste, respectively. One other polar group of compounds is that of elenolic acid and derivatives that, was preferentially enriched in wastewater from both cultivars.

Finally, triterpenes remained preferentially in the solid samples and, particularly, in olive pomace. With these premises, this would the most suited material for isolation of these bioactive compounds due to their concentration and the residual characteristics of the olive pomace.

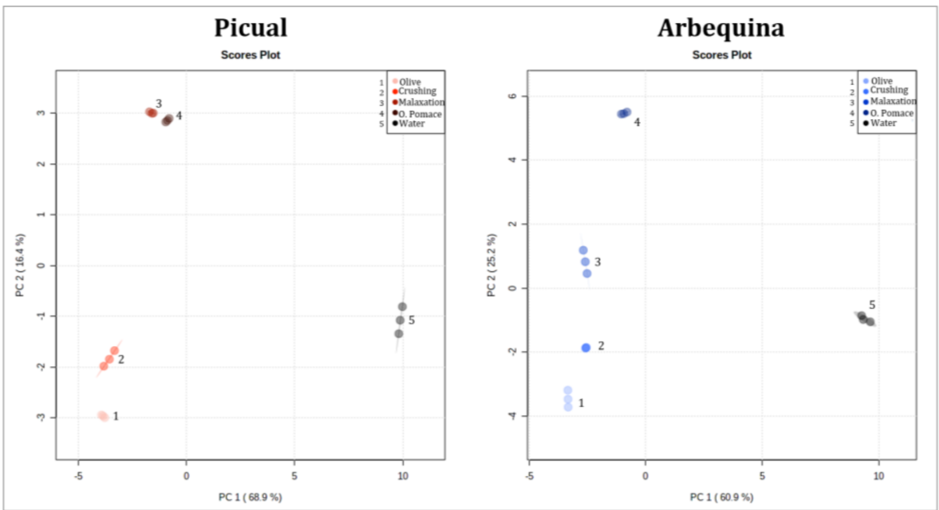

Figure 1. Principal components analysis (PCA) plots showing the distribution of the samples of olives, crushing paste, malaxation paste, wastewater, and olive pomace of 'Picual' and 'Arbequina' cultivars.

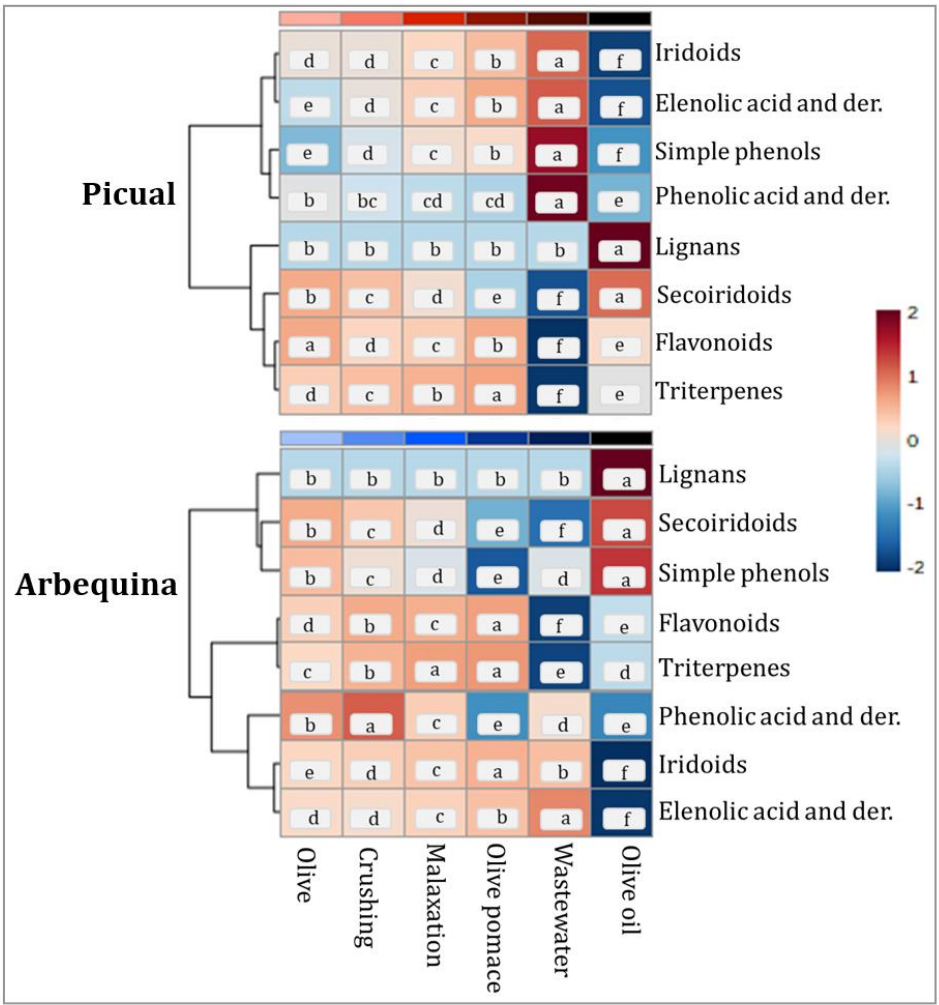

Figure 2. Heat maps showing changes in the concentration of bioactive compounds in olives, olives, crushing paste, malaxation paste, wastewater, and olive pomace of 'Picual' and 'Arbequina' cultivars. Different letters for the same cultivar indicate significant changes in the concentration of bioactive compounds in oil samples obtained in the conventional process (Tukey HSD, p-value < 0.05).

3.3. Monitoring individual bioactive compounds in 'Picual' and 'Arbequina' cultivars during EVOO extraction

Once the behaviour of overall families during the extraction of EVOO was elucidated, we were targeted on specific bioactive compounds detected in the final product. Figure 3 shows the results for main secoiridoids found in olive derived materials. These results can be discussed by comparing between hydroxytyrosol

and tyrosol derivatives. Both oleuropein and ligstroside were detected at low levels in general terms as compared to their derivatives obtained after enzymatic conversions. In addition, oleuropein and ligstroside reported a common pattern in both cultivars. Thus, oleuropein decreased progressively its concentration after fruits crushing and malaxation, whereas ligstroside increased its concentration after olives crushing. Concerning the partitioning of oleuropein and ligstroside, both secoiridoids were mainly enriched in olive pomace, oleuropein was distributed between olive pomace and wastewater, and trace concentrations were found in EVOOs.

The contrary result was identified for oleuropein and ligstroside aglycones, which were mainly enriched in EVOOs as compared to the two wastes. Both phenols, specially ligstroside aglycone, were more concentrated in 'Picual' EVOO as compared to 'Arbequina' EVOO, and this difference was particularly evident in solid pastes. However, a cultivar effect was clearly established by comparing the content of the two aglycones in EVOO and olive pomace. In relative terms, the transfer process seems to be clearly favoured in 'Arbequina' since these phenols were detected at low levels in olive pomace while relevant concentrations were found in 'Picual' olive pomace.

Differences were also found for oleacein and oleocanthal in the two cultivars. For oleacein, maximum concentrations (above 200 mg/kg) in 'Arbequina' and 'Picual' cultivars were found in fruit and crushed paste, respectively. From these samples, the concentration of oleacein described a progressive decay in the two cultivars with the extraction process. Both oleacein and oleocanthal were more concentrated in 'Picual' olive pomace than in 'Arbequina'. However, this situation was completely different for EVOOs, with significantly higher contents in 'Arbequina'. These two phenols were not properly transferred to the oily phase in 'Picual'. Trace concentrations of oleacein and oleocanthal were found in wastewaters. In general terms, wastewater seems not to be a suited residue for isolation of major secoiridoids, while 'Picual' olive pomace would be a preferred option for their isolation.

The performance for simple phenols is well illustrated in Figure 4 for hydroxytyrosol and two conjugates. Hydroxytyrosol was partitioned in both cultivars between the two residues (pomace and wastewater) and EVOO. On the other hand, hydroxytyrosol glucoside was not transferred to EVOO, remaining predominantly in the olive pomace. In turn, hydroxytyrosol acetate was preferentially distributed between EVOO and olive pomace.

The behaviour of flavonoids during the extraction process was explained by differentiating between aglycones and glucoside derivatives (Figure 5). Thus, glucoside conjugates, mainly, apigenin and luteolin derivatives, decreased their concentration in pastes after fruits crushing and they were not detected in EVOOs, remaining preferentially in olive pomace. On the other hand, the aglycone flavonoids were partitioned between EVOOs and olive pomace, being predominantly in the latter. In fact, the maximum concentration of these phenols for both cultivars was found in olive pomace.

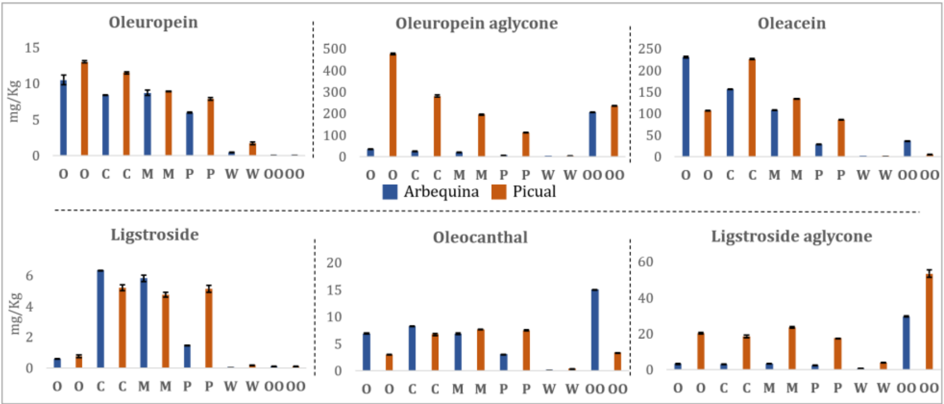

Figure 3. Relative concentrations for secoiridoid compounds expressed as mg/kg equivalents of oleuropein in 'Arbequina' and 'Picual' samples (Olive **O**, crushing paste **C**, malaxation paste **M**, olive pomace **P**, and olive oil **OO**). Three replicates per sample were analyzed.

Verbascoside, nüzhenide and GL3 are other three phenols with bioactive properties that are scarcely found in EVOOs. Thus, verbascoside was only found in fruits and pastes and low detected in wastewaters (Figure 6). Nüzhenide and GL3 reported differences by comparing both cultivars. Both minor phenols were

especially detected in 'Arbequina' fruits while in 'Picual' were particularly detected in solid samples after malaxation, and wastewater.

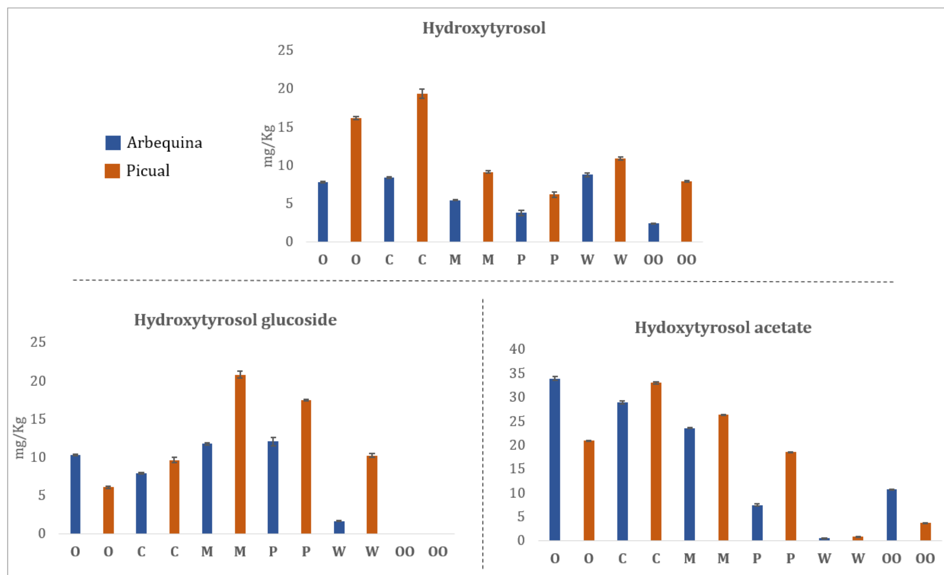

Figure 4. Relative concentrations for hydroxytyrosol and derivative compounds expressed as mg/kg equivalents of oleuropein in 'Arbequina' and 'Picual' samples (Olive **O**, crushing paste **C**, malaxation paste **M**, olive pomace **P**, and olive oil **OO**). Three replicates per sample were analyzed.

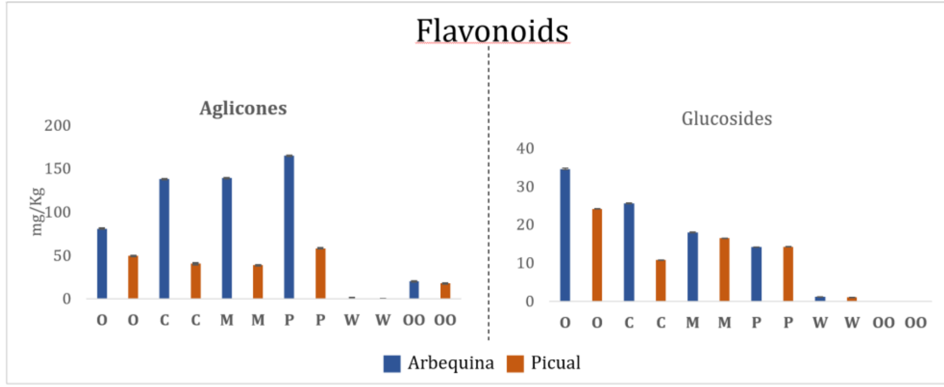

Figure 5. Relative concentrations for flavonoids expressed as mg/kg equivalents of oleuropein in 'Arbequina' and 'Picual' samples (Olive **O**, crushing paste **C**, malaxation paste **M**, olive pomace **P**, and olive oil **OO**). Three replicates per sample were analyzed.

Concerning lignans, 1-hydroxypinoresinol and 1-acetoxypinoresinol were exclusively identified in EVOOs (Supplementary Figure 3). They were not detected in solid pastes and wastewater. Both lignans were more concentrated in 'Arbequina' EVOO as compared to 'Picual' EVOO.

With these results, we can point out that the extraction process affects differently to the main families of bioactive compounds in EVOO. Thus, families such as triterpenic acids or flavonoids remain preferentially in the olive pomace residue, while lignans are exclusively transferred to the oily phase. On the other hand, secoiridoids are mainly partitioned between olive pomace and EVOO. According to their concentration in fruits, secoiridoids can be considered the target bioactive family for implementation of technological solutions to improve their enrichment in EVOOs.

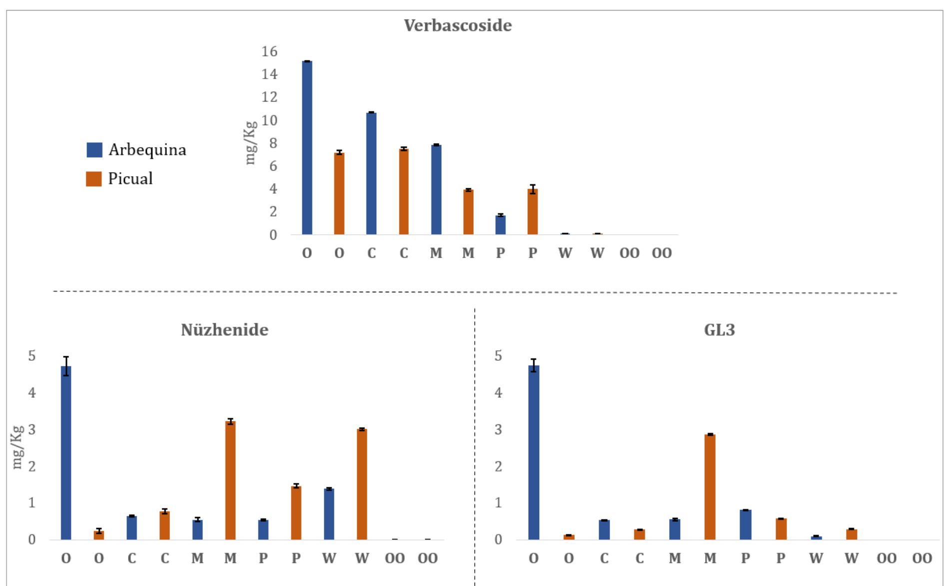

Figure 6. Relative concentrations for verbascoside, nüzhenide, and GL3 expressed as mg/kg equivalents of oleuropein in 'Arbequina' and 'Picual' samples (Olive **O**, crushing paste **C**, malaxation paste **M**, olive pomace **P**, and olive oil **OO**). Three replicates per sample were analyzed.

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Abbreviations Used

EVOO, extra virgin olive oil; 3,4-DHPEA-EDA, oleacein; p-HPEA-EDA, oleocanthal; ESI, electrospray ionization source; LC, liquid chromatography; QTOF MS/MS quadrupole–time-of-flight hybrid mass spectrometer; MF, molecular features; RT, retention time; PCA principal components analysis; BPC, base peak chromatogram

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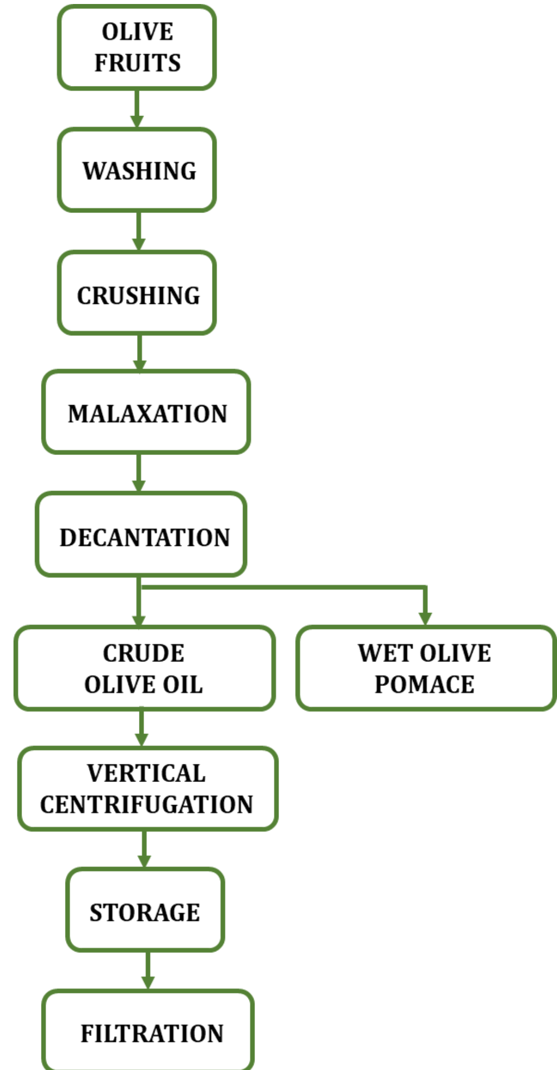
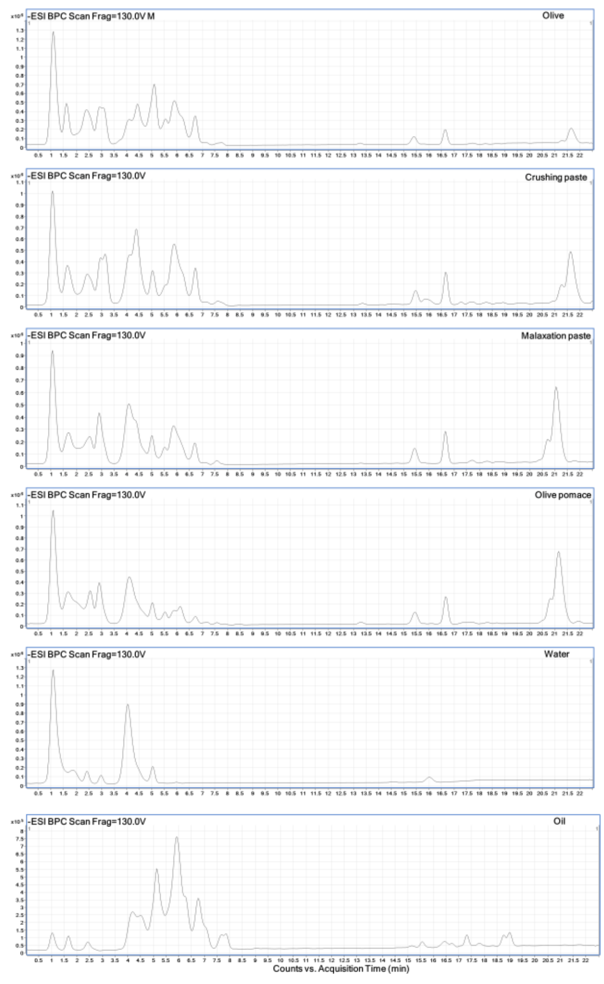


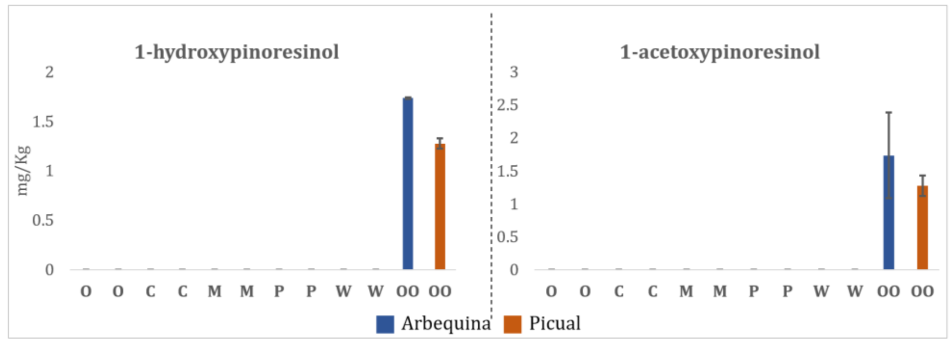
Supplementary Figure 1. Flow diagram of the industrial two-phase process for (E)VOO production.

Supplementary Table 1. Calibration model of oleuropein

Compound	Calibration model	R^2	Calibration range
Oleuropein	y = (2661 ± 42) x – 1739 ± 432	0,9895	1-20 mg/kg



Supplementary Figure 2. Base Peak Chromatograms (BPCs) for 'Picual' cultivar.



Supplementary Figure 3. Relative concentrations for lignans expressed as mg/kg equivalents of oleuropein in 'Arbequina' and 'Picual' samples (Olive **O**, crushing paste **C**, malaxation paste **M**, olive pomace **P**, and olive oil **OO**). Three replicates per sample were analyzed.

SECTION III

Strategy for analysis of diet adherence as a function of the fat source

Metabolomics is an interesting tool for assessing the nutritional status of subjects, the food consumption, the biological consequences of following a nutritional intervention, or the study of metabolic mechanisms associated with a disease in response to a diet depending on a particular metabolic phenotype. Section III of this PhD Book is devoted to offer a complementary strategy to evaluate adherence of diets by evaluating the fat source. This could be perfectly applied to monitor interventions based on olive oil consumption. Previous studies have been focused on the analysis of the fatty acids profile in plasma/serum using the conventional transesterification method without discriminating among lipid families. This method tends to favour kinetically some specific families, particularly, triglycerides. The strategy proposed in Chapter 7 involves also considering the profiling analysis of glycerophospholipid fatty acids by implementing a selective SPE step that allows fractionating these lipid families. The method has been applied in different collaboration studies with research groups pertaining to the Spanish National Center of Epidemiology and the Carlos III Health Institute to associate the glycerophospholipid fatty acids profile to the occurrence of pathologies such as breast cancer. These collaborations have led to three publications that are presented as annexes.

CHAPTER 7

Profiling analysis of phospholipid fatty acids in serum as a complement to the comprehensive fatty acids method

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Profiling analysis of phospholipid fatty acids in serum as a complement to the comprehensive fatty acids method

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Profiling analysis of phospholipid fatty acids in serum as a complement to the comprehensive fatty acids method

Inmaculada Criado-Navarro, A. Mena-Bravo, Mónica Calderón-Santiago, Feliciano Priego-Capote

Abstract

Fatty acids (FAs) are mostly found in blood as triglycerides, phospholipids (PLs) and cholesteryl esters. Determination of FAs is typically carried out in serum or plasma by a comprehensive method (known as the classical FAMEs method since FAs are determined as Fatty Acids Methyl Esters), which is based on liquid–liquid extraction, derivatization by transesterification, and determination by gas chromatography (GC) coupled to a suited detection technique. However, this method does not favor the determination of FAs that are chemically conjugated in PLs due to kinetics impediment. For this reason, we have developed a selective method to determine the FAs profile of PLs in serum based on solid-phase extraction (SPE) for isolation of PLs and determination of the FAME derivatives by GC–mass spectrometry (GC–MS). The method was applied to serum samples collected from twenty-five individuals to compare the FAs profile versus that provided by the non-selective protocol based on liquid–liquid extraction of lipid families. Statistical analysis revealed compositional changes in the FAs profile with special emphasis on the content of saturated (SFAs) and monounsaturated FAs (MUFAs). Thus, SFAs passed from 34.0% with the classical method to 49.3% in PLs while MUFAs went from 24.4% to 11.4%. This study proves that the proposed method provides complementary results to the comprehensive method and, therefore, both methods can be combined to evaluate the effect of intervention diets and their connection to metabolic diseases.

Keywords: Fatty acids; Phospholipids; FAMEs; solid-phase extraction; Mass spectrometry; Gas chromatography

1. Introduction

The determination of the fatty acids (FAs) profile in blood and adipose tissue is a widely implemented experimental tool in nutritional studies. The FAs profile may report an objective estimation of the fat quality that is proportionally consumed by an individual, with special attention paid to the distribution of saturated fatty acids (SFA), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) [1]. Several studies have evidenced dietary patterns by determination of the FAs profile [2, 3].

Fatty acids are mostly present in blood in conjugated forms as triglycerides (TGs), phospholipids (PLs) and cholesteryl esters (CEs) [4]. These three families are highly concentrated in blood, TGs and PLs typically at 150–250 mg/dL, while CEs are around 300 mg/dL [5]. Despite FAs have been selectively determined in the three lipid families, most of the studies involving FAs determination have been addressed by a comprehensive method —known as the classical FAMEs method, since FAs are determined as Fatty Acids Methyl Esters— that does not include a fractionation step [6-8]. Nevertheless, several studies pointed out that the determination of FAs independently in these three chemical families reveals different information [9-11]. Thus, FAs in serum TGs reflect the dietary fat consumed at short-term, from hours to a few days, while CEs and PLs expect to extend the period by a few days earlier [12]. On the other hand, adipose tissue provides long-term information of dietary fat with special emphasis on exogenous FAs due to their slow turnover [13]. Additionally, the distribution of FAs varies in different tissues: MUFAs seem to be more abundant in adipose tissue while plasma and whole blood are richer in PUFAs [14].

Studies targeted at determination of blood PLs are gaining interest in the clinical field [15-18]. Phospholipids constitute a heterogeneous group of small amphiphilic molecules. They represent a class of membrane lipids with a glycerol backbone in which the sn-1 and sn-2 hydroxyl groups are esterified by fatty acids, whereas the third hydroxyl group is phosphorylated. The phosphate group can be esterified by several polar molecules, particularly amino alcohols and alcohols such

as ethanolamine, choline, inositol or glycerol, among others [19]. PLs are involved in cellular signaling, maintenance of membrane integrity and stability [20], cell proliferation and survival [21]. Thus, the analysis of PLs is considered an alternative to more complex applications dealing with the evaluation of metabolic connections by analysis of tissue membranes [22].

Routinely, gas chromatography (GC) is the preferred separation technique used for the analysis of FAs, commonly separated as FAME derivatives. Mass spectrometry (MS) increases the selectivity as detection technique since it provides structural information. Thus, the combination of GC and MS (GC–MS) is a powerful tool to ensure the correct identification of FAs by elucidating the chemical structure [4, 7, 23].

Sample preparation is the critical step to allow the discrimination of FAs from the different chemical families. Most methods targeted at FAs determination in serum/plasma are based on a derivatization step by transesterification and subsequent liquid–liquid extraction, generally with n-hexane. This protocol reports a comprehensive profile of FAs as it is not discriminating between chemical families and, thus, interpretation of results is a challenge. The bulk of the complex lipids consists of TGs (49%), PLs (24%), and CEs (16%) [24]. In fact, Brenna *et al*. have pointed out that TGs and CEs have considerable influence on the serum/plasma FA composition, which is not occurring for PLs [25]. In addition, it is well-known that PLs in aqueous medium form micelles with inner organization of the hydrophobic tails. These structures would theoretically limit the access of the derivatization agent to the ester group. Therefore, the contribution of PL-FAs to the quantitative response would be reduced, being apparently dominated by the contribution of other lipid families. For discrimination of the FAs profile associated to independent chemical classes, it is mandatory the implementation of fractionation steps. According to the literature, separation protocols have been mainly based on thin-layer chromatography (TLC) [18, 26-28] and adsorption chromatography [29, 30]. Despite TLC can be used at routine level, this presents limitations as a low chromatographic resolution, the potential oxidation of the analytes and a high solvent consumption. In the case of adsorption chromatography, handling of the

sorbent and adsorbent phases may be a difficult task. In any case, both separation techniques are tedious and time-consuming and, therefore, they are not a practical solution in studies dealing with large cohorts of samples.

The aim of this research was to develop a fast and selective method to determine the PL-FAs profile in serum by GC–MS. For this purpose, a sample preparation method involving solid phase extraction (SPE) with a selective sorbent (zirconia coated silica) should allow the isolation of PLs from other chemical families by setting a selective interaction between the phosphate moiety and Zr atoms. The derivatization of the resulting extract by transesterification should lead to the PL-FAs. The overall method has been applied to a group of individuals to compare the results versus those provided by the comprehensive classical protocol based on liquid–liquid extraction of lipid families and subsequent derivatization.

2. Materials and methods

2.1. Reagents and samples

Ammonia and MS grade methanol, acetonitrile and formic acid from Scharlab (Barcelona, Spain) were used for sample preparation. HPLC grade hexane and potassium hydroxide solution (5 N) in methanol were from Fischer (Madrid, Spain). Deuterated phosphatidylcholine (16:0/16:0)-d62 from Sigma-Aldrich (Madrid, Spain) was used as internal standard.

Blood samples were donated by 25 healthy volunteers for serum isolation and determination of FAs. Venous blood was collected into plastic serum Vacutainer® tubes (Becton Dickinson) with spray-coated silica and a polymer gel to favor serum separation. All collection tubes were processed by centrifugation for 15 min at 2000 ×*g* for gel tubes. A serum pool was prepared by mixing aliquots from blood donors.

2.2. Sample preparation

Two sample treatments were established to analyze the FAs profile in total serum or in the PLs fraction. Firstly, a 100 μL aliquot of each serum sample is added

to a 1 mL of methanolic potassium hydroxide to convert FAs into their more volatile FAMEs which is required prior to individual GC separation. The mixture was vortexed for 1 min and equilibrated for 10 min to complete the derivatization process. Then, 1 mL of hexane was added, and the biphasic system was vortexed 5 min. The two phases were easily separated, and the upper, containing the FAMEs, was evaporated and the residue reconstituted with 50 μL of hexane prior to injection into the CG–MS system. This protocol provides the total FAs profile in serum.

For PL-FAs, 100 μL aliquot of serum was deproteinized by addition of 300 μL of methanol 0.1 % (v/v) formic acid. The mixture is vortexed for 5 min and incubated at -20 °C for 5 min, then centrifugated at 4 °C for 10 min (20672 ×*g*) to isolate the supernatant, approximately 400 μL. Phospholipids were extracted using 30 mg HybridSPE®cartridges from Supelco (Bellefonte, PA, USA). The active packed material retains selectively PLs by their interaction to zirconia-bonded silica particles. Vacuum was applied to the SPE manifold to favour the pass of the loading solution through the SPE sorbent cartridge. Then, the sorbent was washed twice with 500 μL acetonitrile acidified with 1% (v/v) formic acid. Phospholipids were eluted by changing the pH in two steps using 1mL of methanol with 5% (v/v) ammonium hydroxide. Finally, the extract was derivatized as previously mentioned.

2.3. GC–MS analysis of FAMEs

Chromatographic separation was carried out by a gas chromatograph (Agilent 7890B) coupled to a mass spectrometer (Agilent 5977A), using a SPTM 2560 fused silica column (100m × 0.25 mm, 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA). The GC–MS analysis was performed according to the following conditions: injector temperature, 250 °C; injection in splitless mode; gas flow, 0.6 mL min^{-1} and injection volume, 1 μL. The oven temperature was programmed as follows: initial temperature 100 °C, hold for 5 min; ramp at 4 °C min^{-1} up to 240 °C, hold for 20 min. The total analysis time was 60 min, with 4 additional min necessary for re-establishing the initial conditions. The single quadrupole mass spectrometer was operated in the full scan mode, with the instrumental temperatures set at 250,

250, and 180 °C for transfer line, source, and quadrupole, respectively. The electron energy was set at 70 eV, data acquisition was carried out in an m/z range from 45 to 750 m/z and with a solvent delay for 14.5 min. Supplementary Table 1 lists the parameters for detection of FAs as FAMEs by GC–MS.

2.4. Identification and confirmatory analysis of FAs

Qualitative Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to process the data obtained by GC–MS. Treatment of raw data files started by deconvolution of potential molecular features (MFs) by the algorithm included in the software, which considered all ions exceeding 3000 counts for the absolute area parameter. The NIST Mass Spectral Search Program v.11.0 (NIST, Washington, DC, USA) was used for spectral search (Mainlib and Replib libraries). Tentative identification was reported when the correlation between experimental and database spectra was above 0.85 in normal search mode. Confirmatory analysis was carried out by analysis of a FAMEs multistandard from Sigma-Aldrich (Supelco 37 component FAME mix).

2.5. Data treatment

Quantitation was carried out by integrating the peak area for each considered FA. The relative amount of each fatty acid was expressed as percentage of total peak area considering all FAs. MetaboAnalyst (www.metaboanalyst.ca) (version 4.0) was used for paired t-test analysis to detect significant differences in the relative content of FAs provided by the two methods.

3. Results and Discussion

3.1. Proof-of-concept of the SPE process for isolation of PLs

Several sorbents have been used for SPE isolation of PLs from biological matrices such as serum. Among them, it is worth mentioning the aminopropyl type sorbents, which have been used by different research groups for analysis of PL-FAs [31-33]. Aminopropyl phase retains free FAs and PLs by ionic interactions, which are not highly selective since any polar lipid can be retained in this material. In this research, we have selected a sorbent formed by zirconia bonded silica particles that

has been widely described and applied in the literature for isolation of PLs in liquid biological samples [34-36]. The main benefit of zirconia-based sorbent for isolation of PLs as compared to other alternatives is the selective strong interaction between Zr atoms and phosphate group.

The SPE protocol with zirconia bonded sorbent can be adapted depending on the final aim, which can be targeted at the characterization of PLs as such or their removal to favor the determination of other compounds owing to their high suppression effect in MS detection. In this research, the SPE is used for the characterization of the PL-FAs profile in serum since the comprehensive method for determination of FAs by transesterification is not selectively favored for PLs due to kinetics impediment.

The experimental protocol used in this research is detailed in Figure 1. This protocol is initiated with a first step for protein precipitation by adding MeOH acidified with 0.1% (v/v) formic acid. The supernatant is loaded in the SPE cartridge for selective retention of PLs in acid medium. A frit inserted into the cartridge avoids the elution of residual proteins while a non-polar membrane prevents from the release of neutral lipids, especially triglycerides, which also suppress ionization of coeluting compounds. Then, a clean-up step with acetonitrile acidified with 1% (v/v) formic acid is carried out to wash out interfering species. Finally, the elution of PLs is favored by a pH change using 5% (v/v) ammonium hydroxide in MeOH.

The proof-of-concept for PLs purification is the analysis of the fractions collected before and after the SPE process. A first test was based on the analysis of the supernatant collected after protein precipitation that should contain all the lipid families present in blood. As previously mentioned, the result of the first non-purified sample is a comprehensive FAs profile, which represents mainly those lipid families with a kinetically favored derivatization. On the other hand, the analysis of the fraction collected after SPE purification should selectively report the PL-FAs profile. Differences in the FAs profile can be perfectly visualized in the two chromatograms shown in Figure 1. Thus, the PL-FAs profile was dominated by palmitic acid and linoleic acid as the two most concentrated FAs, while palmitic acid,

oleic acid and linoleic acid stood out as the most concentrated FAs in the profile reported by the comprehensive method. The selectivity of the proposed method is supported on the high purification efficiency of the SPE process. On the other hand, the comprehensive method provides a non-selective FAs profile, which represents mainly TGs and CEs due to their favored reaction kinetics.

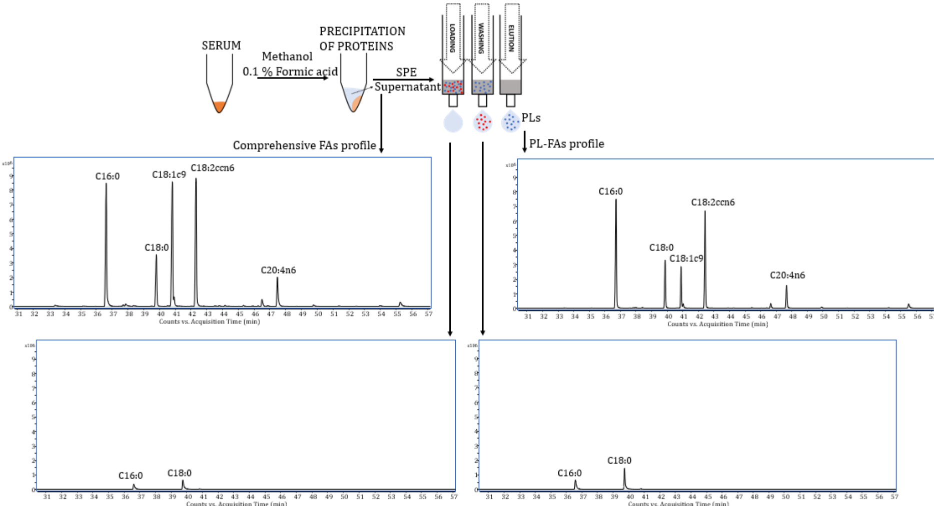

Figure 1. Experimental protocol used for selective isolation of PLs from serum. Total ion chromatograms by GC–MS analysis of the different fractions are illustrated to prove the efficiency of this sample preparation step.

3.2. Optimization of the SPE process for isolation of PLs

An optimization study was designed to evaluate the influence of two critical steps of the SPE protocol, washing and elution, on the purification efficiency. For this purpose, the fractions collected after loading the supernatant in the cartridge and after washing were analyzed to detect the presence of FAs conjugated species. Both fractions led to a similar result since only palmitic acid and stearic acid were scarcely detected in both fractions (Figure 1), which means that the retention/elution dual mechanism operated in an efficient manner. With these premises, TGs or other neutral lipids were mainly retained in the membrane, and PLs were efficiently isolated since they were not indirectly detected in the intermediate fractions collected prior to elution.

One additional test involved the evaluation of the quantitative performance of the retention/elution steps. For this purpose, three serum aliquots were

analyzed, and three consecutive 1-mL eluted fractions were collected for each aliquot with 5% (v/v) ammonium hydroxide in MeOH. PLs were massively eluted in the first step as can be deduced in Table 1 that shows the concentration expressed in percentage of the most concentrated PL-FAs considering the three eluted fractions. The behaviour of most FAs fit that observed for oleic acid, linoleic acid and arachidonic acid, which were quantitatively eluted in the first fraction (E1). On the other hand, two highly concentrated saturated FAs, palmitic and stearic acids, required a second step to elute above 90%. For this reason, two 1-mL consecutive elution steps were adopted in the process.

Table 1. Relative concentration expressed in peak area percentage (%) of the main FAs eluted in three consecutive eluted fractions (E1, E2 and E3).

	E1	E2	E3
Palmitic acid C16:0	83.6	13.6	2.8
Stearic acid C18:0	72.8	17.6	9.6
Oleic acid C18:1c9	100	0	0
Linoleic acid C18:2ccn6	96.1	3.9	0
Arachidonic acid C20:4n6	100	0	0

Quantitative analysis of FAs is typically carried out by using the peak area as response for estimation of the concentration expressed in percentage to the total peak area. This strategy avoids the utilization of internal standards since all potential sources of variability would affect similarly to all compounds. In the proposed method, a SPE step is implemented to fraction PLs from the other lipid families. For this reason, we evaluated the precision of the method by testing the response of a stable isotopically labeled phospholipid (SIL-PL). Particularly, phosphatidylcholine (16:0/16:0)-d62 was spiked as quality control at the same concentration level to three serum aliquots in order to evaluate the variability of the method. The analysis of spiked samples by the complete protocol led to the detection of a unique chromatographic peak corresponding to deuterated palmitic acid (C16:0-d31) at the same retention time detected for the non-deuterated palmitic acid. The ratio between the peak area for each FA and that measured for C16:0-d31 was used as quantitative response. Table 2 shows the variability results expressed as relative standard deviation in percentage considering or not the SIL-

PL response. As the three aliquots were analyzed in the same day, this estimation fits the within-day variability. Experimental variability was corrected for all FAs when the SIL-PL was considered by calculating peak area ratios. The correction effect was particularly substantial for low concentrated fatty acids such as palmitoleic acid, elaidic acid, vaccenic acid, gondoic acid and linolenic acid, but also for palmitic acid, which is one of the most concentrated FA in serum. In these cases, experimental variability in the detection was decreased from values above 30% to maximum values of 15%. As previously mentioned, quantitative analysis of FAs is typically estimated in relative terms in percentage and, therefore, an internal standard is not required in this context. However, the SIL-PL offers benefits as quality control to detect anomalous results and its use is required when the aim is to quantify FAs in absolute terms.

3.3. Application of the proposed method to serum samples and comparison to the comprehensive method

The protocol for determination of PL-FAs was applied to twenty-five samples donated from healthy volunteers to obtain a preliminary range of variability. Table 3 lists the concentration profile of PL-FAs found in this set of samples expressed in percentage. The total content in saturated fatty acids was close to 50%, which was dominated by palmitic acid and stearic acid with concentrations of 33.5 ± 1.5 and 15.3 ± 1.1%, respectively. Palmitic acid was in fact the most concentrated FA in PLs. The total content in MUFAs was 11.4%, with high contribution of oleic acid found at 9.3 ± 1.5%. The total content in PUFAs was 39.3%, distributed in 35.7 and 3.6% for n6 and n3 PUFAs, respectively. The most concentrated n6 PUFAs were linoleic acid and arachidonic acid with 23.2 ± 3.1 and 10.2 ± 1.8%, respectively. On the other hand, the n3 PUFAs content was mostly distributed between eicosapentaenoic acid and docosahexaenoic acid with 0.6 ± 0.5 and 3.0 ± 0.9%, respectively. This PL-FAs profile was in concordance with those previously determined in human plasma by Wang *et al*. and Zheng *et al*. [37, 38]. In these two cases, the PL-FAs profile was dominated by the same FAs. Concretely, in both cases the major FAs were palmitic acid, followed by linoleic acid and stearic acid.

Table 2. Intra-day variability expressed as relative standard deviation (RSD) obtained by analysis of three serum aliquots without or with the use of a SIL-PL. The quantitative parameter was the analyte peak area or analyte peak area/SIL-PL peak area.

	RSD without SIL-PL (%)	**RSD with SIL-PL (%)**
Myristic acid C14:0	5.5	1.2
Pentadecanoic acid C15:0	5.2	4.0
Palmitic acid C16:0	46.7	4.4
Margaric acid C17:0	6.6	4.4
Stearic acid C18:0	13.6	0.6
Arachidic acid C20:0	17.9	7.4
Palmitelaidic acid C16:1t	21.4	6.8
Palmitoleic acid C16:1c	45.7	14.9
Heptadecenoic acid C17:1c	12.8	12.0
Elaidic acid C18:1t9	32.6	1.7
Oleic acid C18:1c9	26.6	8.7
Vaccenic acid C18:1t6	39.3	2.7
Gondoic acid C20:1c9	37.8	2.4
Linoleic acid C18:2ccn6	15.3	9.0
γ-Linolenic C18:3n6	1.1	1.1
Eicosadienoic acid C20:2n6	19.8	6.2
Dihomo-γ-linolenic acid C20:3n6	13.2	4.0
Arachidonic acid C20:4n6	24.3	1.3
Linolenic acid C18:3n3	32.3	1.6
Eicosapentaenoic acid C20:5n3	19.0	3.3
Docosahexaenoic acid C22:6n3	23.7	0.3

The same group of individuals was analyzed with the comprehensive protocol for non-selective determination of FAs, frequently used in nutritional intervention studies. The obtained results (Table 3) can be compared with those provided by the method proposed in this research. Important differences can be observed in the FAs profiles determined with the two methods. The main difference can be attributed to the total content of SFAs and MUFAs. Thus, the total content in SFA was 34.0% also with palmitic acid and stearic acid as dominant SFAs, with 22.4 ± 1.6% and 10.5 ± 1.1%, respectively. One other difference detected in the two FAs

profiles was ascribed to myristic acid, that was found at 0.1 ± 0.0% in PLs while its concentration increased up to 0.4 ± 0.1% with the comprehensive method.

Concerning MUFAs, its concentration was increased up to 24.4% in serum analyzed with the comprehensive method. This variation particularly affected to oleic acid that was detected at 20.6 ± 2.7% versus 9.3% found in PLs. Palmitoleic acid also experienced a similar change, since its concentration in PLs was 0.3 ± 0.1% while its level in the total profile was 1.2 ± 0.5%. The trans MUFAs (C16:1t + C18:1t) also reported differences in the two profiles, with values of 0.2% for PLs and 0.5% for the non-selective profile.

The differences for the PUFAs were less relevant as the two protocols reported quite similar concentrations, 41.6% in the total profile as compared to 39.3% in PLs. In the case of the non-selective profile, the PUFAs content was divided into 37.3% for n6 PUFAs and 4.3% for n3 PUFAs. Similar to the PLs profile, linoleic acid and arachidonic acid were the most concentrated PUFAs with 23.6 ± 2.8% and 10.2 ± 1.8%, respectively, which were close to values found in PLs. On the other hand, the content in n3 PUFAs was quite similar to those measured in PLs, being eicosapentaenoic acid and docosahexaenoic acid the most concentrated n3 PUFAs, at 3.3 ± 1.0% and 0.8 ± 0.5%, respectively.

The difference in the two estimated profiles, total FAs and PL-FAs, was also evaluated considering the intra-individual variability. To address this study, a paired *t*-test was carried out in order to evaluate the differences in the two FA profiles for each individual. The application of this test, setting the cut-off *p*-value at 0.01, allowed detecting significant differences in 18 out of the 21 determined FAs (Table 3), which is indicative of relevant differences between the two profiles. These changes affected considerably to SFAs, MUFAs and PUFAs. All monitored SFAs reported significant differences in concentration terms except for arachidic acid, which corresponds to the less concentrated SFA detected in serum. Concerning MUFAs and PUFAs, all monitored FAs led to significant differences in the total profile as compared to the PLs profile except for the two most concentrated PUFAs, linoleic acid and arachidonic acid.

Table 3. Relative concentration of the comprehensive FAs and PL-FAs profiles expressed in percentage.

	Total FAs	PL-FAs	*p*-value
Myristic acid C14:0	0.4 ± 0.2	0.1 ± 0.0	0.0001
Pentadecanoic acid C15:0	0.2 ± 0.1	0.1 ± 0.0	0.0001
Palmitic acid C16:0	22.4 ± 1.6	33.5 ± 1.5	0.0001
Margaric acid C17:0	0.3 ± 0.1	0.2 ± 0.0	0.0001
Stearic acid C18:0	10.5 ± 1.1	15.3 ± 1.1	0.0001
Arachidic acid C20:0	0.1 ± 0.0	0.1 ± 0.0	0.681
Total SFAs	34.0	49.3	
Palmitelaidic acid C16:1t	0.3 ± 0.1	0.1 ± 0.0	0.0001
Palmitoleic acid C16:1c	1.2 ± 0.5	0.3 ± 0.1	0.0001
Heptadecenoic acid C17:1c	0.1 ± 0.0	0.0	0.0001
Elaidic acid C18:1t9	0.2 ± 0.1	0.1 ± 0.0	0.0001
Oleic acid C18:1c9	20.6 ± 2.7	9.3 ± 1.5	0.0001
Vaccenic acid C18:1t6	1.8 ± 0.2	1.4 ± 0.2	0.0001
Gondoic acid C20:1c9	0.2 ± 0.1	0.1 ± 0.0	0.0001
Total MUFAs	24.4	11.4	
Linoleic acid C18:2ccn6	23.6 ± 2.8	23.2 ± 3.1	0.193
γ-Linolenic C18:3n6	0.3 ± 0.1	0	0.0001
Eicosadienoic acid C20:2n6	0.3 ± 0.1	0.1 ± 0.0	0.0001
Dihomo-γ-linolenic acid C20:3n6	2.8 ± 0.8	2.1 ± 0.7	0.0001
Arachidonic acid C20:4n6	10.2 ± 1.8	10.2 ± 1.8	0.945
Total PUFAs n6	37.3	35.7	
Linolenic acid C18:3n3	0.2 ± 0.1	0	0.0001
Eicosapentaenoic acid C20:5n3	0.8 ± 0.5	0.6 ± 0.5	0.0001
Docosahexaenoic acid C22:6n3	3.3 ± 1.0	3.0 ± 0.9	0.0001
Total PUFAs n3	4.3	3.6	

These statistical differences can be visualized in Supplementary Figure 1 that shows the box-and-whisker plots for each FA. MUFAs and PUFAs were in all cases at a higher concentration in the comprehensive profile than in PLs. This situation also occurred for minor SFAs, C14:0, C15:0 and C17:0. However, palmitic acid and oleic acid showed the opposite behaviour because they were at a higher

concentration in PLs. Based on these results, the PL-FAs profile is noticeable for the concentration of SFAs as compared to the comprehensive FAs profile, the latter standing out for its high concentration of MUFAs and PUFAs. This increased concentration of SFAs in PL-FAs is attributed to the fact that PLs in most cases have an SFA at *sn*-1 position and an unsaturated FA at *sn*-2 position of the glycerol. This aspect explains the relative content close to 50% of SFA in PLs. The profile of FAs in the PL bilayer of membranes determines their fluidity, flexibility, permeability and communication. Thus, unsaturated FAs confer membranes flexibility, which affects the insertion of glucose transporters into cell membranes and reduces hypoxia [39]. Therefore, the PL-FAs profile could be used to monitor the progression of metabolic diseases.

Despite the observed differences in the FAs profiles determined by the comprehensive method and the selective approach developed in this research, a high correlation in the relative levels of most FAs was found (Supplementary Table 2). Thus, Pearson correlation analysis ($R>0.6$; p-value<0.005) revealed significant association in 15 out of 21 FAs, which included dominant FAs such as palmitic acid, stearic acid and linoleic acid, but also low concentrated FAs such as n3 and n6 PUFAs. On the other hand, it is worth mentioning the cases of oleic acid ($R = 0.585$) and pentadecanoic acid ($R = 0.554$) that were close to significant correlation. This high individual correlation between most FAs in serum and in the PL fraction is attributed to dietary effects. Nevertheless, it is not possible to predict the PL-FAs profile from the comprehensive profile and, for this reason, the application of the selective approach proposed in the present research would be mandatory.

4. Conclusions

A method for profiling analysis of PL-FAs in serum has been developed by SPE for selective isolation of PLs, transesterification and GC–MS analysis. The method is proposed as a complement to the comprehensive method for FAs analysis in serum, which is based on a non-selective liquid–liquid extraction of lipid families and determination of FAME derivatives. In fact, significant differences in the FAs profile have been detected by application of both methods to a group of healthy

individuals, particularly, in the content of SFAs and MUFAs. This complementary method can be implemented in studies to evaluate dietary patterns at long term, but also to associate quantitative changes in PL-FAs to the evolution of metabolic disorders.

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Supplementary Table 1. Retention time and ions monitored for detection of FAs by GC–MS.

	RT (min)	Extracted ion (m/z)
Myristic acid C14:0	33.0	74
Pentadecanoic acid C15:0	34.8	74
Palmitic acid C16:0	36.6	74
Margaric acid C17:0	38.2	74
Stearic acid C18:0	39.7	74
Arachidic acid C20:0	42.7	74
Palmitelaidic acid C16:1t	37.6	55
Palmitoleic acid C16:1c	37.8	55
Heptadecenoic acid C17:1c	39.4	55
Elaidic acid C18:1t9	40.5	55
Oleic acid C18:1c9	40.8	55
Vaccenic acid C18:1t6	40.9	55
Gondoic acid C20:1c9	43.8	55
Linoleic acid C18:2ccn6	42.4	67
γ-Linolenic C18:3n6	43.6	79
Eicosadienoic acid C20:2n6	45.5	67
Dihomo-γ-linolenic acid C20:3n6	46.7	79
Arachidonic acid C20:4n6	47.8	79
Linolenic acid C18:3n3	44.2	79
Eicosapentaenoic acid C20:5n3	50.1	79
Docosahexaenoic acid C22:6n3	56.0	79

C14:0
Normalized concentration (%)
1.00
0.75
0.50
0.25
0.00
FAs
PL-FAs

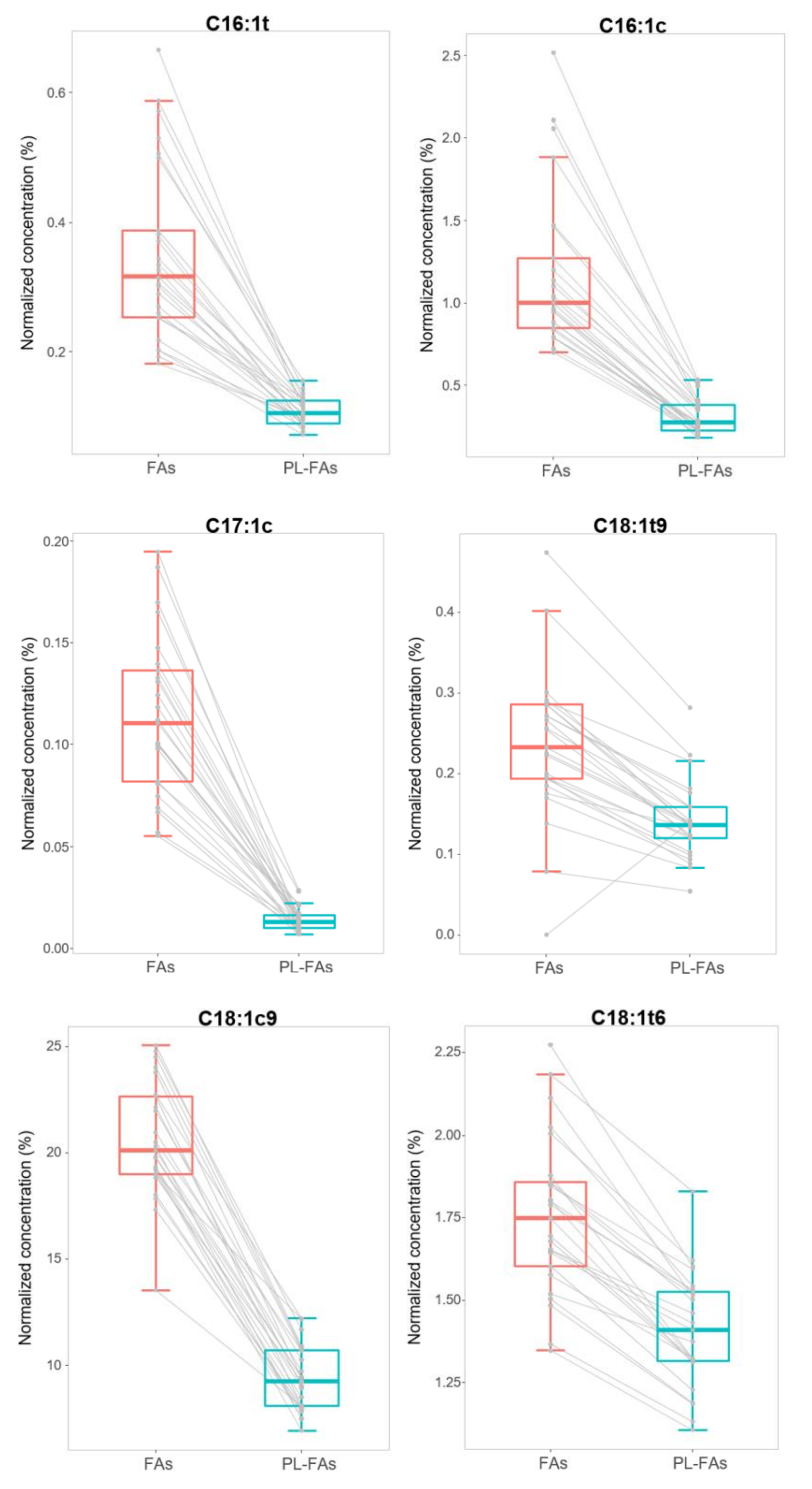
C15:0
Normalized concentration (%)
0.3
0.2
0.1
FAs
PL-FAs

C16:0
Normalized concentration (%)
35
30
25
20
FAs
PL-FAs

C17:0
Normalized concentration (%)
0.4
0.3
0.2
FAs
PL-FAs

C18:0
Normalized concentration (%)
15.0
12.5
10.0
FAs
PL-FAs

C20:0
Normalized concentration (%)
0.10
0.05
FAs
PL-FAs


C16:1t
Normalized concentration (%)
0.6
0.4
0.2
FAs
PL-FAs
C16:1c
2.5
2.0
1.5
1.0
0.5
C17:1c
0.20
0.15
0.10
0.05
0.00
C18:1t9
0.4
0.3
0.2
0.1
0.0
C18:1c9
25
20
15
10
C18:1t6
2.25
2.00
1.75
1.50
1.25

C20:1c9

Normalized concentration (%)

0.4 0.3 0.2 0.1

FAs PL-FAs

C18:2ccn6

Normalized concentration (%)

27.5 25.0 22.5 20.0 17.5

FAs PL-FAs

C18:3n6

Normalized concentration (%)

0.4 0.2 0.0

FAs PL-FAs

C20:2n6

Normalized concentration (%)

0.4 0.3 0.2 0.1

FAs PL-FAs

C20:3n6

Normalized concentration (%)

4 3 2 1

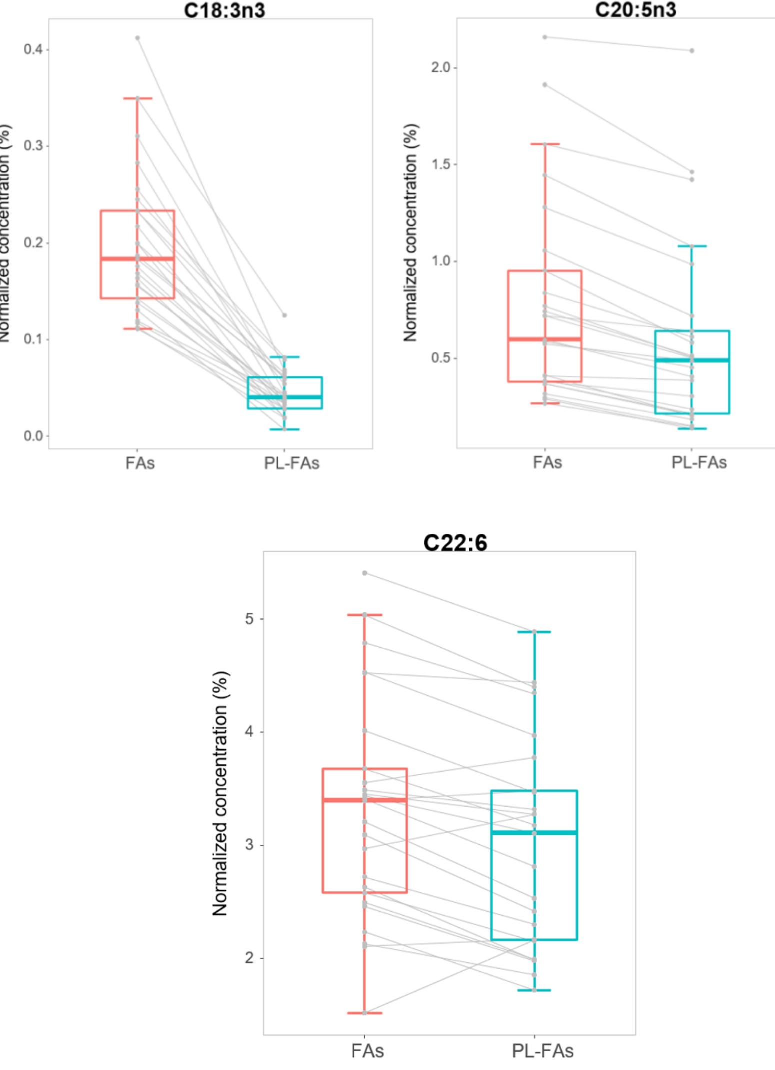
FAs PL-FAs

C20:4n6

Normalized concentration (%)

14 12 10 8 6

FAs PL-FAs



Supplementary Figure 1. Box-and-whisker plots reporting the differences in the relative concentration of FAs measured in a group of 25 individuals by the comprehensive method (red colour) and the selective approach proposed in this research for determination of PL-FAs (blue colour).

Supplementary Table 2. Pearson correlation analysis applied to FAs relative concentration obtained by the comprehensive method and the proposed method. Significance was considered for R>0.6 and p-value<0.005

	R	*p*-value
Myristic acid C14:0	0.787	0.0001
Pentadecanoic acid C15:0	0.554	0.004
Palmitic acid C16:0	0.600	0.002
Margaric acid C17:0	0.612	0.001
Stearic acid C18:0	0.649	0.001
Arachidic acid C20:0	0.427	0.033
Palmitelaidic acid C16:1t	0.566	0.003
Palmitoleic acid C16:1c	0.839	0.0001
Heptadecenoic acid C17:1c	0.405	0.045
Elaidic acid C18:1t9	0.773	0.0001
Oleic acid C18:1c9	0.585	0.002
Vaccenic acid C18:1t6	0.845	0.0001
Gondoic acid C20:1c9	0.545	0.005
Linoleic acid C18:2ccn6	0.836	0.0001
γ-Linolenic C18:3n6	0.711	0.0001
Eicosadienoic acid C20:2n6	0.865	0.0001
Dihomo-γ-linolenic acid C20:3n6	0.940	0.0001
Arachidonic acid C20:4n6	0.900	0.0001
Linolenic acid C18:3n3	0.631	0.001
Eicosapentaenoic acid C20:5n3	0.981	0.0001
Docosahexaenoic acid C22:6n3	0.937	0.0001

DISCUSSION OF THE RESULTS

The present Thesis Book is based on the format of articles compilation (published or next to publication) regulated by University of Córdoba. Therefore, articles were included as such. A joint discussion of the results obtained according to the objectives initially planned is necessary to provide a global vision of the main results derived from the Doctoral Thesis. The research in this Thesis Book is based on the determination of minor families present in (E)VOO and on the explanation of their variability under different conditions. Methods and studies developed here were aimed at reinforcing the competitiveness of (E)VOO from a dual perspective: quality and health value. To obtain the maximum level of analytical information, metabolomics strategies were implemented by using MS detection in targeted and untargeted methods.

The reserarch was divided into three different sections as a function of the pursued objectives. Thus, Section I, constituted by Chapters 1-to-4, was devoted to a methodological update for characterization of two minor families, phenols and GPLs, which can be strictly related to health properties and quality of (E)VOO. Section II, constituted by Chapters 5 and 6, deals with untargeted metabolomics analysis to study changes in the concentration of two families of bioactive compounds (phenols and triterpenes) during the extraction process of EVOO through the analysis of different phases involved. By contrast, Section III focused a strategy to evaluate diet adherence by evaluating the fat source, with special emphasis on olive oil.

In this part of the Doctoral Thesis Book, the most relevant results obtained throughout the experimental development proposed in the different chapters are presented. One common link among sections is the main detection technique:MS. The analytical sensitivity, selectivity, accuracy, precision, and resolution of MS make this technique the most preferred detection tool for targeted and untargeted metabolomic analysis.

Section I:

This section is devoted to a methodological update for characterization of two minor families, GPLs and phenolic compounds, in (E)VOO. Low resolution MS,

essentially with a QqQ configuration, is an ideal tool to undertake quantitative and confirmatory analysis in metabolomics and, therefore, to work in targeted analysis. Moreover, the QqQ mass spectrometer offers additional features as a detector to play an important role in qualitative analysis. For this purpose, in all chapters of this section, LC–QqQ MS/MS was used to determine phenolic compounds and GPLs in oil samples.

The health benefits of (E)VOO are associated to its FAs profile (with predominance of oleic acid) and to the minor components that include phenols, among others. This last fraction, studied in Chapters 1-to-3, differentiates (E)VOOs from lower-quality olive oils due to this family is partially or totally removed in thermal and/or physical-chemical treatments applied in the refining process. Phenols are also responsible for the only health claim of olive oil reported in the Commission Regulation (EU) 432/2012 [1]. The health claim for phenols is specific of olive oil since secoiridoids, the main phenolic family, are only found in plants of the *Oleaceae* family. Thus, the healthy index refers to the mass of phenols expressed in mg consumed with a daily intake of 20 g of oil. A positive healthy index is considered above 5.0 mg/20 g of oil. Furthermore, the European Food Safety Authority (EFSA) pointed out other biological activities of olive oil phenols such as their anti-inflammatory properties and their contribution to maintaining suitable cholesterol levels, normal blood pressure, respiratory health, normal gastrointestinal tract function, and immune system strengthening [2].

Despite this health claim is well-known, this is scarcely used by producers to highlight an added value of high phenolic (E)VOOs. The main limitation is found in the lack of reference analytical methods with capability to respond to the health claim. LC–MS/MS is considered a gold-standard technique for quantitative analysis due to its high sensitivity and selectivity levels. According to these facts, one of the main objectives of this section was to characterize the phenolic content of (E)VOOs to study the healthy value and the influence of different factors such as the crop season, cultivar, harvesting period, orchard location, storage, and heating conditions, on the concentration of these compounds. For this purpose, a LC–MS/MS quantitative method was applied in Chapters 1-to-3 to characterize the individual

phenols in absolute terms and, thus, with capability to respond to the health claim. Monitored phenols included the most concentrated species described in (E)VOO. These are hydroxytyrosol, oleacein, oleocanthal, oleuropein aglycone, oleomissional, ligstroside aglycone, oleokoronal, luteolin, apigenin and oleocanthalic acid. All mentioned phenols except fot the two flavonoids and oleocanthalic acid were used for estimation of the healthy index in the evaluated samples.

One special point to consider in this objective was the analysis of a high number of samples provided by producers. This fact was supported by involvement of the research group in the Aristoil Project, an European project granted by the Interreg-Med. It is worth mentioning that after the analysis of 1239 (E)VOO samples obtained in the 2017/2018 and 2018/2019 crop seasons in Spain, more than 85% reported a concentration above 250 mg kg^{-1}. Therefore, a high proportion of analyzed samples resulted in a phenolic concentration to take benefits from the health claim with the recommended daily consumption. The variability of the phenolic profiles was also estimated by definition of the *f* and *h* factors for first time. The *f* factor was calculated as the concentration ratio between the sum of aglycone isomers of oleuropein and ligstroside and that of oleocanthal and oleacein. Complementarily, the *h* factor was calculated as the ratio between the sum of concentrations of hydroxytyrosol and tyrosol secoiridoid derivatives [3].

The initial phenolic content in (E)VOO is affected by climatological conditions, cultivar, harvesting period and orchard location, as it was studied in Chapter 1. Thus, the average of phenolic content was lower in a season with an intense drought period, as it was the 2018/2019 season; and the maximum phenolic concentrations were obtained in early ripening (E)VOOs, produced in October and November. One of the main factors contributing to the variability of phenols in (E)VOO is the cultivar [4]. In this section, the most widespread cultivars in Spain were evaluated. These were 'Picual', 'Hojiblanca', 'Arbequina', and 'Cornicabra'. 'Picual' (E)VOOs were characterized by a clear predominance of oleuropein and ligstroside aglycones. On the other hand, 'Arbequina' (E)VOOs stood out for a totally different profile with a significant relative predominance of oleocanthal and

oleacein. These results agreed with previous studies carried out with lab-produced samples [5]. The aglycone forms were found at the highest concentration in 'Picual' (E)VOOs, while 'Hojiblanca' and 'Cornicabra' were the oils reporting the highest levels of oleacein and oleocanthal. Thus, 'Arbequina' (E)VOOs have a lower probability to take benefits from the health claim than the other three cultivars. These results support the genotype predisposition of olive cultivars to produce (E)VOOs with a particular phenolic profile and with more possibilities to fulfil the health claim.

In Chapters 2 and 3, the decrease of the (E)VOO quality after storage and frying condition was studied based on the healthy index. The results revealed that the changes in the phenolic content of (E)VOO samples stored for 12 months in absence of light exposition and under atmospheric temperature depended on the initial phenolic profile, and therefore, on the cultivar. After storage for 12 months, (E)VOO phenolic concentration was decreased 42.0 ± 24%. (E)VOOs with predominance in oleacein and oleocanthal experienced a larger decrease in phenolic content than oils enriched in other phenols. These results emphasized the relevance of the phenolic profile, both in absolute and relative terms, in the stability of the (E)VOO. The phenolic profile seems to condition the decay in phenolic concentration and, therefore, in the health benefits. The presence of aglycone forms of oleuropein and ligstroside reduces the decay in phenolic content during storage. In addition, two other phenolic compounds, hydroxytyrosol and oleocanthalic acid (a derivative of oleocanthal produced by oxidation), experienced a significant concentration increase after storage, which can be considered markers of oil ageing. The result observed for oleocanthalic acid was previously proposed as marker in this context in a collaboration of the research group with the University of Athens [6].

In Chapter 3, we evaluated the impact of frying conditions at 180 °C for 90 min on the phenolic concentration of four monocultivar EVOOs. The degradation in total phenolic content (including the concentration of all measured phenols except for oleocanthalic acid) after the complete frying process ranged from 54.7% in 'Arbequina' to 77.9% in 'Hojiblanca'. A correlation pattern was found in oleomissional/oleokoronal degradation to the increase of oleacein/oleocanthal

concentration during the first frying cycles (10 min), which allowed proposing a direct conversion of these open aglycone isomers to oleacein and oleocanthal. In this context, the initial phenolic profile of EVOOs also seems to be determinant to explain the degradation of these compounds after frying. Complementarily, hydroxytyrosol and oleocanthalic acid also increased significantly after frying and, by analogy to the previous study, these two compounds can be considered markers of olive oil alteration during to heating, and they can also provide information about quality or stability. As previously mentioned, oleocanthalic acid was previously proposed as alteration marker. Hydroxytyrosol, released from conjugated forms, is proposed here for first time as marker of EVOO alteration.

Within minor families, GPLs constitute a chemical family in the saponifiable fraction of vegetable oils. There is evidence on their biological activity such as the influence on the antioxidant capacity and oxidative stability. GPLs seem to exert a synergistic role on the stability of edible oils since they allow regeneration of other antioxidants such as phenols or tocopherols [7-9]. Despite their importance, GPLs have been scarcely studied in edible oils owing to the lack of sensitive and selective analytical methods for their characterization. Previous studies just provided information of total GPLs or by families [10,11]. In Chapter 4, a sensitive and selective analytical method for identification, confirmation, and relative quantitation of GPLs in vegetables oils was developed. The resulting method was applied to edibles oils such as EVOO, VOO, OO, pomace olive oil (POO), high-oleic sunflower oil (HOSO), and sunflower oil (SO).

The method is based on SPE for isolation of GPLs and determination by LC–MS/MS. The two most critical steps in the SPE process (the washing and elution steps) were evaluated, concluding that three washes and one elution were the optimum for quantitative isolation of this fraction. The identification of the GPLs was carried out by combination of different acquisition modes (product ion and neutral loss), considering the known fragmentation pattern for each GPL family, in both positive and negative ionization modes. After the presence of GPL families was verified, an MRM method was created with a list of tentative precursor ions corresponding to detected families. The list was prepared by considering the most

abundant FAs in the targeted oils. Finally, a comparison of GPL profiles in the olive oil categories was made to find compositional changes in the GPL fraction associated to the olive oil category, which could be explained by factors such as the quality of fruits and the extraction process.

A Venn diagram revealed that EVOO and VOO were characterized by common GPL profile in qualitative terms. On the other hand, clear differences in GPLs composition were found in OO and PO *versus* EVOO and VOO. These differences particularly affected to phosphatidylglycerides (PGs), glycerophosphatidic acids (PAs), and phosphatidylcholines (PCs). PCA applied to all the samples clearly revealed a discrimination trend as a function of the olive oil category. Thus, three main groups corresponding to POO, OO and EVOO+VOO samples were perfectly distinguishable Families such as PAs and PGs were remarkable because of their capability to discriminate VOOs from the rest of categories. These results open a door to additional studies targeted at the identification of olive oil quality by monitoring these lipids. The hypothesis here is that the quality of the fruits used for oil extraction explains the variability in the GPL profile.

Section II:

One of the main challenges of metabolomics is to improve the detection capacity and, subsequently, the identification of metabolites. This is especially relevant in untargeted analysis. In Section II, two main families of olive oil bioactive compounds were monitored by LC–QTOF MS/MS, phenolic compounds and terpenic acids. In the context of the Nutradaf Project for valorization of by-products, we studied the formation and partition of the two families in the different phases, both pastes and liquids, involved in the process for extraction of EVOO, which is produced from fresh and healthy olive fruits exclusively by mechanical and physical processes. The importance of the phenolic compounds was mentioned above, and most of the studies on olive oil processing are focused on them, disregarding other minor compounds like terpenes. However, the number of studies focused on the bioactivity of EVOO triterpenes is increasing [12, 13]. In Chapters 5 and 6, the changes occurring in both families during the two-phase extraction process were evaluated.

The extraction based on two-phase centrifugal approach has been widely accepted [14]. This system consists of olive fruit collection, washing, crushing, malaxation, decantation, and vertical centrifugation. This system is characterized by two inputs, olive paste and water, and two outputs with three by-products: pomace and wastewater-oil. Destoning before crushing in the process of olive oil extraction is gaining interest since it could enhance the working capacity. Despite most studies of destoning have been targeted at quality evaluation, contradictory results are found in literature mainly related to genetic factors of different cultivars [15]. With these premises, 'Picual' and 'Arbequina' were the cultivars selected in this Section for the study of the influence of extraction processes on bioactive compounds due to their significant different profiles.

After processing the experimental data obtained by LC–QTOF MS/MS, a total of 42 bioactive compounds were tentatively identified in all samples, including oils in Chapter 5 (decanter, centrifuge, and final oils); and olives, pastes, wastewater, and olive pomace in Chapter 6. The identified compounds were grouped by chemical families in flavonoids; lignans; iridoids; secoiridoids; triterpenes; simple phenols; phenolic acids and derivatives; and elenolic acid and derivative. Eighteen out of them were confirmed by injection of analytical standards. The concentration of compounds was expressed as mg kg^{-1} equivalents of oleuropein.

In Chapter 5, the results from the analysis of decanter, centrifuge, and final oils obtained from the extraction of intact olives were that minor families of bioactive compounds such as flavonoids, simple phenols, lignans, phenolic acids and triterpenes in 'Arbequina' decreased their concentration from the decanter up to the final oil. On the other hand, secoiridoids increased slightly their overall concentration. In turn, 'Picual' final oil was more enriched in these minor families as compared to the decanter and centrifuge oils. Secoiridoids in 'Picual' samples showed a reduced variation along the production process.

The results of the final oils after destoning stood out a seriously decrease in the concentration of secoiridoids in 'Arbequina'. An explanation to this effect was that destoning in 'Arbequina' fruits could reduce the activity of β-glucosidases and

esterases enzymes. Less significant was the increase in the concentration of secoiridoids found in 'Picual' VOO obtained from destoned fruits. We detected a non-significant variation in the healthy index of 'Picual' oil when fruits were previously destoned, from 14 to 15 mg/20 g. On the other hand, in 'Arbequina' the healthy index substantially decreased from 11 mg/20 g in the conventional process to 5 mg/20 g with destoned olives. These results confirm the undesirable effect of destoning in 'Arbequina' oils from the health perspective. According to these results, destoning of olive fruits does not affect equally to 'Arbequina' and 'Picual' cultivars.

Phenolic compound changes occurring from olives to EVOOs during the extraction process was deeply studied in Chapter 6. It was observed that simple phenols and phenolic acids were detected at high levels in wastewater, with low presence in the oil due to their hydrophilic nature. Due to the less polar properties of secoiridoid compounds, they were found at higher concentration in oils as compared to wastewaters. On the other hand, main secoiridoid glucosides, oleuropein and ligstroside, were found at trace concentrations in oils. Iridoids, precursor of secoiridoids, followed a common pattern to oleuropein and ligstroside, and they were practically undetected in oil samples. In turn, lignans, one other phenolic class, were mainly detected in olive oils. since their concentration in 'Picual' decreased in the final oil as compared to previous samples.

Changes occurring in the concentration of bioactive compounds were evaluated by unsupervised analysis by PCA. In fact, there was a sequential discrimination following the extraction process: olive fruits, crushing paste, malaxation paste and olive pomace. These groups can be explained by metabolic transformations occurring during the extraction process but also by partitioning in the involved phases. Heat maps allow visualizing these effects for both cultivars. Thus, the concentration of iridoids was maximum in olive pomace and wastewater while minimum levels were found in olive oil, which is clearly attributed to the polar profile of this family. On the other hand, secoiridoids were decreased after malaxation to reach minimum levels in olive pomace and wastewater, but the maximum concentration was found in olive oil. Flavonoids were preferentially found in solid samples while minimum levels were detected in wastewaters. Low

concentrations of these phenols were also found in olive oils as compared to solid material. The contrary scenario was found for lignans since minimum concentrations were detected in fruits, solid pastes and wastewaters and maximum levels were found in olive oil. Significant differences were found for simple phenols and phenolic acids. These two groups were at higher concentration in wastewater derived from 'Picual' olive oil extraction, while in 'Arbequina' simple phenols and phenolic acids were more concentrated in olive oil and crushing paste, respectively. One other polar group of compounds is that of elenolic acid and derivatives that was preferentially enriched in wastewater from both cultivars. Finally, triterpenes remained preferentially in the solid samples and, particularly, in olive pomace. With these premises, this would the most suited material for isolation of these bioactive compounds due to their concentration and the residual characteristics of the olive pomace.

The behaviour of specific compounds was also evaluated, standing out the secoiridoids. Both oleuropein and ligstroside were detected at low levels in general terms as compared to their derivatives obtained after enzymatic conversions. The contrary result was identified for oleuropein and ligstroside aglycones, which were mainly enriched in EVOOs. In relative terms, the transfer process seems to be clearly favoured in 'Arbequina' since these phenols were detected at low levels in olive pomace while relevant concentrations were found in 'Picual' pomace. For oleacein, maximum concentrations (above 200 mg kg^{-1}) in 'Arbequina' and 'Picual' cultivars were found in fruit and crushed paste, respectively. Oleacein and oleocanthal were not properly transferred to the oily phase in 'Picual'. Verbascoside, nüzhenide and GL3 are three phenols with bioactive properties that are scarcely found in EVOOs. Thus, verbascoside was only found in fruits and pastes and low detected in wastewaters. Nüzhenide and GL3 reported differences by comparing both cultivars. With these results, secoiridoids can be considered the target bioactive family for implementation of technological solutions to improve their enrichment in EVOOs.

Section III:

Section III of this PhD Book was devoted to offer a complementary strategy to estimate adherence of diets by evaluating the fat source. This could be perfectly

applied to monitor interventions based on olive oil consumption. The determination of the FAs profile in blood and adipose tissue is a widely implemented experimental tool in nutritional studies. Several studies have evidenced dietary patterns by determination of the FAs profile [16, 17]. FAs are mostly present in blood in conjugated forms as triglycerides (TGs), phospholipids (PLs) and cholesteryl esters (CEs) [18]. Several studies pointed out that the determination of FAs independently in these three chemical families reveals different information [19-21]. The analysis of PLs is considered an alternative to more complex applications dealing with the evaluation of metabolic connections by analysis of tissue membranes [22]. Sample preparation is the critical step to allow the discrimination of FAs from different chemical families. For this purpose, in this section was developed a fast and selective method involving SPE with a selective sorbent (zirconia coated silica), to determinate the PL-FAs profile in serum by GC–MS. The overall method has been applied to a group of individuals to compare the results *versus* those provided by the comprehensive classical protocol based on liquid–liquid extraction of lipid families and subsequent derivatization. The method developed in this section was used in different collaboration studies with the Spanish National Center of Epidemiology and the Carlos III Health Institute. These collaborations have led to three publications that are presented as annexes.

Blood samples were donated by twenty-five healthy volunteers for serum isolation and determination of FAs. The fractions collected before and after the SPE process were studied. The comprehensive method provides a non-selective FAs profile, which represents mainly TGs and CEs due to their favored reaction kinetics [23].

An optimization study was designed to evaluate the influence of two critical steps of the SPE protocol, washing and elution, on the purification efficiency. For this purpose, the fractions collected after loading the supernatant in the cartridge and after washing were analyzed to detect the presence of FAs conjugated species. Both fractions led to a similar result since only palmitic acid and stearic acid were scarcely detected in both fractions, which means that the retention/elution dual mechanism operated in an efficient manner. One additional test involved the

evaluation of the quantitative performance of the retention/elution steps. PLs were massively eluted in the first step. On the other hand, two highly concentrated saturated FAs, palmitic and stearic acids, required a second step to elute above 90%. For this reason, two 1-mL consecutive elution steps were adopted in the process. We evaluated the precision of the method by testing the response of a stable isotopically labelled phospholipid (SIL-PL). The ratio between the peak area for each FA and that measured for C16:0-d31 was used as quantitative response. The correction effect was particularly substantial for low concentrated fatty acids.

As mentioned above, the protocol for determination of PL-FAs was applied to twenty-five samples. The major FAs in the PL-FAs were palmitic acid, followed by linoleic acid and stearic acid. The same group of individuals was analyzed with the comprehensive protocol for non-selective determination of FAs, frequently used in nutritional intervention studies. The main difference can be attributed to the total content of SFAs and MUFAs. Thus, the total content in SFA was 34.0% compared to 49.3% of PL-FAs. This increased concentration of SFAs in PL-FAs is attributed to the fact that PLs in most cases have an SFA at *sn-1* position and an unsaturated FA at *sn-2* position of the glycerol [24]. This aspect explains the relative content close to 50% of SFA in PLs. Concerning MUFAs, its concentration increased up to 24.4% compared to 11.4% of PL-FAs.

A paired t -test was carried out to evaluate the differences in the two FA profiles for each individual. The application of this test, setting the cut-off p-value at 0.01, allowed detecting significant differences in 18 out of the 21 determined FAs, which is indicative of relevant differences between the two profiles. Nevertheless, it is not possible to predict the PL-FAs profile from the comprehensive profile and, for this reason, the application of the selective approach proposed in Chapter 7 would be necessary.

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CONCLUSIONES

La investigación realizada en esta Tesis Doctoral tenía como objetivo determinar familias minoritarias presentes en AOV y AOVE y estudiar su variabilidad asociada a diferentes factores para reforzar la competitividad de ambos productos a partir de dos pilares fundamentales: calidad y valor saludable.

Las conclusiones más destacadas de este trabajo, de acuerdo con los objetivos inicialmente propuestos, se resumen a continuación:

1. Evaluar la influencia de diferentes factores en el contenido fenólico del AOV(E) y, por tanto, sobre su valor saludable atendiendo a la declaración saludable recogida en el Reglamento Europeo 432/2012.

    (i) Se ha caracterizado el contenido fenólico en 1239 muestras de AOV(E) procedentes directamente de productores y obtenidas en dos campañas agronómicas consecutivas para establecer su valor saludable en base a la declaración saludable incluida en el Reglamento Europeo 432/2012. Se pudo comprobar que 85% de las muestras analizadas tuvieron un contenido fenólico superior a 250 mg kg^{-1}, contenido establecido como límite para poder hacer uso de dicha declaración.

    (ii) Se ha estudiado la influencia de factores agronómicos y geográficos tales como la variedad, la fecha de producción y la localización sobre la variabilidad en el contenido fenólico del AOV(E). De esta forma, se pudo caracterizar la variabilidad en contenido fenólico asociada a estos tres factores, destacando por su especial relevancia la variedad y la fecha de producción.

    (iii) La disminución en el contenido fenólico del AOV(E) durante su almacenamiento en condiciones ideales (temperatura ambiente y en material de vidrio opaco) depende del perfil fenólico inicial. La predominancia de determinados fenoles tales como oleaceína y oleocantal incrementa la caída en concentración fenólica tras 12 meses de almacenamiento. Además, se comprobó que la concentración de hidroxitirosol y ácido oleocantálico aumentaron

después del almacenamiento pudiendo considerarse como marcadores de deterioro de AOV(E).

(iv) Se ha estudiado el deterioro del valor saludable de AOVEs sometidos a fritura a 180 °Cdurante 90 min. Se encontró un patrón de correlación en la degradación oleomisional/oleocoronal con el aumento de la concentración de oleaceína/oleocantal. Además, la concentración del ácido oleocantálico aumentó, destacando de nuevo como posible marcador de alteración del aceite.

2. Caracterizar la fracción de glicerofosfolípidos en diferentes categorías de aceite de oliva y en otros aceites vegetales refinados aprovechando la combinación entre sensibilidad y selectividad del analizador de triple cuadrupolo.

(v) Se ha propuesto un método para la determinación cualitativa y cuantitativa de glicerofosfolípidos en aceites vegetales mediante LC–MS/MS con una SPE previa para aislar de forma selectiva dicha fracción. Como prueba de concepto, el método fue aplicado a muestras de aceite de oliva de diferentes categorías y se encontraron diferencias cuali- y cuantitativas en el perfil de glicerofosfolípidos, dando lugar a su propuesta como método para el control de calidad del producto.

3. Estudiar el comportamiento de dos familias de componentes bioactivos del AOVE (fenoles y triterpenos) durante el proceso de extracción de este a partir del análisis de las diferentes fases implicadas: masas sólidas, aceite y residuos.

(vi) El deshuesado del fruto puede afectar al contenido fenólico del AOVE producido y, por tanto, a su valor saludable. Este efecto puede depender de la variedad como se deduce de la comparativa de resultados entre ‘Arbequina’ y ‘Picual’. Así, se vio que el deshuesado reduce el contenido fenólico en ‘Arbequina’ mientras que no afecta en ‘Picual’. En cuanto a componentes individuales, el

deshuesado en 'Arbequina' dio lugar a AOVE con un contenido significativamente menor en isómeros agliconas de oleuropeína y ligustrósido que en el mismo producto obtenido a partir de fruto intacto.

(vii) El proceso de extracción del AOVE juega un papel clave en la distribución de componentes bioactivos entre las fases implicadas. La caracterización de estas fases, concretamente, pastas, AOVE y residuos, resulta clave para la propuesta de mejoras tecnológicas que pudieran mejorar el enriquecimiento de estos componentes en AOVE. En el estudio planteado se pudo ver que los secoiridoides fueron extraídos preferentemente al AOVE, aunque el enriquecimiento en términos relativos fue superior en 'Arbequina' que en 'Picual'. Los lignanos no se detectaron cuantitativamente en los residuos (alperujo y aguas de vegetación) mientras que los ácidos terpénicos y los flavonoides se encontraron mayoritariamente en los residuos, destacando, por tanto, su reducida transferencia al AOVE final.

4. Proponer una estrategia que permita evaluar la adherencia de dietas con aceite de oliva como componente a partir de la determinación de ácidos grasos enlazados a triglicéridos y glicerofosfolípidos.

(viii) Se ha desarrollado un método para la determinación del perfil de ácidos grasos enlazados a glicerofosfolípidos utilizando SPE y GC–MS. Con este método se consigue aislar esta fracción lipídica polar y, por tanto, la derivatización por transesterificación se produce de forma selectiva en estos compuestos. Con este método, se planteó una estrategia doble para monitorizar la adherencia de dietas en función de su perfil graso, consistente en un doble análisis: por un lado, del perfil de ácidos grasos convencional (representativo de triglicéridos y ésteres de colesterol) y, por otro, del perfil enlazado a glicerofosfolípidos. De esta forma, se puede evaluar la adherencia dietética a corto y largo plazo.

CONCLUSIONS

The research conducted in this Doctoral Thesis was aimed at determining minor families present in VOO and EVOO and to study their variability associated to different factors to reinforce the competitiveness of both products based on two fundamental pillars: quality and healthy value.

The most relevant conclusions drawn from this work can be summarized as follows according to the objectives:

1. To evaluate the influence of different factors on the phenolic content of (E)VOO and, therefore, on its health benefits, according to the Commission Regulation (EU) 432/2012 Health Claim.

    (i) The phenolic content has been characterized in 1239 samples of (E)VOO provided directly by producers and obtained in two consecutive agronomical seasons to establish their healthy value based on the health claim included in European Regulation 432/2012. It was found that 85% of the analyzed samples had a phenolic content above 250 mg kg^{-1}, a content established as a limit to take benefits from the health claim.

    (ii) The influence of agronomical and geographical factors such as cultivar, harvesting period and location on the variability in the phenolic content of (E)VOO has been studied. Thus, it was possible to characterize the variability in phenolic content associated with these three factors, highlighting the cultivar and the harvesting period for their special relevance.

    (iii) The decrease in phenolic content of (E)VOO during storage under optimal conditions (room temperature and opaque glassware) depends on the initial phenolic profile. The predominance of certain phenols such as oleacein and oleocanthal increases the drop in phenolic concentration after 12 months of storage. In addition, it was found that the concentration of hydroxytyrosol and oleocanthalic acid increased after storage and could be considered as markers of (E)VOO deterioration.

(iv) The healthy index deterioration of EVOOs subjected to frying at 180 °C during 90 min has been studied. A correlation pattern was found in oleomissional/oleokoronal degradation to the increase of oleacein/oleocanthal concentration. Also, the concentration of oleocanthalic acid increased, again standing out as a possible marker of oil alteration.

2. To characterize the glycerophospholipid fraction in different categories of olive oil, and in other refined vegetable oils, using the combination of sensitivity and selectivity offered by the QqQ system.

(v) A method has been proposed for the qualitative and quantitative determination of glycerophospholipids in vegetable oils by LC–MS/MS with a previous SPE to selective isolation of mentioned fraction. As proof of concept, the method was applied to olive oil samples of different categories and qualitative and quantitative differences were found in the glycerophospholipid profile, that could be a promising approach for product quality control.

3. To study the behaviour of two families of bioactive compounds of EVOO (phenols and triterpenes) during the extraction process through the analysis of different phases involved: pastes, oil, and residues.

(vi) The fruit destoning can affect the phenolic content of the EVOO produced and, therefore, its healthy index. This effect may depend on the cultivar as can be deduced from the comparison of results between 'Arbequina' and 'Picual'. Thus, destoning reduced the phenolic content in ‘Arbequina’ while it did not affect ‘Picual’. Regarding individual compounds, destoning in 'Arbequina' produced an EVOO with a significant lower content of oleuropein and ligstroside aglycone isomers than in the same product obtained from intact fruit.

(vii) The EVOO extraction process plays a key role in the distribution of bioactive compounds between the phases involved. The

characterization of these phases, specifically, paste, EVOO and residues, is important to propose technological improvements that could enhance the enrichment of these compounds in EVOO. In the proposed study, it was remarked that the secoiridoids were preferentially extracted in EVOO, although the enrichment in relative terms was higher in 'Arbequina' than in 'Picual'. Lignans were not detected quantitatively in the residues (olive pomace and wastewater) while terpenic acids and flavonoids were found mainly in the residues, highlighting, therefore, their reduced transfer to the final EVOO.

4. To propose a strategy that allows evaluating the adherence of diets with olive oil as a component by the determination of fatty acids linked to triglycerides and glycerophospholipids.

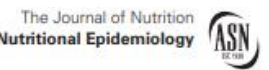
    (viii) A method has been developed for the determination of the fatty acids profile bound to glycerophospholipids using SPE and GC–MS. With this method it is possible to isolate this polar lipid fraction and, therefore, the derivatization by transesterification occurs selectively in these compounds. With this method, a double strategy was proposed to monitor the diet adherence based on their fat profile, consisting of a double analysis: on the one hand, the conventional fatty acid profile (representative of triglycerides and cholesterol esters) and, on the other hand, the profile bound to glycerophospholipids. In this way, short- and long-term dietary adherence can be assessed.

ANNEXES

ANNEX I

Other publications co-authored by PhD student

The Journal of Nutrition
Nutritional Epidemiology



Serum Phospholipid Fatty Acids and Mammographic Density in Premenopausal Women

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ABSTRACT

Background: The role of fatty acids (FAs) on mammographic density (MD) is unclear, and available studies are based on self-reported dietary intake.

Objectives: This study assessed the association between specific serum phospholipid fatty acids (PLFAs) and MD in premenopausal women.

Methods: The cross-sectional study DDM-Madrid recruited 1392 Spanish premenopausal women, aged 39–50 y, who attended a screening in a breast radiodiagnosis unit of Madrid City Council. Women completed lifestyle questionnaires and FFQs. Percentage MD was estimated using a validated computer tool (DM-Scan), and serum PLFA percentages were measured by GC-MS. Multivariable linear regression models were used to quantify the association of FA tertiles with MD. Models were adjusted for age, education, BMI, waist circumference, parity, oral contraceptive use, previous breast biopsies, and energy intake, and they were corrected for multiple testing.

Results: Women in the third tertile of SFAs showed significantly higher MD compared with those in the first tertile (β_{T3vsT1} = 7.53; 95% CI: 5.44, 9.61). Elevated relative concentrations of palmitoleic (β_{T3vsT1} = 3.12; 95% CI: 0.99, 5.25) and gondoic (β_{T3vsT1} = 2.67; 95% CI: 0.57, 4.77) MUFAs, as well as high relative concentrations of palmitelaidic (β_{T3vsT1} = 5.22; 95% CI: 3.15, 7.29) and elaidic (β_{T3vsT1} = 2.69; 95% CI: 0.59, 4.79) *trans* FAs, were also associated with higher MD. On the contrary, women with elevated relative concentrations of n–6 (ω-6) linoleic (β_{T3vsT1} = −5.49; 95% CI; −7.62, −3.35) and arachidonic (β_{T3vsT1} = −4.68; 95% CI: −6.79, −2.58) PUFAs showed lower MD. Regarding desaturation indices, an elevated palmitoleic to palmitic ratio and a low ratio of oleic to steric and arachidonic to dihomo-γ-linolenic acids were associated with higher MD.

Conclusions: Spanish premenopausal women with high relative concentrations of most SFAs and some MUFAs and *trans* FAs showed an increased MD, whereas those with high relative concentrations of some n–6 PUFAs presented lower density. These results, which should be confirmed in further studies, underscore the importance of analyzing serum FAs individually. *J Nutr* 2020;150:2419–2428.

Keywords: breast density, fatty acids, desaturation index, premenopause, DDM-Madrid, biomarkers, fat, breast cancer, epidemiology

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Article

Serum Phospholipid Fatty Acids Levels, Anthropometric Variables and Adiposity in Spanish Premenopausal Women

María del Pilar del Pozo [1], Virginia Lope [2,3,*], Inmaculada Criado-Navarro [4,5], Roberto Pastor-Barriuso [2,3], Nerea Fernández de Larrea [2,3], Emma Ruiz [2,3], Adela Castelló [2,3,6], Pilar Lucas [2], Ángeles Sierra [2,3], Isabelle Romieu [7,8], Véronique Chajès [9], Feliciano Priego-Capote [4,5], Beatriz Pérez-Gómez [2,3] and Marina Pollán [2,3]

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Abstract: This study investigates the still uncertain association between serum phospholipid fatty acids (PL-FA), and anthropometric and adiposity variables. A cross-sectional study was conducted with 1443 Spanish premenopausal women. Participants answered an epidemiological and a food frequency questionnaire. Anthropometric variables were measured using a bioimpedance scale. Serum PL-FAs levels were determined by gas chromatography–mass spectrometry. The association between body mass index (BMI), weight gain, body fat percentage, visceral fat index, and waist circumference with serum PL-FAs and desaturation indices was evaluated using multivariable linear regression models. BMI was positively associated with the relative concentration of saturated fatty acids (SFAs) ($\beta = 0.94$, q-val = 0.001), and with palmitoleic, dihomo-γ-linolenic (DGLA), arachidonic (AA) and α-linolenic acids, and was inversely associated with oleic, gondoic, trans-vaccenic, linoleic and γ-linolenic acids. Total fat percentage was positively associated with DGLA and AA, and inversely with linoleic and γ-linolenic acids. Low relative concentrations of some SFAs and high levels of n-6 PUFAs were associated with greater waist circumference. While the oleic/stearic and AA/DGLA acid ratios were inversely associated with BMI, DGLA/linoleic acid ratio was positively related to almost all variables. In addition to BMI, total fat percentage and waist circumference were also associated with certain individual fatty acids.

Keywords: fatty acids; desaturation index; obesity; body mass index; fat

Article

Serum Phospholipids Fatty Acids and Breast Cancer Risk by Pathological Subtype

Virginia Lope [1,2,3,*], Ángel Guerrero-Zotano [3,4], Ana Casas [3,5], José Manuel Baena-Cañada [3,6,7], Begoña Bermejo [3,8,9,10], Beatriz Pérez-Gómez [1,2,3], Inmaculada Criado-Navarro [11,12,13], Silvia Antolín [3,14], Pedro Sánchez-Rovira [3,15], Manuel Ramos-Vázquez [3,16], Antonio Antón [3,17], Adela Castelló [1,2,18], José Ángel García-Saénz [3,19], Montserrat Muñoz [3,20,21], Ana de Juan [3,22], Raquel Andrés [3,23], Antonio Llombart-Cussac [3,24], Blanca Hernando [3,25], Rosa María Franquesa [3,26], Rosalia Caballero [3], Feliciano Priego-Capote [11,12,13], Miguel Martín [3,10,27,28,†] and Marina Pollán [1,2,3,†]

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Abstract: This study evaluates whether serum phospholipids fatty acids (PL-FAs) and markers of their endogenous metabolism are associated with breast cancer (BC) subtypes. EpiGEICAM is a Spanish multicenter matched case-control study. A lifestyle and food frequency questionnaire was completed by 1017 BC cases and healthy women pairs. Serum PL-FA percentages were measured by gas chromatography-mass spectrometry. Conditional and multinomial logistic regression models were used to quantify the association of PL-FA tertiles with BC risk, overall and by pathological subtype (luminal, HER2+ and triple negative). Stratified analyses by body mass index and menopausal status were also performed. Serum PL-FAs were measured in 795 (78%) pairs. Women with high serum levels of stearic acid (odds ratio $(OR)_{T3vsT1}$ = 0.44; 95% confidence interval (CI) = 0.30–0.66), linoleic acid (OR_{T3vsT1} = 0.66; 95% CI = 0.49–0.90) and arachidonic to dihomo-γ-linolenic acid ratio (OR_{T3vsT1} = 0.64; 95% CI = 0.48–0.84) presented lower BC risk. Participants with high concentrations of palmitoleic acid (OR_{T3vsT1} = 1.65; 95% CI = 1.20–2.26), *trans*-ruminant palmitelaidic acid (OR_{T3vsT1} = 1.51; 95% CI = 1.12–2.02), *trans*-industrial elaidic acid (OR_{T3vsT1} = 1.52; 95% CI = 1.14–2.03), and high oleic to stearic acid ratio (OR_{T3vsT1} = 2.04; 95% CI = 1.45–2.87) showed higher risk. These associations were similar in all BC pathological subtypes. Our results emphasize the importance of analyzing fatty acids individually, as well as the desaturase activity indices.

Keywords: breast neoplasm; breast cancer subtypes; desaturation indices; fats; EpiGEICAM

ANNEX II

Oral and poster communications in national or international meetings

ORAL COMMUNICATION

ASIA-OCEANIA METABOLOMIC FORUM. MERLION METABOLOMICS SYMPOSIUM 2019 (AOMF-MMS 2019)

Early Career Presentation

Ms. Inmaculada Criado

PhD student,
University of Córdoba, Spain.

Visitor Student,
Department of Chemistry,
National University of Singapore.

Determination of Glycerophospholipids in Vegetable Edible Oils: Proof of Concept to Discriminate Olive Oil Categories

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Glycerophospholipids (GPLs) constitute a chemical family within the saponifiable fraction of vegetable oils. GPLs have been scarcely studied in edible oils owing to the lack of sensitive and selective analytical methods. We have developed a method for identification, confirmation and relative quantitation of GPLs in vegetable oils. The method is based on solid-phase extraction (SPE) for isolation of GPLs and determination by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). As proof of concept, the approach has been applied to characterize GPLs in different olive oil categories, thus revealing compositional changes, which could be explained by factors such as the quality of fruits and the extraction process. Families such as glycerophosphatidic acids and phosphatidylglycerides are remarkable because of their capability to discriminate virgin olive oils from the rest of categories. These results open a door to additional studies targeted at the identification of olive oil quality by monitoring these lipids.

Biography

Inmaculada Criado Navarro was born and raised in Cordoba, Spain. She obtained her undergraduate degree in chemistry at the University of Cordoba in 2016, studying the third year in Czech Republic. She joined the Analytical Chemistry Department in 2017 to pursue a Ph.D. focusing on metabolomic under the advisement of Prof. Feliciano Priego Capote. Currently she is a visitor student at NUS in Chemistry Department as part of Prof. Sam Li Fong Yau group.

26 AOMF - MMS 2019

ORAL COMMUNICATION

VIII CONGRESO CIENTÍFICO DE INVESTIGADORES EN FORMACIÓN DE LA UNIVERSIDAD DE CÓRDOBA

UNIVERSIDAD DE CÓRDOBA

idep

La Vicerrectora de Posgrado e Innovación Docente de la Universidad de Córdoba

ACREDITA que:

CRIADO NAVARRO, INMACULADA

ha presentado en la modalidad de **COMUNICACIÓN ORAL** el trabajo que lleva por título:

Determinación de glicerofosfolípidos en aceites vegetales comestibles: Prueba de concepto para discriminar entre categorías de aceite de oliva

en el **VIII Congreso Científico de Investigadores en Formación de la Universidad de Córdoba,** organizado por la Escuela de Doctorado de la Universidad de Córdoba, celebrado en Córdoba los días 18 y 19 de febrero de 2020.

Y para que así conste, se expide y firma este certificado en Córdoba, a 19 de febrero de 2020

Fdo: JULIETA MÉRIDA GARCÍA

Vicerrectora de Posgrado e Innovación Docente de la Universidad de Córdoba

Rectorado de la Universidad de Córdoba – Avd. Medina Azahara 5, 14071 CÓRDOBA

POSTER

IV CONGRESO VIRTUAL DE LA FEDERACIÓN ESPAÑOLA DE SOCIEDADES DE NUTRICIÓN, ALIMENTACIÓN Y DIETÉTICA (FESNAD) 2020

ABREVIATURAS/ABBREVIATIONS

AOV	Aceite de oliva virgen.
AOVE	Aceite de oliva virgen extra.
LC–MS/MS	Cromatografía líquida con detección por espectrometría de masa en tándem.
QqQ	Triple cuadrupolo.
SPE	Extracción en fase sólida.

3,4-DHPEA-EA	Oleuropein-aglycone mono-aldehyde; (Oleuropein-aglycone major form); 3,4-DHPEA-Elenolic acid mono-Aldehyde.
3,4-DHPEA-EDA	Oleacein; 3,4-DHPEA-Elenolic acid Di-Aldehyde Oleuropein-aglycone di-aldehyde; (Decarboxymethyl oleuropein-aglycone major form).
a.s.l.	Above sea level.
ACN	Acetonitrile.
ANOVA	One-way analysis of variance.
Arb	Arbequina
AUC	Area under the curve.
BPC	Base peak chromatogram.
C	Crushing paste.
CE	Capillary electrophoresis.
CEs	Cholesteryl esters.
CI	Confidence interval.
CID	Collision induced dissociation.
COPO	Crude olive pomace oil.
D	Decanter.
DAG	Diacylglycerol.
DI-MS	Direfct infunsion mass spectrometry.
EFSA	European Food Safety Authority.
EICs	Extracted ion chromatograms.
ESI	Electrospray Ionization.
EVOO	Extra virgin olive oil.

F	Final oil.
FA	Fatty acid.
FAO	Food and Agriculture Organization.
FDR	False discovery rate.
FFA	Free fatty acid.
FI	Flow-injection.
FID	Flame ionized detector.
FTICR	Fourier transform ion cyclotron resonance.
FWHM	Full width at half maximum.
GC	Gas chromatography.
GC-MS	Gas chromatography–tandem mass spectrometry.
GCxGC	Two-dimensional gas chromatography.
GPL	Glycerophospholipid.
HDL	High density lipoprotein.
HOSO	High-oleic sunflower oil.
HSD	Honestly significant difference.
IOC	International Olive Council.
IS	Internal standard.
Kp	Partition coefficient.
LC	Liquid chromatography.
LC–MS/MS	Liquid chromatography–tandem mass spectrometry.
LCxLC	Two dimensional liquid chromatography.
LDL	Low density lipoprotein.
LLE	Liquid–liquid extraction.
LOD	Limit of detection.
LOQ	Limit of quantitation.
M	Malaxation paste.
MAG	Monocylglycerol.
MeOH	Methanol.
MFs	Molecular features.
MRM	Multiple reaction monitoring.
MS	Mass spectrometry.

MUFA	Monounsaturated fatty acid.
NIST	National Institute of Standards & Technology.
NLS	Neutral losses.
NMR	Nuclear magnetic resonance.
O	Olive.
OO	Olive oil.
OPO	Olive pomace oil.
P	Olive pomace.
PAs	Glycerophosphatidic acids.
PCA	Principal Component Analysis.
PCs	Phosphatidylcholines.
PEs	Phosphatidylethanolamines.
PGs	Phosphatidylglycerides.
p-HPEA-EA	Ligstroside-aglycone mono-aldehyde; (Ligstroside-aglycone major form); p-HPEA-Elenolic acid mono-Aldehyde.
p-HPEA-EDA	Oleocanthal; p-HPEA-Elenolic acid Di-Aldehyde; Ligstroside-aglycone di-aldehyde; (Decarboxymethyl ligstroside-aglycone major form).
Pic	Picual.
PIs	Glycerophosphatidylinositols.
PIS	Product ion
PL	Phospholipid.
POD	Peroxidase.
POO	Pomace olive oil.
PPO	Polyphenoloxidase.
PS	Phosphatidylserines.
PUFA	Polyunsaturated fatty acid.
QC	Quality control.
QqQ	Triple quadrupole.
QTOF	Quadrupole time of flight.
R	Regression coefficient.
ROC	Receiver operating characteristic curve.

ROO	Refined olive oil.
ROPO	Refined olive pomace oil.
RSD	Relative standard deviation.
RT	Retention time.
SD	Standard deviation.
SFA	Saturated fatty acid.
SIL-PL	Stable isotopically labelled phospholipid.
SO	Sunflower oil.
SPE	Solid phase extraction.
SPME	Solid phase micro extraction.
SRM	Selected reaction monitoring.
TAG	Triacylglycerol.
TGs	Triglycerides.
TICs	Total ion chromatograms.
TLC	Thin-layer chromatography.
TOF	Time of flight.
VC	Vertical centrifuge oil.
VOO	Virgin olive oil.
WHO	World health organization.

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