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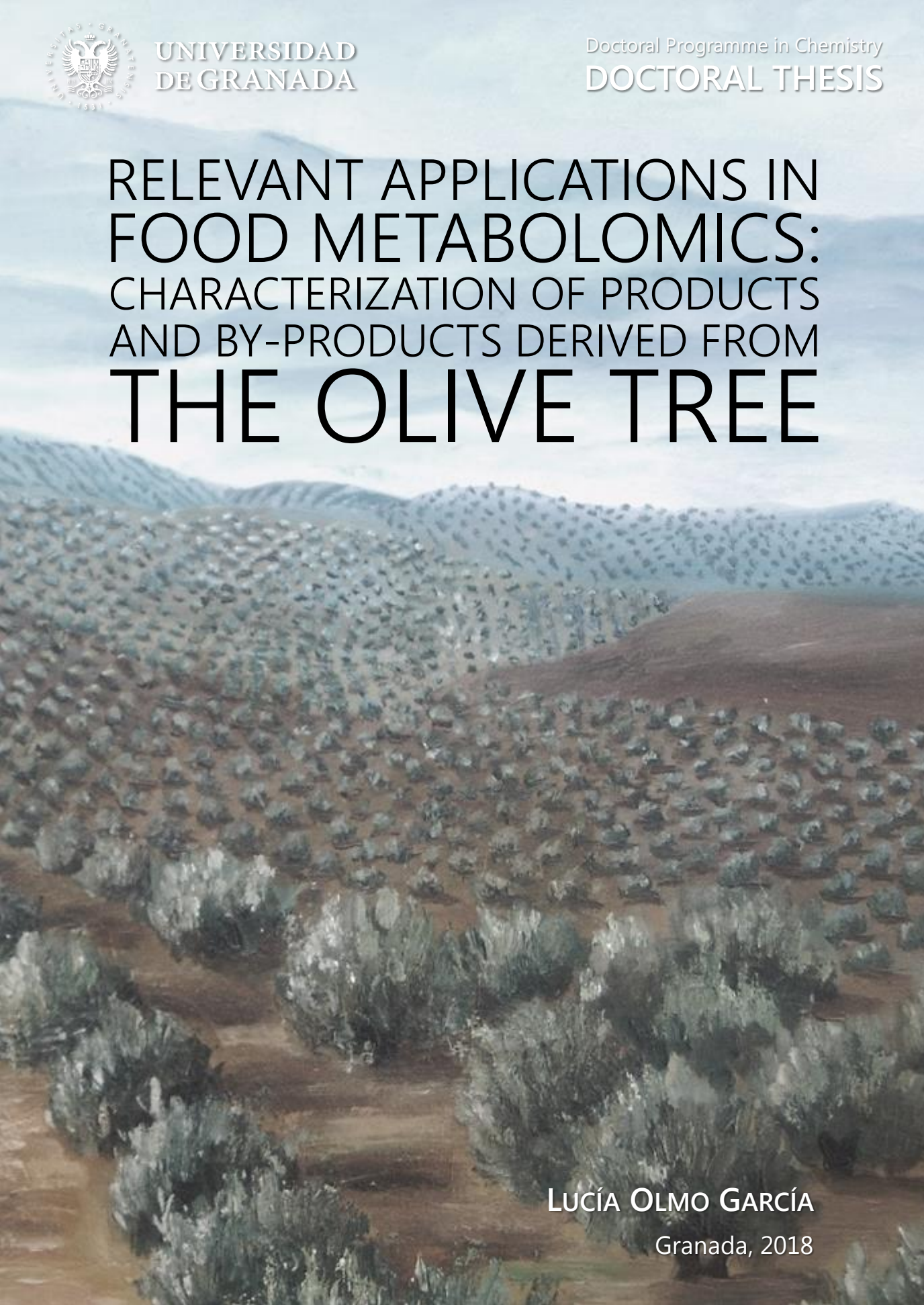
Doctoral Programme in Chemistry

DOCTORAL THESIS

RELEVANT APPLICATIONS IN FOOD METABOLOMICS: CHARACTERIZATION OF PRODUCTS AND BY-PRODUCTS DERIVED FROM THE OLIVE TREE



LUCÍA OLMO GARCÍA

Granada, 2018

UNIVERSIDAD
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Departamento de Química Analítica

Grupo de investigación FQM-297 "Control Analítico, Ambiental, Bioquímico y Alimentario"

Programa de Doctorado en Química

RELEVANT APPLICATIONS IN FOOD METABOLOMICS: CHARACTERIZATION OF PRODUCTS AND BY-PRODUCTS DERIVED FROM THE OLIVE TREE

APLICACIONES DE INTERÉS EN EL CAMPO DE LA METABOLÓMICA DE ALIMENTOS: CARACTERIZACIÓN DE PRODUCTOS Y SUBPRODUCTOS DERIVADOS DEL OLIVAR

Memoria presentada por **Dña. Lucía Olmo García** para optar al grado de Doctora Internacional en Química por la Universidad de Granada

Tesis dirigida por

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El trabajo que se presenta en esta Tesis Doctoral, con el título "APLICACIONES DE INTERÉS EN EL CAMPO DE LA METABOLÓMICA DE ALIMENTOS: CARACTERIZACIÓN DE PRODUCTOS Y SUBPRODUCTOS DERIVADOS DEL OLIVAR", que ha sido realizado bajo mi dirección, reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Lucía Olmo García pueda optar al grado de Doctor Internacional en Química.

Esta Tesis ha sido realizada en los laboratorios del grupo FQM-297 en el Departamento de Química Analítica de la Universidad de Granada y, parcialmente, en el Centro de Instrumentación Científica de la Universidad de Granada, en el Olive Center de la Universidad de California, Davis (Estados Unidos) y en la sede de Bremen de la empresa Bruker Daltonik GmbH (Alemania).

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El trabajo que se presenta en esta Tesis Doctoral, con el título "APLICACIONES DE INTERÉS EN EL CAMPO DE LA METABOLÓMICA DE ALIMENTOS: CARACTERIZACIÓN DE PRODUCTOS Y SUBPRODUCTOS DERIVADOS DEL OLIVAR", que ha sido realizado bajo mi dirección, reúne todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Lucía Olmo García pueda optar al grado de Doctor Internacional en Química.

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La presente Tesis Doctoral ha sido realizada con la financiación recibida a través de una beca predoctoral del Subprograma de Formación de Profesorado Universitario (FPU) dentro del Programa Estatal de Promoción del Talento y su Empleabilidad, procedente del Ministerio de Educación, Cultura y Deporte (ref. FPU13/06438).

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Objectives

Objetivos

During the last decades, olive oil has been considered as a clear example of functional food, existing a strong association between its consumption and the reduction of the risk of suffering certain diseases, particularly those caused by oxidative stress. These healthy properties, together with its distinctive organoleptic characteristics that give it a high culinary value, have prompted the expansion of its consumption throughout the world. Thus, the globalization of the olive oil sector and the emergence of new growing areas beyond the Mediterranean Basin is pushing producers to seek the differentiation of their products in an increasingly competitive market. Some of the proposed strategies are based on the use of labelling declaring the geographical or varietal origin of the oil, or the exploitation of health claims associated to some of its bioactive compounds. In any case, to have powerful analytical tools that are able to authenticate the origin or exhaustively characterize the compositional profile of virgin olive oil is mandatory.

On the other hand, although the oil is the main product derived from olive cultivation, it is not the only one. In fact, the transformation and valorization of the olive by-products are currently considered as parts of the same integral cycle of exploitation. Until now, the whole industry emerged around the olive tree has been based on the complete crushing of the fruits to extract the olive oil (by pressing or centrifugation), which leads to the simultaneous generation of large amounts of contaminant wastes. Nowadays, different strategies are being used to achieve the valorization of the olive by-products and new processing procedures that avoid their generation are being developed, for example, seeking the integral use of each part of the fruit (skin, pulp, stone and seed). As a result, new products derived from the olive tree with potential applications as ingredients in functional foods and cosmetics are emerging; their composition has not been assessed yet. To encourage the use of these products at industrial level, it is essential to deeply characterize them, what could also help to understand their physiological effects. Further study on olive oil and these new products/by-products from the olive grove could assist the search of natural sources of bioactive compounds and, at the same time, the results of these investigations can bring with them the economic boost of the olive sector in our region.

In this context, the analytical methodologies applied in the olive field have clearly evolved over the last decade. That has lead to the introduction of omics strategies, among which chromatographic techniques coupled to mass spectrometry have a leading role (in particular, in the area of Metabolomics).

Therefore, the main goal of this Doctoral Thesis has been to evaluate the potential of different metabolomic approaches and complementary tools to obtain information on the metabolome of products and by-products derived from the olive tree (either to authenticate their geographical or botanical origin, to accurately quantify some of its most relevant metabolites, or to carry out the characterization of previously unexplored matrices). In order to achieve this general purpose, we proposed a series of partial aims that could assure the successful achievement of the main one described above:

- To carry out an appropriate experimental design and global planning of each study, with a rigorous selection of representative samples that ensure the consistency of the results and allow us to reach reliable conclusions.
- To optimize extraction systems of the analytes under study from the selected matrices (plant tissues and oils) and the most relevant variables to carry out their determination using analytical platforms of different nature (LC-DAD, LC-FLD, LC-MS and GC-MS).
- To exhaustively validate the developed methods and to apply them in metabolomic studies (including both targeted and untargeted approaches) on samples derived from the olive tree (oils and other vegetable matrices).
- To identify and quantify, when appropriate, the metabolites found in the samples, and/or to use chemometric techniques to extract useful information from the acquired data in the most effective and reliable possible way.

En las últimas décadas, el aceite de oliva ha sido considerado como un claro ejemplo de alimento funcional, existiendo una fuerte asociación entre su consumo y la disminución del riesgo de padecer ciertas enfermedades, particularmente las provocadas por el estrés oxidativo. Este motivo, sumado a sus distintivas características organolépticas que le otorgan un alto valor culinario, ha producido la expansión de su consumo por todo el mundo. Así, la globalización del sector del aceite de oliva y la aparición de nuevas áreas productoras más allá de la Cuenca del Mediterráneo, está empujando a los productores a buscar la diferenciación de sus productos en un mercado cada vez más competitivo. Algunas estrategias empleadas se basan en utilizar etiquetado que haga alusión al origen geográfico o varietal del aceite, o bien, en intentar explotar la declaración de propiedades saludables asociadas a algunos de sus compuestos bioactivos. En cualquier caso, es necesario disponer de potentes herramientas analíticas que sean capaces de autentificar el origen o caracterizar de manera exhaustiva el perfil composicional del aceite de oliva virgen.

Por otro lado, si bien el aceite es el principal producto derivado del cultivo del olivo, no es el único. De hecho, actualmente el proceso de transformación de la aceituna y la valorización de sus subproductos son considerados partes de un mismo ciclo integral de aprovechamiento. Hasta ahora, toda la industria surgida alrededor del cultivo del olivo ha estado basada en la completa trituración del fruto para poder extraer el aceite de oliva (mediante prensado o centrifugación), lo que conlleva la generación simultánea de grandes cantidades de residuos contaminantes. En la actualidad, se están empleando distintas estrategias para conseguir la valorización de los subproductos del olivar, y se están desarrollando nuevos procedimientos de procesado que eviten su generación, por ejemplo, buscando el aprovechamiento integral de cada una de las partes del fruto (piel, pulpa, hueso y semilla). Como consecuencia, están apareciendo nuevos productos derivados del olivo con potenciales aplicaciones como ingredientes en alimentación funcional y en cosmética, cuya composición, por el momento, no ha sido investigada. Para facilitar la utilización de estos productos a nivel industrial es imprescindible caracterizarlos profundamente, lo que además podría ayudar de manera considerable a comprender sus efectos fisiológicos. Ahondar en el estudio del aceite de oliva y caracterizar estos nuevos productos/subproductos del olivar puede resultar muy interesante en la búsqueda de fuentes de compuestos bioactivos de origen natural. Al mismo tiempo, los resultados de estos estudios, pueden traer consigo el impulso económico del sector del olivar en nuestra región.

En este contexto, la evolución de las metodologías analíticas de uso en el ámbito oleícola es clara, desembocando en la última década en el empleo de estrategias ómicas, donde las técnicas cromatográficas acopladas a espectrometría de masas tienen un papel absolutamente protagonista (en particular, en el área de la Metabolómica).

Por tanto, el objetivo principal de esta Tesis Doctoral ha sido la evaluación del potencial de diferentes aproximaciones y herramientas metabolómicas, complementarias entre sí, para obtener información sobre el metaboloma de productos y subproductos derivados del olivar (ya sea para

autentificar su origen geográfico y botánico, para ser capaces de cuantificar de forma precisa algunos de sus metabolitos más relevantes, o para llevar a cabo la caracterización de nuevas matrices prácticamente inexploradas). Para la exitosa consecución del mismo, se plantearon una serie de objetivos parciales:

- Llevar a cabo un diseño experimental y planificación global de cada uno de los estudios, lo más adecuados posible, con una rigurosa selección de muestras representativas que asegurasen la consistencia de los resultados y el alcance de conclusiones fiables.
- Optimizar los sistemas de extracción de los analitos de interés de las matrices bajo estudio (tejidos vegetales y aceites) y las variables más relevantes para llevar a cabo su determinación empleando plataformas analíticas de distinta naturaleza (LC-DAD, LC-FLD, LC-MS y GC-MS).
- Validar de forma exhaustiva los métodos desarrollados y aplicarlos en estudios metabolómicos (incluyendo tanto aproximaciones *targeted* como *untargeted*) sobre muestras derivadas del olivo (aceites y otras matrices vegetales).
- Identificar y cuantificar (cuando procediese) los metabolitos presentes en las muestras, y/o emplear herramientas estadísticas para poder extraer información útil del modo más efectivo y fiable posible de los datos adquiridos.

Summary

Resumen

This dissertation shows the results achieved within the frame of the Doctoral Thesis entitled "RELEVANT APPLICATIONS IN FOOD METABOLOMICS: CHARACTERIZATION OF PRODUCTS AND BY-PRODUCTS DERIVED FROM OLIVE TREE". The report has been divided into two main blocks: the **Introduction**, which offers a general overview of the subject and contextualize the presented work; and the **Experimental part, Results and Discussion**, which contains the experimental work carried out within this PhD, the achieved results and outstanding conclusions.

In the **Introduction**, the first part includes a brief review on the botanical origin and the compositional characterization of the olive tree, and introduces the four most important groups of *Olea europaea* L. phytochemicals with health-promoting effects (phenolic compounds, pentacyclic triterpenes, tocopherols and sterols). The second part of the introductory section gives an outline of the olive oil and its composition, shows different production methods and defines the generated by-products. The third section presents the concept Metabolomics, lists some of the main applications related to the field of this Doctoral Thesis and describes the different stages that make up the usual workflow in this kind of studies.

The second block of the manuscript refers to the **Experimental part, Results and Discussion** and is divided into two thematic sections, related to the analytical approaches applied to the characterization of the samples under study. It is important to mention that the chapters included in this part of the thesis are consecutively numbered, not necessarily following a chronological order.

Section I deals with the development and application of **"single-class" methodologies**, which are focused on the determination of a limited number of compounds belonging to a specific chemical family. It consists of six chapters:

- **Chapter 1** describes the development of a LC method coupled to three different detectors for the determination of triterpenic acids and alcohols in extracts from olive tree-derived tissues (leaves, skin and olive pulp). DAD, ESI-IT MS and APCI-QTOF MS were used as detection systems to give different alternatives to perform the accurate determination of these analytes. A comparative study was carried out to evaluate their analytical performance, advantages and drawbacks, and to check whether the quantitative results achieved by the three platforms were in good agreement.
- **Chapter 2** includes the development of a LC-MS method for determining olive oil triterpenic acids and dialcohols, giving an alternative to the widely used GC-FID/MS methodologies. First, the sample treatment (ultrasound-assisted extraction) was optimized and then, the method was applied to the analysis of six triterpenic compounds in monovarietal oils. The obtained results were compared with those provided by the reference GC-MS method and the direct injection of diluted olive oil samples into the LC-MS system was also tested, in an attempt to proffer an even simpler sample treatment.
- **Chapter 3** is devoted to the optimization of an LC-FLD method for the determination of phenolic compounds in olive oil with the aim to propose an appropriate alternative to LC-MS

methodologies. The use of the "multi-emission" fluorimetric detection mode allowed the selective quantification of a total of 23 phenolic compounds (plus 7 isomers) belonging to different families (simple phenols, phenolic acids, secoiridoids and lignans).

- **Chapter 4** presents an exhaustive comparison between specific (LC-MS) and global methods (Folin-Ciocalteu method, International Olive Council HPLC method and an approach based on the hydrolysis of complex phenols and subsequent detection by HPLC) for the determination of phenolic compounds in olive oil. All of them were applied to the analysis of 50 extra virgin olive oils (covering all the possible quantitative ranges of these substances). The obtained results were meticulously discussed and the main points of reflection were identified, with special emphasis on the need to reformulate the health claim associated to hydroxytyrosol and its derivatives.

- **Chapter 5** includes the characterization of the phenolic profile of virgin olive oils produced in the region of Maipú (Mendoza, Argentina) by means of a rapid LC-MS method. The results of the analysis of 25 commercial samples allowed the establishment of a correlation between flavonoids content and botanical variety and pointed out some distinctive features of the Arauco variety (Argentinean autochthonous variety).

- In the last chapter of this section, **Chapter 6**, the exhaustive characterization of the phenolic profile of olive leaves from Morocco is presented. The study aimed to contribute to implement the valorization of valuable compounds as a way to increase the profitability of the sector in growing regions. 55 samples belonging to 11 different varieties were analyzed by LC-MS and the application of chemometrics on phenolic compounds quantitative data allowed a good discrimination of the selected samples according to their varietal origin.

Section II of the **Experimental part, Results and Discussion** is devoted to the development and application of **"multi-class" methods** in metabolomic studies. The objective of this type of analytical approach is the determination of as many compounds as possible in a single analysis. That greatly increases throughput and reduces cost since it avoids the need to perform several "single-class" determinations. Five chapters are grouped in this section:

- **Chapter 7** describes the development of two methods (LC-MS and GC-MS) capable of simultaneously determining more than 40 compounds belonging to the olive oil minor fraction in a single run. In the first place, a non-selective and highly efficient sample treatment was optimized. Then, the separation and detection conditions were adjusted so as to cover phenolic and triterpenic compounds, free fatty acids and tocopherols by LC-MS, plus sterols and hydrocarbons by GC-MS, in 31 and 50 min, respectively. In addition, both methods were compared in terms of analytical performance, easiness, cost and adequacy to the analysis of each chemical class.

- **Chapter 8** presents the demonstration of the effectiveness of the GC-MS method proposed in the previous chapter to carry out the quantitative-profiling of the olive oil minor fraction. More

than 40 minor compounds were quantified in of 32 samples from 8 different varieties cultivated in the same experimental orchard (Davis, CA) and, subsequently, the obtained results were subjected to supervised and unsupervised statistical methods to achieve the botanical discrimination of the samples.

- **Chapter 9** includes the exhaustive characterization of two new products obtained from olive fruits (oil and flour) by means of a novel processing method aiming the reduction of the amount of by-products generated by the traditional system. It involves stages of stoning, dehydration of the pulp and cold pressing. A total of 135 samples (including virgin olive oils too for comparative purposes) were analyzed by applying the LC-MS methodology developed in Chapter 7. Concentration ranges were established for 57 metabolites and the effect of the dehydration temperature in the composition of the resulting products was investigated.

- **Chapter 10** shows the evaluation of the potential of untargeted metabolomic approaches to achieve the authentication of the declared geographical origin of 126 extra virgin olive oil samples. For that purpose, two different platforms (LC-ESI-QTOF MS -in positive and negative polarity- and GC-APCI-QTOF MS -in positive mode-) were used to analyze samples from six different geographical indications. Chemometric tools included within the software MetaboScape 3.0 (Bruker Daltonik) allowed to establish classification models and to point out potential origin markers, which were identified to a large extent thanks to the annotation tools included in the mentioned software and to the use of pure standards and isolated olive oil fractions.

- Finally, **Chapter 11** describes the application of "multi-class" methods to study the metabolic profile of eight matrices derived from the olive tree, including plant tissues (leaves, stems, skin, pulp and olive seed) and oils (seed oil, virgin olive oil and oil obtained from stoned and dehydrated fruits). All the experimental conditions were selected to ensure the maximum coverage of the metabolome in a single analysis. The use of LC and GC coupled to high resolution MS (through different ionization sources, ESI and APCI) and the annotation strategies included within MetaboScape 3.0 allowed the identification of around 150 compounds in the profiles, showing great complementarity between the tested analytical platforms. This chapter also includes a semi-quantitative comparison of the content of triterpenes, tocopherols, sterols, phenols, etc. in the selected matrices.

En esta memoria se presentan los resultados obtenidos durante la realización de la Tesis Doctoral titulada "APLICACIONES DE INTERÉS EN EL CAMPO DE LA METABOLÓMICA DE ALIMENTOS: CARACTERIZACIÓN DE PRODUCTOS Y SUBPRODUCTOS DERIVADOS DEL OLIVAR". La misma contiene dos apartados principales: la **Introducción**, que trata de ofrecer una visión general de los temas tratados en la Tesis y contextualizar el trabajo presentado; y la **Parte experimental, Resultados y Discusión**, que recoge todo el trabajo experimental llevado a cabo, los resultados más relevantes, conclusiones de mayor interés y perspectivas futuras.

En la **Introducción**, la primera parte incluye una breve reseña sobre el origen botánico y la caracterización composicional del olivo y presenta los cuatro grupos de fitoquímicos más importantes hallados en él (compuestos fenólicos, tocoferoles, triterpenos pentacíclicos y esteroles), a los que se han asociado distintos efectos beneficiosos para la salud humana. La segunda parte de la sección introductoria, se centra en el aceite de oliva, describiendo su composición y presentando diferentes métodos de obtención y los subproductos que generan. Un tercer apartado se ocupa de introducir el área de la Metabolómica, centrándose en sus aplicaciones en el ámbito de esta Tesis, así como en las distintas etapas que componen el flujo de trabajo habitual en estudios metabolómicos.

El segundo gran bloque de la memoria hace referencia a la **Parte experimental, Resultados y Discusión** y está dividido en dos secciones temáticas, relacionadas con las aproximaciones analíticas puestas a punto para la caracterización de las muestras objeto de estudio. Es importante mencionar que los capítulos recogidos en esta parte de la Tesis están numerados de manera consecutiva sin seguir necesariamente un orden cronológico.

La **Sección I** versa sobre el desarrollo y aplicación de **metodologías "single-class"** o lo que es lo mismo, centradas en la determinación de un número limitado de compuestos pertenecientes a una determinada familia química. Está formada por seis capítulos:

- El **Capítulo 1** describe el desarrollo de un método LC acoplado a tres detectores distintos para la determinación de ácidos y alcoholes triterpénicos en extractos de tejidos derivados del olivo (hojas, piel y pulpa de aceituna). DAD, ESI-IT MS y APCI-QTOF MS se utilizaron como sistemas de detección con el objetivo de ofrecer distintas alaternativas para llevar a cabo la determinación de estos analitos. Se evaluaron las prestaciones analíticas, ventajas y desventajas de cada uno de los métodos desarrollados y se comprobó que los resultados cuantitativos alcanzados mediante las tres plataformas eran estadísticamente equivalentes.
- El **Capítulo 2** recoge el desarrollo de un método LC-MS para la determinación de ácidos y alcoholes triterpénicos en aceite de oliva alternativo a las metodologías GC-FID/MS ampliamente empleadas hasta el momento. Primero se optimizó el tratamiento de muestra (extracción de los analitos de interés asistida por ultrasonidos) y, posteriormente, el método se aplicó al análisis de aceites monovarietales y se compararon los resultados obtenidos con los proporcionados por el método GC-MS de referencia. También se evaluó la inyección

directa de los aceites (tras una simple dilución) en el sistema LC-MS, como propuesta de tratamiento de muestra aún más sencillo.

- El **Capítulo 3** detalla el desarrollo de un método LC-FLD para la determinación de compuestos fenólicos del aceite de oliva, con el objetivo de ofrecer una alternativa válida y más asequible a las metodologías LC-MS. El empleo de un modo de detección fluorimétrico "multi-emisión" permitió cuantificar de forma selectiva un total de 23 compuestos fenólicos (más 7 isómeros) pertenecientes a las familias de fenoles simples, ácidos fenólicos, secoiridoides y lignanos.

- En el **Capítulo 4** se presenta una comparación exhaustiva entre métodos específicos (LC-MS) y globales (método de Folin-Ciocalteu, método HPLC propuesto por el Consejo Oleícola Internacional y un método basado en la hidrólisis de los fenoles complejos y subsecuente deteción mediante HPLC) de determinación de compuestos fenólicos en aceite de oliva. Todos ellos se aplicaron a un conjunto de 50 muestras seleccionadas expresamente para cubrir todos los posibles rangos de concentración. Los resultados obtenidos se discutieron meticulosamente y se identificaron los principales puntos de reflexión, haciendo especial énfasis en la necesidad de reformular la declaración de propiedades saludables asociada al contenido de hidroxitirosol y derivados.

- El **Capítulo 5** incluye la caracterización del perfil fenólico de aceites de oliva vírgenes producidos en la región de Maipú (Mendoza, Argentina) mediante la aplicación de un rápido método LC-MS. Este trabajo condujo a la identificación de algunas características distintivas de la variedad Arauco (variedad autóctona argentina) y permitió establecer una relación entre el contenido de flavonoides y la variedad botánica.

- En el último capítulo de esta sección, el **Capítulo 6**, se muestra la caracterización exhaustiva del perfil fenólico de hojas de olivo procedentes de Marruecos, donde la valorización de este subproducto podría suponer un impulso para la economía de las regiones productoras. Se analizaron 55 muestras pertenecientes a 11 variedades distintas mediante LC-MS y se demostró la utilidad de los compuestos determinados para conseguir la discriminación varietal de las muestras estudiadas mediante la aplicación de herramientas quimiométricas.

La **Sección II** de la **Parte experimental, Resultados y Discusión** está dedicada al desarrollo y aplicación de **metodologías "multi-class"** en estudios metabolómicos. El objetivo de este tipo de aproximaciones analíticas es la determinación del mayor número posible de compuestos en un único análisis, lo que evita la necesidad de realizar varias determinaciones "single-class" y conlleva la reducción de costes y una mejora del rendimiento en los laboratorios. Cinco capítulos constituyen esta sección:

- El **Capítulo 7** describe el desarrollo de dos métodos (LC-MS y GC-MS) capaces de determinar simultáneamente más de 40 compuestros pertenecientes a la fracción minoritaria del aceite de oliva en un solo análisis. En primer lugar, se optimizó un tratamiento de muestra muy poco selectivo y altamente eficiente. Las condiciones de separación y detección se ajustaron de

modo que posibilitasen la determinación de compuestos fenólicos y triterpénicos, ácidos grasos libres y tocoferoles mediante LC-MS, además de esteroles e hidrocarburos por GC-MS, en 31 y 50 min, respectivamente. Asimismo, se realizó una comparativa de ambos métodos en términos de prestaciones analíticas, facilidad, coste y adecuación al análisis de cada familia química.

- El **Capítulo 8** recoge la demostración de la efectividad del método GC-MS propuesto en el capítulo anterior para llevar a cabo la cuantificación de 41 compuestos minoritarios del aceite de oliva, con la intención de caracterizar y conseguir la discriminación botánica de muestras provenientes de 8 variedades distintas. Para ello, se procedió al análisis de 32 muestras procedentes de un mismo campo de cultivo experimental situado en Davis (California) y, posteriormente, los resultados obtenidos se trataron por medio de métodos estadísticos supervisados y no supervisados.

- El **Capítulo 9** está dedicado a la caracterización exhaustiva de dos nuevos productos derivados de la aceituna (aceite y harina), obtenidos a través de un novedoso método de procesamiento que incluye etapas de deshuesado, deshidratación de la pulpa y prensado en frío, con el objetivo de reducir la cantidad de subproductos generados por el sistema tradicional. Se analizaron (aplicando la metodología LC-MS desarrollada en el Capítulo 8) un total de 135 muestras (incluyendo también aceites de oliva vírgenes, con fines comparativos) y se establecieron rangos de concentración para 57 metabolitos evaluando, al mismo tiempo, el efecto de la temperatura de deshidratación en la composición de los productos resultantes.

- En el **Capítulo 10** se presenta la evaluación del potencial de aproximaciones metabolómicas *untargeted* para conseguir la autentificación del origen geográfico declarado de 126 muestras de aceite de oliva virgen extra comerciales. Para ello, se usaron dos plataformas distintas (LC-ESI-QTOF MS -en polaridad positiva y negativa- y GC-APCI-QTOF MS -en modo positivo-) para analizar muestras provenientes de seis indicaciones geográficas diferentes. Las herramientas quimiométricas incluidas en el software MetaboScape 3.0 (Bruker Daltonik) permitieron establecer modelos de clasificación y señalar potenciales marcadores varietales, que fueron identificados en gran medida gracias a las herramientas de anotación incluidas en el mencionado software y al uso de patrones puros y fracciones aisladas del aceite de oliva.

- El **Capítulo 11** recoge la aplicación de métodos "multi-class" para estudiar el perfil metabólico de ocho matrices derivadas del olivo, incluyendo tejidos vegetales (hojas, tallos, piel, pulpa y semilla de aceituna) y aceites (aceite de semilla de olivo, aceite de oliva virgen y aceite de oliva obtenido de frutos deshuesados y deshidratados). Todas las condiciones experimentales se seleccionaron para asegurar la máxima cobertura del metaboloma de las muestras bajo estudio en un solo análisis. El empleo de LC y GC con MS de alta resolución (a través de diferentes fuentes de ionización -ESI y APCI-) y las estrategias de anotación incluídas en el software MetaboScape 3.0 permitieron identificar alrededor de 150 compuestos en los perfiles,

mostrando una gran complementariedad entre las plataformas analíticas evaluadas. Este capítulo también incluye una comparación semi-cuantitativa del contenido de triterpenos, tocoferoles, esteroles, fenoles, etc. de las matrices seleccionadas.

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Introduction

1. *OLEA EUROPAEA* L.

1.1. The culture of the olive tree

Since ancient times, the olive tree has played a key role in the life of Mediterranean communities in the religious, economic, political and cultural fields. Considered as a Gods' gift in the classical mythology, this emblematic tree has been recognized as a symbol of peace, abundance, wisdom and health. Throughout history, olive tree derived products have had diverse uses, from building material or fuel to medicinal and bodycare ointments [1,2]. But, undoubtedly, their culinary use is the most important one; virgin olive oil and olives are indeed considered as staple foodstuffs in the Mediterranean diet [2,3].

It is generally accepted that olive tree domestication occurred in Asia Minor (the region currently located between Syria, Lebanon and Israel) approximately 6000 years ago. Later on, Phoenicians, Greeks and Romans spread its cultivation throughout the Mediterranean basin. After the discovery of the New World in the 15th century, it was propagated to the American continent [4–6]. Nowadays, olive tree is cultivated in 57 countries all over the world, mainly located in regions that have Mediterranean-like climate conditions as shown in Fig. 1 (between the 30th and 45th parallels in both hemispheres) [7,8].

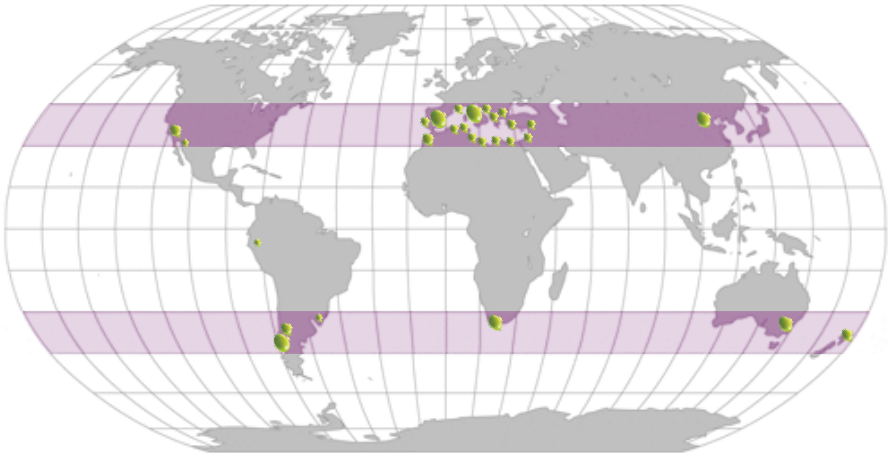


Figure 1. World distribution of olive tree cultivation.

There are around 11.5 million hectares of olive groves that annually produce almost 18000 million tons of olive fruits, with an estimated economic turnover of 13000 million euros and employing more than 35 million people [8]. Approximately, 90% of the olives globally produced are processed to obtain olive oil, while the remaining 10% are consumed as table olives. As a result, between 2 and 3 million tons of olive oil and an average of 2.5 million tons of table olives are produced each crop year. According to the International Olive Council, around 97% of the worldwide olive oil and 91% of table olives were produced by Mediterranean countries in 2017/2018 [9,10].

The economic importance of the olive sector in our country is undeniable. Spain is the world's largest producer, accounting for nearly 50% and 30% of olive oil and table olives annual production, respectively. It also leads the exportations, with more than 100 destination countries. Olive tree dominates the rural landscape of many Spanish regions, where it has a marked socio-economic impact, generating nearly 50 million labor days per season [8–10].

1.2. Botany and morphology

The olive tree (*Olea europaea* L.) belongs to the *Oleaceae* botanical family. The *Olea europaea* subspecies *europaea* includes wild (*Olea europaea* subsp. *europaea* var. *sylvestris*) and cultivated olives (*Olea europaea* subsp. *europaea* var. *europaea* or *sativa*) [11].

The cultivated olive tree is an evergreen, slow-growing and long-lived species with a life span of several centuries. It can grow up to 8-15 m tall but the common pruning practices keep the tree's height low (between 3 and 5 m). It has a short gnarled trunk, and multiple branches with cascading limbs (Fig. 2a). Its silvery green leaves have oblong shape (4–10 cm long and 1–3 cm wide). Flower bud inflorescences develop in the axil of each leaf and contain 15-30 small hermaphrodite flowers, yellow-white in color (Fig. 2b). The fruit is a drupe 2-2.5 cm long, green to purple or black depending on the ripening stage (Fig. 2c) [11,12].

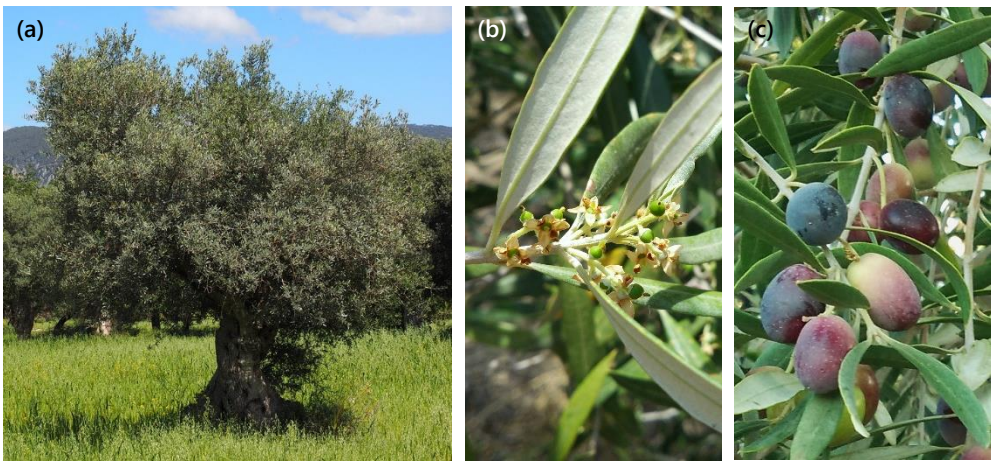


Figure 2. *Olea europaea* L. (a) tree, (b) flowers with incipient fruits and (c) drupes.

There are more than 2500 known cultivars worldwide that displays huge diversity based on fruit size and morphology. General information about the main cultivated olive varieties can be found in the *'World catalogue of olive varieties'* edited by the International Olive Council [13]. Table 1 lists the most prevalent cultivars in the three major olive growing countries. Apart from those mentioned in the table, other relevant varieties from countries with increasing importance in olives production are, for instance, *Picholine Marocaine* in Morocco, *Aglandau and Picholine du Languedoc* in France, *Arauco* in Argentina and *Mission* in California [14].

Table 1. Main olive varieties cultivated in the three major olive-producing countries [14].

Country	Number of cultivars	Most important cultivars in order of decreasing olive-growing surface area	Additional information
Spain	262	*Picual, Cornicabra, Hojiblanca, Manzanilla de Sevilla, Arbequina, Morisca de Badajoz, Empeltre, Manzanilla, Cacereña, Lechín de Sevilla, Picudo, Lechín de Granada, Verdial de Badajoz, Morrut, Sevillenca, Villalonga, Castellana, Farga, Verdial de Huevar, Blanqueta, Gordal Sevillana, Verdial de Velez-Malaga, Aloreña, Changlot Real, Alfafara*	The listed cultivars account for 96% of the total olive-growing area (the three cultivars at the top of the list account for 63%)
Italy	538	*Coratina, Ogliarola salentina, Cellina di Nardò, Carolea, Frantoio, Leccino, Ogliarola barese, Moraiolo, Bosana, Cima di Mola, Dolce di Rossano, Ogliarola messinese, Ottobratica, Sinopolese, Nocellara del Belice, Canino, Carboncella, Itrana, Moresca, Rotondella, Taggiasca, Tondina, Grossa di Gerace, Nocellara etnea*	The listed cultivars account for 58% of the total olive-growing area
Greece	52	*Koroneiki, Kalamata, Mastoidis*	90% of the total olive-growing area

1.2.1. Olive fruit

As already mentioned, the olive fruit is an elliptic drupe of 2-12 g weight. Anatomically, it consists of three parts (Fig. 3): the skin, so-called epicarp (1-3% of the drupe weight), the pulp or flesh, also named mesocarp (70-80% of the whole fruit), and the stone, referred to as woody endocarp (18-22% of the olive weight), which contains the seed [15].

Figure 3. Cross schematic section of the olive fruit (a) and the olive stone (b).

The olive fruit chemical composition is mainly determined by genetic factors (cultivar), but the ripening index, pedoclimatic conditions and agronomic practices also have a great influence in the biosynthesis and possible degradation of several compounds [15]. Average chemical composition of the olive fruit is presented in Table 2.

Fiber and polysaccharides are the most important components of the olive stone (cellulose in the wooden shell and hemicellulose in the seed). In the pulp, the fiber determines its texture and digestibility [16]. Regarding olive fruit constituents, they are usually classified in major (triglycerides) and minor components (aliphatic compounds, polycyclic triterpenes and phenols) [15]. The triglyceridic composition of the pulp differs from that of the seed: while monounsaturated fatty

acids (mostly oleic acid) prevail in pulp triglycerides, seed ones contain more polyunsaturated fatty acids (linoleic and linolenic acids) [17].

Table 2. Olive pulp, stone and seed composition (% w/w) [18].

	Pulp	Stone	Seed
Water	50-60	9.3	30
Oil	15-30	0.7	27.3
Sugar	3-7.5	41	26.6
Cellulose	3-6	38	1.9
Protein	2-5	3.4	10.2
Ash	1-2	4.1	1.5
Other compounds	2-2.5	3.5	3.5-4

1.2.2. Olive leaf

Olive leaves are thick, leathery and oppositely arranged around the stem. Each leaf grows over a 2-3 year period before shedding. They are accumulated in high quantities during tree pruning (25 kg per olive tree) and can be found in high amounts in the olive oil industry (5-10% of the total harvested weight) [19].

The chemical composition of olive leaves and twigs also depends on many factors such as cultivar, pruning period, tree age, etc. They have highly variable dry matter content (46-96%). Neutral detergent fiber (*i.e.* lignin, hemicellulose and cellulose) accounts for 33-56%, crude protein for 6-13% and fat for 4-11% of the total dry weight [20]. Phenolic and triterpenic compounds can reach 6.5% and 4.6%, respectively [20,21].

1.3. *Olea europaea* L. bioactive compounds

Bioactive compounds are essential and non-essential compounds (*e.g.* vitamins and different classes of phytochemicals) that typically occur in small quantities in foods and provide health benefits beyond the basic nutritional value of the product [22]. In spite of its simple definition, the term 'bioactive compound' is not free of criticism. On the one hand, as those compounds are mainly present in vegetables, it is not always clear if some effects are caused by an isolated compound or, otherwise, the responsible of the bioactivity is the synergistic action of several compounds from the vegetal matrix. On the other hand, although mechanisms of action can be established *in vitro*, the efficacy of bioactive compounds on human health must also be demonstrated *in vivo*. Moreover, their bioavailability, dose–effect levels and toxicity are sometimes under-evaluated [22,23].

Regardless of the controversial terminology, the focus of this section will be on the four main chemical families from *Olea europaea* L. that are considered as bioactive compounds: phenolic compounds, tocopherols, pentacyclic triterpenes and phytosterols.

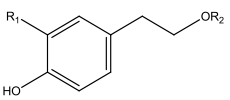
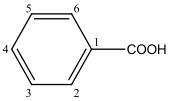
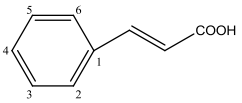
1.3.1. Phenolic compounds

Phenolic compounds are one of the most numerous and ubiquitous groups of phytochemicals in the plant kingdom. Among other functions, they act as protective agents against UV light, take part in growth and reproduction and are components of pigments, essences and flavors. They are secondary metabolites synthesized by plants via two main pathways: the shikimate and the acetate pathways. These substances can have diverse structures and functional groups, but all of them possess, at least, an aromatic ring with one or more hydroxyl substituents (including ester and glycosidic derivatives). Many of them are conjugated to sugar molecules (mainly glucose), and occasionally, they are linked to carboxylic acids, amines, lipids and other phenols [24,25].

Phenolic compounds can be classified according to the number of aromatic rings and the type and number of substituents. As listed in Table 3, the following subgroups have been found in olive tree derived matrices: simple phenols, phenolic acids, coumarins, flavonoids, lignans and secoiridoids [26,27]. Most of them can be found as glycosylated and aglyconic forms.

Simple phenols are formed with an aromatic ring substituted by an alcohol in one or more positions. *Phenolic acids* have a similar structure, with the alcohol moiety replaced by a carboxyl substituent. *Coumarins* belong to the benzopyrones group, which consist of a benzene ring joined to a pyrone. *Flavonoids* have a common skeleton, consisting of two benzene rings connected by a 3-carbon chain forming a closed pyran ring with one of the benzenes. *Lignans* are compounds derived from two *β-β'*-linked phenylpropanoid units. Lastly, *secoiridoids* are complex phenols characterized by the presence of elenolic acid (in its glycoside or aglycone form) [28].

Table 3. Classification of olive phenolic compounds (including some relevant examples).

Compound	Substituent (MW)	Structure
Simple phenols		
tyrosol	R_1-H, R_2-H (138)	
hydroxytyrosol	R_1-OH, R_2-H (154)	
hydroxytyrosol acetate	R_1-OH, R_2-$COCH_3$ (196)	
hydroxytyrosol glucoside	R_1-OH, R_2-glucosyl (316)	
Phenolic acids		
Benzoic acids and derivatives		
p-hydroxybenzoic acid	4-OH (138)	
gentisic acid	2,5-OH (154)	
vanillic acid	3-OCH_3, 4-OH (168)	
gallic acid	3,4,5-OH (170)	
siringic acid	3,5-OCH_3, 4-OH (198)	
Cinnamic acids and derivatives		
p-coumaric acid	4-OH (164)	
caffeic acid	3,4-OH (180)	
ferulic acid	3-OCH_3, 4-OH (194)	
sinapinic acid	3,5-OCH_3, 4-OH (224)	

Compound	Substituent (MW)	Structure
Coumarins		
aesculetin	R-H (178)	
aesculin	R-glucosyl (340)	
Flavonoids		
apigenin	R_1-H, R_2-OH, R_3-H (270)	
luteolin	R_1-H, R_2-OH, R_3-OH (286)	
quercetin	R_1-OH, R_2-OH, R_3-OH (302)	
luteolin 7-O-glucoside	R_1-H, R_2-glucosyl, R_3-OH (448)	
rutin	R_1-rutinosyl, R_2-OH, R_3-OH (610)	
Lignans		
(+)-pinoresinol	R_1-H, R_2-H (358)	
(+)-1-acetoxipinoresinol	R_1-$OCOCH_3$, R_2-H (416)	
syringaresinol	R_1-H, R_2-OCH_3 (418)	
Secoiridoids		
oleoside	R-H (390)	
secologanoside	R-H (390)	
comselogoside	R-*p*-coumaroyl (536)	
Elenolic acid linked to phenyl ethyl alcohols		
ligstroside	R_1-glucosyl, R_2-H (524)	
ligstroside aglycone	R_1-H, R_2-H (362)	
oleuropein	R_1-glucosyl, R_2-OH (540)	
oleuropein aglycone	R_1-H, R_2-OH (378)	
Dialdehydic forms of secoiridoids		
decarboxymethyl ligstroside aglycone or *oleocanthal*	R_2-H (320)	
decarboxymethyl oleuropein aglycone or *oleacein*	R_2-OH (304)	

elenolic acid (EA) *EA aldehydic form*

EA dialdehydic form

Phenolic compounds are among the most important components of virgin olive oil unsaponifiable fraction. They act as natural antioxidants that contribute to olive oil stability against auto-oxidation, increasing its shelf life. They also have a predominant role in virgin olive oil organoleptic properties (bitterness, pungency and astringency) [26]. Furthermore, over the last decades, they have attracted much attention from the scientific community because of their health-promoting effects. Numerous (*in vitro* and *in vivo*) studies have shown that certain phenolic compounds exhibit protective capacity against different illnesses, particularly those caused by oxidative stress, such as cardiovascular disease and metabolic disorders. Other benefits of phenolic compounds are related to infections, cancer, modulation of adipogenesis and autoimmune and neurodegenerative processes. This topic has been object of several reviews whose main conclusions are summarized in Fig. 4 [29–35].

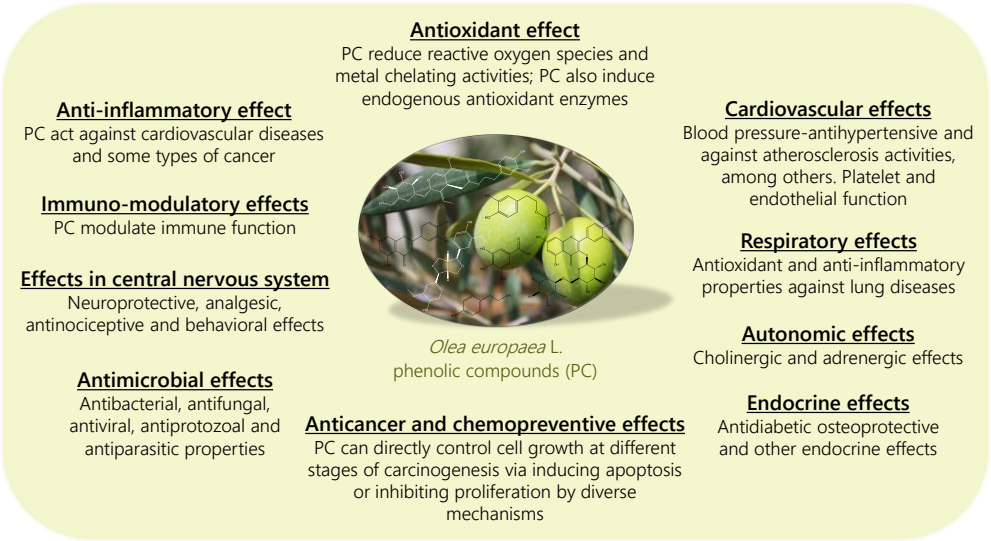


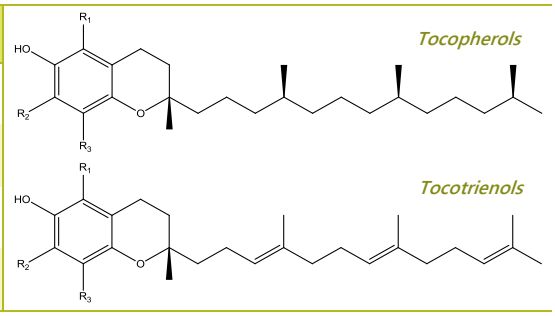
Figure 4. Health-promoting effects associated to olive phenolic compounds (adapted from [32]).

1.3.1. Tocopherols

The so-called vitamin E is a fat-soluble antioxidant that comprises eight natural occurring members (four tocopherols and four tocotrienols). They are monophenols with two main parts in their structure: a chroman complex ring and a geranylgeranyle side chain. Tocopherols have an unsaturated side chain, whilst tocotrienols have three double bonds at positions 3', 7' and 11'. Within each group, the vitamers (designated as α, β, γ and δ) differ in the number and position of the methyl groups in the chromanol ring (Table 4). All of them are known as tocols and are considered as a special and independent group of phenolic compounds [36,37].

Table 4. Chemical structure of tocopherols and tocotrienols.

Name	R_1	R_2	R_3
α	CH_3	CH_3	CH_3
β	CH_3	H	CH_3
γ	H	CH_3	CH_3
δ	H	H	CH_3



They are synthesized and stored in plant leaves and seeds, but are essential for the normal physiological functions of animal organisms and, therefore, must be obtained through the diet. Tocols are mainly found in vegetable oils, where they act as antioxidants, protecting polyunsaturated fatty acids from oxidation. As can be seen in Table 5, olive oil contains α-

tocopherol, minor amounts of *β- and γ-* tocopherols and trace levels of *δ*-tocopherol and two tocotrienols [38].

Table 5. Tocols content (mg/100g of oil) in olive oil and some of the most consumed vegetable oils worldwide (adapted from [38]).

	α-T	*β*-T	*γ*-T	*δ*-T	*α*-T3	*β*-T3	*γ*-T3	*δ*-T3
Olive oil	11.9–17.0	nd–0.27	0.89–1.34	nd–tr	nd–tr	nd	nd	nd–tr
Palm oil	6.05–42.0	nd–0.42	tr–0.02	nd–0.02	5.70–26.0	nr–0.82	11.3–36.0	3.33–8.00
Soybean oil	9.53–12.0	1.00–1.31	61.0–69.9	23.9–26.0	nd	nd	nd	nd
Sunflower oil	32.7–59.0	tr–2.40	1.40–4.50	0.27–0.50	0.11	nd	tr	tr
Peanut oil	8.86–30.4	nd–0.38	3.50–19.2	0.85–3.10	nd	nd	nd	nd

T: tocopherol; T3: tocotrienol; nd: not detected; tr: trace

Tocopherols and tocotrienols consumption has been associated with different health benefits, mainly those related to oxidative stress protection, as the prevention of certain types of cancer and cardiovascular diseases. In addition, tocols seem to participate in important biochemical mechanisms involved in diabetes and obesity [38,39]. Nevertheless, some criticism has arisen regarding the lack of *in vivo* evidence of the vitamin E effects and the disappointing or discrepant results derived from several clinical trials [37].

1.3.2. Triterpenoids

Triterpenoids are a large and structurally diverse group of natural products that arise from cyclization of squalene or related acyclic 30-carbon precursors. Most triterpenoids are 6-6-6-5 tetracycles, 6-6-6-6-5 pentacycles or 6-6-6-6-6 pentacycles. *Olea europaea* L. triterpenoids with well-characterized biological activities include pentacyclic triterpenes and phytosterols [40].

Pentacyclic triterpenes

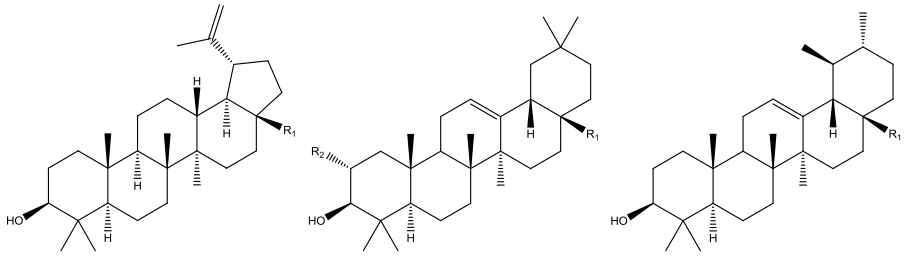
Pentacyclic triterpenes have a common skeleton of five 5- or 6-membered cycles substituted by different functional groups. They are secondary metabolites excreted by plants as protection agents. Therefore, they are found in abundance in stem bark and surface cuticular waxes of leaves and fruits [41,42]. Table 6 lists several substances (alcohols and carboxylic acids) from lupane, oleanane and ursane families, which have been isolated from olive tree derived matrices.

Oleanolic and maslinic acids are the most prevalent triterpenic compounds found in olive fruits and leaves. Concentrations higher than 2000 mg/kg of triterpenic acids have been found in natural black olives, which are only preserved in brine. In other types of table olives, the NaOH debittering treatment causes the solubilization of these substances in the alkaline solutions and leads to significant losses in the final product [43].

Pentacyclic triterpenes content in virgin olive oil depends on the oil quality and the cultivar, ranging from 10 to 120 mg/kg for the alcohols, and up to 200 mg/kg for the acids [44]. Olive pomace oils, which are obtained from the waste of the mechanical extraction of virgin olive oil, have much higher concentrations of triterpenic acids (up to 10000 mg/kg), although their content drastically decreases during the refining process [45].

Table 6. Classification of *Olea europaea* L. pentacyclic triterpenes [42].

Family	Triterpene	R_1	R_2	MW
lupane	*lupeol*	CH_3		426
	betulin	CH_2OH		442
	betulinic acid	COOH		456
oleanane	*β-amyrin*	CH_3	H	426
	erythrodiol	CH_2OH	H	442
	oleanolic acid	COOH	H	456
	maslinic acid	COOH	OH	472
ursane	*uvaol*	CH_2OH		442
	ursolic acid	COOH		456



lupane *oleanane* *ursane*

In recent years, intense pharmacological and mechanistic studies have been carried out on natural pentacyclic triterpenes and much scientific evidence demonstrating their bioactive properties, bioavailability and low toxicity can be found in literature [46–48]. Several interesting reviews have compiled all the available information regarding the bioactivity of olive tree triterpenic compounds, including anti-oxidant, anti-inflammatory, anti-diabetic, anti-atherogenic, anti-tumor, anti-proliferative, anti-viral, anti-parasitic, analgesic, anti-allodynic, hepatoprotective, neuroprotective and growth-stimulating effects, among others [49–54].

Phytosterols

Phytosterols (plant sterols) are important components of plant cells, which control membrane fluidity and permeability and take part in signal transduction events and enzymatic responses. Their skeleton consists of four rings (three 6-carbon rings in a nonlinear structure fused to one 5-carbon ring) with a flexible side chain. Most of them can occur either in free form or esterified with fatty acids and other conjugates [55].

Phytosterols are among the major components of all vegetable oils unsaponifiable fraction, including olive oil. Its sterolic profile and concentration levels vary depending on several factors (climate, agronomic practices, fruit quality, oil extraction/refining procedures, storage conditions, etc.) and can be used to detect fraud related to adulteration with other edible fats [56]. As a consequence, the European Union established limits for individual and total sterol content in olive oil as purity criteria [57].

Three classes of phytosterols, differing in the number of carbons in the alkyl chain and the position and number of double bonds, have been reported in olive derived matrices. The most prevalent ones (displayed in Fig. 5) belong to the 4-desmethylsterols group (*β*-sitosterol, Δ^5-avenasterol, stigmasterol and campesterol). In addition, 4-monomethylsterols (such as citrostadienol) and 4,4'-dimethylsterols (cycloartenol and 24-methylenecycloartanol) also occur with relative abundance in different olive tissues and olive oil [55,56].

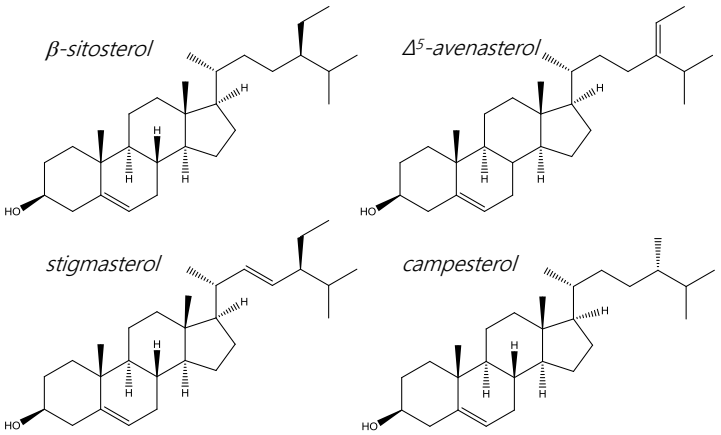


Figure 5. Main phytosterols found in olive tree-related matrices.

Dietary phytosterols play an important role in the regulation of serum cholesterol. Actually, a meta-analysis of 41 trials with various enriched food products, revealed that a dosage of 2 g/day phytosterols resulted in a 10% reduction of low-density lipoprotein cholesterol [58]. It seems that phytosterols displace cholesterol from bile-salt and phospholipid-containing 'mixed micelles', which act as vehicles to pass through intestinal cells. Thus, this competing mechanism makes it difficult for cholesterol to be absorbed into the bloodstream [59].

Additionally, phytosterols have been recognized as cancer preventive substances. Several studies have demonstrated the protective activity of these compounds (especially *β*-sitosterol) against colon, breast and prostate cancer [59].

2. OLIVE OIL

2.1. Olive oil designations and definitions

The International Olive Council, which is the only intergovernmental organization in the world to bring together olive oil producing and consuming stakeholders, defines olive oil as *'the oil obtained solely from the fruit of the olive tree (Olea europaea L.), excluding oils obtained using solvents or re-esterification processes and any mixture with oils of other kinds'* [60].

Olive oils can be classified in different olive oil grades or commercial categories according to specific quality and purity criteria established by the abovementioned entity (Fig. 6) or other regulatory bodies [61]. The quality criteria include the sensory evaluation by a panel test, as well as different physico-chemical parameters, which are considered indicators of hydrolytic modification, oxidation and freshness status of the oil (free acidity, peroxide value and UV specific extinction coefficients, among others). The limits for the main quality parameters, established by the International Olive Council for each olive oil grade, are shown in Fig. 6. The purity criteria are related to the content of diverse groups of chemical compounds such as triacylglycerols, fatty acids, sterols, triterpenic dialcohols and waxes [62].

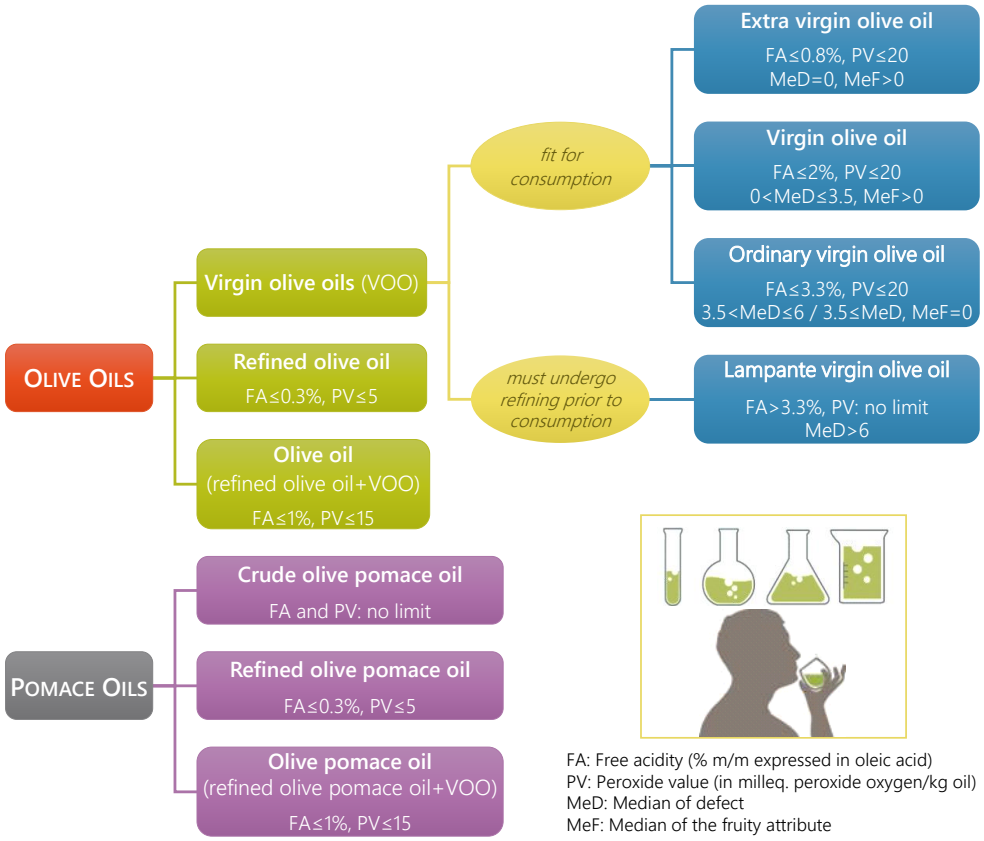


Figure 6. Classification of olive oil and olive pomace oil grades (elaborated with information from [62]).

2.2. Olive oil composition

Virgin olive oil is one of the most appreciated edible oils worldwide, due to its nutritional and gastronomic values. Like the rest of (vegetable and animal) dietary fats, olive oil has a high caloric content (9 kcal/g). However, it is considered a healthy fat because of its fatty acids composition, particularly its high content in oleic acid, and its bioactive minor compounds [63,64]. As a result, this foodstuff is considered as an accurate example of functional food [65].

Olive oil composition is influenced by numerous factors that depend both on the pedoclimatic conditions of the growing location (natural factors) and on all the personal decisions made during the entire production cycle (human factors). The cultivar, the fruits degree of ripeness when harvested, the adopted agronomic practices and the technological extraction processes can be pointed out among the most influential factors that determine the biosynthesis, transformation and degradation of the oil components [66–68].

In general, olive oil constituents can be divided into two main groups, major components (also known as saponifiable fraction) and minor components (unsaponifiable fraction) (See Fig. 7).

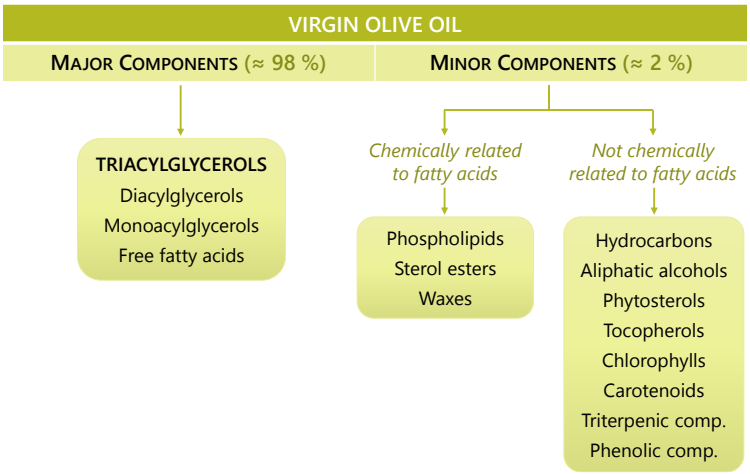


Figure 7. Scheme wich describes the chemical composition of virgin olive oil.

The major components stands for about 98% of the total olive oil weight. This fraction mainly consists of triglycerides and smaller amounts of diglycerides, monoglycerides and free fatty acids. The distribution of fatty acids in olive oil triglycerides (8-14% of saturated fatty acids, 65-83% of monounsaturated fatty acids and 6-16.5% of polyunsaturated fatty acids) is one of its most distinctive features when compared to other vegetable oils [69,70].

The fraction of minor components account for up to 2% of the total olive oil weight. The compounds belonging to this fraction can be grouped into two main categories: those derived from fatty acids (phospholipids, sterol esters and waxes) and those that are not chemically related to fatty acids (hydrocarbons, aliphatic alcohols, free sterols, tocopherols, chlorophylls, carotenoids,

triterpenic and phenolic compounds). Several members of the latter category (already described in section 1.3) are responsible for olive oil oxidative stability, as well as for its organoleptic characteristics and most of their health properties. Some of them are drastically reduced during refining processes, so only virgin olive oils, which, as stated, are obtained solely by mechanical means, keep intact this fraction [69,71].

2.3. Olive oil production

In essence, olive oil extraction consists in breaking the pulp cells to allow the release of the oil from the vacuoles and separating the oily phase from the other fruit components (vegetation water and solids). At industrial scale, the quality of the final product depends on all the required steps from the olive grove to the bottle, which can be grouped into the following stages:

- preliminary operations (olives harvesting, transport, intake, weighing, cleaning and washing),
- fruit processing (crushing, malaxation and pressing or centrifugation),
- storage in cellars and packing.

In order to obtain a high quality olive oil, healthy olive fruits must be harvested at the onset of ripening (veraison), directly from the olive tree better than from the ground, and promptly carried to the olive mill. The olives must be processed as quickly as possible, normally within 24 hours of picking, in order to keep down oxidation and free acidity. In addition, if the olives are stocked for a long time, mold and fermentation products are generated, which can transmit unpleasant flavors to the olive oil [72–75].

Olive fruit processing includes various steps: (i) paste preparation consisting in fruit milling or crushing; (ii) breakage of the oil/water emulsions through malaxation (step in which complex physical and biochemical phenomena with great effects on the extraction yield and on the oil quality occur [76,77]); and (iii) separation of the oil from the vegetation water and the solid phase (organic semisolid components and woody stone fragments).

To carry out the latter step, two main systems are currently used: the traditional system based on mechanical pressing of the olive paste, and the continuous systems in which the separation is performed by centrifugation (using two- or three-phase decanters). Continuous systems differ in the water requirements; in the three-phase mode, water is added to the paste to facilitate the extraction process, whereas in the two-phase decanter, paste centrifugation is directly carried out without adding any water. The obtained oil fraction in the three-phase decanters still contains water droplets in emulsion and insoluble solid impurities, so a further centrifugation step (rotating at high speed in a vertical centrifuge) is required. This last step is not mandatory in the two-phase system, although the separated oil is usually washed with water in a vertical centrifuge too [74,75].

The diagram in Fig. 8 includes the operations involved in each processing system, the main generated by-products and the mass balance.

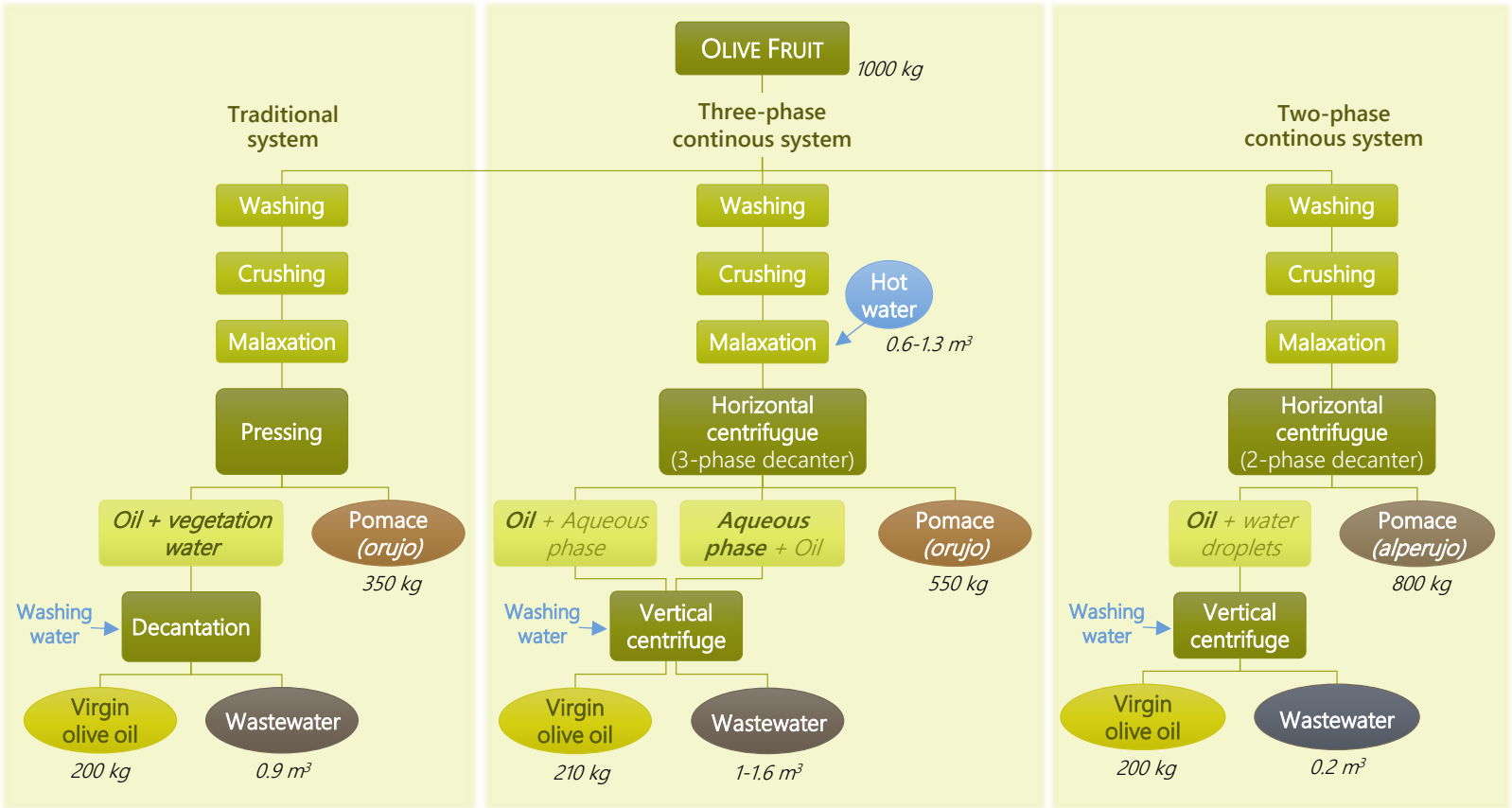


Figure 8. Three main processing systems for olive oil production (adapted from [18,78]).

Last but not least, to control the post-production stage is essential in order to avoid olive oil oxidation by exposition to air, heat, light and metals, and to preserve its organoleptic properties and nutritional value. Virgin olive oil must be stored as soon as it is produced in the mill in containers made of inert material, protected from light and at controlled temperature (13–18 °C). Moreover, if the container is not completely full, the air should be replaced with an inert gas such as nitrogen [72–75].

2.4. Olive oil by-products

The olive oil extraction industry generates large amounts of olive mill wastes, which represent a major environmental issue, particularly in Mediterranean areas, due to their highly toxic organic loads and low pH. As depicted in Fig. 8, the amount and type of generated wastes depend on the olive oil extraction system and can be classified as follows [79,80]:

Mill wastewaters

Liquid effluents from olive mills contain fruits vegetation water together with variable amounts of water added during the oil extraction process. The generated wastewaters will be substantially different depending on the employed system.

The three-phase system wastewater (Fig. 9a), so-called *alpechín* in Spanish, is a dark odorous matrix made up of water (83–94%), inorganic substances (0.4–2.5 %), lipids (0.03–1.1%) and organic matter (4–18%) including carbohydrates, pectins, mucilage, lignin and phenolic compounds. This liquid by-product is characterized by a pH of 3–6 and high chemical oxygen demand (COD: 45–130 g/L) and biochemical oxygen demand (BOD: 35–100 g/L), which are responsible for its phytotoxic and antibacterial effects. Therefore, it is considered as one of the most polluting effluents produced by the agro-food industries [81,82].

In the two-phase system, the liquid effluents mainly come from the washing water added in the last centrifugation step (vertical centrifuge). The toxicity and volume of the produced wastewater are considerably reduced because the major part of fruit vegetation water (and organic substances) remains in the semisolid waste.

Since 1981, the Spanish government prohibits the discharge of untreated olive mill wastewaters into rivers. In our country, they are usually stored in evaporation ponds, but they still generate a serious environmental impact in the areas close to their location, due to bad odors, proliferation of insects, spills and leaks [79,82].

Olive pomace

In the same way as for liquid effluents, the three and two-extraction systems generate different kind of solid by-products, also known as olive pomace or olive cake.

In the three-phase system, solid olive pomace (*orujo*) with 40-45% of water is generated (Fig. 9b). It is made of pulp, skin, seeds and stone fragments and contains 5-8% of oil, which can be extracted with chemical solvents such as hexane and perchlorethylene. The oil obtained in this way is called 'crude pomace oil' and has to be refined to fit for human consumption [80].

The semisolid pomace (*alperujo*) obtained through the continuous two-phase system (Fig. 9c) has a moisture content of 65-75%, since it includes the vegetation water together with the solid parts of the drupes. As a consequence, it is more polluting and difficult to manage by means of the traditional methods applied to the pomace from the three-phase system [78,80]. It also has higher oil content than orujo, so it is usually subjected to a second centrifugation. The resulting oil (*repaso* oil) has physicochemical parameters similar to pomace oil despite the fact that it is mechanically produced [83].

Figure 9. (a) Mill wastewater (*alpechín*); (b) olive pomace (*orujo*); (c) wet olive pomace (*alperujo*)*

Other by-products

Olive stones may be considered a single by-product if they are recovered from fruit paste either before or after oil extraction [84]. Besides, olive leaves are also residues from the olive oil agro-industry; as mentioned in section 1.2.2, up to 10% of the harvested weight can be recovered during the preliminary operations (cleaning step) [85].

2.4.1. Olive oil by-products management

Olive oil by-products management is a challenging issue for the olive oil industry from both economic and environmental perspectives. Over the last years, the interest in looking for sustainable strategies, aiming not only to reduce the amounts of generated wastes, but also to recycle and valorize them, is rapidly growing in an attempt to comply with the environmental regulations and to increase the profitability of the olive sector.

To carry out an exhaustive revision of all the proposed management and valorization strategies is perhaps beyond the scope of this introductory section. For that reason, just some compelling reviews are cited in order to give a general overview of this topic.

* Source: (a) http://olearum.com; (b) http://www.esenciadeolivo.es; (c) http://www.torredonjimeno.net

The most widespread current practices include the use of solid wastes as renewable energy sources (biomass) [86], livestock feed [87] or fertilizers (after composting) [88]. Some special uses have been reported for the recovered olive stones (production of activated carbon, furfural, metal bio-sorbents and plastic filling, among others) [16]. In addition, the seed contained inside unbroken stones has been pointed out as a valuable source of proteins and bioactive peptides [89].

Recently, olive by-products are gaining renewed global recognition as sources of high-added value products such as phenolic and triterpenic compounds, squalene, tocopherols, pectins and oligosaccharides, manitol, etc. [81,90–92]. Some of them have been suggested as promising ingredients in functional foods and beverages, as well as in the cosmetic industry [93–95] .

Other production systems encompassing the total use of by-products during olive oil extraction [96] or strategies aiming the reduction of the generated wastes [97] have also been reported. In this context, *Elayo Group* (a company based in Castillo de Locubín, Jaén) has proposed an integral approach involving the deconstruction of the olive drupe in its different fractions (pulp, stone and seed). In this way, each fruit part can be further processed to obtain new products by using technologies other than those commonly found in the olive oil industry, minimizing the amount of generated wastes at the same time. This company, owns several patents dealing with novel olive fruit processing methods and their resulting products [98–102], which are summarized in Fig. 10.

The procedure began with the fruit stoning (by means of a pitting machine from the table olive industry) to obtain the intact pulp on the one hand and the entire stone in the other. Then, the moisture of the stoned pulp is removed by dehydration. The obtained dried pulp is cold pressed to obtain a new type of olive oil and dehydrated and defatted pulp, which is grinded afterwards to obtain 'olive flour'. The stones are cleaned, dried and cracked, and the wooden shells are sorted from the seeds in a continuous process line. The seeds can be directly consumed or they can be pressed to obtain olive seed oil and olive seed flour. All of these new products contain great amounts of olive bioactive compounds and thus, they can be considered as promising ingredients for nutraceutical, dietary supplements, fortified foods, etc. or cosmetics. The single generated by-product is the wooden part of the stones, which can be used as biomass for the heating devices used in the process or as raw material to make an exceptionally robust and durable chipboard, among other uses.

Some of the products obtained by means of the just described comprehensive approach have been subject of study in several chapters of this Doctoral Thesis as it will be presented in coming sections of the current report.

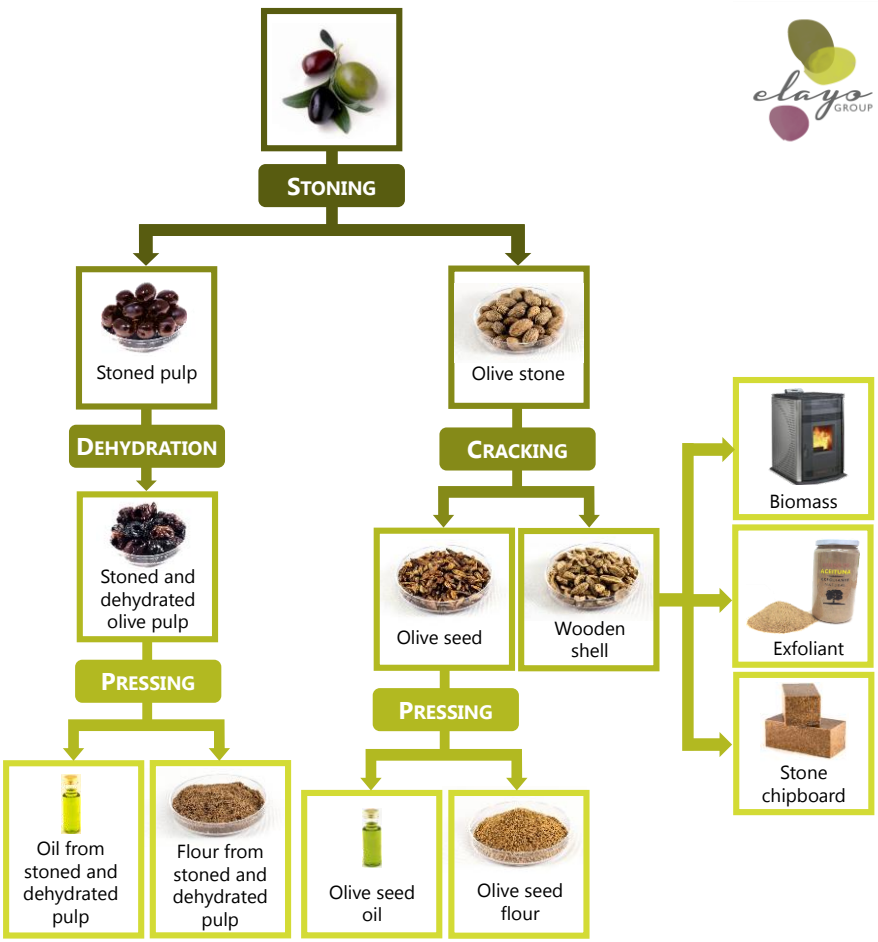


Figure 10. Flowchart of the olive fruit deconstruction approach proposed by *Elayo Group*. It includes all the obtainable products and the main required processing steps.

3. METABOLOMICS

3.1. Terminology, application fields and workflow in metabolomic studies

In the late 20th century, changes in the scientific technologies used in Molecular Biology and Biochemistry led to the development of a series of approaches known as *omics*, aiming the comprehensive characterization of an organism, tissue or cell type [103].

Since the genetic information contained in the DNA –and transcribed through the RNA– determines the synthesis of proteins that regulate the metabolites present in a specific biological system, it could be considered that the metabolome is the final downstream product of the genome (together with exogenous sources). Thus, Metabolomics emerged as a complement to genomics, transcriptomics and proteomics, contributing to the understanding of the complex molecular interactions that take place in living systems [104–106] (Fig. 11).

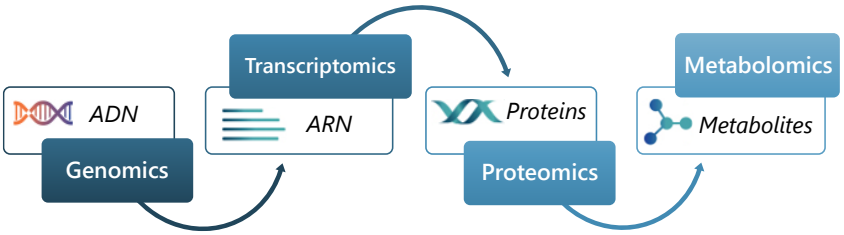


Figure 11. Information flow in biology.

According to Fiehn, one of the first authors who tried to establish its formal definition, Metabolomics is the quantitative and qualitative measurement of the whole low molecular weight metabolites synthesized for a given biological system (organisms, tissues, cells or cell compartments) in a specific biological state [107]. The word Metabonomics is often used interchangeably, though its definition is slightly different; it is the study of how the metabolic profile of a complex biological system changes in response to stresses like disease, toxic exposure, or dietary change [108].

There are different metabolomic approaches that can be classified according to the number of determined metabolites and the data quality, as depicted in Fig. 12 [105,107]:

- **Metabolite targeted analysis** refers to the detection and accurate quantification of a single or small group of metabolites.
- **Metabolic profiling** focus at the quantitative analysis of a related group of metabolites belonging to a class of compounds or which are involved in specific metabolic pathways.
- **Metabolic fingerprinting** involves rapid analysis for complete metabolome comparison without knowledge of compound identification.
- **Metabolomics** is the unbiased overview of whole-biological system metabolic patterns.

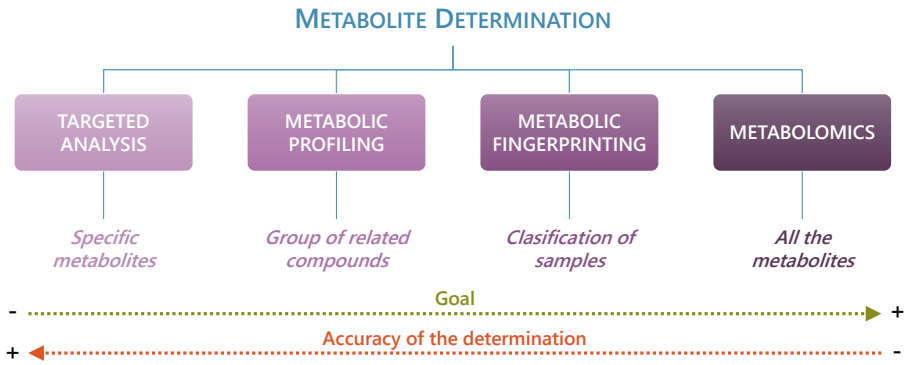


Figure 12. Most used approaches in metabolomic studies.

Metabolomics is a complex transdisciplinary science, encompassing different fields such as organic and analytical chemistry, chemometrics informatics and biosciences. Despite the great technical developments that have taken place during the last years in all of these areas, scientist still have to face some important limitations in metabolomic studies. While genomics, transcriptomics and proteomics study molecules of great structural analogy, Metabolomics' object of study stands out for its complexity and heterogeneity. Firstly, the metabolome of any biological sample includes a broad range of endogenous and exogenous chemical compounds presenting an enormous chemical diversity (peptides, aminoacids, lipids, organic acids, vitamins, phenolic compounds, alkaloids, minerals, carbohydrates, etc.). Thus, the diversity regarding molecular mass, polarity, acid-base properties or volatility, makes necessary the use of multiple complementary tools in an attempt to assure the whole analytical coverage of all the metabolites. Secondly, metabolites can be found in a wide range of concentrations, which hinders the simultaneous quantification of all of them from a technical point of view. Thirdly, a large percentage of the thousands of metabolite signals, which can be detected in a single sample, remains structurally unknown. Therefore, Metabolomics has two great challenges: the development of methodologies capable of determining as many metabolites as possible in a single analysis, and the improvement of chemoinformatic tools for structure elucidation and compound identification [109–112].

3.1.1. Application fields

In spite of its limitations, Metabolomics has demonstrated great applicability in different fields including, but not limited to, cell biology, environmental pollution, plant science, animal health, biomedicine, pharmacology and food chemistry. Since its inception, some of the most relevant applications of this *omics* science have been toxicity assessment, medical diagnosis, biomarkers discovery and drug development [105,113,114]. However, in the last years Metabolomics has emerged as an interesting tool in plant physiology (*e.g.* to identify biomarkers related to fruit growth, ripening or storage) [115] and in food sciences and nutrition research (*e.g.* to achieve a more detailed knowledge about the composition of a foodstuff and the effects of its consumption) [116].

Plant Metabolomics goes beyond the simple identification of plant constituents. It can be used to investigate plant responses to perturbations including environmental changes, physical, abiotic or nutritional stress, mutation and transgenic events. Metabolomics offers new opportunities for the discovery of plant bioactive compounds and the understanding of the biosynthetic mechanisms behind their occurrence, what is a crucial step towards the development of synthetic homologous for the pharmaceutical industry or biocides for use in agriculture. In addition, the metabolic characterization of different parts of the plant –both the tree and fruits (skin, pulp, seed)– is essential for their use in novel plant-based industrial products (polymers, fibers, fuels, biomass and foodstuffs) [117–119].

Plant and Food Metabolomics are closely related since fruit, vegetables and other plant-based foods are consumed as part of the human diet. Over the last years, experts on Nutrition and Food Science and Technology have used metabolomic tools to analyze the relationship between food function and their components, as well as to evaluate food quality and safety. In this context, a new concept has been defined: foodomics, a *"new discipline that studies the food and nutrition domains through the application of advanced omics technologies to improve consumer's well-being, health and confidence"* [116].

Some of the most relevant applications of Food Metabolomics are listed in Fig. 13. They can be grouped in three main areas of study: (i) food composition, (ii) food quality and safety and (iii) nutrition and health [120].

Figure 13. Some of the most relevant application areas in the field of Food Metabolomics.

- ***Food composition***

Food analysis has been traditionally based on the determination of the main macronutrients (carbohydrates, proteins and fats) and micronutrients (vitamins and minerals). Nowadays, the modern metabolomic technologies allow scientists to get a deeper insight into food composition, obtaining detailed information of thousands of metabolites that are responsible for specific aspects such as nutritional value, appearance, flavor, etc. [120,121]. Apart from improving the knowledge about what we eat, Metabolomics also represents a valuable tool for the food industry. For example, the study of the influence of different processing or storage conditions on food composition can greatly support the design and selection of the most favorable preparation and packaging conditions of new food-products [122].

- ***Food quality and safety***

Food quality is a complex parameter that greatly influences consumers' acceptance and involves different factors such as organoleptic properties, freshness and content of several constituents (antioxidants, bioactive compounds, etc.). Its assessment is a difficult task in which metabolomic approaches have proved to be very useful. In addition, some metabolomic strategies are of great help in the evaluation of food traceability, in food authentication and in the detection of adulteration. Regarding food safety, Metabolomics allows the detection of allergens, as well as exogenous contaminants, pathogens and toxins [116,120,122–125].

- ***Nutrition and health***

Modern food-nutrition research aims not only to feed human population, but also to improve individuals' health and prevent disease through diet. Different metabolomic strategies are being used to monitor specific food intake and dietary patterns in humans and to discover dietary biomarkers in food intervention studies [120,121,126,127].

Metabolomics is also assisting scientists to look for bioactive food components that potentially increase life expectancy, reduce weight, enhance physical or mental performance and help to treat diseases related to nutritional 'oversufficiencies' such as obesity, diabetes, chronic inflammation or cardiovascular diseases. Moreover, bioavailability assays or *in vitro* and *in vivo* studies to corroborate the potential effects of these molecules are being carried out by means of the application of different metabolomic tools [128,129].

3.1.2. ***Workflow in metabolomic studies***

Regardless of the application field and the used approach, the typical Metabolomics workflow consists of a sequence of steps that should be carefully designed and executed to provide robust scientific conclusions (Fig. 14). In general, the required stages are: (i) experimental design, (ii) sample preparation and analysis, (iii) data treatment and (iv) biological interpretation of the data [130].

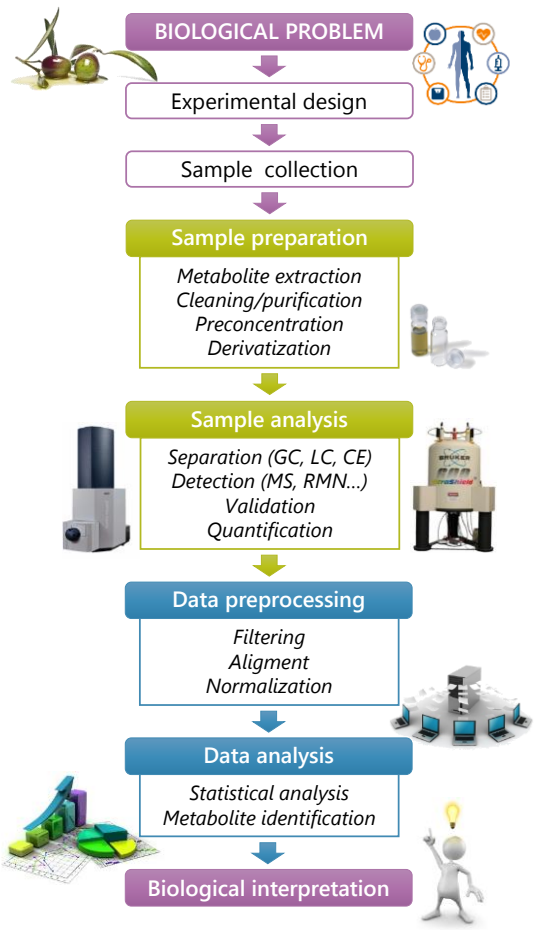


Figure 14. General workflow including all the required stages in any metabolomic study.

Once the biological problem to be addressed has been clearly formulated, the first crucial step is to select the metabolomic approach, samples, analytical platforms and experimental conditions, which will allow the researcher to obtain high-quality information to answer the initial question. The experimental design aims to plan experiments in the most efficient way to obtain data that describe the variability associated with the investigated parameters, and to guarantee the reproducibility of the metabolomic analysis [131].

The next stage involves the collection of representative samples, as well as their appropriate handling and storage. Most of the times, sample treatment is required in order to eliminate interferents that can complicate the subsequent analysis and to achieve a homogeneous liquid phase compatible with the analytical platform to be used. While in untargeted Metabolomics, sample preparation is usually minimal, targeted analysis requires the selective extraction of the metabolites of interest. This stage may comprise processes of cleaning, purification, pre-concentration and derivatization of the extracts [132].

In order to obtain valid and reliable datasets, the suitability of the selected analytical platform should be assessed prior to the analysis of the samples. Method validation procedures for targeted analyses are widely standardized. In contrast, quality assurance in untargeted approaches is particularly challenging. Some of the proposed validation strategies involve the use of interspersed blanks to reveal problems due to impurities in the solvents or contamination of the separations system (if there is any), standard mixtures to assess instrumental accuracy and precision, and quality control samples -analyzed at regular intervals throughout the analytical sequence- to evaluate system stability and general performance of the method during its operation [133,134].

As far as the analytical techniques are concerned, the most commonly employed ones in Food Metabolomics are nuclear magnetic resonance (NMR) and hyphenated techniques combining liquid (LC) or gas (GC) chromatography with mass spectrometry (MS) as detection system [135,136]. Table 7 lists the major advantages and disadvantages of the three of them. The selection of the most suitable technique usually is a compromise solution between speed, selectivity and sensitivity. Moreover, the use of several complementary analytical platforms is sometimes needed to improve metabolite coverage and identification power [137].

Table 7. Comparison of the analytical techniques most widely used in Food Metabolomics (adapted from [121].

Technology	Advantages	Disadvantages
NMR Spectroscopy	Non-destructive Fast (2-3 min/sample) Requires no derivatization Requires no separation Detects all organic classes Allows ID of novel chemicals Large databases for metabolite ID Compatible with liquids and solids	Not very sensitive Requires larger sample size Cannot detect or ID salts and inorganic ions Cannot detect non-protonated compounds Expensive instrumentation Large instrument footprint
GC-Mass Spectrometry	Good sensitivity Modest sample size requirement Excellent separation reproducibility Detects most organic and some inorganic molecules Large databases for metabolite ID Relatively inexpensive	Sample not recoverable May require sample derivatization Requires separation Slow (at least 20-30 min/sample) Novel compound ID is very intricate
LC-Mass Spectrometry	Superb sensitivity Minimal sample size requirement Can be applied without separation (direct injection) Very flexible and versatile technology Detects most organic and some inorganic molecules Potential for detecting larger portion of metabolome (*vs.* the other two)	Sample not recoverable Poorer separation resolution and reproducibility (*vs.* GC) Limited databases for metabolite ID Novel compound ID is difficult Less robust than NMR or GC-MS Expensive instrumentation

The final stage of the Metabolomics workflow is the data treatment, which comprises data pre-processing and data analysis. In this phase, bioinformatics and chemometrics are used to convert

the complex raw data provided by advanced analytical instruments into useful information [138]. The use of multivariate analysis is sometimes required to handle the huge amount of generated data, since it reduces the dimensionality without losing information. Unsupervised methods (*e.g.* principal components analysis (PCA)) give an overview of the dataset and try to identify general trends -without any prior knowledge- by grouping samples that show certain similarities. On the contrary, in supervised methods (*e.g.* partial least squares discriminant analysis (PLS-DA)) groups are known *a priori* and are used to build classification models that, at a later stage, will allow the allocation of new and unknown samples to the most probable class [139]. The latter are very useful to point out markers that are significantly different between sample groups.

Lastly, in untargeted metabolomics, metabolite identification may also be required for data interpretation. When using LC-MS and GC-MS methods, due to the difficulty of the task, only the most relevant compounds (markers) are normally identified. Recently, databases containing MS/MS information and new software with sophisticated annotation tools are being developed. Nevertheless, structure elucidation is still considered a major bottleneck in this kind of studies [112,140].

3.2. Metabolomics applied to the study of *Olea europaea* L. derived matrices

Considering the large amount of examples of Metabolomics applications in the study of olive tree related matrices and their effects on health, this section does no aim to carry out an exhaustive revision of all the published reports, but just to give a general overview of the topic. In general terms, the existing applications can be assorted into three major areas of study (Fig. 15).

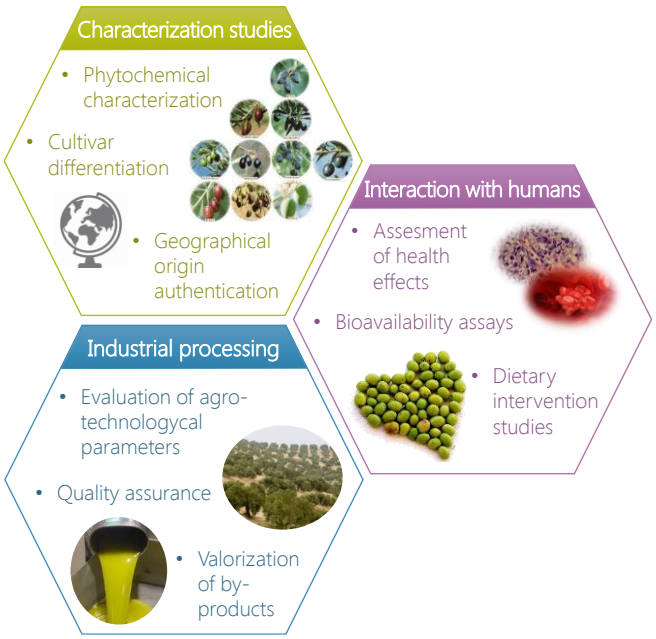


Figure 15. Main applications of Metabolomics in the study of olive tree related samples.

Starting with the characterization studies, during the last decade, olive vegetal tissues (olive fruits, leaves, barks, stems and roots) [141–145], olive oil [146–148] and different olive by-products [149–151] have been object of study of targeted and profiling approaches, mainly focused on their secondary metabolites. The influence of the olive cultivar and the degree of ripening on different families of metabolites such as sterols, phenolic and triterpenic compounds has been also evaluated [44,152–155]. Moreover, the study of olive oil volatile and phenolic profiles have also proved to be a useful tool for geographical origin authentication [156–158].

Regarding olive fruit processing, different metabolomic tools have been used to find the best agronomical practices [159,160] and technological parameters [161–163] to obtain high quality olive oils. In addition, several processing conditions affecting table olives metabolites have been also assessed through targeted approaches [164–167]. Besides, untargeted MS-based metabolomic approaches have been employed to appraise olive oil quality [168–170] and traceability [171]. Metabolomics is also assisting the development of new valorization strategies for olive by-products [172,173].

The search of specific metabolites from olive tree derived matrices, which could prevent or treat human diseases, is one of the most reported applications of Metabolomics in this field. As stated in previous sections, different olive phytochemicals found in olive oil, leaves or olive by-products can be considered as clear examples of potential bioactive compounds. Their occurrence, as well as the study of their beneficial effects both *in vitro* [129,174–177] and *in vivo* [178–180], have been widely investigated by means of different metabolomic approaches. Furthermore, the modern metabolomic tools offer the possibility to monitor olive-based foods intake in dietary intervention studies [181,182] and to assess the bioavailability of the metabolites of interest [183,184], which could support the approval of health claims by regulatory bodies [128].

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Experimental Part, Results and Discussion

In the coming block, each chapter will be presented using the sections distribution, general formatting, abbreviations, units, etc. of the journal in which the different studies have been published or, hopefully, are going to be published. In all the chapters, the references are independent and have been added by using a number between brackets.

SECTION I

"Single-class" Methodologies

This section includes all the chapters involving "single-class" methodologies. The first three chapters are focused on the development and validation of methods for the targeted determination of triterpenic (**Chapters 1** and **2**) and phenolic compounds (**Chapters 3**); **Chapter 4** presents a discussion on different analytical approaches for the measurement of the olive oil phenolic content; and **Chapters 5** and **6** comprise two examples of applications of LC-MS methods for virgin olive oil and olive leaf phenols profiling, respectively.

Chapter 1

Evaluating the potential of LC coupled to three alternative detection systems (ESI-IT, APCI-TOF and DAD) for the targeted determination of triterpenic acids and dialcohols in olive tissues

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Abstract: Herewith the development of a rapid and powerful LC methodology (with three different detectors) is presented to determine triterpenic acids and dialcohols in extracts from *Olea europaea* tissues (olive skin, pulp and leaves). After the proper optimization of the LC, DAD and MS conditions (and the comprehensive characterization of the behaviour of each analyte in ESI and APCI (with accurate *m/z* signals and, in ESI, with MS/MS data too), the method was fully validated. DAD, ESI-IT MS and APCI-QTOF MS were used as detection systems to give different alternatives to carry out the accurate determination of these analytes, evaluate their analytical performance, advantages and drawbacks, and check whether the quantitative results achieved by the three platforms were in good agreement. ESI-IT MS gave the lowest detection limits (3-455 µg/L) followed by APCI-QTOF MS (22-408 µg/L); in contrast, DAD (83-600 µg/L) had the widest dynamic range. The %RSD values for *inter*-day repeatability were found below 11.82% in all the cases. No statistically significant differences were found among the quantitative results from the three detectors. Olive leaves showed the highest concentration levels of ursolic acid (1.8 mg/g), erythrodiol (1.6 mg/g) and uvaol (1.2 mg/g), whereas the olive skin was the richest matrix in terms of maslinic (80 mg/g), betulinic (0.20 mg/g), and oleanolic (26 mg/g) acids. Concentration values of triterpenic acids were established by first time for skinless olive pulp, and were found around 65, 1.2, 55 and 4.4 µg/g for maslinic, betulinic, oleanolic and ursolic acids, respectively.

Keywords: pentacyclic triterpenes; olive tissues; liquid chromatography; mass spectrometry; food metabolomics.

1. INTRODUCTION

Over the last years, a rich in fruits and vegetables diet has been associated to a lower incidence of diseases related to chronic damage and growth dysregulation, such as obesity, diabetes, cancer or cardiovascular disease. This is largely due to the phytochemicals found in food plants at different concentration levels [1]. Some of these compounds are triterpenoids, an important group of natural products with numerous biological effects, which are being used as ingredients in dietary supplements, medicines and healthcare products [2,3]. In particular, pentacyclic triterpenes have been identified as the main components of medicine plants [4], and have shown, among others, analgesic, hepatoprotective, anti-tumor, anti-diabetic, anti-inflammatory and antioxidant effects [5–8]. They are secondary plant metabolites which arise from cyclization of squalene, and have a common skeleton of five 5 or 6-membered cycles substituted by different functional groups [9]. Such substances are excreted by plants as protection agents, so they use to be part of the cuticular waxes that surround plant surfaces (leaves, stems, flowers and fruits) [10].

Olea europaea is a valuable source of this kind of compounds, since different triterpenic acids and alcohols have been described in olive industry-related products (olive leaves, fruits, oil and pomace) [11–13]. Bioactive properties of pentacyclic triterpenes from *Olea europaea* have been systematically reviewed by different authors [14–19], and some protocols for obtaining their pure

extracts have been patented [20–23]. Obtaining these components from olive-industry by-products could be a way to economically upgrade the sector. The use of these compounds as ingredients in new products leads to the need of developing appropriate methods for their determination in a growing variety of samples (raw materials or final products). The optimization of these methods, which can be implemented in routine laboratories to ensure the safety and quality of these new products, represents a considerable challenge.

In recent years, in parallel with the discovery of the biological effects of triterpenoids, many studies have been carried out trying to achieve the best possible determination procedure. Their extraction from vegetal tissues is the first step to be optimized in order to achieve an accurate quantification; in this regard, different strategies have been evaluated [24,25]. In the case of *Olea europaea* tissues, after water removal, analytes of interest have been extracted with ethyl acetate in a Soxhlet apparatus [26], by maceration with ethanol [27], by solid-liquid extraction with a mixture of methanol/ethanol (1:1, v/v) [28] or by microwave assisted extraction with ethanol/water (80:20 v/v) as extractant mixture [29]. Other extraction techniques, such as ultrasonic assisted extraction [30] or supercritical fluid extraction [31], have been also applied to different plants.

Triterpenoid fraction in plant matrices is quite complex, in particular because the coexistence of some structural isomers, therefore, their quantification is quite difficult, making almost mandatory the use of a separation technique before their detection. In any case, interesting examples, which do not imply previous separation, can be found. For instance, discrimination and quantification of oleanolic and ursolic acids in plant matrixes has been achieved using two-dimensional nuclear magnetic resonance spectroscopy [26]. Nevertheless, as stated before, in most of the proposed methods, their separation becomes the key to the success of the analytical process, since commonly used detectors are not capable of distinguish them. So, the analysis of prepared extracts has been commonly made with multiple separation techniques coupled to different detectors. Gas chromatography coupled to FID [27,32,33] or MS [34,35] has been extensively employed to this end. As a way of overcoming the tedious derivatization process (necessary step to increase the volatility of the triterpenoids) liquid-based methods such as capillary zone electrophoresis [36] or liquid chromatography have been also developed. As far as LC is concerned, it has been coupled to photodiode array [37], evaporative light scattering [38] or MS detectors whether for identification or quantification purposes. Fluorescence detection has been also used coupled to LC, but it requires a previous derivatization step [39–41]. In mass spectrometry, different interfaces (ESI, APCI and APPI [42]) and analyzers (IT [43], Q [28], QqQ [29], QTOF [38] and Orbitrap [44]), both in positive and negative polarities, have been employed.

In LC, a great variety of mobile phases has been used, mostly in isocratic methods, although some gradients has been also proposed for triterpenoids separation [38]. Because of the relatively low polarity of these compounds, organic solvents (methanol and/or acetonitrile) mixed with low proportions of water (usually acidified) have been commonly employed [28,43,45]; the effect of some modifiers such as cyclodextrins [46] or triethylamine [47] have been also tested. The use of

neutral [48] and basic [29,49] chromatographic conditions has been reported in few communications, even though they have not been so commonly used.

The aim of this work has been to develop and validate a rapid and powerful analytical method for the determination of pentacyclic triterpenes (maslinic, betulinic, oleanolic and ursolic acids, erythrodiol and uvaol) offering different alternatives (in terms of detection systems) to carry out their accurate determination. Three detectors were selected: DAD, for being the more likely available one in a routine analysis olive oil laboratory; and two MS detectors (one of them with ESI interface and an analyzer of low resolution but very fast switching polarities and the other with an APCI source and a high resolution analyzer), since MS is continuously growing, has a great potential and is becoming a kind of mandatory. We evaluated their analytical performance, discussed their drawbacks and advantages, and checked whether the quantitative results obtained by the three platforms were in good agreement. To achieve this purpose, different olive tissues (olive skin, pulp and leaves) were selected and their triterpenoid content assessed. To the best of our knowledge, the triterpenes levels of one of the matrices under study have been never evaluated before.

2. MATERIALS AND METHODS

2.1. Chemicals and standards

All reagents were of analytical grade and used as received. Acetonitrile and methanol of LC-MS grade from Prolabo (Paris, France), and deionised water from a Millipore Milli-Q (Bedford, MA, USA) water purification system, were used for preparing chromatographic mobile phases. Ammonium formate and ammonium hydroxide from Sigma-Aldrich (St. Louis, MO, USA) were used as buffer components in the aqueous mobile phase. This phase was vacuum filtered with a Nylaflo™ 0.45 µm nylon membrane filter from Pall Corporation (Ann Arbor, MI, USA) before entering into the chromatographic system. Ethanol from J.T. Baker (Deventer, The Netherlands) was used for the extraction of the triterpenic compounds from the selected tissue samples. Pure standards of maslinic acid (MA), betulinic acid (BA), oleanolic acid (OA), ursolic acid (UA), erythrodiol (ER), and uvaol (UV) were all supplied by Sigma-Aldrich. A methanolic stock standard solution containing 200 mg/L of each compound was first prepared by dissolving the appropriate amount of each analyte in methanol and, then, serially diluted to working concentrations. All solutions were stored at −20 ◦C. All the samples and stock solutions were filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

2.2. Samples and extraction procedure

Olive tissues samples were supplied by a local company. Olive skin (mix of varieties not specified by the supplier) was treated as received (it came dried from the olive mill). Olive pulp

(Picudo *cv.*) was obtained manually, after peeling the olive fruits and removing their stones, and then, it was frozen to be further freeze-dried. Olive leaves (Picual *cv.*) were oven-dried at 35ºC until their weight remained constant. After water removal, pulp and leaves tissues were ground to powder before the extraction of triterpenic compounds.

Compounds of interest were isolated by ultrasonic assisted extraction according to the method described by Goulas and Manganaris [24], adapted from those of Lee *et al.* [50] and Li *et al.* [51]. Briefly, 0.5 g of dried tissue and 20 mL of ethanol were put inside a falcon tube which was left in an ultrasonic bath from J.P. Selecta (Barcelona, Spain) for 30 min. The ultrasonic bath characteristics were: 6 L of capacity, dimensions of 15, 30 and 14 cm of height, width and depth of usable bath, respectively, with a generator power of 150 W, a total power capacity of 360 W and a fixed frequency within the range 50-60 Hz. Afterwards, the tube was centrifuged at 9500 rpm for 5 min. Finally, the supernatant was evaporated to dryness and redissolved in 10 mL of methanol.

In order to evaluate the recovery percentage of the extraction system, the first part of the described procedure was repeated three times for each matrix, as follows: after taking the obtained supernatant from the first step, another 20 mL ethanol were added to the solid residue, being left into the ultrasonic bath for 30 min. This was repeated once more. In this way, we could establish the percentage of the total amount of each analyte that remained into the sample after going through the first extraction stage. Thus, when quantifying the analytes of interest in the samples, a correction factor (including the recovery and the dilution factor) was obviously applied to properly calculate the final concentration values of the compounds in the analyzed tissues.

A mixture of the extracts coming from the olive skin, pulp and leaves samples under study (mixing an equivalent volume of each extract) was used as quality control sample to evaluate the repeatability of the method (apart of doing it with a mixture of pure standards). The quality control sample was injected every five analyses (after a blank) in each sequence.

2.3. LC-DAD/MS analysis

2.3.1. Apparatus

In the current study, the analyses were performed by reversed-phase LC coupled to three different detectors, using two different platforms. The first one was an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector (DAD) coupled to a Bruker Daltonic Esquire 2000™ ion trap mass spectometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization (ESI) interface. The second one was a Waters Acquity UPLC™ H–Class system (Waters, Manchester, UK) coupled to a Q-TOF SYNAPT G2 mass spectrometer (Waters) equipped with an atmospheric pressure chemical ionization (APCI) ion source. Additionally, an Acquity UPLC™ H–Class system coupled to a micrOTOF-Q II™ mass spectrometer (Bruker Daltonik) by means of an electrospray source was used only with qualitative purposes, in order to compare the behavior of the analytes in both interfaces by studying the accurate *m/z* signals produced by each ionization source (APCI and ESI).

The first platform was chosen to optimize the chromatographic separation, because it allowed the simultaneous monitoring of the eluent with DAD and ESI-IT MS detectors. Indeed, the fact that triterpenic acids were better detected in negative polarity whilst alcohols needed positive polarity, made IT MS the most appropriate and useful MS detector (of those evaluated within this study), because it could detect all the analytes within a single injection, as it can easily switch polarity during a run.

The developed method was finally applied for analyzing the selected samples by means of DAD, ESI-IT and APCI-QTOF detectors. As stated before, the detectors selection was made in such a way as to fulfill different requirements: 1) to include both cheap and accessible detectors and other more sophisticated and powerful ones; 2) to cover different MS interfaces to check their suitability for properly ionizing pentacyclic triterpenes; and obviously 3) the detectors´ availability within our facilities.

The analytical performance of the three detectors was evaluated, but the aim was not carrying out a comprehensive comparison, but giving to the reader an idea about parameters such as detection and quantification limits, calibration range, accuracy and possible matrix effect. Moreover, in order to fairly compare the two used interfaces, we should have selected the same analyzer for both platforms.

Chromatographic data acquisition and DAD peaks integration in the first system was performed by using ChemStation B.04.03 software (Agilent Technologies). Instrument control of Waters chromatographic systems was carried out using the software Acquity UPLC Console (Waters), and the data processing of the Waters spectrometer was made with the software MassLynx 4.1 (Waters). Finally, Bruker mass spectrometers were controlled using the software Esquire Control and the resulting files were treated with the software Data Analysis 4.0 (Bruker). Statistical analyses (ANOVA test) to compare the quantitative results achieved by the three tested detectors, were carried out by using STATGRAPHICS Centurion XVII.

2.3.2. Chromatographic conditions

Regardless of the detection system, the compounds under study were separated by using a Zorbax Extend C18 analytical column (4.6 x 100 mm, 1.8 µm particle size) (Agilent Technologies), which can be used in a wide pH range (2.0 to 11.5), operating at 25ºC. The mobile phases were 1.5 mM ammonium formate in water (adjusted to pH 9.6 with ammonium hydroxide) (Phase A) and acetonitrile/methanol (60:40, v/v) (Phase B). Analytes were isocratically eluted (10% Phase A and 90% Phase B) at a flow rate of 1.2 mL/min and the injection volume was 10 µL. Run time was 6 minutes with one additional post run minute before the subsequent injection.

In order to evaluate the effect of the different chromatographic conditions tested during the development of the method not only on the separation itself but also in the MS signals, the signal to noise ratio and maximum intensity (in counts) -in the IT MS detector- of the analytes under study were calculated for each tested LC condition. Very similar behavior was shown by the six

analytes, and therefore, results from betulinic acid have been selected as example to be shown in the graphics. Moreover, the number of theoretical plates (N) for this compound was calculated from the LC-ESI-IT MS data as $N = 5.54\ (t_r/w_{1/2})^2$, where both retention time (t_r) and peak width at half height ($w_{1/2}$) were expressed in minutes.

2.3.3. *Detection conditions*

Bearing in mind the previously published results and the maximum absorbance wavelengths observed in the spectra of the individual pure standards, the optimum wavelength for the determination of the triterpenic compounds in the diode-array detector was set at 210 nm.

Besides, mass spectrometric conditions were optimized for each triterpenic compound (in ESI and APCI ionization sources, respectively) by continuous infusion of standard solutions (at a concentration level of 20 mg/L approx.). In ESI-IT MS, analyses were made in negative ion mode until minute 4 (for triterpenic acids detection) and in positive polarity from 4 to 6 min (for triterpenic alcohols detection), with a scan range from 400 to 600 *m/z*, regardless of ion polarity, which enhanced the ion detection selectivity and gave higher intensities. The end plate offset voltage was set at -500 V, and the capillary voltage at +3500 V in negative polarity, and -4000 V in positive polarity. Optimum values for the ESI source parameters were: 300ºC of drying gas temperature, 9 L/min of drying gas flow and 30 psi of nebulizer pressure. These parameters were then transferred to the ESI-QTOF spectrometer. Nevertheless, two injections per sample (one for each ion mode) were needed, because polarity changes while running are not recommended in the micrOTOF-Q II that we were using, since the switching needs some time and additional calibration could be required. In APCI-QTOF MS, two injections were needed too, because the used system is unable to switch polarity in the middle of a run. In this instrument, corona, sampling cone and extraction cone voltages were -5000 V, 20 V and 5 V, respectively, in positive polarity, and +3000 V, 60 V and 5 V, in negative polarity. Regardless of the ion mode, source and probe temperatures were set at 100 and 500ºC, respectively, and 30 L/h of cone gas flow and 600 L/h of desolvation gas flow were used.

2.4. Method validation

Solutions containing pure standards of 6 triterpenoids in methanol at 8-10 different concentration levels over the range of 0.1–100 mg/L for MA and OA, and 0.1-50 mg/L for the rest of the analytes, were employed to check linearity and to establish the calibration curves which allowed their quantification in the samples. External calibration curves were established for each standard by performing a linear regression by the least-squares method. Each point of the calibration graph corresponded to the mean value from three independent injections. Detection (LOD) and quantification limits (LOQ) for each individual compound of the standard solution were calculated; thus, the signal to noise ratio of the standards at the lowest concentration level injected (for every analyte) in the three detectors was obtained, and LOD and LOQ were estimated by calculating the concentration that generate a signal to noise ratio equal to 3 and 10, respectively

[52]. Standards at concentrations below 0.1 mg/L (compound dependent) were injected to corroborate the theoretically obtained LODs and LOQs.

Method accuracy was assessed by determining precision under repeatability conditions and trueness. *Intra-day* repeatability was expressed as the relative standard deviation (%RSD) obtained for 4 injections of the quality control mix (which, as stated above, was a sample mixture of extracts of the three tissues under study), carried out within the same sequence. *Inter-day* repeatability was calculated as %RSD of 8 injections (belonging to 4 different sequences carried out over 4 days) of 8 different extracts coming from the same olive leaves sample; in such a way that the *inter-day* repeatability values could give to the reader an estimation about the overall repeatability of the method. The same strategy was followed using olive skin and pulp samples (data not shown).

Trueness was expressed as recovery, and it was estimated by analyzing a sample of each matrix extracted before and after the standard addition (using low, intermediate and high concentration levels (within the linear dynamic range) of pure standards) and calculating the difference between the results obtained.

Finally, the presence/absence of matrix effect was assessed in all the commodities under evaluation, since different samples may exhibit matrix effects of variable magnitude. Several methods have been proposed to this end, but most of them need a blank sample, which was not available in this study. Therefore, the matrix effect evaluation was made following the same strategy as Kmellár et all. [53], which consist in applying the standard addition method to the different kind of samples, and comparing the slope of the external calibration function (in solvent) and the slope of the standard addition calibration curve (which compensates for any matrix effect). A matrix effect coefficient was calculated for each compound in each matrix (leaves, skin and pulp), following the equation given by these authors:

$$\textit{Matrix effect coefficient (\%)} = (1-(\textit{slope matrix}/\textit{slope solvent}))*100$$

3. RESULTS AND DISCUSSION

3.1. Optimization of chromatographic conditions

Since the analytes under study have very similar chemical structures (being some of them mass isomers), particular attention has been paid to assure their appropriate chromatographic separation and, therefore, their accurate determination. Two pairs are particularly problematic (oleanolic and ursolic acids, and erythrodiol and uvaol), so the chromatographic method optimization has tried to achieve the maximum peak resolution between them in the shortest possible time.

As mentioned before, this LC optimization step of the study was carried out in the LC-DAD/ESI-IT MS system. A mixture of standards prepared in methanol was used. A univariate optimization was carried out, changing one of the parameters and evaluating its influence, while keeping

constant the other variables. The composition of mobile phase was, firstly, optimized. Two organic solvents with different polarities (acetonitrile and methanol) were mainly tested both individually and mixed in different proportions, under isocratic conditions. Fig. 1a shows the effect of acetonitrile, methanol and their mixtures in mobile phase B on the retention times of all the analytes, number of theoretical plates and signal to noise ratio (S/N) of betulinic acid peak. Methanol produced faster analyses, but it was a detriment to resolution. It can be observed that the higher the percentage of acetonitrile, the better the separation among the analytes; however, increasing proportions of acetonitrile also produced lower S/N values (ionization was less efficient) and a reduction of N. Acetonitrile/methanol (60:40, v/v) was pointed out as the optimum composition of Phase B.

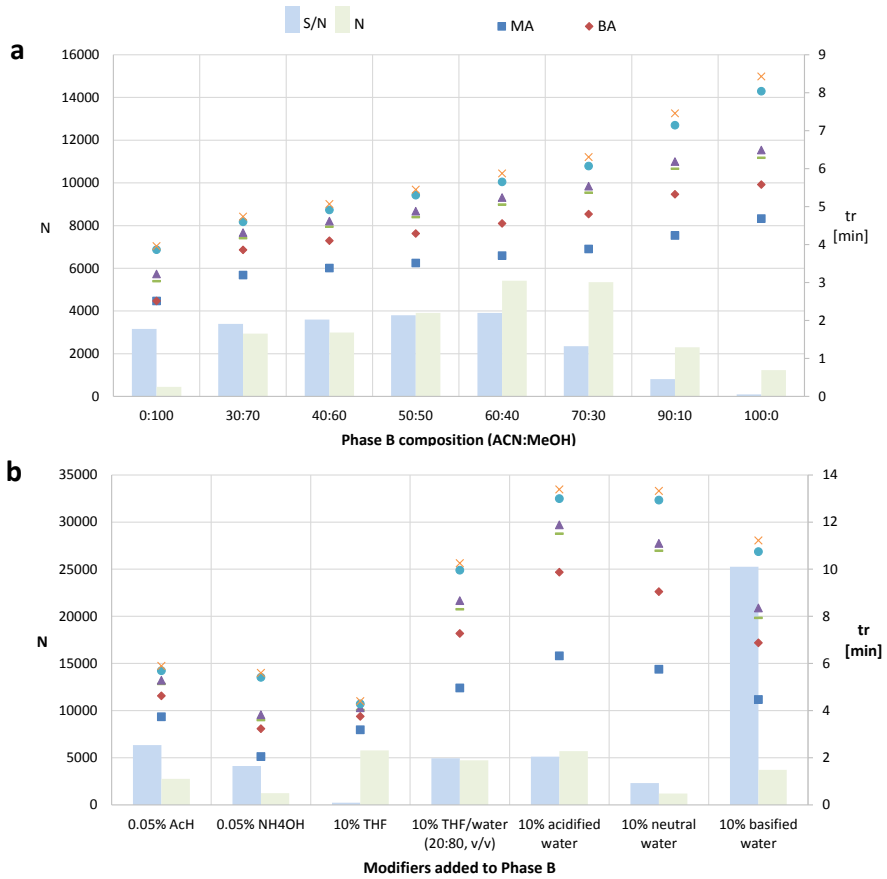

Figure 1. a) Graphical representation of the influence of composition of Phase B on: retention times of all the analytes, number of theoretical plates and signal to noise ratio of betulinic acid peak (selected as example to illustrate the influence of the tested parameters). In the Y axis, S/N ratio is normalized to the scale of N to facilitate the display of the data. b) Graphical representation of the influence of the modifiers added to Phase B (tetrahydrofuran (THF), acetic acid (AcH) and ammonium hydroxide (NH_4OH) directly added and diluted in different proportions of water) on the same variables as observed in a). *The results shown in this figure were obtained by using a C18 Zorbax Eclipse Plus column (4.6 x 150 mm, 1.8 µm).*

After that, some modifiers were added to the organic solvents with different purposes: to enhance ionization, to reduce run time and to increase the resolution between the problematic pairs. We tested tetrahydrofuran, triethylamine, acetic acid and ammonium hydroxide directly added in Phase B, and diluted in different proportions of water. Fig. 1b shows that a percentage of 10% of basified water resulted to be the best choice taking into account the variables previously mentioned. In the figure, it is possible to observe that a good separation is achieved among the compounds under study when the added modifiers were 10% of acidified water, 10% of neutral water and 10% of basified water; the resolution was also acceptable for 10% of THF/water, just observing worse separation for the critical pair ER and UV. When S/N was considered, it was clear that 10% of basified water was the most appropriate composition of Phase A (keeping reasonable N values).

Since the pH of the aqueous phase was very critical for the triterpenic acids resolution, it was necessary to carry out its comprehensive optimization. Different MS compatible buffers (ammonium bicarbonate adjusted to the desired pH with acetic acid, and ammonium formiate and ammomium acetate adjusted with ammonium hydroxide) at different concentrations levels (1 to 25 mM) were tested in a pH range between 7 and 11 and, finally, 1.5 mM ammonium formate in water adjusted at pH 9.6 with ammonium hydroxide, was found to be the most appropriate composition of Phase A. After choosing these conditions regarding mobile phase composition, the repeatability was checked carrying out consecutive injections of both standard mix and ethanolic extracts of the matrices under study. When a C18 Zorbax Eclipse Plus column (Agilent Technologies) was used, the observed repeatability was not good enough, fact which can be probably explained due to the high pH at what the column was being subjected, which was in the upper limit of the working conditions range recommended by its manufacturer. To solve this problem two analogous end-capped columns, which are indicated for separating compounds under high pH conditions, were

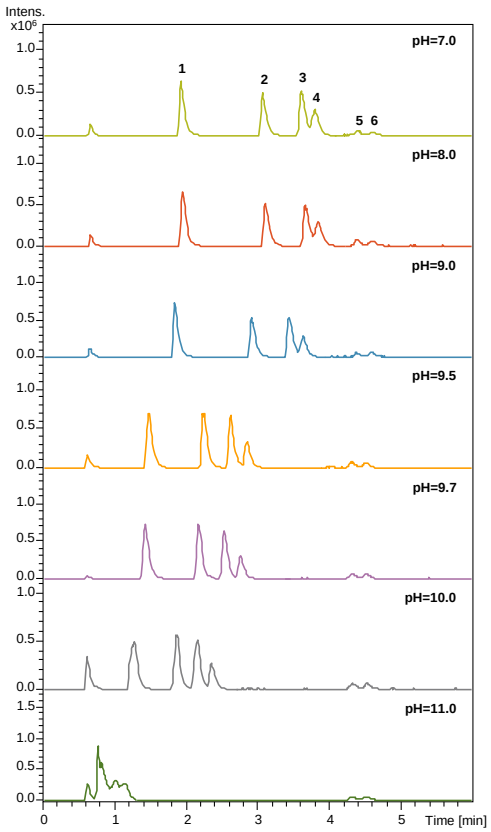

Figure 2. Base peak chromatograms obtained in ESI-IT MS (using negative ionization mode for triterpenic acids and positive polarity for alcohols) showing the influence of the pH value on the separation of the compounds under study. Peak identification numbers: 1, MA; 2, BA; 3, OA; 4, UA, 5, ER, and 6, UV. *The results shown in this figure were obtained by using the Zorbax Extend C18 analytical column (4.6 x 100 mm, 1.8 µm).*

tested: Gemini column (Phenomenex, Torrance, CA, USA) and Zorbax Extend (Agilent Technologies). The last one was finally chosen because of its high stability and retention time repeatability.

To illustrate the effect of the pH, Fig. 2 shows the chromatograms corresponding to its optimization (with the optimum column, Zorbax Extend), using ammonium formate buffer, which was the most effective enhancing the ionization of triterpenes in ESI-IT MS detector. As the pH increases, shorter analysis time and better resolution between the problematic pair (OA and UA) is achieved, but after reaching the maximum resolution at a pH range of 9.5-9.7, these triterpenic acids start to coelute at higher pH values. As can be seen in the figure, pH changes do not practically affect triterpenic alcohols elution (either in terms of resolution or retention time), fact that can be explained because their theoretical pKa is above these pH levels.

To complete the optimization of the separation method, different column temperatures and flow rates were tested. We tried temperatures between 5 and 40ºC and flow rates between 0.8 and 1.5 mL/min; eventually, a compromise solution between run time and resolution was reached at 25 ºC and 1.2 mL/min. The final optimum conditions led to an analysis time of 6 minutes; a total run time which makes our methodology shorter than others previously described where triterpenic acids and alcohols were simultaneously determined in more than 30 minutes [29,48].

Chromatograms resulting from the application of the optimized LC conditions in the three evaluated detectors are shown in Fig. 3. In all the cases, we show the profiles obtained after analyzing a standard mixture containing the following concentrations: 2 mg/L of MA, OA and UA; 1 mg/L of BA; 4 mg/L of ER and UV. These concentration levels were decided keeping in mind the ionization efficiency of each analyte in MS. In ESI-IT MS, MA and BA are better ionized than in APCI-QTOF MS. Besides, in both MS detectors, triterpenic alcohols are poorly ionized, being their relative response in DAD more similar to rest of the compounds under study.

3.2. MS signal characterization

According to Rhourri-Frih *et al.* [42], ESI interface is adequate for ionizing polar and ionic compounds, while more apolar compounds are properly ionized by APCI, so that means that, in principle, APCI would be more suitable for the ionization of pentacyclic triterpenes, especially for uvaol and erytrodiol which only possess one hydroxyl group on their structure. Giménez *et al.* [48] recently described ESI as a not recommendable ion source for detecting triterpenic alcohols (neither in positive nor in negative polarity), but their determination have been previously achieved using electrospray ionization sources by some other authors, such as, for instance, Sanchez-Avila *et al.* [29]. Thus, we tried to do a comprehensive characterization of the accurate MS signals obtained using both ESI and APCI sources. The MS signals achieved for the 6 triterpenic compounds found in *Olea europaea* (when both ionization sources were used) were study in depth in order to evaluate the differences between them. The identification of the 6 chromatographic peaks corresponding to our compounds of interest was achieved by using the information coming

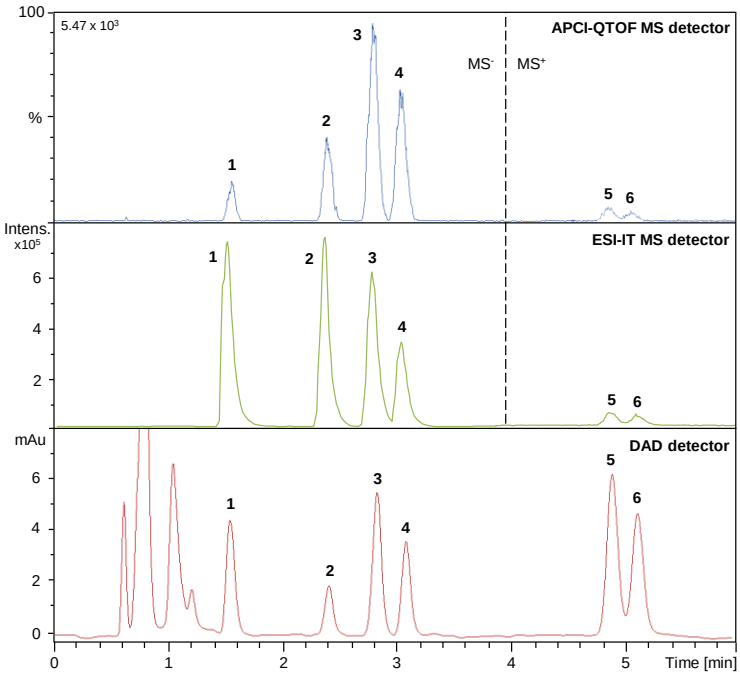

Figure 3. Separation achieved by using the optimal chromatographic and detection conditions (in MS, base peak chromatograms are shown). Two segments were used for MS detection, from the beginning of the analysis to 3.9 min in negative polarity, and from 3.9 till the end of the run, in positive mode. Peak identification numbers with the same meaning as in Fig. 2.

from the QTOF analyzers (which allowed predicting the molecular formula of analytes from their exact mass) and it was logically corroborated comparing their retention times with those of the pure standards.

Both interfaces gave the pseudo-molecular ion in positive and in negative polarity (except for triterpenic alcohols which could be only determined when positive polarity was chosen), although $[M+H]^+$ and $[M-H]^-$ were not the most abundant signals in the spectra in every case. In general, the fragmentation in-source was much more easily observed when positive polarity is used. Moreover, in negative polarity, signals were the same in both ESI and APCI ionization sources, meanwhile in positive polarity, ESI *m/z* signals commonly include alkaline metal adducts, fact which is not observable in APCI, being in this case species such as $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, and $[M+H-COOH]^+$ more prevalent. See Table 1 and 2 SM (supplementary materials) for further details.

Furthermore, since MS^2 could help us to distinguish between molecules with the same molecular formula (in case they have different fragmentation patterns), the MS fragmentation of the six analytes was carried out by means of the ESI-IT-MS detector. Nevertheless, as could be expected considering their chemical structure similarities, olive triterpenic mass isomers showed the same fragmentation patterns too. In negative ion mode, the fragmentation pattern matched

with data previously reported using the same MS configuration [43,54]. The most prevalent signal in mass spectra of triterpenic acids corresponded to the deprotonated molecular ion $[M-H]^-$ (*m/z* 471 for MA, and 455 for BA, OA and UA) which gave a MS/MS fragment corresponding to the carboxylic group loss (m/z 423 for MA, and 407 for BA, OA and UA). Moreover, the removal of the additional hydroxylic group in MA, gave another fragment in its spectrum (*m/z* 393). As mentioned above, the triterpenic alcohols peaks could not be detected in the chromatograms when negative ionization mode was employed. MS detection in positive ionization mode was less sensitive (considering triterpenic acids) than in negative polarity, but allowed UV and ER detection. In positive polarity, the most abundant *m/z* signal in MA spectrum corresponded to the molecular ion accompanied by a sodium adduct (*m/z* 495) which lost the carboxylic group –when was fragmented in MS/MS mode- giving the corresponding fragment with *m/z* 451. Triterpenic acids isomers (BA, OA and UA) gave the same three MS signals (*m/z* 457, 439 and 479) which differed in terms of relative intensities depending on the analyte; the signals were the protonated molecular ion (*m/z* 457), the protonated molecular ion with a water loss (*m/z* 439) and the molecular ion plus a sodium aduct (*m/z* 479). These precursors lost the carboxylic group (which is in agreement with previously reported results [43]) giving the following fragments: *m/z* 411 ($[M + H - COOH]^+$) *m/z* 393 ($[M + H - H_2O - COOH]^+$) and 435 ($[M + Na - COOH]^+$). The MS signal with *m/z* 191 was also detected, which according to Sánchez-Ávila *et al.* [29], is a characteristic fragment from oleane structure. When the triterpenic alcohol isomers (ER and UV) MS spectra were observed, the same three precursor ions were found, with *m/z* 443, 425 and 465, corresponding to $[M + H]^+$, $[M + H - H_2O]^+$ and $[M + Na]^+$, respectively. However, they only can lose alcohol groups, so their fragments are water losses, such as *m/z* 425 ($[M + H - H_2O]^+$) and *m/z* 407 ($[M + H - 2\ H_2O]^+$) or the oleane structure fragment with *m/z* 191.

3.3. Analytical parameters of the method

Table 1 shows the analytical parameters of the proposed method, which give an idea of its suitability for the analysis of the selected samples (for each tested detector). The table includes calibration curves and regression coefficients (r^2), LOD, LOQ, accuracy (expressed as trueness and intra/inter day repeatability), and matrix effect coefficients.

To evaluate the response of each analyte in DAD, ESI-IT MS and APCI-QTOF MS, their peak areas were plotted as a function of their concentration (for both MS platforms, the extracted ion chromatograms of the most intense *m/z* signal were used for quantitative purposes) performing a linear regression by the least-squares method.

All the resulting calibration lines showed good linearity within the indicated concentration ranges, with regression coefficients (r^2) higher than 0.9896. Furthermore, in every tested detector, triterpenic acids had lower detection and quantification limits than triterpenic dialcohols. ESI-IT MS gave the lowest limits followed by APCI-QTOF MS; in contrast, DAD had the widest dynamic range, fact which was found to be inversely proportional to the LOD and LOQ. For some cases, such as

Table 1. Analytical parameters of the developed method.

Compound	Detector	Calibration curve	r^2	LOD (µg/L)	LOQ (µg/L)	Linear range[a]	Accuracy					Matrix Effect Coefficient (%)[e]		
							Intra-day Repeatability[b]	*Inter-day* Repeatability[c]	Trueness[d]					
									Leaves	Skin	Pulp	Leaves	Skin	Pulp
Maslinic acid	DAD	$y = 4.776\ x + 0.704$	0.9970	93	311	80	7.1	8.2	101.2	111.9	110.2	-5.0	-14.6	-10.2
	ESI-IT	$y = 1.804 \cdot 10^6\ x + 1.322 \cdot 10^5$	0.9896	3	9	5	4.3	5.4						
	APCI-QTOF	$y = 131.926\ x - 114.337$	0.9961	65	217	20	9.7	9.8						
Betulinic acid	DAD	$y = 5.462\ x - 0.837$	0.9872	86	288	8	10.8	11.8	109.6	106.1	103.2	1.5	-5.2	-3.3
	ESI-IT	$y = 3.794 \cdot 10^6\ x - 1.568 \cdot 10^5$	0.9897	3	10	1.2	3.4	4.0						
	APCI-QTOF	$y = 226.982\ x + 12.453$	0.9927	41	138	1.2	2.2	4.3						
Oleanolic acid	DAD	$y = 6.292\ x + 0.006$	0.9976	83	277	80	5.2	6.8	98.6	97.2	93.5	-0.9	-2.0	-2.9
	ESI-IT	$y = 1.179 \cdot 10^6\ x + 4.521 \cdot 10^5$	0.9905	3	10	5	5.8	6.4						
	APCI-QTOF	$y = 450.214\ x - 283.256$	0.9941	22	73	20	9.7	10.0						
Ursolic acid	DAD	$y = 4.388\ x - 1.062$	0.9939	441	1471	10	8.3	8.6	106.7	104.7	96.9	-7.5	-2.3	1.3
	ESI-IT	$y = 7.212 \cdot 10^5\ x + 9.236 \cdot 10^4$	0.9950	9	29	5	6.3	7.7						
	APCI-QTOF	$y = 292.5318\ x - 217.414$	0.9911	28	94	20	10.5	10.9						
Erythrodiol	DAD	$y = 3.679\ x - 0.170$	0.9913	480	1600	10	9.4	9.5	98.9	105.0	108.5	-0.9	-4.9	-10.9
	ESI-IT	$y = 3.323 \cdot 10^4\ x + 2.317 \cdot 10^3$	0.9974	226	753	10	3.9	4.2						
	APCI-QTOF	$y = 11.761\ x + 3.230$	0.9971	273	911	6	10.5	11.0						
Uvaol	DAD	$y = 2.833\ x - 0.087$	0.9962	600	2000	10	2.6	4.5	101.5	103.1	101.3	-2.1	-0.9	-1.3
	ESI-IT	$y = 2.855 \cdot 10^4\ x + 5.915 \cdot 10^3$	0.9919	455	1515	10	5.5	6.8						
	APCI-QTOF	$y = 8.608\ x + 2.317$	0.9993	408	1359	6	9.7	9.9						

For MS detectors, negative polarity was used for triterpenic acids and positive, for alcohols.

[a] Linear ranges were established from LOQ to the indicated value (mg/L).

[b] RSD values (%) for peak areas of the analytes under study measured from 4 injections of the quality control mix carried out within the same sequence.

[c] RSD values (%) for peak areas of the analytes under study measured from 8 injections (belonging to 4 different sequences carried out over 4 days) of 8 different extracts from the same olive leaves sample.

[d] Trueness was measured by calculating the recovery (%), and it was estimated by analyzing the samples extracted before and after the standard addition and calculating the difference between the results obtained. The values included on this table are those achieved for the intermediate concentration level.

[e] Matrix effect coefficient (%) = (1−(slope matrix/slope solvent))×100. Matrix effect coefficients are just given for APCI-QTOF MS detector, since similar behaviour was observed in the rest of the tested platforms.

UA, for instance, the LOD values varied a lot depending on the detector: 9 µg/L in ESI-IT, 28 µg/L in APCI-QTOF, and 441 µg/L when DAD was used. However, for ER and UV, the differences among the achieved LODs by the three detectors were not so pronounced. The %RSD values for *intra-day* repeatability were found between 2.6 and 10.8% in DAD, 3.4 and 6.3% in ESI-IT MS detector, and within the range from 2.2 to 10.5 % in the case of the APCI-QTOF MS detector. The %RSD values for *inter-day* repeatability (which were calculated with different extracts of olive leaves extracts measured in different sequences (similar behavior was corroborated for the rest of the evaluated matrices)), were a little worse than those obtained for *intra-day* repeatability, since they include the precision of sample preparation and analysis (from our point of view, these values can be used to give an estimation the global method repeatability). With regard to the retention time repeatability, the %RSD was less than 1.6% for intra-day repeatability and less than 2.2% for inter-day repeatability in the worst-case scenario (data not shown in Table 1). As far as trueness is concerned, Table 1 shows the recoveries obtained for each individual analyte in the three matrixes which were found between 93.5 % (for OA in pulp) and 111.9 % (for MA in skin). This means that the proposed method is truthful, according to the AOAC guidelines [55], which establishes a good trueness from 80% to 115%.

Matrix effect was also evaluated according to the procedure described in section 2.4. Calculated matrix effect coefficient for each compound in each type of sample fluctuated between−14.6% and +1.4%, falling within the range described by Kmellár *et al.* [53] in which there is a mild signal suppression or enhancement effect (from -20 to +20%). Only the calculated matrix effect coefficients for APCI-QTOF MS detector are shown in Table 1, since similar behaviour (proving that there is no need to use standard addition calibration to achieve a proper quantification) was observed for the rest of the tested platforms. In almost all the cases, the matrix effect coefficients were below ±10% (only one case exceeded this value); indeed, for every analyte in the three matrices, the coefficients were actually below ±7.5%. Bearing in mind these results, it is possible to claim that the magnitude of possible matrix effect was not significant; therefore, the quantification was carried out by using external calibration equations.

Bearing in mind the just described performance of each detector and in an attempt of carrying out a systematic description of the purpose of using each platform, their most remarkable characteristics, advantages/drawbacks and the global major achievements, Table 2 is presented. As stated before, this work does not intend carrying out a comprehensive comparison of the analytical performance of the tested detectors (we are absolutely aware about the fact that in order to fairly compare the two used interfaces, we should have selected the same analyzer for both platforms), but offering different useful alternatives to accomplish a reliable determination of pentacyclic triterpenes in olive tissues, which, from our point of view, represents an interesting and challenging application in the field of Food Metabolomics.

The necessary number of injections in each case, as well as the quantitative results obtained will be thoroughly discussed in the following section.

Table 2. Comparison of the overall performance of the different platforms used within this study.

	Agilent 1260 LC system with DAD and -ESI-Ion Trap (Bruker Daltonic)		Waters Acquity UPLC coupled to APCI-Q TOF (Waters)
	DAD	**ESI-IT**	
Purpose of using it	Quantitative purposes	Quantitative & qualitative purposes	
LOD (µg/L)	83-600	3-455	22-408
Linear dynamic range* (mg/L)	8 (for BA) 10 (for UA, ER, UV) 80 (for MA, OA)	1.2 (for BA) 5 (for MA, OA, UA) 10 (for ER, UV)	1.2 (for BA) 6 (for ER, UV) 20 (for MA, OA, UA)
%RSD (intra-day)**	2.6-10.8	3.4-6.3	2.2-10.5
Number of injections needed	1 injection (widest linear dynamic range)	2 injections to make sure that a proper quantification is achieved (within the linear range)	4 injections: 2 to make sure that the quantification is done within the linear range, and 2 (one in each polarity) to properly detect both triterpenic acids and alcohols
Other remarkable characteristics	- More difficulties to identify the analytes under study (need of spiked samples)	- Used to obtain the fragmentation pattern of each analyte in MS^2 - Very fast switching polarities - Positive and negative polarities within the same run	- Used to understand the signal/behaviour of each analyte in APCI with accurate *m/z* signals and to quantify - No switching polarity within the same run
	If the complete platform is evaluated (LC-DAD/ESI-IT MS *on-line*): very advisable coupling, since it combines the benefits of both detectors		
Cost***	+++	++	+
Major achievements	- Optimization of a rapid (6 min) LC methodology (with proper analytical figures of merit) of application in Food Metabolomics - Quantitative results from 3 detectors in good agreement - Establishment of triterpenic acids and dialcohols levels for olive skin, pulp (not described before) and leaves		

*From LOQ to the indicated value (mg/L)
**It could be considered as the instrumental repeatability
***The more convenient, the higher number of "+"

3.4. Method application

Once it was optimized and validated, the developed LC-DAD/MS method was applied to the quantification of the six pentacyclic triterpenes under study in samples of three kinds of olive tissues. Olive leaves and olive skin were chosen because they have been described as very rich matrices in terms of those compounds; indeed, in general, fruit peel and, especially, fruit cuticular waxes have been identified as promising and highly available triterpenoid-rich plant tissues [10]. Skinless pulp seemed to be another interest matrix because, to the best of our knowledge, its triterpenic content has not been previously established. Guinda *et al.* (2010) [27], in a very interesting study, evaluated the pentacyclic triterpenoids content from olive fruit and leaves and their results indicated that maslinic and oleanolic acids were exclusively located in the epidermis,

being below their detection limits in the flesh and seed of the olive drupe. As stated before, in the current study, we wanted to compare the concentration levels of triterpenoids in olive skin, leaves and pulp (even though the latter was expected to be very low in comparison), in order to provide some useful information which could help to understand more in depth their metabolism and distribution over the different issues of *Olea europaea*.

In a first screening, the method was applied to some sample extracts to roughly estimate the concentration of each metabolite, observing that their relative concentration levels differed a lot for each analyte (being some of them at very low levels and some others at very high concentrations). This fact is illustrated in Fig. 4 and, obviously, turned the quantification of the triterpenoids into a very complicated task. Therefore, two injections per extract were necessary in MS detectors to ensure that all the compounds of interest could be measured in their linear calibration ranges. Nevertheless, although two injections were also registered in DAD detector (because it is *on line* with the LC – IT MS platform) just one injection was absolutely necessary due to its wider dynamic range.

Quantitative results for each detection method (DAD, ESI-IT and APCI-QTOF) and matrix are shown in Table 3. They are presented as the average of four replicates accompanied by the %RSD. The final results are the interpolated concentration values multiplied by the estimated recovery (which, as stated before, was calculated dividing the concentration of the analytes in the first extract between the total content of each analyte as the sum of the found amounts in the first, second and third extracts) and the dilution factor.

The found values in the three tested detectors were in good agreement as no significant statistical differences (at a 95% confidence level, $p < 0.05$) were found among them. The concentration levels of the six triterpenes analyzed in the olive leaves were found around 3.7 mg/g for MA, 18 mg/g for OA, 1.8 mg/g for UA, 1.6 mg/g for ER and 1.2 mg/g for UV, which generally were into the ranges previously reported by Sanchez-Avila *et al.* [29] and Peragon [54], but slightly lower than those reported by Guinda *et al.* [27] and much higher than the achieved results by Stiti *et al.* [56] (who quantified with respect to the internal standard). The quantitative composition of olive leaves could be strongly affected by agronomic factors, such as cultivar or ripening degree [10]; this fact could explain some differences in the described concentration levels. Besides, BA, which has not been previously quantified in olive leaves, was found at a concentration level around 0.12 mg/g.

As far as olive skin is concerned, it is possible to stand out that the triterpenoid found contents were around: 80 mg/g of MA, 0.20 mg/g of BA, 26 mg/g of OA, 0.14 mg/g of UA, 0.78 mg/g of ER and 0.30 mg/g of UV. In this case, a direct comparison with literature data cannot be done, because skin pentacyclic triterpenes levels have not been previously reported. It is well-know that these compounds are mainly located in the epicarp of the olive fruit, but a scarce number of reports

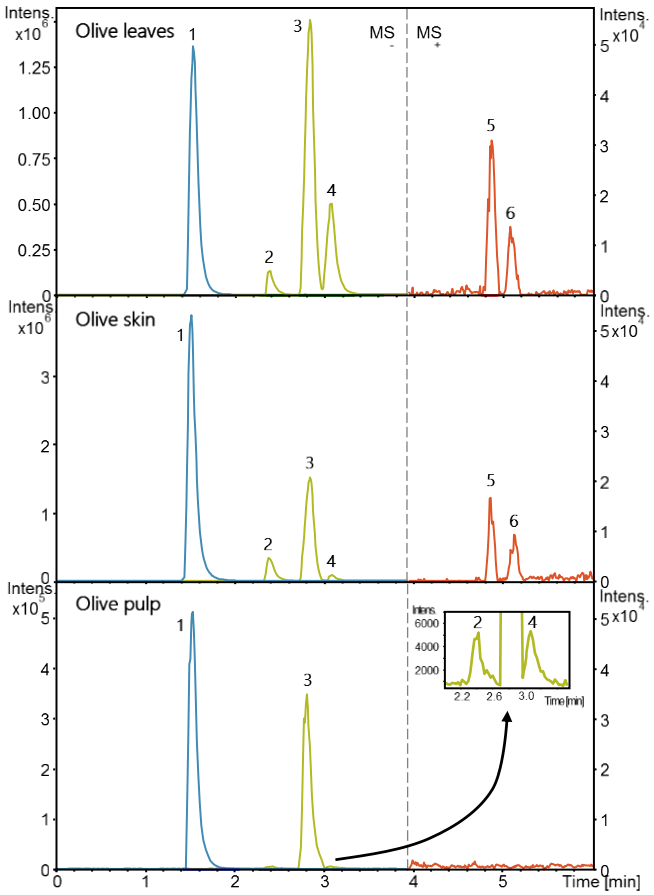

Figure 4. Extracted ion chromatograms (EICs) obtained when the different matrices selected within this study were analyzed in LC-ESI-IT MS platform. Peak identification numbers with the same meaning as in Fig. 2. The EICs shown are those corresponding with *m/z* 471 for peak number 1; *m/z* 455 for peaks 2-4; and *m/z* 425 for compounds 5 and 6.

explicitly stating its content can be found, since the entire fruits (including skin and pulp) are often analyzed [27,48,54,56]. In some other interesting applications, the composition of the waxy material covering the surface of olive fruits is given expressing the amounts with regard to the total weight of the fruit [34,57].

Even though these compounds have not been detected so far in such matrix [27], the described method allowed quantifying little amounts of triterpenic acids in olive pulp (around 65, 1.2, 55 and 4.4 µg/g for MA, BA, OA and UA, respectively).

Betulinic acid, which has been, to a certain extent, used as internal standard when quantifying triterpenic compounds in *Olea europaea*-related samples [29,32,48], was determined in the three studied matrices (by means of the three tested detectors), confirming its presence in surface waxes of olive leaves and fruits, reported by Bianchi *et al.* [13,57].

Table 3. Quantitative results (mg analyte/kg dry sample) obtained for the three different *Olea europaea* tissues under study by using the LC developed method coupled to DAD, ESI-IT MS and APCI-QTOF MS.

Sample	Compound	APCI-QTOF MS	ESI-IT MS	DAD
Olive leaves	MA	3904 ± 340	3469 ± 169	3698 ± 268
	BA	123 ± 11	111 ± 5	130 ± 9
	OA	18629 ± 1624	18515 ± 902	18149 ± 1314
	UA	1869 ± 163	1880 ± 92	1760 ± 127
	ER	1491 ± 130	1638 ± 80	1677 ± 121
	UV	1204 ± 105	1303 ± 63	1257 ± 91
Olive skin	MA	88343 ± 7703	74202 ± 3616	76787 ± 5560
	BA	216 ± 19	205 ± 10	210 ± 15
	OA	27970 ± 2439	26755 ± 1304	25364 ± 1836
	UA	140 ± 12	137 ± 7	158 ± 11
	ER	723 ± 63	843 ± 41	779 ± 56
	UV	304 ± 26	297 ± 14	298 ± 22
Olive pulp	MA	67 ± 6	66 ± 3	63 ± 4
	BA	1 ± 0.1	1 ± 0.1	1 ± 0.1
	OA	57 ± 5	53 ± 3	54 ± 4
	UA	4 ± 0.4	4 ± 0.2	4 ± 0.3

Every result is the average of four independent (sample preparation and injection) determinations (n = 4).
The results are given by the mean value ± SD.
No statistical significant differences among three tested detector were found (95%; $p < 0.05$).

4. CONCLUSIONS

The optimization of a liquid chromatography method using three different detectors (DAD, ESI-IT MS and APCI-TOF MS) and its potential application in the field of Food Metabolomics have been discussed in the current study; in particular for the determination of pentacyclic triterpenes in olive tissues. The method was fully validated and the analytical performances of the different detectors were described. The concentrations of the triterpenic compounds under study were established in three different matrices (triterpenic acids levels were evaluated by first time for olive pulp) and no statistically significant differences among the quantitative results achieved by each platform were observed, so they could be interchangeably used. Nevertheless, LC-DAD and LC-ESI-IT MS were able to detect all the compounds under study within a single run, whilst the LC-APCI-QTOF MS used platform needed two injections (one for each ion polarity mode) in order to detect both triterpenic acids and alcohols. Moreover, because of the wider linear range of DAD, it was the only one capable of quantify all the analytes by using a single dilution in every matrix (the other two, needed one additional dilution for MA and OA in leaves and skin).

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Table 1 SM. High resolution MS data of the six pentacyclic triterpenes found in *Olea europaea* in both ESI-QTOF MS and APCI-QTOF MS detectors.

	Compound	t_r (min)	MS mode	*m/z* experimental	Pseudo-molecular formula	*m/z* theoretical	Error (ppm)	mSigma value	Pseudo-molecular ions* and other signals
ESI-QTOF	**Maslinic acid**	1.5	-	471.3491	$C_{30}H_{47}O_4$	471.3474	-3.5	11.3	471.3491 $[M-H]^-$ (100); 539.3357 $[M-H+NaHCO_2]^-$ (5.5)
			+	473.3630	$C_{30}H_{49}O_4$	473.3631	0.2	80.4	495.3488 $[M+Na]^+$ (100); 409.3456 $[M+H-H_2O-COOH]^+$ (99.6); 490.3936 $[M+NH_4]^+$ (7.7); 473.3630 $[M+H]^+$ (4.9)
	Betulinic acid	2.4	-	455.3550	$C_{30}H_{47}O_3$	455.3525	-2.5	27.8	455.3550 $[M-H]^-$ (100); 523.3408 $[M-H+NaHCO_2]^-$ (2.8)
			+	457.3686	$C_{30}H_{49}O_3$	457.3682	-0.9	30.3	457.3686 $[M+H]^+$ (100); 479.3621 $[M+Na]^+$ (97.8); 439.3528 $[M+H-H_2O]^+$ (60.7); 495.3596 $[M+K]^+$ (5.1)
	Oleanolic acid	2.8	-	455.3534	$C_{30}H_{47}O_3$	455.3525	-1.9	36.7	455.3534 $[M-H]^-$ (100); 523.3386 $[M-H+NaHCO_2]^-$ (3.7)
			+	457.3669	$C_{30}H_{49}O_3$	457.3682	2.8	39.2	439.3532 $[M+H-H_2O]^+$ (100); 479.3617 $[M+Na]^+$ (63.8); 495.3599 $[M+K]^+$ (31.8); 457.3669 $[M+H]^+$ (4.8)
	Ursolic acid	3.1	-	455.3532	$C_{30}H_{47}O_3$	455.3525	-1.5	79.3	455.3532 $[M-H]^-$ (100); 523.3420 $[M-H+NaHCO_2]^-$ (4.4)
			+	457.3670	$C_{30}H_{49}O_3$	457.3682	2.5	167.7	479.3622 $[M+Na]^+$ (100); 439.3550 $[M+H-H_2O]^+$ (98.5); 495.3278 $[M+K]^+$ (28); 457.3670 $[M+H]+$ (21.2)
	Erythrodiol	4.8	+	443.3879	$C_{30}H_{51}O_2$	443.3889	2.3	24.4	425.3769 $[M+H-H_2O]^+$ (100); 465.3699 $[M+Na]^+$ (94.5); 443.3879 $[M+H]^+$ (18.5); 407.3672 $[M + H - 2\ H_2O]^+$(5.5)
	Uvaol	5.1	+	443.3876	$C_{30}H_{51}O_2$	443.3889	2.9	21.2	465.3703 $[M+Na]^+$ (100); 425.3773 $[M+H-H_2O]^+$ (53.8); 443.3876 $[M+H]^+$ (32.8); 407.3679 $[M + H - 2\ H_2O]^+$(5.5)

*Pseudo-molecular ions m/z signals are also included in this column to indicate their relative intensity.

	Compound	t_r (min)	MS mode	*m/z* experimental	Pseudo-molecular formula	*m/z* theoretical	Error (ppm)	mSigma value	Pseudo-molecular ions* and other signals
ESI-QTOF	**Maslinic acid**	1.5	-	471.3476	$C_{30}H_{47}O_4$	471.3474	0.4	28.1	471.3476 $[M-H]^-$ (100); 539.3330 $[M-H+NaHCO_2]^-$ (2.0)
			+	473.3628	$C_{30}H_{49}O_4$	473.3631	-0.6	35.9	409.3438 $[M-H_2O-COO]^+$ (100); 473.3628 $[M+H]^+$ (72.5); 437.3359 $[M+H-2H_2O]^+$ (61.8); 490.3752 $[M+NH_4]^+$ (14.6)
	Betulinic acid	2.4	-	455.3529	$C_{30}H_{47}O_3$	455.3525	0.9	43.1	455.3529 $[M-H]^-$ (100); 523.3370 $[M-H+NaHCO_2]^-$ (2.1)
			+	457.3683	$C_{30}H_{49}O_3$	457.3682	0.2	11.4	439.3559 $[M+H-H_2O]^+$ (100); 457.3683 $[M+H]^+$ (12.0)
	Oleanolic acid	2.8	-	455.3524	$C_{30}H_{47}O_3$	455.3525	-0.2	71.9	455.3524 $[M-H]^-$ (100)
			+	457.3679	$C_{30}H_{49}O_3$	457.3682	-0.7	79.1	439.3573 $[M+H-H_2O]^+$ (100); 411.3618 $[M+H-COOH]^+$ (7.0); 457.3679 $[M+H]^+$ (4.6)
	Ursolic acid	3.1	-	455.3525	$C_{30}H_{47}O_3$	455.3525	0.0	72.9	455.3525 $[M-H]^-$ (100)
			+	457.3669	$C_{30}H_{49}O_3$	457.3682	-2.8	68.1	439.3563 $[M+H-H_2O]^+$ (100); 411.3595 $[M+H-COOH]^+$ (28.9); 457.3669 $[M+H]^+$ (19.9)
	Erythrodiol	4.8	+	443.3893	$C_{30}H_{51}O_2$	443.3889	0.9	97.7	425.377 $[M+H-H_2O]^+$ (100); 407.3667 $[M+H-2H_2O]^+$ (41.4); 443.3893 $[M+H]^+$ (18.7)
	Uvaol	5.1	+	443.3889	$C_{30}H_{51}O_2$	443.3889	0	100.5	425.3773 $[M+H-H_2O]^+$ (100); 407.3669 $[M+H-2H_2O]^+$ (46.2); 443.3878 $[M+H]^+$ (44.0)

*Pseudo-molecular ions' *m/z* signals are also included in this column to indicate their relative intensity.

Table 2 SM. ESI-IT MS signals produced by each analyte in both positive and negative polarities and MS/MS mode.

Compound	MS mode	Major *m/z* signals in MS	Major ion signal in MS	Precursor ions and fragments in MS/MS			Cut off energy	Amplitude (V)
Maslinic acid	-	471.3	$[M-H]^-$	**471.3**	423.3	393.4	130	1.25
	+	495.4	$[M+Na]^+$	**495.4**	451.3		137	0.8
Betulinic acid	-	455.4	$[M-H]^-$	**455.3**	407.3		126	1.3
	+	457.4	$[M+H]^+$	**479.4**	435.3		132	0.8
				457.4	439.2	411.3	126	0.8
				439.4	393.4	191.2	121	0.8
Oleanolic acid	-	455.4	$[M-H]^-$	**455.2**	407.3		126	1.25
	+	439.4	$[M+H-H_2O]^+$	**479.4**	435.3		132	0.8
				457.4	439.2	411.3	126	0.8
				439.4	191.2	393.4	121	0.8
Ursolic acid	-	455.4	$[M-H]^-$	**455.3**	407.3		126	1.25
	+	479.4	$[M+Na]^+$	**479.4**	435.3		132	0.8
				457.4	439.2	411.3	126	0.8
				439.4	191.2	393.4	121	0.8
Erythrodiol	+	425.4	$[M+H-H_2O]^+$	**465.4**	407.3		128	0.7
				425.4	191.2	407.3	117	0.7
				443.4	425.3	191.2	122	0.7
Uvaol	+	465.4	$[M+Na]^+$	**465.4**	407.3		128	0.7
				425.4	191.2	407.3	117	0.7
				443.4	425.3	191.2	122	0.7

In bold letter we indicate the precursor ion. Fragments appear in decreasing order of intensity in the MS/MS spectrum. In every case, width in MS/MS was set at 4 units of *m/z*.

Chapter 2

Development and validation of LC-MS-based alternative methodologies to GC-MS for the simultaneous determination of triterpenic acids and dialcohols in virgin olive oil

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Abstract: Pentacyclic triterpenes are minor, but very relevant compounds found in virgin olive oil (VOO). A rapid and reliable LC-MS method for determining the triterpenic acids and dialcohols (after ultrasound assisted extraction) from VOO has been developed, giving an alternative to the widely used GC (FID/MS) methodologies. The analytical parameters of the proposed method were exhaustively checked, establishing limits of detection (from 1 to 95 µg/l) and quantification, precision (%RSD values for *inter*-day repeatability were found between 4.2 and 7.3% considering area values), trueness (within the range 92.7 and 100.5%) and evaluating possible matrix effect (which was no significant). The method was applied to the analysis of six triterpenic compounds in 11 monovarietal VOOs and the results compared with the quantitative GC-MS data. Moreover, the direct injection (after a simple dilution) of the samples into the LC-MS system was also tested, in an attempt to proffer an even simpler sample treatment.

Keywords: pentacyclic triterpenes; virgin olive oil; liquid chromatography; gas chromatography; mass spectrometry; direct injection.

1. INTRODUCTION

It has been demonstrated that VOO consumption brings along beneficial effects on human health, being the high content of monounsaturated fatty acids together with its non-glyceridic components the main responsible of its benefits [1]. Among the minor components of VOO, phenolic compounds and pentacyclic triterpenes have been capturing lots of researchers´ attention in the last decades because of their interesting biological properties. Phenolic content, for instance, has been widely assessed in VOOs produced by using a great diversity of agro-technological parameters, coming from different varieties and geographical origins [2,3]. On the contrary, VOO triterpenic content has been scarcely reflected in literature. Even though several stimulating reports carrying out the quantification of the most abundant pentacyclic triterpenes in olive oils (from different categories, varieties and obtained by different processing methods) have been published [4–9], the analytical methods used so far generally do not give an estimation of their absolute content, since they carry out the quantification based on the response factor of another triterpenic compound. Triterpenic dialcohols are commonly determined as the percentage of total sterols, since it is a recognized authenticity index to detect possible fraudulent mixtures with olive-pomace oils) [10].

The limited number of published analytical methods to determine pentacyclic triterpenes in olive oil does not match with the proliferation of research studies about their bioactivity. In the last years, some interesting reviews providing an overview of the biological activities (anti-inflammatory, antitumoral, cardioprotective and antidiabetic, among others) of triterpenes from *Olea europaea* have been written [11–16]. Bearing this in mind, finding analytical methods to determine them (easily and reliably) seems imperative, in order to allow consumers, as well as olive oil industry, to know their concentration levels.

Gas chromatography (GC) coupled to flame ionization (FID) [4,8,9,17] or mass spectrometry (MS) [5,6] detectors have been the most applied platforms for the determination of triterpenoids in olive oil so far. Only one reference can be found in literature about the use of liquid chromatography (LC) coupled to diode array detection (DAD) for the determination of two triterpenic acids in olive-pomace oil [7]. Nevertheless, LC-DAD and LC-MS have been used for the identification and quantification of these compounds in other matrixes such as plant materials [18–21] and biological fluids [22–24].

Both GC and LC have in common a previous step to assure the isolation of these analytes from the matrix. Extraction of triterpenic acids from olive oil has been commonly carried out by using solid phase extraction (SPE) according to a method firstly proposed by Pérez-Camino and coworkers [4]. Alternatively, two liquid-liquid extraction (LLE) protocols have been proposed: one with a methanol/ethanol mixture (1:1, v/v) as extractant agent [7]; and the other with methanol [5], for the simultaneous extraction of triterpenic acids and phenolic compounds from VOO. Triterpenic dialcohols have been frequently determined according to the method proposed by the European Regulation 2568/91 [25], which involves a tedious saponification process [8,9,26].

The main aim of this work has been to propose an alternative LC-MS method for the determination of pentacyclic triterpenes in olive oil, avoiding the need of a derivatization step, which is one of the main disadvantages of the GC methods. Based on the previous experience of our research group in determining these compounds in plant matrixes [21], the main challenge has been to find a simple sample treatment and to adapt the chromatographic separation to the oily matrix. The proposed method was logically validated and then, applied to the analysis of six triterpenic compounds in 11 monovarietal VOO samples, comparing the results with those obtained by GC-MS data. Moreover, the direct injection (DI) of the samples in the LC-MS system after a simple dilution was also explored, trying to simplify even further the sample treatment. Bring the results achieved by using the three chosen strategies into comparison could give, from our point of view, more reliability to the outcomes of our study, making possible to discuss in depth the advantages/drawbacks of each approach.

2. MATERIALS AND METHODS

2.1. Chemicals and standards

All reagents were of analytical grade and used as received. Methanol (MeOH) tetrahydrofuran, acetone and isopropanol (gradient grade) from Prolabo (Paris, France) and ethanol absolute (EtOH) from Panreac (Barcelona, Spain) were used for the sample preparation. Chromatographic mobile phases were prepared with acetonitrile and MeOH (LC-MS grade) from Prolabo, and deionised water (obtained by using a Milli-Q system from Millipore (Bedford, MA, USA)). Aqueous phase was daily prepared and filtered with a Nylaflo™ 0.45 µm nylon membrane filter from Pall Corporation (Ann Arbor, MI, USA) before entering into the chromatographic system. N,O-

bis(trimethylsilyl)trifluoroacetamide plus 1% of trimethylchlorosilane (BSTFA+TMCS, 99:1), used as derivatization reagent in GC, and the buffer components of the aqueous mobile phase in LC (ammonium formate and ammonium hydroxide) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standards of maslinic (MA), betulinic (BA), oleanolic (OA) and ursolic (UA) acids, as well as erythrodiol (ER) and uvaol (UV), were also supplied by Sigma-Aldrich. Methanolic stock solutions of 100 mg/L for each standard were first prepared by dissolving the appropriate amount of each analyte in MeOH and then, they were serially diluted to working concentrations (within the range 0.1 – 25 mg/L). All the samples and stock solutions were stored at −20 ◦C and filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) before injection into the instrument.

2.2. Samples and sample treatment

The VOO samples used within this study came from 11 different cultivars grown in the experimental olive grove of the Agro-pôle Olivier National School of Agriculture of Meknès, Morocco. Fruits samples with a ripening index between 3.0 and 3.5 were randomly hand-picked from the selected trees and monovarietal oils were further extracted using an Oliomio laboratory mill (Toscana Enologica Mori, Tavernelle Val di Pesa-Fl, Italy) simulating a two-phase commercial oil-extraction system. A mixture of all the samples under study (prepared by mixing an equivalent volume of each one) was used for the extraction procedure optimization. Then, it was also used as a quality control sample (QC) for ensuring the proper performance of the systems as well as for evaluating the analytical parameters of the methods (repeatability, recovery and matrix effect). Moreover, commercial sunflower oil was used as a blank matrix for preparing DI calibration curves.

2.2.1. Extraction of triterpenic compounds

The isolation of the triterpenic compounds under study from the VOO samples was achieved by ultrasound assisted extraction (UAE). A portion of 0.2 (± 0.01) g of VOO were weighed in a conical centrifuge tube and mixed with 5 mL of the extractant agent (MeOH, MeOH/EtOH (1:1, v/v) or EtOH/H_2O (90:10, v/v) depending on the experiment) by vortexing during 1 min (MeOH was finally pointed out as the solvent giving the best results). Then, the tube was left in an ultrasonic bath for 30 min and centrifuged at 5000 rpm for 6 min. These steps were repeated twice and both supernatants were collected together. Thereupon, the solvent was evaporated to dryness under reduced pressure by using a rotary evaporator at 35ºC and the obtained residue was redissolved in 1 mL of MeOH.

During the sample treatment optimization, two alternative extraction protocols were also tested. In the first one, the compounds of interest were isolated by SPE according to a previously described protocol [4]. Briefly, 0.2 (± 0.01) g of VOO dissolved in 1 mL of hexane were put into a properly conditioned bonded aminopropyl phase SPE cartridge (500 mg, 3 mL) from Agilent. After successive washes, the triterpenic compounds were eluted with diethyl ether/acetic acid (98:2, v/v). Finally, the eluate was evaporated and reconstituted in 1 mL of MeOH. The other alternative

extraction protocol was based on the use of microwave assisted extraction (MAE), and the optimum conditions were reached after a preliminary optimization and keeping in mind previously published reports [27,28]. In short, 0.2 ± 0.01 g of VOO and 10 mL of the pertinent extractant agent (of the three tested ones) were put into the extraction vessel which was placed in the microwave system with the following irradiaton power ramp: 0-400W (80ºC) in 5 min, holding it constant over 10 min. Once the vessel was cooled to room temperature, its content was centrifuged at 5000 rpm for 6 min, and the supernatant was evaporated and redissolved in 1 mL of MeOH.

2.2.2. Cleaning step and derivatization of the extracts for GC analyses

Prior to the injection into the gas chromatograph, both the standard solutions and the extracts obtained by the UAE protocol slightly modified (see below) were derivatized in order to increase the volatility of the analytes under study and making them suitable for being analysed by GC. Before that, triterpenic extracts had to be further cleaned and preconcentrated. The sample preparation was performed by UAE with MeOH as reported in section 2.2.1., but once the combined extracts were evaporated in the rotary evaporator, the resulted residue was reconstituted in 500 µL of acetonitrile; this solvent exhibits a lower miscibility in hexane than the MeOH; fact which could facilitate the following cleaning step where hexane was used to dissolve the oily interferences. After being washed with 1 mL of hexane and filtered, 200 µL of the extract were evaporated to complete dryness with a stream of N_2. Then 50 µL of the derivatization reagent (BSTFA+TMCS, 99:1) were added to the dried residue and vortexed during 1 min (final preconcentration of 4:1, v/v). The trimethylsilylation reaction was performed at room temperature for 30 min. For calibration curves, aliquots of 50 µL of the methanolic standard solutions of each concentration level were evaporated and derivatized by addition of 50 µL of (BSTFA+TMCS, 99:1) following the above mentioned procedure.

2.2.3. Sample dilution for DI

The preparation of the VOO samples for DI into the LC-MS system was carried out as follows: 0.2 (±0.01) g of VOO were weighed in a volumetric flask of 1 mL and diluted to the mark with acetone. Blank matrix calibration (used for quantification of DI analyses) was made in sunflower oil. Every concentration level was prepared weighing 0.2 g ±0.01 of sunflower oil in a volumetric flask of 1 mL, spiking it with the appropriate volume of methanolic standard solution of the six triterpenes under study, and diluting to the mark with acetone after evaporating the MeOH with a stream of N_2.

2.3. LC-MS methodology

The LC-MS analyses were performed with an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker Daltonics Esquire 2000™ ion trap mass spectometer (Bruker, Bremen, Germany) by an electrospray ionization source.

Regardless the sample preparation (extraction or oil dilution), the applied LC-MS method was a modification of a previous one proposed by our research group[21]. The triterpenic compounds under study were separated by using a Zorbax Extend C18 analytical column (4.6 x 100 mm, 1.8 µm particle size) (Agilent Technologies), operating at 20ºC. The mobile phases were 1.5 mM ammonium formate in water (adjusted to pH 9.6 with ammonium hydroxide) (Phase A) and acetonitrile/MeOH (60:40, v/v) (Phase B). Analytes were isocratically eluted (10% Phase A and 90% Phase B) during 5 min; this step was followed by a column cleaning with 100% Phase B. Therefore, the LC method can be described as follows: 0 to 5 min, 90% B; 5.5 min, 100% B; 12.5 min, 100% B; 13 min, 90% B, with 2 min additional post run time before the subsequent injection. The flow rate was 1.2 mL/min and the injection volume was 10 µL for the extracts and standards and 3 µL from every vial when DI was the used strategy.

Concerning the ESI-IT MS conditions, analyses were made using to different MS segments; in negative ion mode from the beginning to min 4 and in positive polarity until the end of the run, with a capillary voltage of +3500 V and -4000 V, respectively. The end plate offset voltage was set at -500 V, drying gas temperature at 300ºC, drying gas flow at 9 L/min, and nebulizer pressure at 30 psi. A scan range from 400 to 600 m/z was selected.

2.4. GC-MS methodology

An Agilent 7890A gas chromatograph coupled to a Waters QUATTRO™ mass spectrometer (Waters, Manchester, UK) operating as a single quad, was used for GC analyses.

The separation of the analytes in this instrument was carried out in a fused silica capillary column coated with (5%-Phenyl)-methylpolysiloxane (HP-5MS) (30 m x 0.25 mm i.d., 0.25 µm) from Agilent. After the optimization process, a temperature gradient was applied for the triterpenic compounds analysis: the oven temperature was initially kept at 200◦C for 2 min, then it was increased until 300◦C at 14◦C/min and held for 15.5 min. The operating conditions were 250◦C and 300ºC for injector and transfer line temperatures, respectively, with He as a carrier gas at a flow rate of 1 mL/min. 1 µL of sample volume was injected in splitless inlet mode. Electron impact (EI) spectra were acquired at 70 eV in total ion monitoring mode (mass range from 50 to 600 m/z) operating in positive polarity, with a source temperature of 210ºC. A solvent delay of 11 min was set at the beginning of each run to avoid damaging the filament of the MS because of solvent peaks and/or some other VOO compounds found in the extracts which could saturate the detector.

2.5. Auxiliary equipment and software

An ultrasonic bath from J.P. Selecta (Barcelona, Spain) was used for triterpenic compounds extraction from VOO samples. Its characteristics were: 6 L of capacity, dimensions of 15, 30 and 14 cm of height, width and depth of usable bath, respectively, with a generator power of 150 W, a total power capacity of 360 W and a fixed frequency within the range 50-60 Hz. Besides, a Preppy™ vacuum manifold for SPE (Supelco, Bellefonte, PA, USA) and a START E Microwave Extraction

System (230V/50 Hz) from Milestone (Bergamo, Italy) were used during the extraction procedure optimization.

ChemStation B.04.03 (Agilent) and Esquire control (Bruker), for LC-MS analyses, and Acquity UPLC Console and MassLynx 4.1 (Waters), for GC-MS analyses, were the software used for instrument control and file acquisition. The treatment of the data coming from both systems was carried out with the software Data Analysis 4.0 (Bruker), after exporting in compatible format the data coming from GC-MS. Statistical analyses (ANOVA test) to compare the quantitative results achieved by the different methods used within this study, were carried out by using STATGRAPHICS Centurion XVII (Statpoint Technologies, Inc., Warrenton, VA, USA).

3. RESULTS AND DISCUSSION

3.1. Extraction procedure optimization

Trying to find the simplest sample treatment with the highest recovery percentages for all the analytes under study, three extraction techniques were selected to be evaluated (SPE, UAE and MAE) in a first step of the optimization, keeping in mind some previous published works. A SPE extraction procedure [4] (very widely used since its publication), was compared with two assisted LLE methods. Moreover, three different solvents or mixtures (MeOH, MeOH/EtOH (1:1, v/v) and EtOH/H_2O (90:10, v/v)) were tested as extractant agents in the LLE-based methods. The experimental design was directed towards having comparable results so, in all the cases, 0.2 (±0.01) g of VOO were subjected to extraction and led to a final volume of 1 mL of MeOH. That means that the yield of each experiment could be easily compared in terms of peak area in the chromatograms for the 6 analytes under study.

Fig. 1 presents the results of the first step of the optimization experiments for MA and OA, which are the two most abundant triterpenic compounds found in VOO. That was the reason to pick these two analytes to illustrate the results (similar behavior was shown for the other compounds under study). The figure shows MA and OA peak areas (average values of three independent sample preps) in the extracts prepared by using the three tested techniques employing the different solvents enumerated in section 2.2.1., just for UAE and MAE. In both bars graphics, it can be observed that the extraction protocol displaying the highest recoveries was UAE with MeOH, solvent which has been previously reported for the simultaneous extraction of phenols and triterpenes from VOO [5]. The extraction of MA was considerably affected by the physico-chemical properties of the solvent used; indeed, for the extraction of this compound, the chosen solvent had a more significant impact than for the rest of the analytes. SPE gave good recoveries for BA, OA and UA, but as far as MA and the alcohols are concerned, the other two LLE procedures seemed to be more effective. UAE with MeOH was finally pointed out as the protocol with better performance (higher recoveries, easier use and lower cost of consumables).

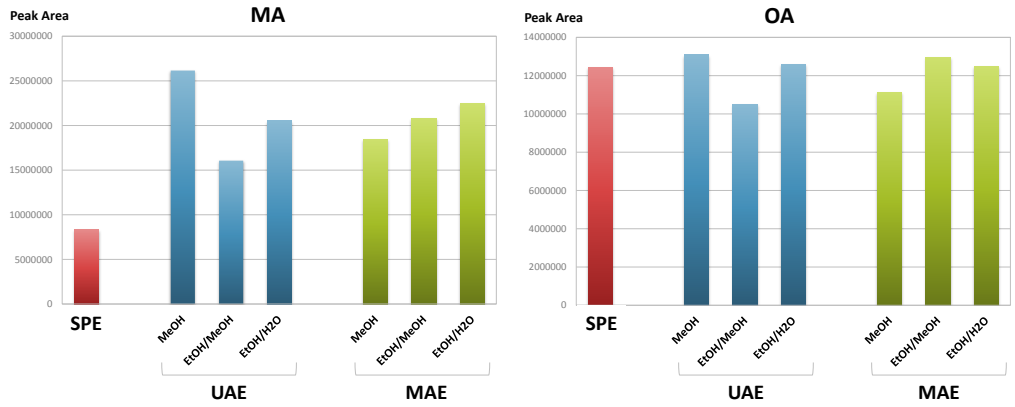

Figure 1. Bars graphs representing the peak area of the two most abundant triterpenic compounds found in VOO (MA and OA) in the SPE, UAE and MAE experiments carried out during the first stage of the sample treatment optimization. UAE was applied by using MeOH, MeOH/EtOH (1:1, v/v) and EtOH/H_2O (90:10, v/v), respectively, and MAE (with the same solvents as for UAE).

In a subsequent step, new experiments were designed, trying to achieve the best operating conditions for the UAE MeOH-based procedure. Extraction time (15, 30, 45 or 60 min), extractant agent volume (5 or 10 mL), and number of extraction cycles (1, 2 or 3 times) were carefully optimized. Being 15 min a not enough extraction time, no differences were found between lengths higher than 30 min; so the shortest possible UAE extraction time was chosen. Concerning the MeOH volume, 10 mL gave higher recoveries than 5 mL in every case; and with regard to the number of extraction cycles, a second step was always needed (significant amounts of all the analytes were found in the oil after the first extraction cycle).

At the end, 10 mL of MeOH left in an ultrasound bath over 30 min, repeating it twice, were the preferred operating conditions. In order to establish the percentage of the total amount of each analyte which remained into the sample after the two extraction stages and give and estimation of the recovery of the extraction protocol, a third repetition of the extraction was carried out, finding that less than 0.5% of every triterpenic compound remained in the sample.

The optimized extraction conditions in UAE were used for preparing the VOO extracts both for LC-MS and GC-MS analyses.

3.2. Analysis of the UA extracts by LC-MS

3.2.1. Optimization of chromatographic conditions in LC

The extracts obtained by using the optimum protocol just described above, were firstly analysed with a chromatographic method previously reported by our research team [21]. The compounds under study were properly separated, but after few analyses, some experimental issues started to show up. They can be enumerated as follows: appearance of big peaks coeluting with the analytes, slightly shorter retention times, as well as a considerable decrease in the MS intensity. At this point, we considered as mandatory to modify the method and lengthen the run

time by adding a column cleaning step, which could assure the elution of the most apolar oily compounds before the subsequent injection. 7 min at 100% Phase B and 1 additional min to reach initial conditions, followed by 2 min of stabilization were enough to achieve good repeatability inter-sequence, as described in the following paragraphs.

3.2.2. LC-MS method characterization

Before carrying out the analysis of the samples, the performance of the whole methodology was obviously assessed, so the main analytical parameters, which give an idea of the linearity, sensitivity, accuracy and matrix effect of the method, were calculated. Both the standard mixture containing the 6 triterpenic compounds and the QC sample (fortified at different concentration levels) were used for validation purposes. The results of the validation studies are summarized in Table 1.

In order to check the linearity of the method, external calibration curves were established for each pure standard by plotting the peak area as a function of its concentration (12 different concentration levels over the range 0.1-15 mg/L, injected in duplicate). For MA and OA which were found in the samples in a wide concentration range, two calibration curves were used: one for the lowest concentration levels and the other for the highest ones. The responses fitted well to a straight line with regression coefficients (r^2) higher than 0.9909 in every case. Instrumental signal to noise ratio (S/N) was measured for each standard at the lowest concentration level injected, in order to calculate detection (LOD) and quantification (LOQ) limits, which were considered as the concentrations that generated a S/N equal to 3 and 10, respectively. As shown in Table 1, LOD were found within the range from 1 to 95 µg/L and LOQ varied between 3 and 317 µg/L (for UA and UV, respectively). In general, LOD and LOQ were found at concentration levels of few ppbs for triterpenic acids, whilst they were of several hundred ppbs approx. for the two alcohols, which are poorly ionized in comparison.

Method accuracy was evaluated in terms of precision and trueness. Precision was expressed as repeatability by calculating the relative standard deviation (%RSD) of peak areas and retention times (Rt) of the analytes under study measured from 4 injections of the QC carried out within the same sequence (intra-day repeatability) and from 8 injections of the QC belonging to 4 different sequences carried out over 4 days (inter-day repeatability). %RSD was lower than 3.4% and 5.1% for Rt and peak area, respectively, for *intra*-day repeatability, and lower than 4.0% and 7.3% for *inter*- day repeatability. Trueness was determined as recovery (%), which was estimated by analysing the QC extracted before and after the standard addition at three concentration levels (0.25, 0.5 and 1 mg/L) and calculating the difference between the obtained results. Good recoveries for all the analytes (between 92.7% for UV and 100.5% for OA, at the intermediate concentration level) were found. Similar values were achieved for the other concentration levels, demonstrating the suitability of the extraction system.

Table 1. Analytical parameters of the LC-MS developed method.

Compound	*m/z* signal used	Calibration curve	r^2	Linear range (mg/L)	LOD (µg/L)	LOQ (µg/L)	Repeatability[b]				Trueness[c]	Matrix Effect Coefficient (%)
							Intra-day		*Inter-day*			
							Area	*Rt*	*Area*	*Rt*		
Maslinic acid	471 ($[M-H]^-$)	$y = 7.5 \cdot 10^5 x + 4.7 \cdot 10^5$ $y = 4.6 \cdot 10^5 x + 2.2 \cdot 10^6$	0.9985 0.9962	2[a] 2-15	2	8	3.4	3.4	5.3	4.0	99.3	-1.2
Betulinic acid	455 ($[M-H]^-$)	$y = 2.0 \cdot 10^6 x - 6.1 \cdot 10^4$	0.9993	1[a]	2	8	2.4	2.3	4.2	2.6	99.7	10.9
Oleanolic acid	455 ($[M-H]^-$)	$y = 8.6 \cdot 10^5 x + 1.4 \cdot 10^5$ $y = 5.2 \cdot 10^5 x + 8.5 \cdot 10^5$	0.9944 0.9916	2[a] 2-10	1	3	3.5	1.9	6.4	2.2	100.5	7.2
Ursolic acid	455 ($[M-H]^-$)	$y = 7.2 \cdot 10^5 x + 4.4 \cdot 10^5$	0.9909	2[a]	3	11	5.1	2.0	5.9	2.0	100.2	3.0
Erythrodiol	425 ($[M+H-H_2O]^+$)	$y = 7.5 \cdot 10^4 x + 9.3 \cdot 10^2$	0.9968	6[a]	95	317	3.4	1.1	4.6	1.1	94.9	6.5
Uvaol	443 ($[M+H]^+$)	$y = 1.2 \cdot 10^5 x - 1.9 \cdot 10^4$	0.9953	6[a]	74	245	4.2	1.0	7.3	1.2	92.7	4.4

[a] Linear ranges were established from LOQ to the indicated value.

[b] %RSD values

[c] The values included on this table are those recoveries (%) achieved for the intermediate concentration level.

In order to make a choice of the most appropriate kind of calibration methodology to achieve accurate quantitative results, matrix effect was evaluated according to a previously proposed strategy [29]. Consequently, a matrix effect coefficient was calculated by applying the following equation:

$$\textit{Matrix effect coefficient (\%)} = (1-(\textit{slope matrix}/\textit{slope solvent})) \times 100$$

where slope matrix was the slope of a standard addition calibration curve (prepared by fortifying a QC extract at 3 concentration levels over the range 0.25-1 mg/L) and slope solvent was the slope of the external calibration function prepared in MeOH. The resulting coefficients fluctuated between -1.2% for MA (very slight signal suppression) and 10.9% for BA (mild enhancement effect), so they were found within the range in which the matrix effect is negligible (from -20% to +20%), according to Kmellár *et al.* Therefore, the external standard (solvent-based) calibration could be considered as a fitting calibration strategy to properly quantify the triterpenic compounds in the samples, as the presence of VOO matrix did not practically interfere the response of the analytes.

3.2.3. Application of the LC-MS method to the analysis of the samples

Once the developed method was validated, it was applied to the quantification of the six triterpenic compounds under study in the 11 selected monovarietal VOO samples. In Table 2, the results for each analyte are organized in three different tables (a, b and c); the first one (Table 2a) shows the quantitative data obtained from the analysis of the extracts with the LC-MS method. These data were achieved interpolating the peak area of three independent replicates (each one injected in duplicate) in the calibration curves presented in Table 1.

In the following sections the results included in the other two tables (2b and 2c) will be introduced. A discussion regarding the comparison of the quantitative data achieved by the application of the different strategies will be presented in section 3.5.

3.3. Analysis of the UA extracts by GC-MS

3.3.1. Optimization of the chromatographic conditions in GC

As highlighted before, GC has been considered the reference analytical technique for the analysis of pentacyclic triterpenes in VOO. Bearing that in mind, a very appropriate way to validate a possible alternative to GC could be to compare the quantitative results obtained with both methodologies (the new one (LC-MS) and the one contemplated as the gold standard in the field). With this aim, the sample set was treated again according to the protocol mentioned in section 2.2.2 and the derivatized extracts were injected into the gas chromatograph. The applied separation conditions described in section 2.4 were the result of the slight modifications which were made to previously reported methods [4,5,30,31], in order to have reasonable retention times together with adequate chromatographic efficiency.

Table 2. Quantitative results (mg analyte/kg olive oil) obtained for the olive oils under study by using the different approaches tested (LC-MS with UAE, GC-MS after UAE, and LC-MS after a simple dilution of the sample).

a. LC-MS UAE	**Arbequina**	**Arbosana**	**Cornicabra**	**Frantoio**	**Hojiblanca**	**Haouzia**	**Koroneiki**	**Langedoc**	**Manzanilla**	**Picholine**	**Picual**	**QC**
MA	52 ± 2	25 ± 1	41 ± 2	20 ± 1	16 ± 1	27 ± 1	71 ± 2	13.2 ± 0.6	24 ± 1	19 ± 1	22 ± 1	29 ± 2
BA	0.34 ± 0.02	0.21 ± 0.01	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OA	24 ± 0.4	15.5 ± 0.5	24 ± 1	7.1 ± 0.2	5.6 ± 0.2	16.8 ± 0.8	36 ± 2	4.3 ± 0.2	9.8 ± 0.4	7.3 ± 0.4	10.2 ± 0.4	13.7 ± 0.7
UA	nd	1.2 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.13 ± 0.01
ER	2.1 ± 0.2	3.9 ± 0.3	1.9 ± 0.1	1.3 ± 0.1	0.55 ± 0.05	2.9 ± 0.2	7.8 ± 0.8	0.9 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	2.2 ± 0.2
UV	nd	1.0 ± 0.1	nd	nd	nd	nd	1.4 ± 0.1	nd	nd	nd	nd	nd

b. GC-MS UAE	**Arbequina**	**Arbosana**	**Cornicabra**	**Frantoio**	**Hojiblanca**	**Haouzia**	**Koroneiki**	**Langedoc**	**Manzanilla**	**Picholine**	**Picual**	**QC**
MA	55 ± 3	24 ± 1	38 ± 2	22 ± 1	17 ± 1	26 ± 1	68 ± 4	14 ± 1	25 ± 1	20 ± 1	24 ± 1	29 ± 1
BA	0.31 ± 0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OA	24.4 ± 0.9	15.8 ± 0.9	26 ± 1	7.1 ± 0.4	6.0 ± 0.3	17 ± 1	36 ± 2	4.6 ± 0.2	9.2 ± 0.5	7.4 ± 0.4	11.0 ± 0.5	13.9 ± 0.7
UA	nd	1.2 ± 0.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
ER	2.3 ± 0.2	3.8 ± 0.3	1.1 ± 0.1	1.4 ± 0.1	0.50 ± 0.04	2.7 ± 0.2	7.5 ± 0.6	0.9 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	2.4 ± 0.2
UV	nd	nd	nd	nd	nd	nd	1.3 ± 0.1	nd	nd	nd	nd	nd

c. LC-MS DI	**Arbequina**	**Arbosana**	**Cornicabra**	**Frantoio**	**Hojiblanca**	**Haouzia**	**Koroneiki**	**Langedoc**	**Manzanilla**	**Picholine**	**Picual**	**QC**
MA	56 ± 3	25 ± 2	42 ± 3	20 ± 1	17 ± 1	28 ± 2	68 ± 4	15 ± 1	27 ± 2	22 ± 1	24 ± 1	31± 2
BA	0.28 ± 0.02	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
OA	24.5 ± 0.7	16 ± 1	27 ± 2	7.2 ± 0.5	5.8 ± 0.3	17 ± 1	38 ± 3	4.1 ± 0.4	10.2 ± 0.7	7.7 ± 0.5	10.9 ± 0.7	14.6 ± 0.9
ER	2.5 ± 0.2	4.2 ± 0.3	nd	nd	nd	3.1 ± 0.2	7.4 ± 0.7	nd	1.5 ± 0.1	nd	nd	2.0 ± 0.2

Every result included in this table is the average of three independent replicates (each one injected in duplicate). The results are given as the mean value ± SD.
No statistical significant differences among the three tested strategies were found (95%; $p < 0.05$).
nd: non detected

3.3.2. GC-MS method characterization

To make sure that the comparison between LC-MS and GC-MS quantitative results was fair and properly carried out, trueness was considered as the crucial parameter when the GC-MS method validation was done, although linearity and instrument repeatability were logically evaluated too. Trueness was assessed by means of the analysis of different replicates of a blind sample; since suitable Certified Reference Materials are not available, standard mix of pure standards (at different concentration levels) were prepared by the technical assistants of our lab (not involved in this project) and analysed. Their concentration in terms of all the analytes was calculated and resulting values were compared with the real ones through the Student's t-test. No statistically significant differences were found among them for any of the evaluated concentration levels (at a 95% confidence level, $p<0.05$), what means that the method was very truthful. Besides, all the external calibration curves showed good linearity within the work range ($r^2 > 0.9901$), and intra-day repeatability, calculated as the %RSD of the peak areas of the six triterpenes in 4 injections of the standard mix carried out within the same sequence, was lower than 8.3% in every case.

3.3.3. Application of the GC-MS method to the analysis of the samples

The quantitative data obtained after the analysis of the 11 VOO samples with the GC-MS method are presented in Table 2b. The m/z signals monitored for each compound were the following ones: 73, 129, 203 and 497 for ER and UV (eluting at 17.1 and 17.7 min, respectively); 73, 129, 203, 320 and 483 for OA and UA (eluting at 18.3 and. 19.2 min, apiece); 73, 129, 189 and 483 for BA (eluting at 18.5 min); and 73, 147, 203, 320 and 571 for MA (eluting at 21.9 min).

3.4. Direct injection of diluted VOO samples in LC-MS

Although the proposed sample treatment gave good recoveries and was easy to perform, any extraction protocol is always reagent and time consuming. Some researchers have focused their efforts on simplifying the sample preparation trying to carry out, to a considerable extent, more rapid and simpler determinations of different analytes in VOO. One possible strategy is the DI of the sample into the liquid chromatograph after a simple dilution, which has been applied to determine triacylglycerols [32] and VOO minor compounds such as phenols [33,34], chlorophylls, pheophytins [35], β-carotene, tocopherols and tocotrienols [36]. To the best of our knowledge, VOO triterpenes have not been determined by using this approach so far; therefore, the determination of triterpenic compounds from VOO by LC-MS after a simple dilution of the sample was explored in another stage of the current study.

Initially, the most adequate solvent used to dissolve the oil samples and the optimum oil/solvent ratio were investigated. Taking into account the previous experience of our research group with VOO DI for phenolic compounds determination [34], three solvents (tetrahydrofuran, acetone and isopropanol) and three ratios (1 g diluted to a final volume of 2, 5 and 10 mL) were tested. 1g of VOO diluted to a final volume of 5 mL with acetone was pointed out as the optimal option, avoiding the rapid soiling of the column with more concentrated preparations. This ratio

could lead to inject concentration levels analogue to those of the methanolic extracts prepared by UAE. However, in order to lengthen the column life, the volume injected into the LC system was reduced from 10 µL (for the extracts and standard mix injected when LC-MS with UAE was used) to 3 µL (for every preparation –diluted samples or standard mix– injected when the DI approach was employed). Nevertheless, column performance, which is one of the most debatable and controversial aspects of this operating mode, was carefully checked. For that purpose, the standard mixture in MeOH (at a concentration level of 1 mg/L) and methanolic blanks were interspersed within the sequence every three and six samples, respectively. A decrease in retention times of about 20% was found in 50-analyses-sequences, although its overall effect was not very drastic, since the separation of the analytes remained acceptable within the sequence. Signal intensity in MS was dropping during the sequence, causing a reduction in peak area of about 20% after 60 injections; problem which was addressed by applying a correction factor to the integrated areas for each compound, considering a lineal decrease in MS signal intensity. Accordingly, a curve was obtained for each analyte by plotting its area in the methanolic standard mix (injected every three analyses) versus the injection number (good linearity was found for these curves, with correlation coefficients higher than 0.9987). Then, a correction factor for each analysis and every substance under study was interpolated in these curves and applied to the integrated areas in all the chromatograms. After each sequence, the column cleaning protocol previously reported by our team [34] was used; this cleaning strategy together with a simple spray shield cleaning process with isopropanol/water (50:50, v/v) brought the retention times and MS signal back to their original values. In other words, the column was returned to its original state after the cleaning and regeneration process.

Calibration curves in blank matrix were established for each analyte with quantitative purposes (sunflower oil was considered as a triterpenoids-free oily sample or blank sample), following a similar approach as the one described by Olmo-García *et al.* in the above mentioned publication. The same concentration levels as those used for external calibration were considered. Once the areas of the analytes in the samples under study were properly corrected, they were interpolated in the corresponding blank matrix calibration curves. The quantitative data obtained by this approach are presented in Table 2c. As can be seen in the table, the minor triterpenoids could not be properly detected with this methodology. Nonetheless, the quantitative results show the potential of DI for the analysis of the main triterpenic compounds found in VOO (MA, OA and ER).

3.5. Overall view

The prevailing goal of this work was to develop a LC-MS method for triterpenic compounds determination in olive oil. As extensively explained in section 3.2., after carrying out the methodological optimization and validating the developed method, it was applied to the analysis of 11 VOO samples. As already stated, to compare the quantitative results reached by LC-MS with the ones achieved by a more standardized method, a GC-MS methodology (based on previous reports) was optimized and applied to the analysis of the same sample set.

Fig. 2 shows the chromatograms of an Arbosana VOO UAE extract and the standard mix containing the six triterpenic compounds under study in both LC-MS and GC-MS. As can be perceived in the figure, both LC and GC led to an adequate separation of the six triterpenic compounds, exhibiting proper resolution and good peak shape, however, the analysis time was shorter in LC (5 min versus 22 min approx.). In the VOO chosen to exemplify the figure -Arbosana sample-, UV could not be detected in the GC-MS chromatogram; fact which can be explained taking into account that LODs were much lower in LC-MS, platform that determined 1.0 mg/kg as UV content. Something similar was observed for BA, which showed a concentration value of 0.21 mg/kg when determined by LC-MS, but it was not detected by GC-MS. By using both platforms, MA, OA and ER were satisfactorily determined in all the oils; however, BA, UA and UV were just found in few examples.

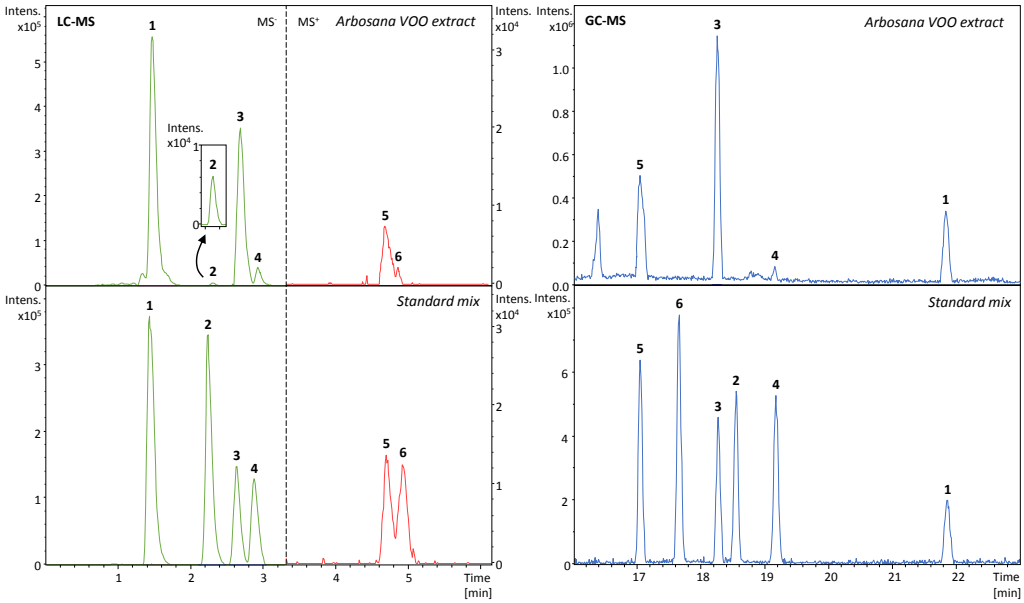

Figure 2. Left: Extracted ion chromatograms obtained in LC-MS (using negative ionization mode for triterpenic acids and positive polarity for alcohols) for an Arbosana VOO extract (upper part of the figure) and a standard mix (lower chromatogram). Right: Base peak chromatograms obtained in GC-MS in positive polarity (samples are the same as for LC-MS). Peak identification numbers: 1, maslinic acid; 2, betulinic acid; 3, oleanolic acid; 4, ursolic acid, 5, erythrodiol, and 6, uvaol.

Besides, the DI of VOO (after a simple dilution in acetone) in LC-MS was proposed as an alternative with a clear advantage: the easiness of the sample preparation. Table 2c summarizes the found amounts by DI of each analyte in the 11 samples under evaluation. MA and OA were found in every sample, BA was determined just in Arbequina VOOs, and ER was at concentration levels beyond the LODs for Arbequina, Arbosana, Haouzia, Koroneiki and Manzanilla. UA and UV were not determined by this strategy in any sample. In general, this DI strategy was somewhat less sensitive than GC-MS and LC-MS (the two last ones combined with UAE).

All the quantitative data of Tables 2a, b and c were subjected to a statistical data treatment to evaluate the similarity among the results coming from the three methodological approaches used within this study. ANOVA test demonstrated that no statistical differences (at a 95% confidence level, $p < 0.05$) were found among the values obtained by the three alternatives (when detected in all of them). This fact led us to make these assertions: i) the herein proposed LC-MS method is a reliable and tangible alternative to GC, which can be even faster and avoid the need of derivatization; and ii) DI strategy could represent another promising and trustworthy resource, in particular when the analyst is interested on establishing the concentration levels of the most abundant triterpenic acids and dialcohols.

Apart from determining the analytes in the 11 selected samples, the olive oil mix composed by equivalent volumes of all the VOOs (QC sample) was also analysed by the three strategies. Results are included in Tables 2a, b and c and it is worthy to underline that -for the compounds detected with all the methodologies- the concentration levels were in good agreement. Additionally, the found amounts were very similar to the theoretical or putative values presupposed for the QC for each compound (estimation which can be made averaging the measured amount of each analyte in the 11 selected oils).

Having a look at the quantitative results of Table 2a, b and c, Koroneiki was the variety showing the highest content of triterpenic compounds, whereas Langedoc exhibited the lowest concentration levels. MA and OA were found in every sample. MA values fluctuated between 13.2 and 71 mg/kg, in Langedoc and Koroneiki (LC-MS with UAE data), respectively. OA was found at levels oscillating between 4.3 and 36 mg/kg in the same varieties. Arbosana was the only VOO sample in which the six triterpenic compounds under study could be determined. The triterpenic acids found at undermost levels were UA (merely found at upper levels than LOD in Arbosana, with 1.2 mg/kg), and BA (which was just determined in Arbequina (0.34 mg/kg) and Arbosana (0.21 mg/kg)). To the best of our knowledge, this is the first time that BA concentration has been determined in an olive oil sample. In fact, in some previously published works, this compound was used as internal standard [4,8,9,17]. As mentioned in the introductory section, few references containing data about the triterpenic content of VOO can be found in literature, but the pentacyclic acids are very scarcely quantified in terms of the pure standard of each analyte (MA, UA and OA) [4]. In the just quoted work, the concentration ranges were slightly higher than those presented in Table 2. Similar contents for the major triterpenic acids were described by Allouche and coworkers for monovarietal VOOs [8] and for oils prepared under different technical conditions [9]. However, they found significantly higher amounts of triterpenic dialcohols (quantified with respect to betulin pure standard). The ranges of ER and UV found in another interesting publication [26] -although expressed in terms of cholestanol- are much closer to those presented in Table 2. All the existing results agree in the prevalence of ER over UV. Indeed, UV was only determined in Arbosana and Koroneiki, with 1.0 and 1.4 mg/kg, respectively, whilst ER values where higher in all the cases, as expected, varying from 0.9 mg/kg in Langedoc to 7.8 mg/kg in Koroneiki VOO.

4. CONCLUSIONS

The relevance of triterpenic acids and dialcohols from *Olea europaea* is unquestionable nowadays, finding numerous reports describing and demonstrating their biological activities. GC with FID or MS as detectors is considered the gold standard tool in this field. Few examples can be also found in literature regarding their determination in the mentioned matrix by LC-MS, but generally without giving quantitative data of each analyte in terms of their own standard (but referring them to another analyte) and not determining both triterpenic acids and dialcohols within a single run. A faster and reliable alternative (which no need of any derivatization step) has been developed and validated in the current contribution, demonstrating its applicability to VOOs coming from 11 olive varieties and proving that the obtained data are in good agreement with those achieved by GC-MS. Besides, the achieved data were also comparable to those derived from the use of a third strategy (DI), proffering an additional methodology for the accurate determination of the most abundant pentacyclic triterpenes.

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Chapter 3

Potential of LC coupled to fluorescence detection in Food Metabolomics: Determination of phenolic compounds in virgin olive oil

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Abstract: A powerful chromatographic method coupled to a fluorescence detector was developed to determine the phenolic compounds present in virgin olive oil (VOO), with the aim to propose an appropriate alternative to liquid chromatography-mass spectrometry. An excitation wavelength of 285 nm was selected and four different emission wavelengths (316, 328, 350 and 450 nm) were simultaneously recorded, working therefore on "multi-emission" detection mode. With the use of commercially available standards and other standards obtained by semipreparative high performance liquid chromatography, it was possible to identify simple phenols, lignans, several complex phenols, and other phenolic compounds present in the matrix under study. A total of 26 phenolic compounds belonging to different chemical families were identified (23 of them were susceptible of being quantified). The proposed methodology provided detection and quantification limits within the ranges of 0.004–7.143 $\mu g \cdot mL^{-1}$ and 0.013–23.810 $\mu g \cdot mL^{-1}$, respectively. As far as the repeatability is concerned, the relative standard deviation values were below 0.43% for retention time, and 9.05% for peak area. The developed methodology was applied for the determination of phenolic compounds in ten VOOs, both monovarietals and blends. Secoiridoids were the most abundant fraction in all the samples, followed by simple phenolic alcohols, lignans, flavonoids, and phenolic acids (being the abundance order of the latter chemical classes logically depending on the variety and origin of the VOOs).

Keywords: fluorescence detection; olive oil; phenolic compounds; secoiridoids; food metabolomics.

1. INTRODUCTION

Virgin olive oil (VOO), the juice of the olive obtained by pressing, is one of the few oils that are consumed without any further refining process. For that reason, it contains several bioactive molecules (vitamins, carotenoids, tocopherols, phenolic compounds, and some other natural antioxidants), which may act, by different mechanisms, as an effective defense against reactive oxygen substances [1]. Among its several minor constituents, phenolic compounds attract considerable attention because of their connection with some healthy benefits, including the prevention of chronic diseases such as cancer, obesity, diabetes, or coronary diseases [2,3]. Moreover, they contribute to the stability of VOO against auto-oxidation and have an important role in its organoleptic properties (bitterness, pungency, and astringency) [4,5]. These metabolites are also important from a commercial point of view, since the phenolic profile of a VOO can be a very useful distinctive feature to assure, for instance, its geographical origin [6,7] or authenticate its variety [8,9], which are two criteria considered by the international protection labels of geographical indications (*e.g.* Protected Designations of Origin and Protected Geographic Indications). Bearing in mind all the reasons just given, the importance of this group of secondary metabolites seems undeniable; indeed, over the last decade, these compounds have been considered as relevant targets in the field of Food Metabolomics.

In addition, declaring the phenolic content of a VOO in its label can represent a strategy for attracting consumer's attention, since international organisms such as the European Food Safety Authority (EFSA) have approved the use of health claims concerning olive oil phenolic compounds. Their scientific panel has recognized a relationship between the consumption of olive oil phenolic compounds and the protection of LDL particles from oxidative damage [10]. The European Union (EU), as the world leading olive oil producer, established in 2012 a list of permitted health claims made on foods and restricted the use of the just mentioned claim to olive oils which contain at least 5 mg of hydroxytyrosol (HTY) and its derivatives (*e.g.* oleuropein complex and tyrosol (TY)) per 20 g of olive oil [11].

Nevertheless, there are some issues that are depriving the producers and consumers of the benefits derived from this regulation. As stated in a very interesting recent report [12], two main problems should be addressed: (a) the lack of clarity in terminology ("olive oil" and "polyphenols" are terms which are sometimes not properly used; even the EU conditions of use of the claim are not clearly formulated); and (b) the absence of a suitable analytical protocol for the determination of the bioactive compounds behind the claim. A more comprehensive discussion concerning the first specified problem is perhaps beyond the scope this manuscript. With regard to the second one, different approaches have been used so far: determination of the total content on phenolic compounds (colorimetric methods, using the Folin–Ciocalteu reagent); assessment of total HTY and TY content by liquid (LC) or gas chromatography after appropriate sample preparation (hydrolysis of bound forms); application of nuclear magnetic resonance procedures; profiling and individual determination of the phenolic compounds by using powerful chromatographic methodologies, etc. LC methods can be pointed out among the preferred ones, because they allow the individual determination of phenolic compounds and are simple, repeatable and easily adapted to routine laboratories. The lack of commercially available pure standards (only accessible for some of the phenolic compounds found in VOO) is a serious problem, since this fraction is considerably complex. It is composed by a heterogeneous mixture of compounds belonging to different families with varying chemical structures (simple phenolic alcohols, phenolic acids, flavonoids, lignans, secoiridoids, etc.). At least 32 structurally distinct phenolic compounds have been identified in this matrix [1].

Although LC has been used coupled to different detectors, mass spectrometry (MS) is becoming almost mandatory to overcome the above-mentioned issue (absence of appropriate pure standards) [1,13]. Unfortunately, this detector is not always available in routine laboratories due to its high acquisition and maintenance costs. Some other less expensive detection techniques such as UV absorption or fluorescence (FL) [1,14] could be, therefore, good alternatives. FL has been used considerably less than UV detection, although it shows, in some cases, higher selectivity and sensitivity, so it could be offered as a robust and reliable alternative to MS detection systems.

A limited number of LC-FL methods in the field of olive oil analysis have been published, and they have been mainly focused on the determination of few compounds belonging to some

particular families such as lignans [15,16], phenyl alcohols and phenolic acids [17–20] or phenyl alcohols and secoiridoid derivatives [21–24]. In fact, the use of additional detectors was imperative in most of the cases to determine a major number of phenolic compounds. Nevertheless, the molecular structure of phenolic compounds makes them potentially detectable by a fluorescence detector (FLD), as they are natural fluorophores (that typically contain aromatic groups, or combined π bonds) which absorb energy of a specific wavelength and emit it at another particular higher wavelength (with less energy), depending on their structure and chemical environment [25–27].

The aim of the present work has been to develop a LC-FLD method for the identification and quantification of a noteworthy number of phenolic compounds in VOO samples belonging to different chemical classes. After a deep evaluation of the spectral behavior of the compounds under study (using commercial standards and standards obtained by semipreparative high performance liquid chromatography (HPLC)), a multi-emission wavelengths strategy was pointed out as optimum, and it gave us the possibility of characterizing simple phenols, lignans, several complex phenols and other phenolic compounds within a single run. This is the first method implying the use of a FLD, which is able to achieve qualitative and quantitative information of about 23 phenolic compounds.

2. RESULTS AND DISCUSSION

2.1. Preliminary FLD Study and Compounds Identification

The FLD method development obviously began with a literature review, trying to identify a promising starting point regarding excitation and emission wavelengths. Apart from the interesting information included in previously published manuscripts [2–5], we took into account the fact that an absorption spectrum is a good starting point for selecting the excitation wavelength of an analyte, because the spectrum indicates which energy is absorbed to excite an electron to a higher quantum state. The absorption maximum, quite often, is pretty similar to the excitation maximum, so 240 and 280 nm were selected in this case in a first stage of our study, as excitation wavelengths to start the deep and rigorous fluorescence characterization of the substances under evaluation. In a subsequent step, zero order emission mode was used (fixing the excitation first at 240 nm and, then, at 280 nm). This kind of emission mode sets the monochromator so that all light emitted from the sample will be reflected onto the detector regardless of the emission spectrum. In such a way, we collected relevant information about the maximum emission wavelengths (325, 360 and 450 nm), afterwards fixing those values to carry out zero order-excitation spectra, for re-evaluating therefore the suitability of the initially selected excitation features (maximum λ_{exc}). In this sort of excitation mode, the full spectrum of light from the Xenon lamp illuminates the flow cell and each compound can absorb its distinctive wavelength of light and then emit maximum fluorescence.

Bearing in mind the complexity of the phenolic fraction under study and the number of analytes which composed the extracts, the criteria considered to select the optimum excitation

and emission wavelengths were: (1) to have the possibility of determining in VOO samples as many compounds as possible (belonging to different chemical classes) within a single run; (2) to increase, if possible, the selectivity by using proper wavelengths in multi-excitation or multi-emission conditions; and (3) to favor the achievement of a method with the best possible sensitivity and enhanced analytical parameters.

After the preliminary studies and trying to sweep the whole excitation range, four zero order-emission spectra were recorded, fixing 210, 236, 285 and 300 nm as λ_{exc}. As a result of their evaluation, 285 nm seemed to be the most adequate excitation wavelength to start with (further details in Section 2.2). Setting this value, a 3D plot was acquired with zero order emission; this kind of plot displays a three dimensional image of the data file including spectra. The x-axis represents the retention time, the y-axis the wavelength and the z-axis the emission signal of the sample. The 3D plot shows peaks belonging to every fluorescent compound in the sample, which appear at different depth in the z-axis depending on its emission wavelength. It gives to the analyst the chance to explore the spectral landscape from a complete run, since by tilting, swiveling or turning the graphic it is possible to reveal hidden analyte characteristics in complex mixtures.

At this point and with the aim of achieving the identity of as many peaks as possible within the profiles, before keep going with the FLD optimization, we used different kind of standards (commercially available standards and the previously isolated phenolic compounds by semi-preparative HPLC), as well as a MS detector coupled to the HPLC-FLD instrument. Therefore, the identities of the phenolic compounds were established by comparison of these 3D plots with MS chromatograms for standard mix solutions, VOO samples and fortified samples (taking into account the retention time of each peak). Fig. 1 shows, in the upper part, a 3D FL chromatogram (λ_{exc} = 285 nm and zero order emission) of a fortified extra-VOO extract; and in the lower part, the equivalent MS chromatogram (in both cases, peaks are identified with the pertinent numbers). In this figure, apigenin (Api) peak can only be found in the MS chromatogram (it does not appear in FLD 3D plot) because this compound does not fluoresce with enough intensity (regarding fluorescence efficiency, the same was observed for the isolated standards of elenolic acid (EA) and decarboxymethyl oleuropein aglycon (DLA) whose FL signal was undetectable).

Table S1 (supplementary) shows the retention time, molecular formula, chemical structure, the family of compounds at which they belong to, and the optimum spectral parameters (obtained in methanol) of the phenolic compounds which could be quantified by our method. The table also includes the FL emission channel at which each compound will be more satisfactorily detected (this point is extensively explained in the following section). Apart from the compounds identified in the sample in Fig. 1, three more compounds can be seen in the Table S1: oxidized hydroxytyrosol (OxHTY), hydroxytyrosol acetate (AcHTY) and decarboxymethyl oleuropein aglycon (DOA). In total, 23 compounds (and seven isomers) are presented in this table, which were further quantified in the samples. In addition to the compounds that were quantified, hydroxy elenolic acid,

syringaresinol and 10-hydroxy oleuropein aglycone, which have not been previously reported using FLD, were found in some of the samples.

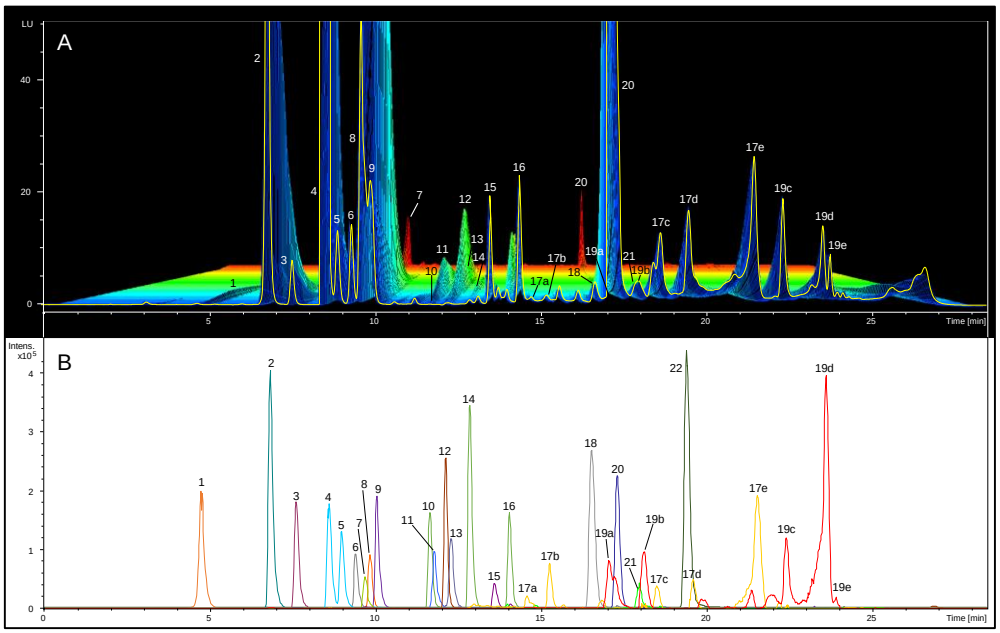

Figure 1. (A) 3D-plot of a fortified extra-VOO extract with eighteen phenolic compounds at a final concentration level of 10 µg·mL^{-1}, when excitation wavelength is set at 285 nm and the zero order emission spectra is recorded; (B) Extracted ion chromatograms (EICs) of the known phenolic compounds for the same fortified extract as in (A), obtained in electrospray ionization–ion trap MS (ESI-IT MS) detector (using negative ionization mode).

Peak identification numbers: (1) Gallic acid (Gal); (2) HTY; (3) 3,4-dihydroxyphenylacetic acid (DOPAC) (used as internal standard (IS)); (4) TY; (5) 4-hydroxybenzoic acid (4-HBA); (6) 4-hydroxyphenylacetic (4-HPA); (7) vanillic acid (Van); (8) syringic acid (Syr); (9) homovanillic acid (Hmvan); (10) *p*-coumaric acid (*p*-Cou); (11) vanillin (Val); (12) sinapic acid (Sin); (13) ferulic acid (Fer); (14) *m*-coumaric acid (*m*-Cou); (15) oleuropein (Ole); (16) *o*-coumaric acid (*o*-Cou); (17) oleuropein aglycon (OleAgly) and isomers; (18) luteolin (Lut); (19) ligstroside aglycon (LigAgly) and isomers; (20) (+)-pinoresinol (Pin); (21) acetoxypinoresinol (AcPin); and (22) Apig. As far as the different isomers of OleAgly and LigAgly are concerned, they are identified by adding a letter (a–e) to the number assigned for the main isomer.

2.2. Optimization of Detection Conditions

After the preliminary evaluation of the FL conditions and achieving the identification of an important number of analytes within the profiles, we got ready to face the final optimization of the conditions for fluorescence detection (obviously under the optimum LC conditions), as well as to select the most appropriate fluorescence mode (segments with time programming, multi-channels, etc.). For that purpose, the standard mix solution, the isolated phenolic compounds, the quality control (QC) and fortified sample extracts were used.

As stated in the previous section, the establishment of a proper starting value for excitation wavelength was the first stage of the detection method development; 285 nm was the one that generated a chromatogram with the major number of peaks. The zero order emission spectrum obtained while setting excitation wavelength at 285 nm was studied in depth in order to find the emission maximum for each phenolic compound (apart from those logically found in the preliminary study). As many zero order excitation and emission spectra as necessary (selecting different wavelengths and conditions) were done in order to identify the maximum λ_{exc} and λ_{em} for each compound. The resulting values are included in Table S1.

After corroborating the excitation and emission wavelengths that produced the most promising FL chromatograms, it was required to choose the most appropriate operational mode. Our FLD was able to work in the following modes: 3D spectral mode (for the rapid online acquisition of full excitation and emission spectra); time programming of spectral (where a timetable is created by the analyst defining different segments where diverse FL conditions are applied (excitation and emission wavelengths, PMT gain, bandwidth, attenuation, etc.)); and multi-emission (where an excitation wavelength is fixed and different emission wavelengths are simultaneously recorded) or multi-excitation mode. The potential of the first mentioned mode (3D spectral mode) was already used in previous stages of the current study. The usefulness of the second one—the creation of segments with different emission wavelengths during the run—was investigated in order to adequate the detection conditions in each segment for the compounds eluting at those retention times and, therefore, to be capable of determining all the phenolic compounds in only one chromatogram; in other words, defining different analytical windows within the same chromatographic trace. When this mode was selected, it was necessary to overcome several difficulties: (1) great number of segments needed (at least 20) to assure that each phenolic compound was determined by using the optimum wavelengths; (2) the fact that the change of the conditions between two segments was sometimes drastic and it caused the loss of information when the peaks next to the edge of the segment were too close (the instrument requires some time to change the operational parameters); and (3) the remarkable fluctuations of the baseline when using different FL conditions, fact which made the obtained chromatograms very difficult to process.

The third operational mode, multi-channel (in particular, multi-emission mode), was then used. A wavelength of 285 nm had been clearly pointed out as optimum excitation wavelength. Considering the results included in Table S1 regarding the maximum λ_{em} and trying to look for a compromise solution limiting the number of selected wavelengths for this stage of the optimization, four λ_{em} were chosen: 316, 328, 350 and 450 nm. These values allowed the determination of an important number of analytes with an adequate intensity (at very close emission wavelengths to the optimum found for each substance) with the best possible selectivity. In this mode the FLD was able to monitor phenols separation simultaneously in four channels with different emission wavelengths (while maintaining constant the excitation wavelength). In addition,

when the emission of an analyte saturates the detector, its quantification can be made in a less sensitive wavelength. This is an important advantage to determine analytes whose concentration range varies a lot depending on the selected sample (as happens with phenolic compounds extracted from different VOOs).

Fig. 2 shows the chromatograms of a standard mix with 18 phenolic compounds and a sample of olive oil from Picual variety; four traces are presented, corresponding to the four emission channels in the FLD. Although some of the analytes under study could be detected by using different chromatographic traces, they have been just numbered in the most adequate one (which is also presented in Table S1). Nevertheless, Ole and TY are numbered in the traces that have been finally used for their quantification (traces achieved by using a λ_{em} value that does not match with their maxima λ_{em} shown in Table S1). Ole was detected at 316 nm instead of 328 nm because all secoiridoid derivatives, whose optimum λ_{em} was found at 316 nm, were quantified with respect to its calibration curve and it seemed to be more adequate to detect all of them at the same λ_{em}. With regard to TY, its optimum λ_{em} was found at 316 nm but, as signal saturation at low concentration levels was observed, it was more favourable to quantify it at 350 nm. In the extract of Picual olive oil, 19 compounds (with 7 isomers; 5 of OleAgly and 2 of LigAgly) could be detected; 14 of them could be quantified if found within the linear dynamic range of the method (in terms of their own standards (if available) or using for that purpose another structure-related compound). The list of the compounds identified in the caption to figure by using Roman numbers, includes three substances which have not been previously determined by FLD (hydroxy elenolic acid, syringaresinol and 10-hydroxy oleuropein aglycone) and two mass isomers of HTY and Pin, respectively, which were found at min 10.0 and 18.7 but whose identity has not been confirmed.

Concerning the fluorescence behavior of the different compounds, it seems convenient to mention that cyclic molecules may have activators, such as –OH and –OR groups substituted in *o*- or/and *p*-positions, which produce an enhanced fluorescence, and deactivators such as –COOH, –COOR, –CHO and –COR groups substituted in *m*- positions, which induce a decrease of the fluorescence [28]. Therefore, in general, phenyl alcohols (OxHTY, HTY, TY and AcHTY) and lignans (Pin and AcPin) which have an activator group in *p*-position, are very susceptible to be detected by FL, producing peaks of considerable intensity within the profiles; and secoiridoid derivatives (with a phenyl alcohol group within their structure) can be also properly detected. A lower relative response is observed in those structures that contain a dialdehydic group in the open elenolic acid ring, since minor planarity of the molecule gives reduced fluorescence intensity [22]. Flavonoids also showed a very low fluorescence intensity (Api was not detected even at high concentrations), but this fact should not be considered as an issue or a factor with detrimental effect on the potential of the method, since it is well known that these compounds can be easily quantified at 330 nm with a UV detector (we indeed had diode-array detector (DAD) connected in series). The spectral characteristics of the phenolic acids included in our standard mix logically depends on their structure, so we could group these acids in two main classes: compounds with a benzoic-like

structure (Gal, 4-HBA, Van, and Syr) whose optimum λ_{em} is 350 nm, and compounds with a hydroxycinnamic-like structure (*p*-Cou, Sin, Fer, *m*-Cou, and *o*-Cou) whose optimum λ_{em} is 450 nm. Nevertheless, 4-HPA which has a different kind of structure and Hmvan which is one exception with benzoic-like structure, have their optimum λ_{em} at 316 nm.

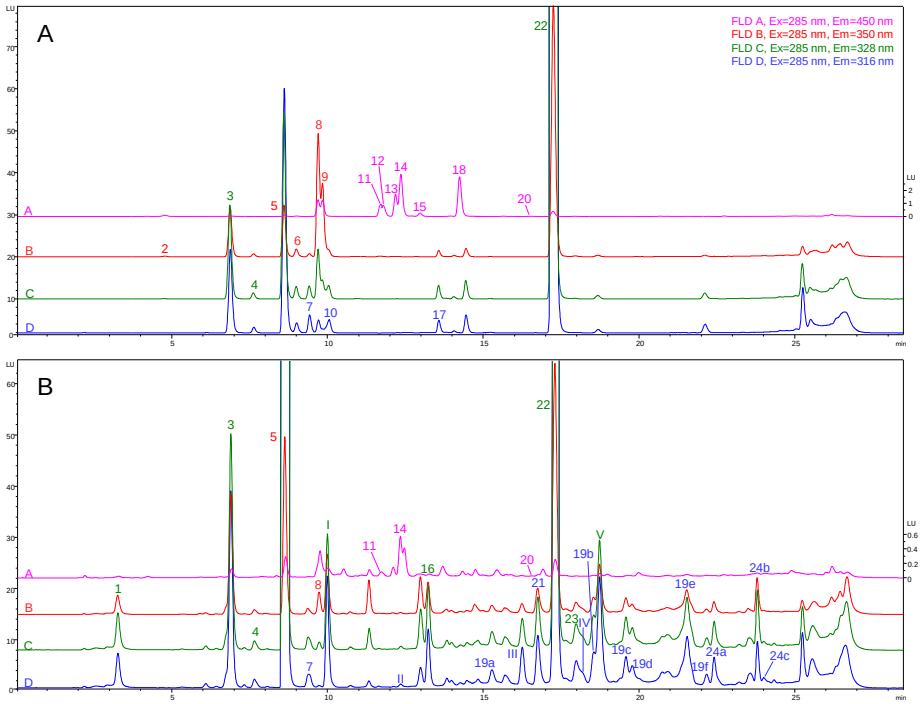

Figure 2. Chromatograms of: the standard mix with eighteen phenolic compounds at a concentration level of 10 µg·mL^{-1} (A); and a sample of olive oil from Picual variety (B).

Peak identification numbers: (1) OxHTY; (2) Gal; (3) HTY; (4) DOPAC (IS); (5) TY; (6) 4-HBA; (7) 4-HPA; (8) Van; (9) Syr; (10) Hmvan; (11) *p*-Cou; (12) Val; (13) Sin; (14) Fer; (15) *m*-Cou; (16) AcHTY; (17) Ole; (18) *o*-Cou; (19) OleAgly isomers (20) Lut; (21) DOA; (22) Pin; (23) AcPin; and (24) LigAgly isomers. As far as the different isomers of OleAgly and LigAgly are concerned, they are identified by adding a letter (a–f) to the number assigned for the main isomer. Peak identification codes for those compounds which were not quantified: (I) hydroxytyrosol mass isomer; (II) hydroxy elenolic acid; (III) syringaresinol; (IV) 10-hydroxy oleuropein aglycone; and (V) pinoresinol mass isomer.

After deciding to maintain four emission channels, the very last step in the FL conditions optimization consisted of trying to maximize the fluorescent signals achieved. In this regard, we can stand out that an increase of one unit in the PMT gain parameter doubled the signal of the analytes, but also affected to the noise and declined the signal to noise ratio by more than a 30%. A PMT of 10 was the value finally selected for this parameter.

2.3. Method Validation

Validation studies were carried out to check the analytical performance of the developed method. Both the standard mixture containing the 18 phenolic compounds and the QC sample

(serially diluted or fortified at different concentration levels) were used at this stage of the study. The calculated analytical parameters of the method are summarized in Tables 1 and 2.

Table 1. Analytical parameters related to the evaluation of accuracy and matrix effect of the developed LC-FLD method.

Compound	Accuracy					Matrix Effect		
	Intra-Day Repeatability (%RSD)[a]		*Inter*-Day Repeatability (%RSD)[b]		Trueness (%)[c]	Solvent Calibration Slope	Matrix Calibration Slope	Matrix Slope/ Solvent Slope
	Area	t_r	*Area*	t_r				
Gal	2.72	0.39	5.70	0.41	92.83	0.127	0.137	1.08
HTY	6.80	0.34	6.91	0.40	103.66	10.226	9.041	0.88
TY	2.17	0.26	3.40	0.47	90.08	6.239	5.257	0.86
4-HBA	3.34	0.17	4.27	0.46	101.98	0.880	0.983	1.12
4-HPA	3.68	0.19	4.66	0.50	85.49	2.012	2.104	1.05
Van	3.68	0.21	3.74	0.51	101.01	14.276	13.384	0.94
Syr	3.49	0.25	4.80	0.54	104.71	6.770	6.748	1.00
Hmvan	9.05	0.24	11.98	0.56	87.53	1.244	1.273	1.02
***p*-Cou**	2.94	0.18	3.51	0.58	86.03	0.272	0.285	1.05
Val	2.94	0.23	4.42	0.53	85.28	0.299	0.323	1.08
Sin	3.75	0.26	4.38	0.63	80.55	0.618	0.678	1.10
Fer	2.35	0.24	2.79	0.61	88.06	1.287	1.410	1.10
***m*-Cou**	3.33	0.21	5.93	0.63	111.81	0.083	0.087	1.05
Ole	4.32	0.34	6.83	0.81	112.28	1.246	1.411	1.13
***o*-Cou**	2.52	0.24	2.92	0.70	84.27	1.170	1.288	1.10
Lut	*	*	*	*	100.74	0.009	0.011	1.16
Pin	2.35	0.36	2.13	0.99	100.46	40.452	44.258	1.09

[a] %RSD values for peak areas or retention times of the analytes under study measured from five injections of the fortified QC carried out within the same sequence; [b] %RSD values for peak areas or retention times of the analytes under study measured from five injections of the fortified QC (belonging to five different sequences carried out over five days); * Lut was found under the detection limits in the fortified QC, so its repeatability could not be measured; [c] Trueness was measured by calculating the recovery (%), and it was estimated by analyzing the same sample extracted before and after the standard addition and calculating the difference between the obtained results. The values included in this table are those achieved for the intermediate concentration level (except for Lut, whose values belong to a higher concentration level).

Method accuracy was assessed in terms of precision and trueness. The first one was evaluated by means of the *intra*-day and *inter*-day repeatability studies, by calculating the relative standard deviation (%RSD) of the retention time and peak area of the analytes under study. The *intra*-day precision was expressed as the %RSD obtained for five injections of the QC carried out within the same sequence and its values were below 0.39% (for retention time) and 9.05% (for peak area), for Gal and Hmvan, respectively. The *inter*-day precision was the %RSD of five replicated injections of the QC belonging to five different sequences carried out over five days. Its values were below 0.99% (for retention time) and 11.98% (for peak area), for Pin and Hmvan, respectively. The trueness was expressed as recovery (%), which was calculated by analyzing the same sample extracted

before and after the standard addition. Three concentration levels (low, intermediate and high) of the pure standards (within their linear range) were tested and resulting recovery values were found between 80.55% and 112.28%, for Sin and Ole, respectively.

Table 2. Comparison between calibration functions, determination coefficients, LODs, LOQs and lineal dynamic ranges for the FLD and the MS detectors.

Analyte	Detector	External Calibration Curve	R^2	LOD ($\mu g \cdot mL^{-1}$)	LOQ ($\mu g \cdot mL^{-1}$)	Linear Range ($\mu g \cdot mL^{-1}$)[a]
Gal	FLD	y = 0.127x + 0.1263	0.9987	0.625	2.083	250
	MS	y = 40519x + 37647	0.9965	0.051	0.171	100
HTY	FLD	y = 10.226x + 2.1124	0.9989	0.035	0.115	20
	MS	y = 52263x + 55720	0.9945	0.017	0.057	50
TY	FLD	y = 6.239x + 3.3395	0.9993	0.009	0.029	100
	MS	y = 20379x + 17714	0.9924	0.058	0.195	50
4-HBA	FLD	y = 0.880x + 2.804	0.9954	0.036	0.121	250
	MS	y = 16457x + 49330	0.9911	0.061	0.204	150
4-HPA	FLD	y = 2.012x + 2.6778	0.9982	0.357	1.190	250
	MS	y = 13242x – 2729.7	0.9938	0.122	0.407	150
Van	FLD	y = 14.276x – 0.4684	0.9989	0.004	0.013	20
	MS	y = 16919x + 11618	0.9956	0.025	0.084	10
Syr	FLD	y = 6.770x + 8.7496	0.9861	0.007	0.023	50
	MS	y = 27088x + 14618	0.9928	0.025	0.083	10
Hmvan	FLD	y = 1.244x + 0.4454	0.9981	0.556	1.852	250
	MS	y = 31576x – 42195	0.9983	0.155	0.515	50
p-Cou	FLD	y = 0.272x + 1.036	0.9932	0.031	0.103	250
	MS	y = 40281x + 21180	0.9941	0.018	0.059	20
Val	FLD	y = 0.299x + 0.631	0.9974	0.090	0.301	250
	MS	y = 12440x + 7246.2	0.9927	0.055	0.182	10
Sin	FLD	y = 0.618x + 0.8065	0.9986	0.039	0.132	250
	MS	y = 58259x + 28312	0.9939	0.021	0.069	10
Fer	FLD	y = 1.287x + 3.1028	0.9965	0.021	0.069	250
	MS	y = 39342x + 13674	0.9949	0.022	0.073	10
m-Cou	FLD	y = 0.083x + 0.122	0.9995	0.306	1.020	250
	MS	y = 58990x + 130287	0.9934	0.013	0.043	100
Ole	FLD	y = 1.246x + 0.1575	0.9998	0.273	0.909	250
	MS	y = 6709x + 86	0.9990	0.050	0.165	20
o-Cou	FLD	y = 1.170x + 3.713	0.9954	0.022	0.072	250
	MS	y = 33291x + 12614	0.9946	0.077	0.255	100
Lut	FLD	y = 0.009x + 0.0064	0.9984	7.143	23.810	250
	MS	y = 201100x + 44947	0.9994	0.005	0.016	10
Pin	FLD	y = 40.452x + 7.795	0.9991	0.005	0.016	10
	MS	y = 34634x + 63107	0.9912	0.032	0.108	50

[a] Linear ranges were established from LOQ to the indicated value.

Matrix effect was also evaluated in order to assess whether interferences may affect the spectral behavior of the phenolic compounds and hinder the proper quantification of the analytes under study. To do it so, a standard addition calibration curve was prepared by fortifying the QC with the standard mix at different concentration levels (within the range from 0.5 to 250 $mg \cdot L^{-1}$). Then, its slope was compared with the one of the external calibration curve, and the slope ratio (slope of the calibration curve in matrix/slope of the external calibration curve) was calculated for each analyte. If matrix does not affect the response of the phenolic compound being studied, the ratio between both slopes ($b_{matrix}/b_{solvent}$) should be between 0.80 and 1.20 [29]. Depending on the value of this ratio, different matrix effects could be observed: a value lower than 0.80 shows matrix suppression effect, whilst a value higher than 1.2 demonstrates matrix enhancement. As the values for the slopes ratio were found between 0.86 and 1.16 (for TY and Lut, respectively), the very low matrix effect observed made the external calibration appropriate for quantifying the phenolic compounds under study.

The linearity of the proposed method was checked by establishing solvent-based standard calibration curves within the range of 0.5 to 250 $mg \cdot L^{-1}$. A linear regression using the least-squares method was performed, and the peaks areas of each analyte (injected in triplicate) were plotted as a function of its concentration (eleven levels of concentration were tested). The responses properly fitted to a straight line with r^2 values between 0.9861 for Syr and 0.9998 for Ole. Additionally, the detection and quantification limits for each analyte were calculated as the concentrations that give a signal-to-noise ratio equal to 3 and 10, respectively. The LODs ranged from 0.005 $\mu g \cdot mL^{-1}$ for Pin to 7.143 $\mu g \cdot mL^{-1}$ for Lut, while the LOQs were found between 0.016 and 23.810 $\mu g \cdot mL^{-1}$, for the same compounds. The linear range was established from LOQ to 250 $\mu g \cdot mL^{-1}$ in most of the cases, except for the most fluorescent compounds (HTY, TY, Van, and Pin), which saturate the detector and, therefore, did not present such wide linear range (more details in Table 1).

As the LC was connected in series to the FLD detector and to an ESI-IT MS system, the validation of the LC-MS method was carried out simultaneously, calculating analogue parameters to those of the LC-FLD method. Some relevant analytical parameters directly related to the quantification, such as calibration curves equations, determination coefficients, LODs, LOQs and linear dynamic ranges are shown in Table 2. In this way, the analytical performance of the method proposed herein as an alternative to the widely used (for the determination of phenolic compounds from VOOs) LC-MS methodologies could be compared with the analytical figures of merit of a method using MS as detection system. As expected, linear ranges in FLD were wider than in MS detector, except for HTY and Pin which promptly produced signal saturation. In MS detector, LODs and LOQs were generally lower than in FLD, but some exceptions can be found in Table 2.

Since the evaluation of the linear range for compounds whose commercial standard is not available (such as secoiridoid derivatives) could not be performed by using the same approach as

the one previously described, it was necessary to use another strategy, which, to a certain extent, could be considered as a third kind of calibration. In this case, the dilution and pre-concentration of QC (between 1% and 200%) was carried out, obtaining eight calibration levels (1%, 10%, 20%, 50%, 75%, 100%, 150% and 200%). Dilution was obviously carried out adding controlled volume of methanol to the QC extract; on the contrary, the pre-concentration was done by evaporating the appropriate amount of QC extract and re-dissolving in the adequate volume. The linearity was then checked by representing the area of the selected compounds (OxHTY, OleAgly (main isomer and isomer 3), DOA, and LigAgly (main peak and its first isomer)) versus the relative concentration of the QC extracts (%). The mentioned peaks were chosen, since they could be properly detected and easily integrated even in the most diluted level. In every case, responses that could be fitted to linear equations were achieved. Indeed, the linear regression equations showed r^2 values between 0.9901 and 0.9993 for OleAgly's main isomer and OxHTY, respectively. The confirmation of this linear behaviour could guarantee that the quantification was being made within a range in which proportional responses for different concentrations of the analytes under evaluation would be obtained; that is a mandatory requirement to assure a correct quantitative determination.

To conclude this section, Table S2 provides an overview of some the main aspects (extraction procedure, instrumental platform used, injections needed, number of determined analytes, and FL wavelengths) of the methodology presented in the current contribution, compared with some other LC-FLD methods previously reported in literature. One of the major achievements of the new method is the great number of analytes that can be identified and quantified (26 and 23 phenolic compounds, respectively, belonging to each and every chemical type of the polyphenols found in olive oil). In contrast, 12 is the highest number of phenols which have been quantified with FLD (16 if it is combined with DAD) [5]. The simplicity of the novel method is quite remarkable too; one simple extraction procedure followed by a single injection is needed in this case, whilst the powerful method developed by Godoy-Caballero *et al.* [5] needs two different protocols for major and minor phenolic compounds extraction (liquid–liquid and SPE, respectively). Moreover, the multi-emission mode allows us to detect each compound, practically, by using the best possible FL conditions, whilst some other previously described methods detect all the phenolic compounds by means of a fixed combination of λ_{em}-λ_{exc} or have to use additional injections in different conditions [2].

2.4. Analysis of Phenolic Compounds in VOO Samples

Once the developed LC-FLD method was validated, it was applied to quantify 23 phenolic compounds in 10 olive oil samples from different varieties and origins. Both good peak shape and proper resolution were achieved for the compounds under study, as it can be corroborated in Fig. 2B, where the chromatograms obtained in the four emission channels for a Picual monovarietal VOO are presented.

Tables 3 and 4 summarizes the concentrations of the measured phenolic compounds in the selected olive oil samples. To reach our main goal and being able to propose a LC-FLD methodology as a proper and powerful alternative to LC-MS, the quantification of the phenolic compounds whose standard is commercially available was made with both detection systems (see Table 3). For the rest, OxHTY, AcHTY, AcPin and secoiridoid derivatives, the quantitative FL data are presented in Table 4; in those cases, MS data are not included, since these analytes are not quantified in terms of their own pure standard (but by using another standard with a related (but different) structure), and therefore, trying to compare FL and MS results is not a very straightforward task. In other words, we cannot try to establish a proper comparison between the fluorescent behavior and the MS ionization efficiency for compounds with different structures.

The levels of the glycosidic form of Oleuropein (Ole) and some phenolic acids (Gal, Syr, Hmvan, Sin, *m*-Cou and *o*-Cou) were found below the detection limits in all the samples belonging to our sample set (information which was corroborated by the two used detectors (data not shown to contain the size of Table 3)).

Taking into account the data included in Table 3, it is possible to say that the quantitative results from both detectors (FLD and MS) were in good agreement. Thus, no statistically significant differences were found regarding the determined amounts of HTY, TY, 4-HBA, 4-HPA, Van and Fer by both detectors. Blend 2 and Picual were the VOOs showing the highest concentrations of HTY and TY, while, in contrast, Arbequina 1 was the one exhibiting the lowest levels of HTY (0.46 $\mu g \cdot g^{-1}$) and, Blend 3 and Hojiblanca, the poorest in terms of TY (with 1.2 and 2.0 $\mu g \cdot g^{-1}$, respectively). 4-HBA was just found in Hojiblanca VOO, with a mean concentration value of 0.29 $\mu g \cdot g^{-1}$; and 4-HPA could be only determined in Picual oils (1.68 $\mu g \cdot g^{-1}$). As far as Van in concerned, Blend 3 was the sample presenting the highest amounts of this compound (0.66 $\mu g \cdot g^{-1}$). Arbequina 2 also exhibited remarkable concentration levels of Van (0.51 $\mu g \cdot g^{-1}$). Fer was found in six samples (its mean concentration levels varied from 0.058 to 0.166 $\mu g \cdot g^{-1}$, in Arbequina 1 and Arauco 1, respectively); in three others, it was not quantified; and Blend 3 was the only one in which it not detected.

FL and MS results were also in good agreement regarding, for instance, *p*-Cou. The sample Arauco 1 showed the highest mean levels of *p*-Cou (0.61 $\mu g \cdot g^{-1}$) and Arbequina 1 the lowest ones (0.11 $\mu g \cdot g^{-1}$). In two samples (Blends 1 and 3), however, its levels were found under the LOQ with FLD, whereas they could be properly quantified by the MS detector. Something similar was observed for Val, with some of the studied VOOs showing levels below LOD and LOQ with FLD, but properly quantified by MS (which is logical anyway bearing in mind the LOD and LOQ of each detection system given in Table 1). Arbequina 2 (0.51 and 0.52 $\mu g \cdot g^{-1}$, respectively, in FL and MS) and Blend 3 (0.66 and 0.70 $\mu g \cdot g^{-1}$, respectively, in FL and MS) were the two examples in which Val was quantified both by FL and MS, achieving statistically equivalent results. Lut could not be quantified in any sample by FL, fact which can be easily understood observing the LOD that this phenolic compound presented (Tables 1 and 2). Lut's MS values, however, are given for all of them

Table 3. Content of phenolic compounds with available commercial pure standard (expressed in $\mu g \cdot g^{-1}$) determined by FL and MS detectors coupled to LC.

Compound	Detector	Arbequina 1	Arbequina 2	Arauco 1	Arauco 2	Changlot	Hojiblanca	Picual	Blend 1	Blend 2	Blend 3
HTY	FLD	0.46 ± 0.03	5.0 ± 0.3	5.8 ± 0.4	4.3 ± 0.3	5.0 ± 0.3	0.66 ± 0.04	11.2 ± 0.8	3.0 ± 0.2	19.2 ± 1.2	1.6 ± 0.1
	MS	0.48 ± 0.03	4.8 ± 0.3	6.3 ± 0.4	4.2 ± 0.3	5.0 ± 0.3	0.74 ± 0.05	11.4 ± 0.5	2.8 ± 0.2	17 ± 1	1.5 ± 0.1
TY	FLD	2.8 ± 0.2	4.9 ± 0.3	13.9 ± 0.8	11.7 ± 0.8	6.8 ± 0.3	2.0 ± 0.1	14.5 ± 0.7	15.3 ± 0.9	24 ± 1	1.2 ± 0.1
	MS	3.1 ± 0.2	4.7 ± 0.2	15 ± 1	10.2 ± 0.7	6.2 ± 0.3	2.3 ± 0.2	15.8 ± 0.7	14.8 ± 0.6	24 ± 1	1.2 ± 0.1
4-HBA	FLD	n.d.	n.d.	n.d.	n.d.	n.d.	0.29 ± 0.01	n.d.	n.d.	n.d.	n.d.
	MS	n.d.	n.d.	n.d.	n.d.	n.d.	0.31 ± 0.01	n.d.	n.d.	n.d.	n.d.
4-HPA	FLD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.68 ± 0.07	n.d.	n.d.	n.d.
	MS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.72 ± 0.07	n.d.	n.d.	n.d.
Van	FLD	0.29 ± 0.02	0.45 ± 0.03	0.91 ± 0.06	0.39 ± 0.02	n.q.	0.43 ± 0.02	0.76 ± 0.04	2.0 ± 0.1	0.61 ± 0.02	n.d.
	MS	0.26 ± 0.02	0.41 ± 0.02	0.82 ± 0.04	0.43 ± 0.02	n.d.	0.40 ± 0.02	0.68 ± 0.04	1.8 ± 0.1	0.58 ± 0.03	n.d.
***p*-Cou**	FLD	0.11 ± 0.01	0.21 ± 0.01	0.61 ± 0.04	0.32 ± 0.02	0.25 ± 0.01	0.28 ± 0.02	0.38 ± 0.02	n.q.	0.15 ± 0.01	n.q.
	MS	0.13 ± 0.01	0.21 ± 0.01	0.57 ± 0.03	0.35 ± 0.02	0.25 ± 0.01	0.25 ± 0.02	0.36 ± 0.02	0.061 ± 0.003	0.17 ± 0.01	0.093 ± 0.005
Val	FLD	n.q.	0.51 ± 0.02	n.q.	n.q.	n.q.	n.q.	n.d.	n.q.	n.q.	0.66 ± 0.03
	MS	0.134 ± 0.007	0.52 ± 0.03	0.102 ± 0.004	0.113 ± 0.006	0.135 ± 0.007	n.q.	n.q.	n.q.	0.143 ± 0.008	0.70 ± 0.03
Fer	FLD	0.058 ± 0.002	0.081 ± 0.004	0.166 ± 0.005	0.076 ± 0.002	0.126 ± 0.005	n.q.	0.071 ± 0.002	n.q.	n.q.	n.d.
	MS	0.055 ± 0.002	0.080 ± 0.004	0.176 ± 0.006	0.074 ± 0.002	0.122 ± 0.005	n.q.	0.069 ± 0.002	n.q.	n.q.	n.d.
Lut	FLD	n.q.	n.q.	n.q.	n.q.	n.d.	n.q.	n.d.	n.d.	n.d.	n.d.
	MS	5.7 ± 0.3	4.4 ± 0.1	6.2 ± 0.3	5.3 ± 0.3	2.6 ± 0.1	7.0 ± 0.4	3.4 ± 0.2	1.22 ± 0.07	1.20 ± 0.06	3.0 ± 0.1
Pin	FLD	2.09 ± 0.09 *	3.0 ± 0.1	0.95 ± 0.04	0.90 ± 0.04 *	2.7 ± 0.1 *	0.81 ± 0.05	4.0 ± 0.2 *	3.1 ± 0.1 *	0.77 ± 0.05	3.3 ± 0.2
	MS	0.91 ± 0.05 *	3.0 ± 0.1	0.87 ± 0.05	0.39 ± 0.02 *	1.6 ± 0.1 *	0.71 ± 0.05	2.4 ± 0.1 *	1.3 ± 0.1 *	0.68 ± 0.05	2.9 ± 0.2

Every result is the average of three independent (sample preparation and injection) determinations (n = 3). The results are given by the mean value ± SD. n.d.: non detected; n.q.: non quantified; * Quantitative results from both FL and MS detectors have statistical significant differences (95%; $p < 0.05$).

Table 4. Content of phenolic compounds whose pure standard is not commercially available (expressed in μg·g^{-1}) achieved by LC-FLD.

Compound		Arbequina 1	Arbequina 2	Arauco 1	Arauco 2	Changlot	Hojiblanca	Picual	Blend 1	Blend 2	Blend 3
OxHTY		0.163 ± 0.008	1.87 ± 0.09	1.91 ± 0.09	0.19 ± 0.01	0.34 ± 0.02	0.122 ± 0.006	2.3 ± 0.1	n.q.	1.9 ± 0.1	0.51 ± 0.02
AcHTY		1.34 ± 0.07	0.47 ± 0.02	1.10 ± 0.06	0.41 ± 0.02	n.d.	0.27 ± 0.01	2.9 ± 0.2	n.d.	9.2 ± 0.5	0.20 ± 0.01
OleAgly	*Isomer 1*	0.74 ± 0.04	1.04 ± 0.05	2.7 ± 0.1	5.4 ± 0.3	n.d.	n.d.	n.q.	10.8 ± 0.5	1.10 ± 0.06	6.1 ± 0.3
	Isomer 2	n.d.	n.d.	n.d.	25 ± 1	19.2 ± 0.9	n.d.	15.8 ± 0.6	115 ± 6	48 ± 2	43 ± 2
	Isomer 3	1.50 ± 0.07	2.6 ± 0.1	11.7 ± 0.6	85 ± 4	58 ± 3	2.1 ± 0.1	n.q.	7.8 ± 0.4	13.3 ± 0.6	3.4 ± 0.2
	Isomer 4	2.0 ± 0.1	2.4 ± 0.2	5.5 ± 0.3	8.9 ± 0.5	3.3 ± 0.2	1.46 ± 0.07	12.7 ± 0.8	10.1 ± 0.5	n.d.	3.9 ± 0.2
	Main isomer	12.7 ± 0.6	7.5 ± 0.4	32 ± 2	147 ± 7	87 ± 4	6.5 ± 0.3	36 ± 2	24 ± 1	28 ± 1	11.1 ± 0.6
	Isomer 5	4.1 ± 0.2	3.1 ± 0.1	1.16 ± 0.04	3.1 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	4.3 ± 0.2	19.1 ± 0.8	3.7 ± 0.2	3.8 ± 0.1
	Total	**21.1 ± 0.6**	**16.6 ± 0.4**	**53 ± 2**	**274 ± 8**	**170 ± 5**	**12.8 ± 0.3**	**69 ± 2**	**187 ± 6**	**94 ± 1**	**72 ± 2**
DOA		12.1 ± 0.6	23 ± 1	11.5 ± 0.6	11.8 ± 0.6	16.6 ± 0.9	5.4 ± 0.2	25 ± 1	18.4 ± 0.9	10.0 ± 0.4	11.6 ± 0.6
AcPin		11.1 ± 0.6	2.5 ± 0.1	1.21 ± 0.06	1.02 ± 0.05	3.2 ± 0.1	0.82 ± 0.05	1.08 ± 0.05	3.2 ± 0.1	1.52 ± 0.08	2.14 ± 0.09
LigAgly	*Isomer 1*	10.6 ± 0.6	2.8 ± 0.2	10.8 ± 0.6	103 ± 6	38 ± 2	5.9 ± 0.4	17 ± 1	19 ± 1	7.1 ± 0.4	4.7 ± 0.2
	Main isomer	7.6 ± 0.4	18.7 ± 0.9	9.6 ± 0.5	11.3 ± 0.6	10.3 ± 0.5	4.4 ± 0.2	18.8 ± 0.9	36 ± 2	6.4 ± 0.3	16.2 ± 0.8
	Isomer 2	6.7 ± 0.3	n.d.	5.9 ± 0.3	5.1 ± 0.3	6.1 ± 0.3	5.8 ± 0.3	8.4 ± 0.4	11.4 ± 0.5	16.1 ± 0.8	n.d.
	Total	**24.8 ± 0.8**	**21.4 ± 0.9**	**26.2 ± 0.8**	**119 ± 6**	**54 ± 2**	**16.1 ± 0.5**	**44 ± 1**	**67 ± 2**	**30 ± 1**	**20.9 ± 0.8**

Every result is the average of three independent (sample preparation and injection) determinations (n = 3). The results are given by the mean value ± SD. OxHTY and AcHTY were quantified in terms of HTY; AcPin, by using the calibration curve of Pin; OleAgly and its isomers, DOA, and LigAgly and its two isomers were quantified in terms of Ole. n.d.: non detected; n.q.: non quantified.

and were found within the range 1.20–7.0 μg·g^{-1}. Hojiblanca VOO was the richest in terms of this flavonoid. The case of Pin deserves to be studied carefully; indeed, in five samples, the quantitative results regarding this lignan, from both FL and MS detectors, showed statistical significant differences (95%; $p < 0.05$). The samples for which contradictory Pin's results (between FL and MS) were found, presented high contents of DLA (also known as oleocanthal (m/z 303)) which coeluted with Pin (m/z 357) and caused ionic suppression in the MS detector. As a consequence, MS detector led to lower Pin´s concentration values than FLD. DLA did not emitted light at 328 nm in FLD, avoiding therefore its presumable interference in that detector and making, from our point of view, more reliable the quantitative value achieved by FLD. In any case, in samples not presenting so remarkably high levels of DLA, MS detector gave results which were in good agreement with those of FL (Aberquina 2 (3.0 μg·g^{-1} in FL and exactly the same value in MS), Arauco 1 (0.95 μg·g^{-1} in FL and 0.87 μg·g^{-1} in MS), Hojiblanca (0.81 μg·g^{-1} in FL and 0.71 μg·g^{-1} in MS), Blend 2 (0.77 μg·g^{-1} in FL and 0.68 μg·g^{-1} in MS) and Blend 3 (3.3 μg·g^{-1} in FL and 2.9 μg·g^{-1} in MS)). Arbequina 2 and Blend 3 were the richest samples in terms of this compound.

With regard to the compounds quantified by using another standard with a related structure (Table 4), it is possible to say that OxHTY was found in all the samples (only in one of them was below the LOQ (Blend 1)), being Picual (2.3 μg·g^{-1}) and Blend 2 (1.9 μg·g^{-1}) those VOOs with highest concentrations. AcHTY was detected in eight samples and, again, the just mentioned two samples were those exhibiting the maximum concentrations (2.9 and 9.2 μg·g^{-1} in Picual and Blend 2, respectively). OleAgly and its isomers were found in almost all the VOOs; isomer 1 was solely not detected in Changlot and Hojiblanca, isomer 2 was not found in Arbequina 1 and 2, Arauco 1 and Hojiblanca, and isomer 4 was not detected in Blend 2. It seems pertinent to make a comment about the fact of detecting multiple isomers of OleAgly (what is also applicable for LigAgly). This fact has already been discussed by our research group in a recent publication [30], where previous findings regarding the formation of "artificial isomers" of secoiridoids (particularly OleAgly and LigAgly) were corroborated. These isomers show up as long as methanol is used as extractant (usually mixed with water) during the sample preparation. As extensively justified in the cited publication, we think that ignoring these isomers would mean underestimating their "native amount", which is the reason for finding the five isomers of OleAgly and two of LigAgly (Table 4). With respect to total OleAgly's concentration, Arauco 2 (274 μg·g^{-1}), Blend 1 (187 μg·g^{-1}) and Changlot (170 μg·g^{-1}) showed considerably high levels when compared to the rest. DOA's upper levels were determined in Picual (25 μg·g^{-1}) and Arbequina 2 (23 μg·g^{-1}), while Hojiblanca was the VOO with the minimum concentration of this compound (5.4 μg·g^{-1}). As expected, since it is one of the most remarkable features of Arbequina VOOs, one of the Arbequina samples (Arbequina 1) displayed the highest amount of AcPin (11.1 μg·g^{-1}). Arbequina 2, Changlot and Blend 1 also exhibited considerable concentrations of this lignan. LigAgly and its two isomers were found in almost all the samples; isomer 2 was the exception, not being found in Arbequina 2 and Blend 3. The three VOOs that were mentioned as the richest in terms of OleAgly were the samples showing

most abundant total LigAgly's content: Arauco 2 (119 $\mu g \cdot g^{-1}$), Blend 1 (67 $\mu g \cdot g^{-1}$) and Changlot (54 $\mu g \cdot g^{-1}$).

Considering the total amount of phenols of each sample (value achieved by adding up all the individual concentrations, just to give an estimation), the sample Arauco 2 was the richest (424 $\mu g \cdot g^{-1}$), while Hojiblanca was the olive oil with the lowest total levels (40 $\mu g \cdot g^{-1}$). In addition, Arauco 2 was also the sample exhibiting the largest number of phenolic compounds (a total of 21 phenolic substances (including OleAgly and LigAgly isomers) were found in the mentioned sample).

From the 23 phenolic compounds susceptible to being quantified with the developed method, 16 (including seven secoiridoid isomers) have been found and properly quantified in the selected VOOs.

3. MATERIALS AND METHODS

3.1. Reagents and Materials

The reagents used in the present work were of analytical grade and used as received. The solvents used for extraction of the analytes under study from the olive oil samples, methanol and *n*-hexane (both of HPLC grade), were purchased from Panreac (Barcelona, Spain). The mobile phases were prepared with acetonitrile (LC-MS grade) acquired from Lab-Scan (Dublin, Ireland), acetic acid (provided by Panreac) and doubly deionized water obtained using a Milli-Q-system (Millipore, Bedford, MA, USA). Standards of Gal, 4-HBA, 4-HPA, Van, Syr, Hmvan, *o*-, *m*- and *p*-Cou, Sin and Fer, as well as HTY, TY, Lut, Api, Val and DOPAC (IS) were supplied by Sigma-Aldrich (St. Louis, MO, USA), Pin was purchased from Arbo Nova (Turku, Finland), whereas Ole was acquired from Extrasynthese (Lyon, France). The stock solutions were prepared weighing the appropriate amount of each phenolic compound and dissolving it in methanol for obtaining a concentration of 500 $\mu g\ mL^{-1}$, and then, they were serially diluted to working concentrations (with concentration levels ranging from 0.5 to 250 $\mu g \cdot mL^{-1}$). Moreover, different solutions containing previously isolated phenolic compounds [31,32] (AcHTY, EA, LigAgly, OleAgly, DOA, DLA, and AcPin) were used with identification purposes. All the samples and stock solutions were stored in glass coloured flasks at −20 °C and, before the injection into the LC system, both standard solutions and sample extracts were filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela Technologies (Wilmington, DE, USA).

3.2. LC-FLD/MS Analysis

An Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, a binary pump, an autosampler, a DAD and a multiple wavelength FLD was used. Apart from the two mentioned detectors, the chromatographic system was coupled to a Bruker Daltonic Esquire 2000™ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) with an ESI interface.

The separation of the target compounds was performed using a Zorbax Eclipse Plus C_{18} analytical column (4.6 × 150 mm, 1.8 µm particle size) protected by a guard cartridge of the same packing, operating at room temperature and a flow rate of 0.8 mL·min^{-1}. The mobile phases used were water with acetic acid (0.5%) (Phase A) and acetonitrile (Phase B), and the solvent gradient changed according to the following conditions [7]: 0 to 10 min, 5%–30% B; 10 to 12 min, 30%–33% B; 12 to 17 min, 33%–38% B; 17 to 20 min, 38%–50% B; 20 to 23 min, 50%–95% B. Finally, the B content was decreased to the initial conditions (5%) in 2 min and the column re-equilibrated for 2.5 min. A volume of 10 µL of the methanolic extracts of olive oil, pure or isolated standards and standard mixtures was injected in each case.

The separated compounds were monitored on-line with the FLD, DAD, and the ESI-IT MS detectors. In the first one, excitation wavelength was set at 285 nm and four channels with the following emission wavelengths λ: 316 nm, 328 nm, 350 nm, and 450 nm, were used in order to obtain four different chromatographic traces per run. Some other important parameters in that detector were 2.31 Hz for signal acquisition rate (0.2 min of peak width which corresponds to a response time of 4 s), 10 units for photomultiplier (PMT) gain, 5% of Zero offset and 100 Lu of attenuation in analog output. Besides, previously optimized conditions for phenolic compounds detection were employed in DAD (240, 280, and 330 nm) (data has not been considered in the manuscript to contain the size of the contribution) and the MS detector [7]. The most relevant ionization source and transferring MS parameters resulted to be: nebulizer pressure, drying gas flow and drying temperature, which were set at 30 psi, 9 L·min^{-1} and 300 °C, respectively; and voltages in the end plate offset and in the capillary, which were −500 V and +3200 V, apiece.

3.3. Samples and Sample Preparation

The VOO samples studied in this work were commercial samples, acquired from Argetinean, Spanish and Moroccan factories. The samples included monovarietal olive oils from different varieties: two Arbequina (one Spanish (number 1 in Table 2) and another Argentinean (number 2)), two Arauco (both Argentinean), one Changlot (Argentinean), one Hojiblanca (Spanish), one Picual (Spanish); and three blends (two Moroccan (numbers 1 and 2) and another Spanish (number 3)). The oils were extracted from olive fruits with maturity index around 3 by a two-phase continuous centrifugation process. All the samples were kept refrigerated in their original containers until their analysis. With the just described sample selection, we logically attempted to demonstrate the potential of our method by using accessible olive oils coming from different countries rather than carrying out an agronomical comparison among the selected samples.

The phenolic compounds were isolated by using a liquid–liquid extraction according to a previously reported procedure [33], which can be briefly described as follows: 2.0 ± 0.1 g of olive oil were weighed in a test tube with a screw cap and 0.025 mL of IS solution (at a concentration of 500 mg·L^{-1}) were added. After solvent evaporation of the IS (using nitrogen), 1 mL of n-hexane was added and the tube was shaken in a vortex. Then, the compounds of interest were extracted

three times, by adding 2 mL of methanol/water (60:40, v/v), shaking during 2 min and centrifuging at 3500 rpm for 6 minutes (each time). The supernatants were put together and evaporated to dryness using a rotary evaporator. The residue was finally redissolved in 1 mL of methanol and filtered through a 0.22 µm membrane filter.

A QC sample was used to assure the stability of the system and to evaluate different analytical parameters. This QC sample was made by mixing equivalent volumes of the extracts of each variety of the selected VOOs. For validation purposes, the mentioned QC sample and the standard mix composed by the 18 compounds previously mentioned in Section 3.1 were used.

4. CONCLUSIONS

In the present work, a LC-FLD methodology was developed for the determination of phenolic compounds in olive oil. The deep evaluation of the fluorescence features of the analytes, the correct selection of emission/excitation wavelengths, and the selection of the most appropriate fluorescence mode (multi-channels) were pivotal steps in the optimization of the methodology. Validation studies were carried out, paying particular attention to precision, trueness and possible matrix effects and the results were very satisfactory. With the final goal of comparing the FL and MS quantitative results, analytical figures of merit were also determined for MS. The applicability of the LC-FLD method was finally evaluated carrying out the analysis of ten olive oil samples (different origins and varieties), and comparing (when possible) the results achieved by FL with those of MS.

The presented methodology allowed the selective determination of a considerable number of compounds (23 (plus seven isomers) which could be quantified and 26 (plus isomers), also counting those three that have been reported for the first time using FLD). It has been demonstrated that FLD is an affordable and powerful tool for the determination of these relevant minor components of the olive oil.

It is imperative to continue working in this direction, searching for simple, repeatable, reliable, affordable and easily adaptable methodologies to be used in routine analytical labs. However, the analytical part is not the only "piece of the puzzle" which has to be addressed, it is also necessary to clarify the terminology in this context and get a consensus among scientists regarding the formulation of the health claim on "olive oil polyphenols" (with regard to wording and even the specified level in the conditions of use of the claim (which absolutely depends on the quantitative approach used)).

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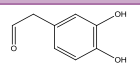
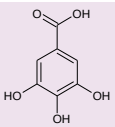
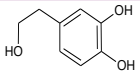
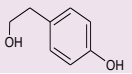
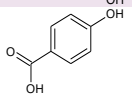
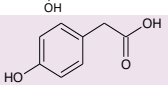
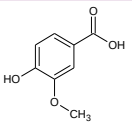
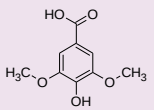
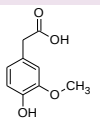
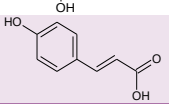
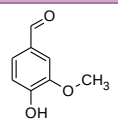
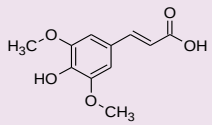
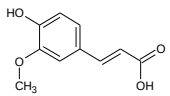
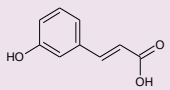
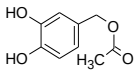
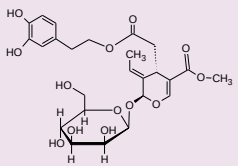
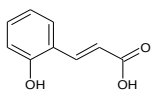
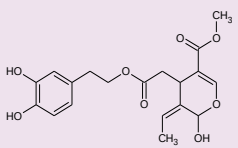
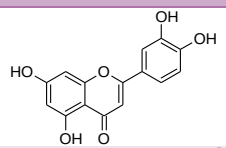
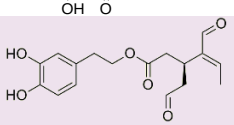
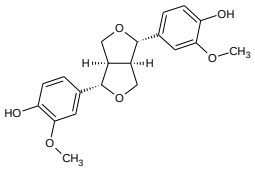
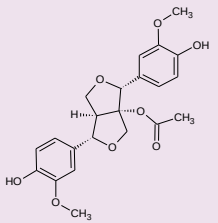
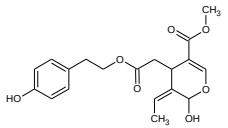
Table 1S. Molecular formula, chemical structure, retention time and fluorescence maxima of the phenolic compounds under study.

Compound	t_R (min)	Molecular formula	Structure	λ_{exc} (nm)	λ_{em} (nm)	Selected λ_{em} (nm)	Group
Oxidized hydroxytyrosol (OxHTY)	3.3	$C_8H_8O_3$		235, 285	324	328	Phenyl alcohols
Gallic acid (Gal)	4.8	$C_7H_6O_5$		278[a]	376	350	Benzoic derivate
Hydroxytyrosol (HTY)	6.9	$C_8H_{10}O_3$		235, 285	324	328	Phenyl alcohols
Tyrosol (TY)	8.6	$C_8H_{10}O_2$		232, 283	313	316	Phenyl alcohols
4-Hydroxybenzoic acid (4-HBA)	9.0	$C_7H_6O_3$		265	328, 340	350	Benzoic derivate
4-Hydroxyphenylacetic acid (4-HPA)	9.4	$C_8H_8O_3$		234, 280	316	316	Other phenolic compounds
Vanillic acid (Van)	9.7	$C_8H_8O_4$		269, 294	356	350	Benzoic derivate
Syringic acid (Syr)	9.8	$C_9H_{10}O_5$		233, 285	362	350	Benzoic derivate
Homovanillic acid (Hmvan)	10.0	$C_9H_{10}O_4$		239, 283, 298[a]	320	316	Benzoic derivate
p-coumaric acid (*p*-Cou)	11.6	$C_9H_8O_3$		239[b], 300[b]	414	450	Hydroxycinnamic derivate

Compound	t_R (min)	Molecular formula	Structure	λ_{exc} (nm)	λ_{em} (nm)	Selected λ_{em} (nm)	Group
Vanillin (Val)	11.8	$C_8H_8O_3$		241[b], 296[b]	415	450	Other phenolic compounds
Sinapic acid (Sin)	12.1	$C_{11}H_{12}O_5$		249[b], 310[b]	448	450	Hydroxycinnamic derivate
Ferulic acid (Fer)	12.3	$C_{10}H_{10}O_4$		250[b], 303[b]	440	450	Hydroxycinnamic derivate
m-coumaric acid (*m*-Cou)	12.8	$C_9H_8O_3$		285[b]	434	450	Hydroxycinnamic derivate
Hydroxytyrosol acetate (AcHTY)	13.2	$C_9H_{10}O_4$		235, 285	323	328	Phenyl alcohols
Oleuropein (Ole)	13.6	$C_{25}H_{32}O_{13}$		236, 285, 328[b]	324	328	Secoiridoids
o-coumaric acid (*o*-Cou)	14.0	$C_9H_8O_3$		284[b], 322[b]	454, 522	450	Hydroxycinnamic derivate
Oleuropein aglycone (OleAgly)	15.2, 18.5, 19.5, 19.8, 21.5 (main isomer), 22.2	$C_{19}H_{22}O_8$		235, 285	321	316	Secoiridoids

Compound	t_R (min)	Molecular formula	Structure	λ_{exc} (nm)	λ_{em} (nm)	Selected λ_{em} (nm)	Group
Luteolin (Lut)	16.5	$C_{15}H_{10}O_6$		255	330, 434	450	Flavonoids
Decarboxymethyl oleuropein aglycone (DOA)	16.7	$C_{18}H_{20}O_4$		240, 284	316	316	Secoiridoids
Pinoresinol (Pin)	17.3	$C_{20}H_{22}O_6$		238, 285	323	328	Lignans
Acetoxypinoresinol (AcPin)	17.9	$C_{22}H_{24}O_8$		240, 284	322	328	Lignans
Ligtroside Aglycone (LigAgly)	22.4, 23.8 (main isomer), 24.0	$C_{19}H_{22}O_7$		232, 281	314	316	Secoiridoids

Maximum excitation wavelengths obtained when λem is set at 325 nm except for a (360 nm) and b (450 nm).
Maximum emission wavelengths obtained when λexc is set at 285 nm.
In both columns of λexc and λem (nm), we highlight in bold letter the most convenient wavelength value when more than one fluorescence maximum were found

Table 2S. Comparison between the methodology described herein and the previously published methods with FL detection for the determination of phenolic compounds in olive oil and related matrixes.

Analytical technique	Extraction procedure of the phenolic compounds	Matrix	Nº needed injections in FLD	Nº analytes using FLD/Total analytes	Purpose	FLD wavelengths (nm) for each analyte	Ref.
LC-DAD-FLD/LC-MS	SPE (1 g olive powder → 5 mL methanol/water (50:50 v/v), dilution 1:10)	Olives	2	6/26	Identification	λ_{exc}=280, λ_{em}=320 (TY, Van, Ole); λ_{em}=320 (chlorogenic acid, caffeic acid (Caf), *p*-Cou)	[17]
LC-DAD-FLD	LLE (0.6 mL olive oil → 1.8 mL DMF)	Olive oil	1	9/14	Quantification of lignans and identif. of the rest	λ_{exc}=280, λ_{em}=320 (HTY, TY, AcHTY, AcPin, Pin, OleAgly, LigAgly, DOA, decarboxymethyl ligstroside aglycon (DLA))	[15]
LC-DAD-FLD	LLE (0.6 mL olive oil → 1.8 mL DMF)	Olive oil	1	9/14	Identification	λ_{exc}=280, λ_{em}=320 (HTY, TY, AcHTY, AcPin, Pin, OleAgly, LigAgly, DOA, DLA)	[16]
LC- FLD	LLE (50 µL rat plasma → 100 µL water)	Rat plasma	1	2	Quantification	λ_{exc}=281, λ_{em}=316 (HTY, Ole)	[21]
LC-DAD-FLD	LLE (90g olive oil → 10 mL); Direct injection (2 g olive oil + 10 mL acetone)	Olive oil	1	7/7	Quantification	λ_{exc}=280, λ_{em}=353 (HTY, TY, OleAgly, DOA); λ_{em}=313 (DLA); λ_{em}=339 (Pin, AcPin)	[22]
CE-DAD-FLD	LLE (5 g olive oil → 1 mL ethanol); Direct injection for HTY, Van, Caf (6 mL olive oil + 6 mL 1-propanol)	Olive oil	1	5/9	Quantification	λ_{exc}=297, λ_{em}=320 (gentisic acid (Gen), Caf, Van, HTY, *o*-Cou)	[20]
CE-DAD-FLD	Direct injection for HTY and Van (6 mL olive oil + 6 mL 1-propanol); SPE (60 g olive oil → 2 mL methanol)	Olive oil	1	6/9	Quantification	λ_{exc}=297, λ_{em}=320 (quercetin, Gen, Caf, Van, HTY, o-Cou)	[18]
LC-DAD-FLD	LLE for HTY and TY (1g → 2 mL ethanol, dilution 1:10); SPE for the rest (15 g → 1 mL methanol/water (50:50 v/v), dilution 1:2)	Olive oil	2	12/16	Quantification	λ_{exc}=300, λ_{em}=330 (4-HPA, 4-HBA); λ_{em}=350 (Van, HTY and TY); λ_{em}=380 (Syr); λ_{em}=450 (Gal, Gen, Fer, *p*-Cou, *o*-Cou)	[19]
LC-DAD-FLD	LLE (3 g olive oil → 4 mL methanol/water (60:40 v/v))	Olive oil	1	4/8	Quantification	λ_{exc}=250, λ_{em}=350 (HTY, TY, Van, OleAgly)	[23]
LC-DAD-FLD	LLE (3 g olive oil → 4 mL methanol/water (60:40 v/v))	Olive oil	1	7/11	Quantification	λ_{exc}=250, λ_{em}=350 (HTY, TY, Van, OleAgly, LigAgly, DOA, DLA)	[24]
LC- FLD	LLE (2 g olive oil → 1 mL methanol)	Olive oil	1	26 (+7 isomers)	Quantification	See Table 1S	This method

Chapter 4

Evaluating the reliability of specific and global methods to assess the phenolic content of virgin olive oil: do they drive to equivalent results?

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Abstract: Despite the huge number of different published strategies, there is no international regulation for the analysis of phenolic compounds from virgin olive oil. Diverse technical issues together with the disparity of criteria regarding results expression cause a lot of confusion. Herewith, a systematic comparison between specific (a powerful and fully validated LC-MS method) and global methodologies (the Folin-Ciocalteau (FC) colorimetric assay, the International Olive Council (IOC) method and hydrolysis plus HPLC-DAD) has been carried out. Thus, these strategies have been applied to the analysis of 50 extra virgin olive oils (covering all the possible quantitative ranges of these substances). This is the first time in which the individual LC-MS quantification of so many phenolic substances is included in this kind of comprehensive comparison. The outcomes of all the strategies have been thoroughly confronted and their equivalence (or divergence) has been carefully evaluated, establishing possible correspondence factors. The LC-MS individual determination with the pure standard of every analyte represented the ideal situation; when only commercial standards were used, a drastic change was observed in the absolute concentrations of oleuropein derivatives (in terms of hydroxytyrosol). Total phenolic content (summing individual levels) proved to be higher (1.9-3.0 times when data was expressed in mg/kg) than the values given by the three non-specific methods (with R^2 from 0.84 to 0.90). In any case, the IOC method, the FC assay and the hydrolysis approach could be considered as feasible strategies when a global value is pursued. Good correlations between their results were found ($R^2 > 0.89$), with the following equivalence factors: $FC_{(mg\ caffeic\ acid/kg)} \approx 0.60\ IOC_{(mg\ TY/kg)}$; $IOC_{(mg\ TY/kg)} \approx 1.27$ Sum acid hydrolysis$_{(mg\ TY+HTY/kg)}$; $FC_{(mg\ HTY/kg)} \approx 1.04$ Sum acid hydrolysis$_{(mg\ TY+HTY/kg)}$.

Keywords: virgin olive oil; phenolic compounds; hydrolysis; Folin-Ciocalteu reagent; liquid chromatography; mass spectrometry.

1. INTRODUCTION

The reality of our current society is that health and wellness concerns continue to increase in importance; one example of this trend is the shift in consumers' focus on the role that diet plays in health. In this context, the unquestionable relevance of virgin olive oil (VOO) within the Mediterranean diet, alongside fresh vegetables and fruits, motivates its inclusion in any sound food meal plan as a dietary staple. Indeed, VOO and health are two terms that, over the last years, have commonly appeared together. As a matter of fact, international organizations such as the European Food Safety Authority or the United States Food and Drug Administration have approved "health claims" relating VOO consumption and reduction of cardiovascular diseases risk [1,2]. Scientific evidences suggest that both unsaturated fatty acids together with VOO phenolic compounds are responsible for those effects. A wide number of compelling reports have been actually published including evidences of VOO phenolic compounds effects on health [3–6]. In addition, even though they appear at relatively low concentrations, phenolic compounds greatly influence VOO quality, contributing to its oxidative stability and organoleptic properties [7].

It is hardly surprising, therefore, that so many analytical methods have been proposed to determine VOO phenolic compounds. Table 1 provides some examples of methodologies for carrying out the characterization of this fraction which imply the application of diverse types of sample treatment and analytical tools, including NMR, colorimetric and separative approaches (CE, LC and GC coupled to different detection systems). Our aim was not to thoroughly review the analytical options, but just to include some interesting examples to illustrate the following methodological classification; that is why, to contain the size of the table, we have not cited some other very commendable instances. They can be classified in two categories: non-specific or global methods, that generate a "total phenolic content" result, and specific methods, that allow the quantification of individual phenolic compounds. All the published options show worthy points, but also some limitations that, together with the disparity of criteria regarding results expression, lead to non-comparable outcomes.

Firstly, in order to compare the results from global methods and those obtained when applying profiling approaches, individual phenols concentrations are usually summed to get an artificially created total number. The validity of this number can be questionable from a mathematical/chemical point of view, but the truth is that there is no other option to enable the comparison. Secondly, regarding individual phenols determination, one of the major issues is the absence of commercially available pure standards for most of them. Thus, their concentration is usually referred to a particular standard with a different response factor depending on its molecular structure and the detector being used [8]. In addition, the complexity and heterogeneity of this family of compounds makes difficult to achieve their chromatographic separation with proper resolution and efficacy, what represents an obstacle to achieve a reliable (individual) quantification. Lastly, quantitative results can also be influenced by the employed experimental conditions, affecting to analytes' recovery or even causing the formation of artifacts (*e.g.* using protic solvents in sample preparation or as mobile phases in LC) [9–11]. Consequently, incomplete phenolic fraction characterizations and contradictory results, which can vary by orders of magnitude in some cases, can be found in literature. As a result, this causes great confusion among VOO producers, consumers and even the legislative bodies.

The link between olive oil consumption, phenolic compounds and health is so solid, that the European Union approved in 2012 a specific health claim on VOOs containing at least 5 mg of hydroxytyrosol (HTY) and derivatives (*e.g.* oleuropein complex and tyrosol (TY)) per 20 g of olive oil [12]. However, this regulation is surrounded in controversy not only due to the ambiguous employed terminology, but also to technical gaps regarding the analysis of the targeted analytes [13,14]. The methodology adopted by the International Olive Council (IOC) for the determination of VOO biophenols [15], which is based on the chromatographic separation of a VOO hydroalcoholic extract followed by UV detection at 280 nm, gives a global result (encompassing simple phenols, secoiridoids, lignans flavonoids and phenolic acids) in terms of an internal

Table 1. Some examples of analytical methods for the determination of VOO phenolic compounds

Sample Treatment	Analytical tool	N analytes (considered families)	Standard/s for quantification	Result expression	Weak points	Ref
			Global methods			
LLE + reduction reaction *(15 min)*	Spectrophotometry	Total antioxidants	caffeic acid	mg caffeic acid/ kg VOO	- Global antioxidants index: any reducing substance may interfere in the assay	[16]
SPE + reduction reaction *(1 h)*	Spectrophotometry	Total *o*-diphenols	pirocatechol	mmol *o*-diphenols/kg VOO	- Partial index: just considering luteolin, HTY and derivatives	[17]
UAE	LC-DAD *(82 min)*	Global result (phenolic acids, simple phenols, secoiridoids, flavonoids, lignans)	syringic acid (IS), TY	mg TY/kg VOO	- Time consuming with regard to the obtained result - Result referred to a unique compound (considering the same response factor for all the analytes)	[15]
LLE + acid hydrolysis *(2 h)*	LC-DAD *(50 min)*	2 analytes (simple phenols and indirect measurement of secoiridoids)	2 pure standards	mg TY and HTY/ kg VOO	- Two steps of sample preparation - Approach involving chemical modification of the targeted analytes - Not considering some relevant phenolic families	[18]
Hydrolysis *(6 h)*	LC-DAD *(70 min)*	2 analytes (simple phenols and indirect measurement of secoiridoids)	2 pure standards	mg TY and HTY/ kg VOO	- Chemical modification of the targeted analytes is required - Some relevant phenolic families are not taken into account	[19]
UAE + hydrolysis *(1 h)* + derivatization *(1 h)*	GC-FID *(42 min)*	2 analytes (simple phenols and indirect measurement of secoiridoids)	2 pure standards	mg TY and HTY/ kg VOO	- Three steps of sample preparation - Hydrolysis produces a chemical modification of the substances under study - Do not allow determining some other relevant phenolic families	[20]
			Specific methods			
SPE	CZE-DAD *(7 min)*	13 analytes (phenolic acids, simple phenols, secoiridoids, flavonoids, lignans)	8 pure standards	mg analyte/ kg VOO	- Coelution of some peaks - Quantification of 5 compounds in terms of a different standard	[21]
LLE + derivatization *(30 min)*	GC-EI-(IT)MS *(55 min)*	27 analytes (phenolic acids, simple phenols, secoiridoids)	sinapinic acid (IS)	mg analyte/ kg VOO	- Results referred to a unique compound (considering the same response factor for all the analytes) - Not considering flavonoids nor lignans	[22]
DI	LC-DAD/FLD *(73 min)*	7 analytes (simple phenols, secoiridoids, lignans)	2 pure standards + 5 isolated compounds	mg analyte/ kg VOO	- Scarce number of determined analytes - Not considering flavonoids	[23]
LLE	LC-ESI-(IT)MS *(25 min)*	20 analytes (phenolic acids, simple phenols, secoiridoids, flavonoids, lignans)	10 pure standards	mg analyte/ kg VOO	- Quantification of 10 compounds in terms of a different standard	[24]
LLE	^{1}H NMR	4 analytes (secoiridoids)	4 isolated compounds	mg analyte/ kg VOO	- Scarce number of determined analytes - Just considering major secoiridoids	[9]

LLE: liquid-liquid extraction; SPE; solid phase extraction; UAE: ultrasound assisted extraction; DI: direct injection; LC/GC: liquid/gas chromatography; CZE: capillary zone electrophoresis; DAD: diode-array detector; FLD: fluorescence detector; MS: mass spectrometry; IS: internal standard; VOO: virgin olive oil; TY: tyrosol; HTY: hydroxytyrosol

standard (IS) which is referred to tyrosol. Hence, it does not seem appropriate to address the analytical requirements of the just mentioned health claim. Some authors have proposed to carry out a simpler approach by measuring the amount of HTY and TY produced after hydrolyzing bound forms as a way to simultaneously measure all the secoiridoid derivatives. Having in mind the differences in molecular weight between secoiridoids and their corresponding phenyl alcohols, the need of a correction factor or the reformulation of the specific conditions of the health claim has also been stated [19,25]. Some authors have compared the results of this approach with more widely used methodologies showing relatively good correlations with the IOC method [18,20] or the Folin-Ciocalteu (FC) colorimetric assay [26].

Over the last years, the general recognition of the problems regarding the determination of phenolic substances from VOO and the concern about the incomparability of quantitative data have also prompted some researchers to carry out very valuable methodological comparisons between LC-profiling strategies and more simple and cost-effective colorimetric methods [27–30]. In all these cited examples, a diode-array detector (DAD) was the detection system of choice (MS was also used in one case with identification purposes), which is very logical since it is the most accessible and widespread one. However, because of the very frequent overlapping within the LC profiles (baseline separation of these substances is unfeasible), DAD exhibits some clear weaknesses regarding individual quantification. To the best of our knowledge, a wholesale and systematic comparison between global and specific methods, including individual comprehensive characterization by LC-MS has not been published so far.

Consequently, this project aims to evidence the difficulties for carrying out a reliable determination of this important kind of minor compounds from VOO achieving comparable results when diverse approaches are applied. To do it so, a specific methodology (LC-MS using various reference pure standards) and three global strategies (FC assay, IOC method and hydrolysis approach) have been used to analyze 50 extra virgin olive oil (EVOO) samples (covering all the possible ranges in terms of phenols concentration) and their outcomes have been very thoroughly compared. Their equivalence (or not) has been carefully evaluated, establishing possible correspondence factors and discussing the advantages and disadvantages of each considered option.

2. MATERIALS AND METHODS

2.1. Chemicals and standards

Deionized water was daily produced with a Milli-Q-system (Millipore, Bedford, MA, USA). Methanol (MeOH) and acetonitrile (ACN) LC-MS grade, supplied from Prolabo (Paris, France), along with acetic and phosphoric acids from Sigma-Aldrich (St. Louis, MO, USA) were used for mobile phase preparation. HCl 37 % (v/v) and Na_2CO_3 were purchased from Panreac (Barcelona, Spain). The FC reagent as well as pure standards of gallic, caffeic, syringic and quinic acids, HTY, TY,

luteolin, apigenin and pinoresinol were acquired from Sigma-Aldrich. Standards of oleuropein aglycone and decarboxymethyl oleuropein aglycone were kindly donated by the research group FQM-367 from the University of Granada (Granada, Spain). Decarboxymethyl ligstroside aglycone was isolated from a VOO extract prepared using the protocol for extraction, purification and characterization developed by Prof. Magiatis' research group (University of Athens, Athens, Greece). Ligstroside aglycone and elenolic acid were isolated by the research group FQM-297 of the University of Granada. All these isolated substances had a purity >95%. Both the standard stock solutions and phenolic extracts were filtered through a 0.22 μm nylon filter from Agela Technologies (Wilmington, DE, USA) before chromatographic analysis.

2.2. EVOO samples

50 EVOO samples were acquired from the Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA) 'Venta del Llano', Mengíbar (Jaén), Spain. Sample selection was done so as to cover low, intermediate and high phenolic content in the studied oils. All the selected oils were stored at -20ºC until their analysis. Randomized numbers were assigned as sample names.

2.3. Methodology

2.3.1. Phenols profiling by LC-MS

The individual quantification of EVOO phenolic compounds was performed using the profiling approach previously reported by Bajoub *et al.* [24] with slight modifications. Phenols fraction isolation was carried out by applying the described LLE protocol, using three 2 mL portions of MeOH/water (60:40, v/v) as extractant agent. After solvent evaporation of the obtained hydroalcoholic extracts, the residue was reconstituted in 1 mL of ACN/water (50:50, v/v) in order to avoid the presence of MeOH in the final extract, which could produce the artificial formation of secoiridoids isomers [10,11]. In this respect, we found that the substitution of MeOH by ACN in the final extracts was enough to avoid the just mentioned effect, not having to remove MeOH from the extractant mixture.

LC-MS analyses were conducted on an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker Daltonics Esquire 2000™ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization interface. 10 μL of the extracts, diluted 1:10 with ACN/water (50:50, v/v), were injected into the system. Analytes were separated in a Zorbax Eclipse Plus C18 column (4.6 × 150 mm, 1.8 μm particle size) at room temperature with acidified water (0.5% acetic acid) and ACN as mobile phases at a flow rate of 0.8 mL/min. The employed mobile phase gradient as well as the MS detection conditions are detailed elsewhere [24]. MS spectra were acquired in negative ion mode within a *m/z* range from 50 to 1000, using a capillary voltage of +3200 V. Quantification of individual compounds was done by external calibration with standards solutions prepared in ACN/water (50:50, v/v) at different concentration levels over the range 0.1-300 mg/L. Every phenol was quantified in terms of its own standard or

the most similar molecule if the pure standard was not available (hydroxytyrosol acetate was quantified in terms of HTY; desoxy elenolic acid and elenolic acid methylester were quantified in terms of elenolic acid; hydroxy decarboxymethyl oleuropein aglycone was quantified in terms of decarboxymethyl oleuropein aglycone; acetoxypinoresinol was quantified in terms of pinoresinol; and diosmetin was quantified in terms of luteolin). For comparison purposes, quantification of secoiridoids was also performed on the basis of the related simple phenol, using HTY calibration curve for oleuropein derivatives and TY calibration curve for ligstroside ones, since it is a common strategy applied to secoiridoids quantification [28,31].

2.3.2. Total phenolic content by the IOC HPLC method

Total phenolic content was determined by the reference method of the IOC [15]. In short, 2 g of EVOO were extracted with 5 mL MeOH/water (80:20, v/v) in an ultrasonic bath for 15 min. Then, the supernatant containing the extracted phenolic compounds was analyzed by means of the same LC system described in section 2.3.1. equipped with DAD. The chromatographic separation was carried out on a Tracer Extrasil ODS-2 column (4.6 × 250 mm, 5 µm particle size) (Teknokroma, Barcelona, Spain) with a mobile phase gradient of acidified water (0.2% H_3PO_4), MeOH and ACN, pumped at 1 mL/min at ambient temperature. Chromatograms were recorded at 280 nm and the sum of the integrated areas of the individual peaks was considered to calculate the final result. The biophenol content was expressed as mg of TY equivalents per kg of EVOO, using syringic acid as internal standard and a TY calibration standard solution prepared in MeOH/water (80:20, v/v). It seems appropriate to mention that the IOC method has a recommended range of measurement from 30 mg/kg to 800 mg/kg.

2.3.3. Total phenolic content by the Folin–Ciocalteu method

Colorimetric determination of the total phenolic content was performed using the FC reagent according to the procedure described by Vazquez *et al.* [16]. In brief, 10 g of EVOO were extracted three times with 20 mL of MeOH/water (60:40, v/v), and an aliquot of the diluted extract was mixed with 2.5 mL of FC reagent and 5 mL of Na_2CO_3 (20% w/v). After 1 hour, absorbance measurements were carried out at 725 nm in a Helios UV-vis spectrophotometer (Thermo Scientific, Bremen, Germany). Four different calibration curves were established from 0.5 to 10 mg/L with caffeic and gallic acids, HTY and TY. The total phenolic content was expressed in mg/kg of the four just mentioned standards.

2.3.4. Total content of HTY and TY derivatives: acid hydrolysis of secoiridoids

Secoiridoids hydrolysis was carried out following the protocol reported by Romero and Brenes [19]. Briefly, 1.5 g of EVOO and 30 mL of HCl (2M) were mixed together in an orbital shaker at ambient temperature for 6 h. Afterwards, the aqueous phase was analyzed by the same LC method used for phenols profiling, with DAD detection at 280 nm. HTY and TY calibration curves were prepared in HCl 2M in a concentration range from 1 to 50 mg/L.

2.3.5. Data treatment

Data acquisition and data processing in the LC-DAD system was done with ChemStation B.04.03 (Agilent Technologies). When LC-MS was employed, Esquire control and Data Analysis 4.4 (Bruker Daltonik) were used for instrument control and MS data treatment, respectively.

3. RESULTS AND DISCUSSION

3.1. Phenols profiling by LC-MS

The first stage of this project involved the comprehensive characterization of the phenolic fraction of the samples under study. A LC-MS method thoroughly validated and widely used in our laboratory for phenols profiling in metabolomic studies [10,24,32–35] was selected to obtain the concentration of the individual phenolic compounds in the samples as accurately as possible. To that end, the use of pure standards of all the targeted analytes would have been the best case scenario. We were very close to the ideal situation; however, the analytical standard of 6 out of the 24 phenolic compounds which were finally determined in the samples, were not commercially available nor accessible as isolated fraction and had to be quantified in terms of the standard with the most similar chemical structure, as specified in section 2.3.3. The slight chemical differences between the analytes lacking the corresponding standard and the compounds used for their quantification would suggest a similar response in the MS detector for both of them. Having in mind this assumption, Table 2 presents the phenolic compounds' concentration found in all the analyzed extracts. Every result is the average of three independent replicates and is expressed in mg of the individual compound per kg of olive oil. The determined analytes were grouped in six families, including organic acids (quinic acid), simple phenols (HTY, HTY acetate and TY), elenolic acid derivatives (two isomers of elenolic acid, desoxy elenolic acid and elenolic acid methylester), secoiridoids (four isomers of oleuropein aglycone, two isomers of decarboxymethyl oleuropein aglycone (also designated as oleacein), hydroxy decarboxymethyl oleuropein aglycone (or hydroxy oleacein), three isomers of ligstroside aglycone and decarboxymethyl ligstroside aglycone (also known as oleocanthal)), flavonoids (luteolin, apigenin and diosmetin) and lignans (pinoresinol and acetoxypinoresinol). In general terms, organic acids, flavonoids and lignans were the less prevalent families of phenolic compounds found in the 50 evaluated samples, representing an average of 0.12, 0.27 and 0.33% of the total phenolic content, respectively. Sum concentrations of the compounds belonging to each family were lower than 6.9, 9.0 and 13.6 mg/kg for quinic acid, flavonoids and lignans, apiece. Simple phenols exemplified another class of phenols which was found at relatively low concentration levels (in comparison with other determined chemical classes); they represented a percentage of 2.54% (mean value) and showed total concentrations within the range from 18.5 to 50.4 mg/kg. Even though elenolic acid derivatives do not formally belong to phenolic compounds category, they are taken into account in some studies when the phenolic fraction is evaluated because they are structural part of the secoiridoids and can be

Table 2. Quantitative data obtained for the 50 EVOO samples under study: individual phenolic compounds'concentration determined by LC-MS (using their reference pure standards or a compound with a highly related chemical structure) and total phenolic content measured through the three evaluated global methods (IOC, FC and hydrolysis approach).

		[M-H]⁻	671	621	670	687	620	689	684	685	699	691	701	686	692	120	698	690
1	Quinic acid	191	1.4	1.4	1.8	1.2	1.4	1.4	2.8	2.7	2.0	1.9	3.0	1.9	1.5	0.2	2.1	2.3
Organic acids			*1.4*	*1.4*	*1.8*	*1.2*	*1.4*	*1.4*	*2.8*	*2.7*	*2.0*	*1.9*	*3.0*	*1.9*	*1.5*	*0.2*	*2.1*	*2.3*
2	Hydroxytyrosol	153	10.2	12.5	12.9	12.9	16.9	10.3	13.8	14.9	15.2	14.8	20.9	12.7	15.4	7.5	11.4	13.3
3	Hydroxytyrosol acetate	195	0.9	2.7	0.7	2.2	3.4	2.4	0.4	0.7	1.4	2.9	2.1	0.7	2.9	15.7	0.9	2.9
4	Tyrosol	137	13.3	7.7	16.8	10.8	12.4	9.1	10.9	11.4	11.5	10.4	12.4	11.0	10.3	6.4	9.2	9.3
Simple phenols			*24.4*	*22.9*	*30.4*	*26.0*	*32.7*	*21.8*	*25.2*	*27.0*	*28.2*	*28.1*	*35.4*	*24.4*	*28.6*	*29.7*	*21.5*	*25.5*
5	Elenolic acid (is I)	241	69.8	112.8	89.0	346.3	184.8	279.7	338.1	334.6	338.6	378.2	326.1	281.8	367.7	208.0	264.8	315.7
6	Elenolic acid (is II)	241	7.4	0.3	8.8	21.0	<0.1	22.6	23.4	22.0	28.0	22.8	29.4	32.0	20.9	22.7	29.0	19.7
7	Desoxy elenolic acid	225	<0.1	2.1	0.3	13.1	2.0	12.1	19.9	20.2	24.8	16.8	28.3	13.7	12.3	99.6	21.9	16.5
8	Elenolic acid methylester	255	0.1	1.1	0.4	26.9	<0.1	23.7	49.7	43.1	40.6	46.6	39.6	29.4	30.8	37.2	23.9	37.3
Elenolic acid derivatives			*77.4*	*116.3*	*98.6*	*407.3*	*186.9*	*338.0*	*431.1*	*420.0*	*432.0*	*464.4*	*423.3*	*356.9*	*431.7*	*367.5*	*339.7*	*389.1*
9	Oleacein (isomer I)	319	8.7	44.0	14.1	56.0	59.1	44.1	41.3	46.7	44.6	71.3	60.8	42.1	69.7	340.2	28.4	55.1
10	Oleacein (isomer II)	319	1.5	2.1	1.1	2.3	3.6	1.8	2.1	2.0	1.9	2.8	2.5	1.7	1.7	43.7	1.9	2.2
11	Hydroxy oleacein	335	1.5	1.9	1.5	2.1	3.8	1.5	1.5	1.5	<0.1	2.0	4.4	1.5	1.3	57.5	1.8	4.3
12	Oleuropein aglycone (is I)	377	0.3	0.6	0.7	7.5	0.2	9.5	8.2	13.5	12.8	10.8	24.8	11.7	10.5	11.2	11.0	12.8
13	Oleuropein aglycone (is II)	377	0.5	10.0	7.4	21.7	13.7	21.7	43.0	49.4	32.1	45.2	61.1	44.9	37.6	7.0	38.8	41.4
14	Oleuropein aglycone (is III)	377	45.0	90.0	71.0	92.8	123.0	84.7	140.9	131.1	152.6	222.5	318.6	115.1	183.5	60.5	96.6	110.9
15	Oleuropein aglycone (is IV)	377	1.0	9.2	6.0	13.9	9.6	14.0	33.6	31.2	30.6	42.2	56.3	32.5	36.6	11.3	23.1	27.0
16	Oleocanthal	303	5.7	7.8	4.3	12.7	12.3	8.1	7.7	9.1	8.2	12.7	9.7	5.8	14.5	186.0	3.8	7.4
17	Ligstroside aglycone (is I)	361	2.3	14.4	7.5	42.8	13.8	58.1	113.9	111.7	66.4	84.9	89.0	88.8	61.8	8.6	83.5	73.3
18	Ligstroside aglycone (is II)	361	56.8	145.1	77.3	186.5	185.9	152.3	288.6	316.6	268.3	239.5	348.1	193.2	203.5	65.4	188.0	161.8
19	Ligstroside aglycone (is III)	361	4.2	24.4	9.3	62.0	30.4	58.5	137.6	137.7	105.9	112.9	132.9	90.9	85.2	20.1	88.2	73.5
Secoiridoids			*127.5*	*349.6*	*200.1*	*500.2*	*455.5*	*454.4*	*818.3*	*850.6*	*723.4*	*846.9*	*1108.3*	*628.3*	*705.9*	*811.6*	*565.2*	*569.9*
20	Luteolin	285	0.3	<0.1	0.4	1.1	0.2	1.7	2.1	2.2	2.1	2.1	2.9	2.1	1.6	5.4	2.0	1.5
21	Apigenin	269	0.1	<0.1	0.2	0.4	<0.1	0.5	0.7	0.8	0.9	0.6	1.1	0.8	0.5	1.1	0.9	0.5
22	Diosmetin	299	0.1	<0.1	0.1	0.3	<0.1	0.4	0.4	0.5	0.7	0.5	0.8	0.4	0.4	1.7	0.6	0.4
Flavonoids			*0.5*	*<0.1*	*0.7*	*1.8*	*0.2*	*2.6*	*3.2*	*3.4*	*3.7*	*3.2*	*4.8*	*3.3*	*2.5*	*8.1*	*3.5*	*2.4*
23	Pinoresinol	357	0.9	0.2	1.0	2.9	0.3	3.3	2.8	2.8	3.1	3.2	3.4	2.7	2.6	2.1	2.8	2.9
24	Acetoxypinoresinol	415	<0.1	<0.1	<0.1	0.3	<0.1	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.1	7.4	0.1	0.2
Lignans			*0.9*	*0.2*	*1.0*	*3.1*	*0.3*	*3.7*	*3.0*	*3.0*	*3.3*	*3.4*	*3.6*	*2.8*	*2.7*	*9.6*	*2.9*	*3.2*
Total (mg each compound/kg)			**232**	**490**	**333**	**940**	**677**	**822**	**1284**	**1307**	**1193**	**1348**	**1579**	**1018**	**1173**	**1227**	**935**	**992**
IOC HPLC method (mg TY/kg)			152	207	213	281	283	325	350	369	409	411	423	425	426	433	438	444
FC method	*(mg caffeic acid/kg)*		108	110	114	227	169	205	298	313	287	278	338	355	291	295	300	288
	(mg gallic acid/kg)		78	81	84	204	142	181	279	295	267	259	320	338	271	277	281	268
	(mg HTY/kg)		120	123	128	274	199	246	365	384	350	340	416	438	356	362	367	352
	(mg TY/kg)		196	202	209	458	330	410	612	645	587	570	699	735	597	607	616	590
HTY from acid hydrolysis			75	162	88	145	165	169	174	192	201	207	262	221	205	191	217	218
TY from acid hydrolysis			88	173	94	179	157	179	223	223	221	192	223	227	193	299	251	196
Sum acid hydrolysis (mg TY+HTY/kg)			163	335	183	324	321	348	397	414	422	398	485	448	398	489	468	413

		117	700	695	619	110	694	121	673	111	109	674	678	672	679	703	680	702
1	Quinic acid	0.3	3.1	2.9	1.3	<0.1	2.7	0.1	1.0	0.2	<0.1	1.6	0.6	1.1	0.4	6.9	0.3	6.0
Organic acids		*0.3*	*3.1*	*2.9*	*1.3*	*<0.1*	*2.7*	*0.1*	*1.0*	*0.2*	*<0.1*	*1.6*	*0.6*	*1.1*	*0.4*	*6.9*	*0.3*	*6.0*
2	Hydroxytyrosol	10.3	16.4	16.9	26.9	7.1	21.2	8.6	16.4	12.5	7.9	18.1	18.6	16.6	21.3	24.0	17.8	23.3
3	Hydroxytyrosol acetate	2.3	1.8	1.9	2.2	10.3	1.6	11.2	0.2	14.4	13.9	0.5	0.3	0.5	0.7	1.5	0.9	1.7
4	Tyrosol	12.1	10.0	12.6	18.6	3.2	13.9	6.8	14.5	7.5	4.0	14.2	11.6	11.4	17.0	15.0	13.1	15.7
Simple phenols		*24.7*	*28.1*	*31.4*	*47.7*	*20.7*	*36.7*	*26.7*	*31.1*	*34.4*	*25.8*	*32.8*	*30.6*	*28.4*	*38.9*	*40.5*	*31.8*	*40.6*
5	Elenolic acid (is I)	299.2	277.5	364.8	261.8	450.1	418.4	161.8	391.4	282.1	323.2	392.2	344.3	325.1	454.6	391.6	371.0	375.3
6	Elenolic acid (is II)	32.6	26.6	23.0	0.4	14.4	17.5	21.4	20.0	14.3	13.1	17.6	14.1	17.9	16.5	27.9	15.7	27.1
7	Desoxy elenolic acid	186.4	26.5	15.9	7.8	58.1	15.0	78.1	10.2	16.7	56.7	10.0	9.8	12.3	8.2	23.2	8.2	24.4
8	Elenolic acid methylester	13.0	49.3	33.1	<0.1	76.1	26.7	12.2	9.0	36.3	47.5	50.7	29.4	34.0	16.2	22.0	22.9	21.3
Elenolic acid derivatives		*531.2*	*380.0*	*436.8*	*270.0*	*598.8*	*477.6*	*273.5*	*430.5*	*349.3*	*440.5*	*470.5*	*397.5*	*389.2*	*495.5*	*464.8*	*417.9*	*448.9*
9	Oleacein (isomer I)	163.7	57.8	56.6	173.3	352.8	61.9	309.4	42.9	288.8	421.4	52.9	54.3	51.2	57.4	57.0	54.7	58.6
10	Oleacein (isomer II)	33.4	2.5	2.2	3.1	34.7	2.9	38.4	2.4	21.7	35.0	3.1	2.6	2.3	3.4	2.2	2.8	2.5
11	Hydroxy oleacein	34.5	4.0	1.8	3.6	50.2	4.7	91.3	2.1	45.4	46.2	2.1	2.5	2.6	2.0	3.1	2.8	1.9
12	Oleuropein aglycone (is I)	12.5	16.2	13.7	3.0	10.4	8.5	10.6	7.7	15.3	9.1	8.6	8.8	7.9	13.2	24.6	11.0	18.0
13	Oleuropein aglycone (is II)	20.3	56.3	46.7	42.1	9.3	38.6	1.3	44.5	13.7	4.6	46.0	54.9	57.3	40.9	55.3	50.3	46.6
14	Oleuropein aglycone (is III)	83.8	209.3	141.3	291.1	46.9	195.3	42.0	252.0	89.4	46.0	170.2	258.8	211.8	195.1	353.2	197.4	302.9
15	Oleuropein aglycone (is IV)	12.6	38.2	27.3	27.3	12.5	35.0	4.0	34.8	14.2	8.7	27.9	35.2	37.4	30.4	41.1	33.5	28.7
16	Oleocanthal	83.2	8.7	8.8	13.8	125.1	8.7	190.9	8.9	84.6	166.8	8.9	9.4	9.0	10.0	9.3	10.2	8.5
17	Ligstroside aglycone (is I)	48.3	100.7	81.9	36.5	8.4	58.7	2.8	129.9	19.1	3.4	103.0	113.2	138.0	77.3	116.5	115.8	107.1
18	Ligstroside aglycone (is II)	194.4	296.9	336.6	353.7	39.0	327.0	46.5	524.5	92.1	30.4	440.0	435.5	428.0	541.6	593.5	450.9	533.9
19	Ligstroside aglycone (is III)	56.8	134.4	134.2	67.4	12.9	110.1	7.1	179.9	27.6	6.5	162.6	172.9	174.0	146.8	173.5	175.7	162.5
Secoiridoids		*743.6*	*925.1*	*851.1*	*1014.8*	*702.1*	*851.7*	*744.3*	*1229.5*	*711.9*	*778.1*	*1025.2*	*1148.1*	*1119.6*	*1118.0*	*1429.3*	*1105.2*	*1271.1*
20	Luteolin	2.4	3.0	1.6	0.1	4.2	1.4	5.6	1.7	4.7	3.4	1.9	1.2	1.7	1.3	3.0	1.4	2.6
21	Apigenin	1.0	1.1	0.6	0.1	0.9	0.6	1.1	0.5	2.9	0.5	0.5	0.4	0.4	0.3	0.9	0.4	0.9
22	Diosmetin	1.1	0.7	0.4	<0.1	1.9	0.3	1.1	0.4	1.2	1.4	0.4	0.3	0.4	0.3	0.7	0.3	0.6
Flavonoids		*4.4*	*4.8*	*2.6*	*0.2*	*6.9*	*2.3*	*7.9*	*2.5*	*8.9*	*5.4*	*2.8*	*1.9*	*2.5*	*2.0*	*4.6*	*2.1*	*4.2*
23	Pinoresinol	1.3	3.2	2.5	0.3	1.7	2.3	2.0	2.7	2.4	2.0	2.4	2.2	2.1	2.2	2.6	2.2	2.7
24	Acetoxypinoresinol	6.9	0.2	0.2	<0.1	9.0	0.2	7.7	<0.1	11.2	9.9	<0.1	<0.1	<0.1	<0.1	0.2	<0.1	0.1
Lignans		*8.2*	*3.5*	*2.7*	*0.3*	*10.7*	*2.5*	*9.6*	*2.7*	*13.6*	*11.9*	*2.4*	*2.2*	*2.1*	*2.2*	*2.7*	*2.2*	*2.9*
Total (mg each compound/kg)		**1312**	**1344**	**1328**	**1334**	**1339**	**1374**	**1062**	**1697**	**1118**	**1262**	**1535**	**1581**	**1543**	**1657**	**1949**	**1559**	**1773**
IOC HPLC method (mg TY/kg)		479	484	485	498	503	504	516	529	545	549	566	584	601	619	625	654	673
FC method	*(mg caffeic acid/kg)*	302	378	327	260	306	282	309	376	329	312	419	445	453	426	405	438	384
	(mg gallic acid/kg)	283	364	309	238	286	262	290	362	310	294	408	435	443	414	391	426	370
	(mg HTY/kg)	370	468	402	315	374	344	379	466	404	383	522	555	565	530	502	545	476
	(mg TY/kg)	620	787	675	527	628	577	636	783	678	643	878	934	951	892	845	918	800
HTY from acid hydrolysis		215	255	229	226	217	243	227	236	251	214	258	274	278	264	292	275	291
TY from acid hydrolysis		359	222	228	188	208	232	346	281	181	214	286	289	292	289	279	288	276
Sum hydrolys. (mg TY+HTY/kg)		574	477	458	415	425	475	573	516	432	428	545	563	570	553	571	563	567

		118	124	113	122	683	675	123	682	676	116	404	119	407	127	402	405	115
1	Quinic acid	0.2	<0.1	<0.1	0.2	0.8	5.3	0.1	0.6	2.2	0.2	0.6	<0.1	0.4	0.1	0.2	0.7	0.1
Organic acids		*0.2*	*<0.1*	*<0.1*	*0.2*	*0.8*	*5.3*	*0.1*	*0.6*	*2.2*	*0.2*	*0.6*	*<0.1*	*0.4*	*0.1*	*0.2*	*0.7*	*0.1*
2	Hydroxytyrosol	10.4	9.6	13.0	7.7	17.3	19.2	10.1	22.0	22.9	20.7	30.1	10.5	24.7	23.0	25.8	28.7	21.8
3	Hydroxytyrosol acetate	2.3	20.4	11.6	12.7	1.3	0.7	15.6	1.7	0.9	2.2	0.1	0.1	0.5	<0.1	<0.1	<0.1	2.0
4	Tyrosol	10.5	6.3	8.9	6.9	10.3	10.4	5.3	11.8	12.4	14.0	20.1	7.8	15.4	20.0	15.1	17.4	19.2
Simple phenols		*23.1*	*36.3*	*33.5*	*27.3*	*28.9*	*30.4*	*31.0*	*35.5*	*36.2*	*36.9*	*50.4*	*18.5*	*40.6*	*43.1*	*41.0*	*46.2*	*42.9*
5	Elenolic acid (is I)	329.6	191.0	289.7	108.1	338.9	329.2	227.3	393.8	425.5	390.2	402.7	381.0	278.6	247.6	281.4	289.8	389.9
6	Elenolic acid (is II)	31.9	21.7	13.8	29.9	14.9	14.2	18.3	16.7	11.5	20.6	18.1	33.8	18.9	42.2	16.3	16.8	17.3
7	Desoxy elenolic acid	188.7	94.2	15.1	100.1	12.8	15.9	103.1	14.6	16.0	57.9	17.3	154.0	18.6	24.3	25.2	22.3	66.0
8	Elenolic acid methylester	35.4	15.1	21.5	8.5	26.8	34.7	31.9	31.6	34.9	60.6	28.8	52.8	46.9	41.5	38.8	11.0	54.9
Elenolic acid derivatives		*585.6*	*322.0*	*340.1*	*246.6*	*393.4*	*394.0*	*380.4*	*456.7*	*487.9*	*529.4*	*466.9*	*621.7*	*363.0*	*355.7*	*361.6*	*339.9*	*528.2*
9	Oleacein (isomer I)	176.4	434.9	345.0	234.9	71.9	67.3	634.7	79.3	71.1	355.5	118.0	394.7	97.1	253.5	73.6	77.9	282.2
10	Oleacein (isomer II)	20.1	36.4	35.8	45.4	2.8	2.4	37.7	2.7	2.9	16.7	5.2	24.1	2.7	21.3	1.7	2.7	16.0
11	Hydroxy oleacein	33.9	103.2	63.7	73.2	2.3	3.4	177.1	3.6	3.0	37.1	13.4	39.6	12.2	75.6	7.4	1.5	32.2
12	Oleuropein aglycone (is I)	14.9	13.9	12.9	12.7	8.3	5.5	12.5	6.4	6.2	13.6	5.9	13.5	9.0	12.4	7.6	6.9	7.7
13	Oleuropein aglycone (is II)	37.3	6.7	11.3	2.8	73.9	80.6	10.5	78.2	76.9	104.9	87.7	93.9	140.9	81.0	124.7	107.9	94.4
14	Oleuropein aglycone (is III)	110.3	64.4	93.3	33.2	238.1	311.7	79.5	357.1	441.2	253.5	430.9	265.9	423.4	292.0	400.4	380.5	225.1
15	Oleuropein aglycone (is IV)	32.3	7.8	10.1	3.4	48.9	72.6	13.7	67.4	71.0	71.4	95.8	84.1	133.8	55.3	120.0	93.1	81.8
16	Oleocanthal	61.1	135.2	110.1	99.5	11.0	10.5	172.6	12.0	11.3	105.8	18.2	122.6	16.6	75.0	13.8	8.9	107.3
17	Ligstroside aglycone (is I)	114.2	5.4	18.5	2.8	135.3	168.0	11.6	169.3	141.5	226.3	178.2	265.8	257.1	187.7	265.4	190.3	266.8
18	Ligstroside aglycone (is II)	207.9	49.5	106.5	26.5	501.6	539.7	58.7	631.9	549.3	687.6	881.1	405.0	864.8	782.8	902.9	794.4	833.6
19	Ligstroside aglycone (is III)	112.8	12.9	26.2	4.8	186.1	205.3	18.2	214.7	192.4	268.4	312.3	228.0	370.0	253.9	338.4	271.4	338.2
Secoiridoids		*921.2*	*870.4*	*833.4*	*539.3*	*1280.2*	*1467.3*	*1226.8*	*1622.5*	*1566.7*	*2140.8*	*2146.7*	*1937.2*	*2327.7*	*2090.6*	*2255.7*	*1935.4*	*2285.2*
20	Luteolin	2.4	2.8	5.1	5.6	1.9	1.7	4.0	2.1	1.5	1.7	2.6	3.6	3.5	2.4	1.5	2.6	1.4
21	Apigenin	1.1	0.3	3.0	1.1	0.4	0.3	0.4	0.4	0.3	0.5	0.6	1.2	1.0	1.0	0.3	0.5	0.3
22	Diosmetin	1.1	0.9	1.0	1.3	0.4	0.3	1.1	0.4	0.3	0.2	0.3	1.5	0.4	0.6	0.2	0.3	0.2
Flavonoids		*4.6*	*4.0*	*9.0*	*8.0*	*2.6*	*2.3*	*5.5*	*2.9*	*2.1*	*2.4*	*3.5*	*6.4*	*4.8*	*3.9*	*2.1*	*3.4*	*1.9*
23	Pinoresinol	1.4	4.1	2.3	2.2	2.3	2.0	3.1	2.4	2.0	0.5	1.7	1.1	1.9	3.0	1.5	1.6	0.3
24	Acetoxypinoresinol	6.4	4.8	10.8	9.5	<0.1	<0.1	4.1	<0.1	<0.1	2.4	<0.1	3.8	<0.1	8.8	<0.1	<0.1	2.1
Lignans		*7.8*	*8.9*	*13.0*	*11.8*	*2.3*	*2.0*	*7.2*	*2.4*	*2.0*	*2.9*	*1.7*	*4.9*	*1.9*	*11.8*	*1.6*	*1.6*	*2.4*
Total (mg each compound/kg)		**1543**	**1242**	**1229**	**833**	**1708**	**1901**	**1651**	**2121**	**2097**	**2713**	**2670**	**2589**	**2738**	**2505**	**2662**	**2327**	**2861**
IOC HPLC method (mg TY/kg)		692	708	738	769	782	783	786	797	829	905	1037	1101	1167	1174	1175	1244	1269
FC method	*(mg caffeic acid/kg)*	459	356	274	378	517	561	513	536	594	687	732	623	748	704	797	813	697
	(mg gallic acid/kg)	449	340	253	362	510	557	505	530	591	690	737	621	753	708	809	823	700
	(mg HTY/kg)	572	440	334	467	647	704	642	671	746	866	924	783	944	888	1009	1028	878
	(mg TY/kg)	964	739	559	785	1090	1187	1081	1131	1258	1461	1560	1321	1594	1500	1705	1737	1483
HTY from acid hydrolysis		236	300	246	325	329	374	458	337	387	457	502	370	523	453	529	562	447
TY from acid hydrolysis		366	263	225	409	298	308	327	292	315	456	445	459	458	507	475	457	562
Sum hydrolys. (mg TY+HTY/kg)		602	563	470	734	627	682	785	629	702	914	947	829	981	961	1003	1019	1009

%RSD LC-MS profiling method < 6.6%; %RSD IOC method < 7.2%; %RSD FC method < 4.9%; %RSD Sum hydrolysis products < 3.4%

perfectly determined in HPLC by using the same conditions. In the current study, we have considered them as a separate group to facilitate global comparisons with other quantification approaches. All members of this family were quantified in terms of elenolic acid and their total concentration ranged from 77.4 to 621.7 mg/kg. They accounted for a mean of 28.25% of total phenols, but their percentage greatly varied depending on the sample (from 13.26 to 44.72%). Finally, secoiridoids family (just including oleuropein and ligstroside derivatives) was the most abundant group of compounds, with sum concentrations ranging from 127.5 to 2327.7 mg/kg. In terms of percentage, they stood for an average of 68.49% of the total phenolic content (fluctuating between 52.4 and 85.0%). Among all the phenolic substances, secoiridoids are those with the most challenging quantification. On the one hand, as discussed in previous reports [9,11,36], the typical analytical procedures for VOO phenolic compounds determination lead to the formation of "artificial peaks" (secoiridoids' isomeric forms, acetals or hemiacetals) when using MeOH (or any protic solvent, in general) in sample preparation or as mobile phase in LC. Therefore, the native amount of secoiridoids could be underestimated if those new peaks are not considered. On the other hand, because of the absence of commercial pure standards and the tedious process needed to achieve their isolation from VOO, secoiridoids' concentration is commonly referred to a different compound (usually with a related structure, such as oleuropein, TY or HTY). These approaches seem valid when applied in the same way to all the considered samples in comparative studies. Nevertheless, they generate questionable results for total secoiridoids concentrations, even if all isomers are taken into account for quantification purposes. To evaluate the effect of the chosen standard (to quantify with) on the obtained results, all oleuropein derivatives were also quantified using the HTY external calibration curve and ligstroside derivatives were referred to the TY standard.

Sums of individual concentrations (mg/kg) of oleuropein and ligstroside derivatives quantified in terms of their pure standard or a highly related molecule, and by comparison with the corresponding related phenolic alcohol (HTY or TY) are shown in Fig. 1 (A). Results presented in Fig. 1 (B) show good correlation between the sum of concentrations of oleuropein (I) and ligstroside (II) derivatives quantified by both strategies. R^2 were 0.9784 and 0.9684 for oleuropein and ligstroside derivatives, respectively, what means that results obtained following both quantitative approaches are highly correlated. However, while in the case of ligstroside derivatives, both results are very close (slope ≈ 1), the use of the individual standards for the quantification of oleuropein derivatives gave concentrations more than three times higher than the approach that refers their concentrations to HTY (slope = 3.31). This effect can be easily corroborated looking at yellow and green bars in Fig. 1 (A). In other words, the use of HTY standard to quantify the secoiridoids derived from oleuropein drastically underestimated their concentration. At this point it seems necessary to assert that this kind of equivalence-factors have the precise stated values when using the LC-ESI-IT MS methodology reported in section 2.3.3; similar factors should be properly calculated when the applied method changes. Different analytes' responses are expected

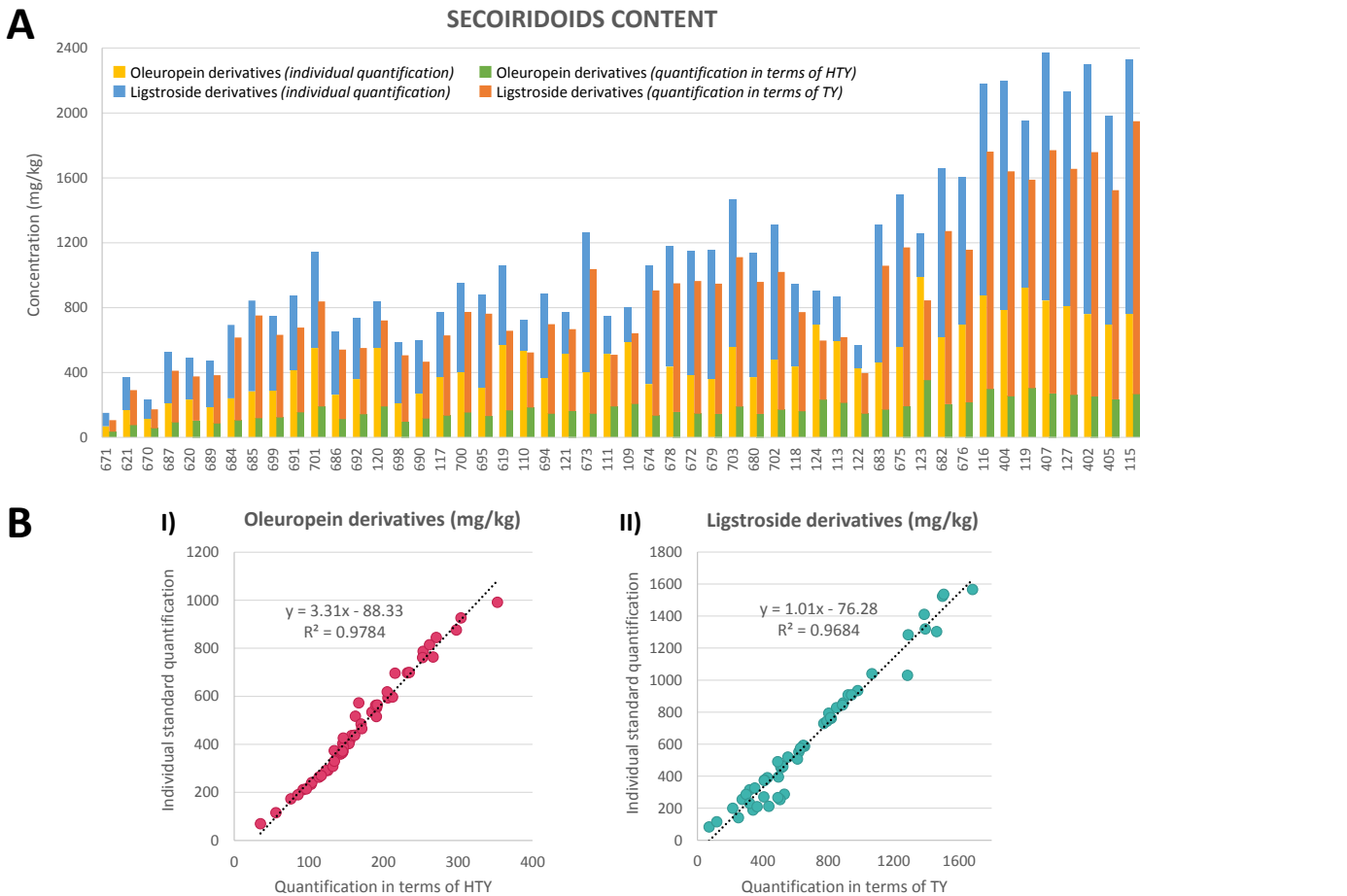

Figure 1. (A) Graph showing secoiridoids total content in the 50 analyzed EVOO samples, quantified in terms of their own standard (or a compound with a similar chemical structure) (left columns) and referred to HTY or TY (right columns). (B) Correlation between the secoiridoids' content (oleuropein derivatives (I) and ligstroside derivatives (II)) quantified following the two just mentioned strategies.

in other detectors or even when modifying ionization conditions in the same MS instrument. These findings highlight the drastic effect of the use of a different standard to determine the absolute concentrations of a given secoiridoid.

3.2. Establishment of correlations between specific and global methods

3.2.1. Sum of individually quantified phenolic compounds vs. Total phenolic indices

Once the phenolic profile of the samples under study was deciphered, the next stage of the project sought to establish correlations with the quantitative data provided by the three non-specific methods evaluated in this study. Table 2 also includes the results of estimating the phenolic content by using the IOC HPLC method, the FC assay (using different standards for quantification) and the secoiridoids' hydrolysis approach. In Fig. 2 (A), results from those global methods (depicted with colored lines, referred to the left axis) are graphically compared to the sum of concentrations found for each determined phenolic compound by the LC-MS profiling method (bars, right axis). Samples are sorted by increasing order of concentration levels determined by the IOC HPLC method (green line).

As stated in the introductory section, the direct comparison between "global results" and the sum of individually quantified phenolic compounds may not be correct in the formal sense, but it seems appropriate to bring to light the discrepancies found in literature. Total phenolic content obtained by summing up individual phenolic compounds' concentration (expressed in mg/kg) proved to be from 2 to 3.3 times higher than the values given by the non-specific methods (that is why two different scales are used in Fig. 2 (A)). In view of this finding, we could state that global methods systematically underestimated phenolic content in the analyzed oils. As far as the correlations between the profiling LC-MS method and the global ones are concerned, they were lower than 0.81 (IOC method and acid hydrolysis of secoiridoids) and 0.88 (FC colorimetric method), respectively, as shown in Fig. 2 (B) I, II and III. A better correlation (R = 0.95) between the result of the FC spectrophotometric method (mg/kg of gallic acid) and the sum of concentrations (mg/kg) of individual phenolic compounds determined by HPLC-DAD was reported in a very complete research conducted by Alessandri *et al.* [29]. This can be partially explained because they used four standards for quantification purposes (tyrosol, hydroxytyrosol, luteolin and oleuropein) and included a multiplication factor to correct the obtained concentrations for each phenolic compound, depending on the reference standard. In another thorough contribution, Garcia *et al.* found lower values of total phenolic content when it was determined by HPLC (expressing the results in mmol/kg EVOO) instead of using FC (R^2 = 0.8752) [28]. The discrepancies are probably caused by i) the number of compounds from the phenolic fraction that they considered within the LC profiles (it is much wider in the current study) and ii) the standards they used for quantification of the individual compounds (tyrosol, hydroxytyrosol, hydroxytyrosol acetate, oleuropein, oleuropein aglycone and oleacein).

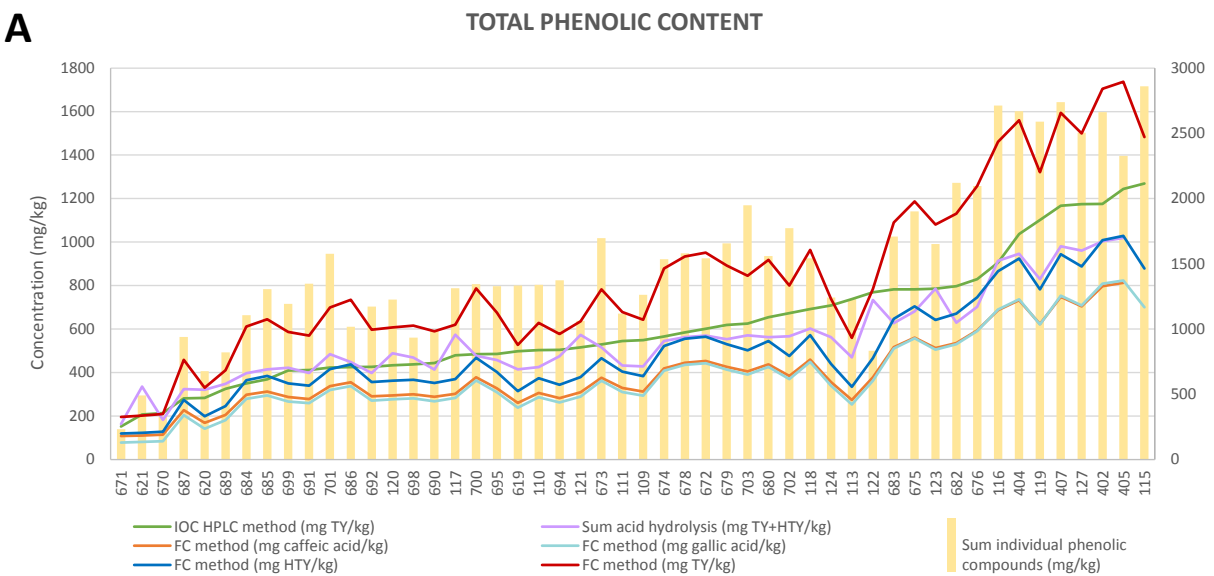

Figure 2. (A) Graph showing total phenolic content determined by the four evaluated methodologies: IOC HPLC method, FC method (using caffeic and gallic acids, TY and HTY calibration curves), sum of HTY and TY resulting from acid hydrolysis of secoiridoids and sum of individual concentrations obtained by the LC-MS profiling method (using their own standard for quantification). Lines show non-specific methods (values referred to left axis) while bars show the sum of the individual compounds obtained by the profiling strategy (right axis).

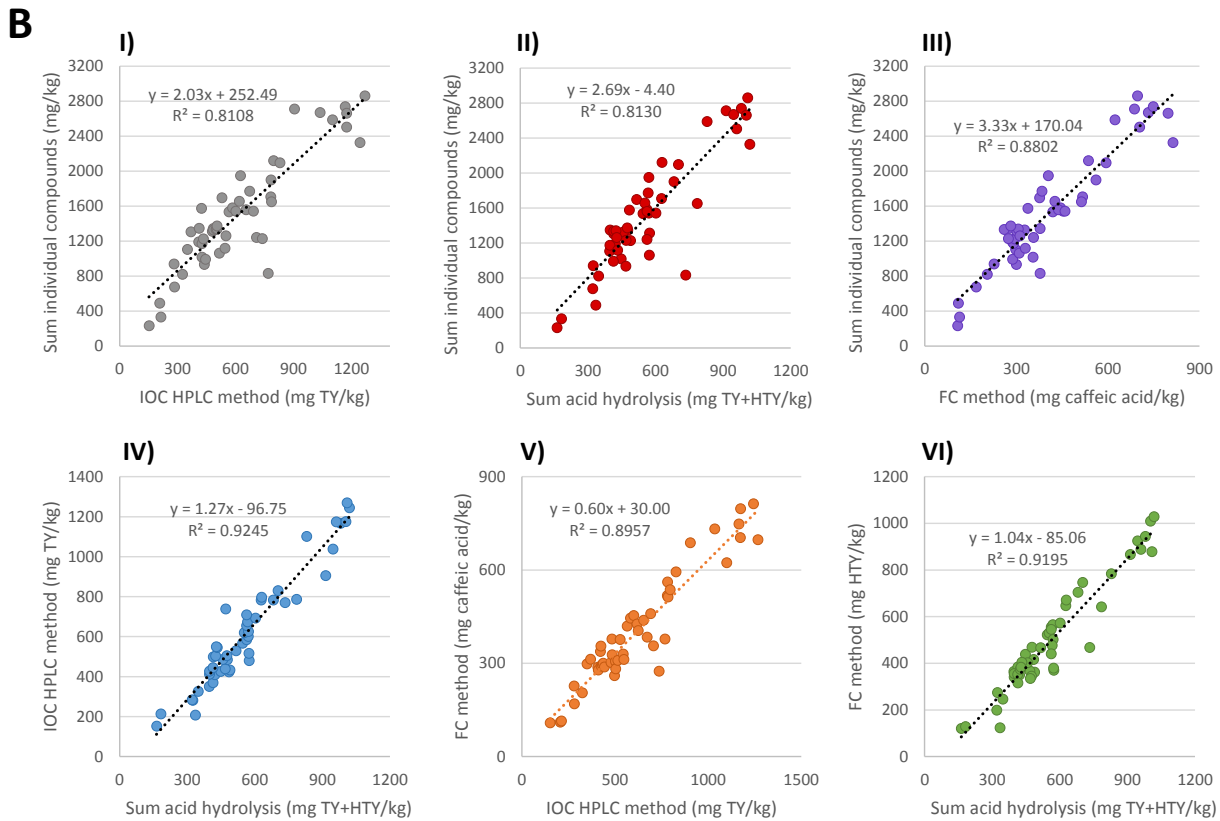

Figure 2. (B) Correlations between total phenolic content determined by different methods for the 50 EVOO samples under evaluation. For more details see the text below.

In our case, if the comparison was made in molar basis, the correlations between the sum of individual compounds and the result from the non-specific methods were slightly lower than in mg/kg. As seen in Fig. 1 SM (I, II and III), the obtained correlation coefficients were R^2 = 0.7931 with the IOC method, R^2 = 0.8541 with the FC assay and R^2 = 0.7953 with the hydrolysis approach: the new achieved equations have been logically included in the figure.

Taking into account that the IOC HPLC method with UV detection at 280 nm does not consider elenolic acid and derivatives in its global result for total phenolic content, the corresponding comparison (leaving them out) was also investigated. Fig. 1 SM (IV) shows the correlation between the sum of individual phenolic compounds excluding elenolic acid derivatives (expressed in mg/kg EVOO) versus the result of the IOC method (expressed in mg of TY/kg EVOO). The achieved equation (y=1.87x-36.75, R^2 = 0.8396) reveals a lightly improved correlation between the profiling strategy and the IOC method when exactly the same compounds are considered in both cases. The sum of individual phenolic compounds in mg/kg (excluding elenolic acid and its derivatives) is still higher than the result provided by the IOC method. The same was done for hydrolysis approach and FC method; the results are displayed in Fig. 1 SM (V) and (VI), respectively. The slopes of the equations were lower but not accurately different to those obtained when elenolic acid derivatives were included in the sum; the coefficients of determination were moderately better (R^2 = 0.8440 and R^2 = 0.9003, for hydrolysis and FC, apiece).

3.2.2. Correlations between non-specific methods

3.2.2.1. IOC method vs. Hydrolysis approach

The logical subsequent phase of the project was to evaluate the possible interconnections between the non-specific methods. Good correlations were actually found when comparing results from global methods. The IOC method gave values around 1.3 times higher (in average) than the result obtained by summing up TY and HTY generated after secoiridoids hydrolysis, with R^2 = 0.9245 (Fig. 2 (B) IV). This good correlation was previously reported by Purcaro *et al.* who found an R coefficient of 0.948 between the sum of HTY and TY determined by GC-FID after acid hydrolysis and the total phenolic content obtained by applying the IOC method [20]. However, they obtained an average ratio Total hydrolyzed (HTY+TY)/Total phenolic content (IOC method) of 1.34, which would correspond to a slope of about 0.75 if we establish a parallelism with our correlation equation. Our findings (*i.e.* obtaining higher values in general for the IOC method in contrast with the hydrolysis plus HPLC-DAD strategy) can be justified considering the fact that along with the simple phenols and secoiridoids derivatives, the IOC method also quantifies flavonoids, lignans and phenolic acids. Nonetheless, total secoiridoids concentration obtained by the hydrolysis method was higher than the total phenolic content reported by the IOC method for a substantial number of samples of those with lower levels than 400-500 mg/kg, as seen in Fig. 2 (A). The latter observation would be in good agreement with the conclusions drawn in the above mentioned publication. Therefore, the influence of minor families (other than secoiridoids), which

constitute a reduced percentage of phenols fraction, was found to be negligible for the VOOs of our sample selection with lower levels of total phenolic content. As shown in Fig. 2 SM (I), equally good correlation (R^2 = 0.9226) was found when the comparison between the total phenolic content determined by the IOC method and the sum of HTY and TY obtained after secoiridoids' hydrolysis was carried out in molar basis; the obtained correlation equation in this case was y=1.34x-0.71.

3.2.2.2. FC method vs. IOC method

Regarding the FC colorimetric assay, it logically provided different concentrations of total phenols depending on the standard used for quantification purposes. Caffeic and gallic acids, which are the most commonly used standards when this assay is applied in routine analysis, drove to practically identical results, with the following correlation equation: $FC_{(gallic)} = 1.06\ FC_{(caffeic)} - 36.21$, $R^2 = 1$. In comparison with them, the results expressed in TY and HTY equivalents were higher but perfectly correlated too ($FC_{(TY)} = 2.18\ FC_{(caffeic)} - 39.29$, $R^2 = 1$; $FC_{(HTY)} = 1.29\ FC_{(caffeic)} - 18.94$, $R^2 = 1$). If compared to the IOC determination by HPLC, the results from the FC assay, expressed in caffeic acid equivalents, were lower than the concentrations obtained by applying the IOC method with R^2 = 0.8957, regardless of whether the comparison was made expressing the results in mg or in mmol (Fig 2 (B) V and Fig. 2 SM (II), respectively). Ricciutelli *et al.* [30] investigated this topic in a paper where they compared an HPLC-DAD method developed in their lab with IOC and FC (gallic acid) procedures. Both HPLC methods were, in general, in good agreement; however, to convert the FC results into similar HPLC values, they should be multiplied by an average factor of 2.3-2.6. In this report, secoiridoid derivatives were quantified in terms of oleuropein (applying a correction factor).

3.2.2.3. FC method vs. Hydrolysis approach

The comparison of the results coming from FC with the obtained HTY and TY levels generated by secoiridoids' bonds breakage gave equations with logically different slopes depending on the standard used for quantification when performing the FC assay. However, the achieved correlations were very similar in every case considering the coefficients of determination ($R^2 \approx 0.92$). When comparing the sum concentrations of HTY and TY after hydrolysis and the FC method expressed as mg HTY/kg EVOO, analogous results were found with appreciably good correlation ($y = 1.04x - 85.06$, $R^2 = 0.9195$), as depicted in Fig 2 (B) VI. This finding was in agreement with a previous publication [26], where the authors reported no significant differences between the total phenolic content determined by acid hydrolysis of an EVOO phenolic extract (with HPLC detection of HTY and TY) and the concentration value achieved by applying the FC assay to the same phenolic extract (with or without hydrolysis step), expressing the results in HTY equivalents. In the current study, a wider sample set has been considered and results given by both methods are supposed to encompass more variability; in spite of that, our results corroborate the potential of the FC method as an alternative to the acid hydrolysis of secoiridoids, to verify the health claim referred to the HTY and derivatives content in EVOO [12].

An additional correlation (shown in Fig. 2 SM (III)) was established taking into account that monophenolic compounds are expected to form half of the color complex and, thus, to display half of the molar absorbance than the diphenolic compounds [27]. Results from the FC assay, expressed in mmol of caffeic acid, were compared to an equivalent value for the products of the acid hydrolysis (½ mmol TY + mmol HTY), obtaining a considerably good correlation (R^2 = 0.9317).

3.3. Secoiridoids analysis

As reported in section 3.1, secoiridoids were the constituents of the phenolic fraction found at highest concentrations in all the analyzed EVOO samples. Apart from the fact of being so profuse, the health claim associated to the content of HTY and its derivatives in the oil makes their accurate determination very pivotal for both producers and consumers [13]. Consequently, it seems worthwhile to carry out a deeper comparison between the individual secoiridoids concentrations determined by the LC-MS method and the amount of HTY and TY generated by the EVOOs acid hydrolysis. By understanding their existing correlations, we could somewhat evaluate if simplifying their analysis is advisable.

At this point, it seems adequate to mention the interesting report authored by Mulinacci *et al.* [18], describing chemical hydrolytic procedures to evaluate the total amount of free and/or linked TY and HTY. They observed a good correlation between the results of the hydrolysis and the total content of secoiridoids molecules revealed by HPLC at 280 nm (quantified in terms of oleuropein and applying molecular weight correction factors) before hydrolysis, the latter being systematically higher (results expressed in mmol). The importance and usefulness of other publications regarding hydrolysis has been already stated [19,20,25,37,38].

Fig 3. (A) depicts the results from both methods for secoiridoids determination. The quantification of phenolic alcohols after hydrolysis included the native amount of these compounds together with the free forms generated by the HTY/TY-elenolic acid bound breakage. For that reason, HTY and TY were summed to oleuropein and ligstroside derivatives, respectively, in order to generate a hydrolysis-homologous value obtained by the LC-MS profiling method. It could be expected that a higher concentration of oleuropein derivatives would mean a higher amount of HTY after hydrolysis and the same for ligstroside derivatives and TY. However, the situation was the opposite in some of the EVOOs included in the sample set, as shown in Fig. 3 (A). Broadly speaking, that circumstance was more commonly observed for samples with high phenolic compounds' global content.

In general, higher levels of TY after the hydrolysis reaction were found in samples presenting higher concentration of oleuropein aglycone-related analytes than ligstroside aglycone derivatives when the oils had a specific compositional pattern containing elevated levels of olecanthal and considerably high amounts of oleacein and hydroxy oleacein. Illustrative examples are EVOOs with numbers 120, 121 and 122. 120 and 121 presented the highest content of oleocanthal; moreover, 120 and 122 were the richest in terms of oleacein (isomer II). Before continuing, it seems necessary

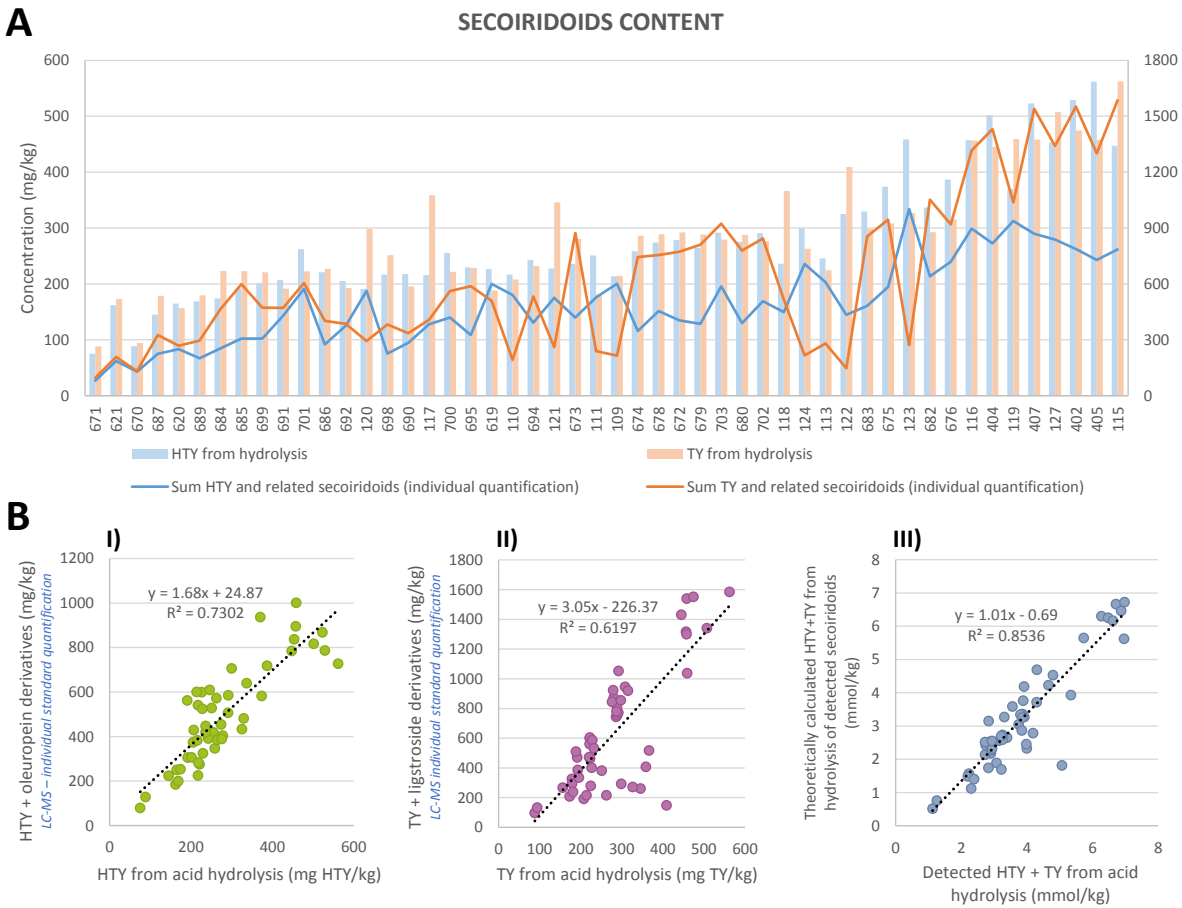

Figure 3. (A) Graph showing HTY and TY content determined after secoiridoids hydrolysis (bars, left axis) together with the sum of oleuropein or ligstroside derivatives and their related phenolic alcohol, determined by the LC-MS profiling method (lines, right axis). (B) Correlations between HTY-homologous compounds (I) and TY-analogous analytes (II) determined by acid hydrolysis and by the LC-MS method, expressed in terms of their own standard; (III) correlation between the theoretically calculated and the actual molar concentration of HTY and TY after hydrolysis.

to make a remark about the establishment of the concentration of oleocanthal and oleacein (the same is applicable to the other analytes quantified by using the non-commercially available standards). The purity of all the standards was tested and demonstrated to be higher than the 95% in every case. However, they were neither analytical standards nor certified reference materials, what means that the ascertainment of their true concentration (very challenging and troublesome task) was carried out by the labs collaborating with us that generously supplied the standards. This fact coexisted with another issue: EVOOs, in general, exhibit a wide range of variable concentrations of each bioactive phenolic compound, what compels to build calibration curves covering a broad concentration range (2 or 3 orders of magnitude). This, in turn, constrained us to use different calibration curves for the same compound (dividing the covered range in sub-ranges) and/or to inject different dilutions of the samples, depending on the concentration level to be determined. In practice, it is more intricate to achieve an accurate quantitative value in MS when the found level is rather high; this drives us to state that we could have slightly underestimated the concentration of some secoiridoids in certain samples. Besides all this, it is pertinent to say that we applied a very useful hydrolysis procedure previously described [19] whose applicability was validated for VOOs ranging from 100 to 400 mg total HTY and TY/kg oil approx. It should be interesting to fully validate the protocol for even richer oils, otherwise the effectiveness of the reaction at very high phenolic levels cannot be positively guaranteed.

Coming back to the core of the matter, an inverse behavior (*i.e.* higher sum of ligstroside derivatives' concentrations and, contradictorily, higher HTY content after hydrolysis) was found in a substantial number of oils, such as 701, 700, 683, 675, 682, 676, 404, 407, 402 and 405. All of them showed outstanding concentrations of secoiridoids (both oleuropein and ligstroside aglycones derivatives) and, specifically, had a very high concentration (or the highest) of, at least, one of the isomers of oleuropein algycone. In this regard, it is suited the argumentation previously formulated, *i.e.* the concentration of those substances could have been somewhat undervalued. The expected behavior (higher levels of HTY and greater concentration of oleuropein aglycone-related substances) was, however, observed for sample 123, which was one of the richest studied EVOOs. In this case, the EVOO had a compositional pattern characterized by predominant appearance of oleacein, hydroxyl oleacein and oleocanthal (together with relatively low levels of both oleuropein and ligstroside aglycones).

As a consequence of all the inconsistencies detected in the above mentioned samples, not very satisfactory correlation values were found between the sum of oleuropein and ligstroside derivatives quantified in terms of their pure standards and the amount of HTY and TY determined after acid hydrolysis (linear least squares regression showed R^2 = 0.7302 and R^2 = 0.6197, respectively) (Fig. 3 (B) I and II). Not much improvement in terms of correlation was found when secoiridoid derivatives were quantified in terms of their related phenolic alcohol by the LC-MS profiling methodology, as seen in Fig. 3 SM (I and II). A comparison in molar basis was also carried out by dividing each individual concentration between the molar mass of the corresponding

compound. In that way, we could compare the number of mmol of HTY and TY determined after the acid hydrolysis with the theoretically calculated number which would be generated from the detected secoiridoids in each sample (having in mind that one mol of any secoiridoid derivative will generate one mol of its phenolic alcohol). Obtained correlations were not very adequate either, as can be observed in Fig. 3 SM (III and IV).

When the molar concentration of "predicted" and measured TY were compared, a R^2 = 0.6338 and a slope of 1.17 were found. In general, the predicted TY concentration was higher than the found level. Some of most unfavorable cases (those exhibiting a worse prediction) were selected to be thoroughly evaluated. The most unsatisfactory predictions were principally made when the EVOO samples contained considerably high levels of oleocanthal and relatively low concentrations of ligstroside aglycone isomers (oils 109, 121 and 123 are clear examples of it, with 166.8, 190.9 and 172.6 mg/kg of oleocanthal, respectively, together with low amounts of isomers of ligstroside aglycone (40.2, 56.3, and 88.5, apiece)). Samples containing marked levels of oleocanthal were better predicted when they had remarkable concentrations of ligstroside aglycone isomers. Examples illustrating the latter are samples 119 (122.6 mg/kg of oleocanthal and 898.8 mg/kg of ligstroside aglycone isomers) and 115 (with 107.3 mg/kg of oleocanthal and 1438.6 mg/kg of ligstroside aglycone isomers).

As far as prediction of HTY is concerned, the measured value was in almost all the cases higher than the estimated one for HTY (slope of the correlation equation of 0.72, with R^2 = 0.6612). Samples such as 120, 402, 404, 405 and 407 can be taken as instances of inadequate predictions. The first one (EVOO number 120) had a very high concentration of oleacein (isomer I and II) and hydroxy oleacein, as stated above, and it seemed that their concentration could have been underrated. 402, 404, 405 and 407 had very elevated values of all the isomers of oleuropein aglycone and abundant levels of oleacein (isomer I). EVOO 407, for example, had 707.2 mg/kg of oleuropein aglycone and 97.1 mg/kg of the isomer I of oleacein. Just in 8 samples, the predicted HTY concentration was higher than the found HTY level after hydrolysis. All of them showed one common feature: high amounts of oleacein and hydroxy oleacein and relatively low levels (in comparison with other EVOOs from the sample set) regarding the isomers of oleuropein aglycone. Some oils exemplifying this are 123, 109, 110, 121, 124 and 113.

When the detected sum of both species (HTY and TY) in mmol/kg and its homologous theoretically calculated molar concentration were compared, equivalent results were achieved (slope of the correlation equation exceptionally close to 1) with an acceptably good correlation (R^2 = 0.8536), as shown in Fig. 3 (B) III, probably compensating the previously HTY and TY divergences.

3.4. Overall view of the achieved results

Bearing in mind the amount of data discussed in the previous sections, it appears imperative to provide room for a kind of summary. Table 3 tries to incorporate the most relevant results regarding the comparison (and establishment of correlations) among the evaluated approaches.

Table 3. Overall overview of the most relevant results regarding the comparison (and establishment of possible correlations) among the evaluated approaches.

Comprehensive LC-MS individual characterization		IOC recommended method		FC assay		Hydrolysis + HPLC-DAD	Secoiridoids from individual analysis *vs* hydrolysis	
- When pure standards are available for almost all of compounds: true individual estimation achieved - TY is an appropriate standard for correctly estimating Lig Agly derivatives - HTY drastically underestimates Ole Agly derivatives (factor ≈ 3.3)		$IOC_{(mg\ TY/kg)} \approx 1.27$ Sum acid hydrolysis$_{(mg\ TY+HTY/kg)}$	$R^2 = 0.9245$	$FC_{(gallic)} \approx 1.06\ FC_{(caffeic)}$ $FC_{(TY)} \approx 2.18\ FC_{(caffeic)}$ $FC_{(HTY)} \approx 1.29\ FC_{(caffeic)}$ (expressed in mg/kg)	$R^2 = 1$ $R^2 = 1$ $R^2 = 1$	- Quite equivalent results to those from FC assay (in terms of HTY) - Hydrolysis leads to faintly lower global results than IOC	HTY + Ole Agly derivatives from individual det.$_{(mg/kg)} \approx$ 1.68 HTY from acid hydrolysis$_{(mg\ HTY/kg)}$	$R^2 = 0.7302$
- Total phenolic content (sum of individual (mg/kg)) > 1.9-3 times if compared with global approaches* - Global methods (using the units that they traditionally use to express the results) systematically underestimate phenolic content		Higher values than the obtained levels applying $FC_{(mg\ caffeic/kg)}$ (approx. 1.5 times higher)		$FC_{(mg\ caffeic/kg)} \approx 0.60\ IOC_{(mg\ TY/kg)}$	$R^2 = 0.8957$		TY + Lig Agly der. Individual det.$_{(mg/kg)} \approx$ 3.05 TY from acid hydrolysis$_{(mg\ TY/kg)}$	$R^2 = 0.6197$
- HPLC-DAD could not be advised for individual profiling; baseline chromatographic separation of these substances is practically unfeasible (chance of properly quantifying very few analytes)								
Sum individual phenolic compounds (excluding EA derivatives)$_{(mg/kg)} \approx 1.87\ IOC_{(mg\ TY/kg)}$	$R^2 = 0.8396$			$FC_{(mg\ HTY/kg)} \approx 1.04$ Sum acid hydrolysis$_{(mg\ TY+HTY/kg)}$	$R^2 = 0.9195$		Theoretical HTY from hydrolysis of detected Ole Agly der.$_{(mmol/kg)} \approx 0.72$ Detected HTY from acid hydrolysis$_{(mmol/kg)}$	$R^2 = 0.6612$
Sum individual phen. Comp.s (excl. EA der.)$_{(mg/kg)} \approx 2.47$ Sum acid hydrolysis$_{(mg\ TY+HTY/kg)}$	$R^2 = 0.8440$			$FC_{(mmol\ caffeic/kg)} \approx 0.88$ Sum acid hydrolysis$_{(mmol\ eq.\ caffeic/kg)}$	$R^2 = 0.9317$			
Sum individual (excl. EA der.)$_{(mg/kg)} \approx 3.04$ FC method$_{(mg\ caffeic\ acid/kg)}$	$R^2 = 0.9003$						Theoretical TY from hydrolysis of detected Lig Agly der.$_{(mmol/kg)} \approx 1.17$ Detected TY from acid hydrolysis$_{(mmol/kg)}$	$R^2 = 0.6338$

*In all the cases, calculations were made (and included within the paper) in mg/kg and in molar basis

Abbreviations used within the current table (in alphabetical order): EA: elenolic acid; FC: Folin-Ciocalteu; HTY: hydroxytyrosol; IOC: International Olive Council; Lig Agly: ligstroside aglycone; Ole Agly: oleuropein aglycone; TY: tyrosol.

A robust LC-MS method thoroughly validated was applied to obtain the concentration of the individual phenolic compounds (24 substances) in the 50 selected samples as accurately as possible. The quantification was done considering two different scenarios: 1) having pure standards of almost all the targeted analytes (ideal context), and 2) possessing just commercially available standards (most common situation). Compositional patterns of the oils were first established simulating both scenarios. Good correlation of the results was observed; however, it was noted that the use of HTY standard to quantify the secoiridoids derived from oleuropein drastically underestimated their concentration (by a factor of about 3.3). It is clear that the most accurate determination is the one which can be achieved within the ideal scenario (logically presuming an appropriate extraction protocol and an exhaustive control of all the technical conditions), however, that is an exceptional situation. In addition, we are aware about the fact that establishing cutting-edge technologies as the recommended or official approaches for determining phenolic compounds from VOO would substantially limit the number of entitled laboratories to carry out such analysis. HPLC-DAD cannot be advised for individual profiling, since the baseline chromatographic separation of these substances is practically unfeasible, giving to the analyst the chance of properly quantifying very few analytes.

When the comprehensive profiling is not required, "global methods" can represent a good option. After all, what every analyst covets is a simple, repeatable, robust and easily adaptable to the conditions of as many analytical laboratories as possible.

For that reason, once the samples were fully characterized, we tried to establish possible correlations between specific and non-specific methods. Total phenolic content obtained by summing up individual phenolic compounds' concentration proved to be from 1.9 to 3.0 times higher (when data were expressed in mg/kg) than the values given by the three global methods (with R^2 fluctuating from 0.84 to 0.90). In view of this finding, we could state that global methods systematically underestimated the phenolic content in the analyzed oils.

Comparing the non-specific strategies, it was perceived that the IOC method gave values around 1.3 times higher (in average) than the result obtained by summing up TY and HTY generated after secoiridoids hydrolysis (R^2 = 0.9245), although for some samples IOC lead to lower values. Results from FC assay with caffeic and gallic acids were equivalents, but slightly varied when HTY was used as reference standard and, more drastically for TY ($FC_{(TY)} \approx 2.18\ FC_{(caffeic)}$, R^2 = 1). If compared to the IOC determination, the results from the FC assay (caffeic acid equivalents) were lower than the concentrations obtained by applying the IOC method (R^2 = 0.8957). As previously stated by other authors, when we tried to correlate the sum concentrations of HTY and TY after hydrolysis and the FC method (in mg HTY/kg EVOO), analogous results were found with appreciably good correspondence; this outcome is in favor of the potential of the FC method as an alternative to the acid hydrolysis of secoiridoids. Nevertheless, FC is obviously unable of giving a separate estimation about the oleuropein and ligstroside derivatives, which can be very interesting.

An independent section of the contribution focused on secoiridoids determination, taking into account the individual secoiridoids concentrations from LC-MS and the amount of HTY and TY generated by the EVOOs acid hydrolysis. Not entirely satisfactory correlation values were found between the sum of oleuropein and ligstroside derivatives (both quantified in terms of their pure standards and their corresponding phenolic alcohol) and the amount of HTY and TY determined after acid hydrolysis (R^2 = 0.7302 and R^2 = 0.6197, respectively). In this regard, the typical features of some samples with discrepant results were defined and exhaustively discussed. A comparison in molar basis was also carried out to note the similarities between the number of moles of HTY and TY determined after the hydrolysis and the theoretically calculated number (estimated from the amount of secoiridoids found in each sample), selecting several specific examples to illustrate the different found situations.

4. CONCLUSIONS

Despite the general recognition of the problems with the quantitative estimation of phenolic compounds from olive oil, no consensus method has been published. The scientific community is making efforts to point at the most convenient analytical approach, but different views and methods of choice coexist so far. This contribution has not attempted to completely unravel the existing issues in this regard, but to present a comprehensive comparison between specific and global methods (applied to the analysis of a varied sample-set), to meticulously discuss the results and to identify points of reflection. The LC-MS individual establishment of absolute concentration values (using pure standards of all the phenolic substances under study) is the optimal and most reliable and accurate situation. Total phenolic content obtained by summing up individual phenolic compounds' concentration resulted to be higher than the values given by the three non-specific methods; in any case, IOC method, FC assay (applied to a properly obtained VOO extract) and hydrolysis approach (using validated and efficient protocols) can represent valid strategies when a global value is pursued. Good correlations between their results have been found. Hydrolysis of bound forms and determination of HTY and TY could be considered as a reasonable compromise solution concerning the health claim on "olive oil polyphenols". In that respect, the current contribution cannot be concluded without mentioning the need of re-formulating the claim (wording, used terminology, compounds to be considered, methodological standardization, expression of the results (units), verification of the phenolic content value to be included in the claim (x mg/20 g oil), etc.). Some colleagues have already taken into consideration this point with an eloquent reasoning; our positioning about this is absolutely the same.

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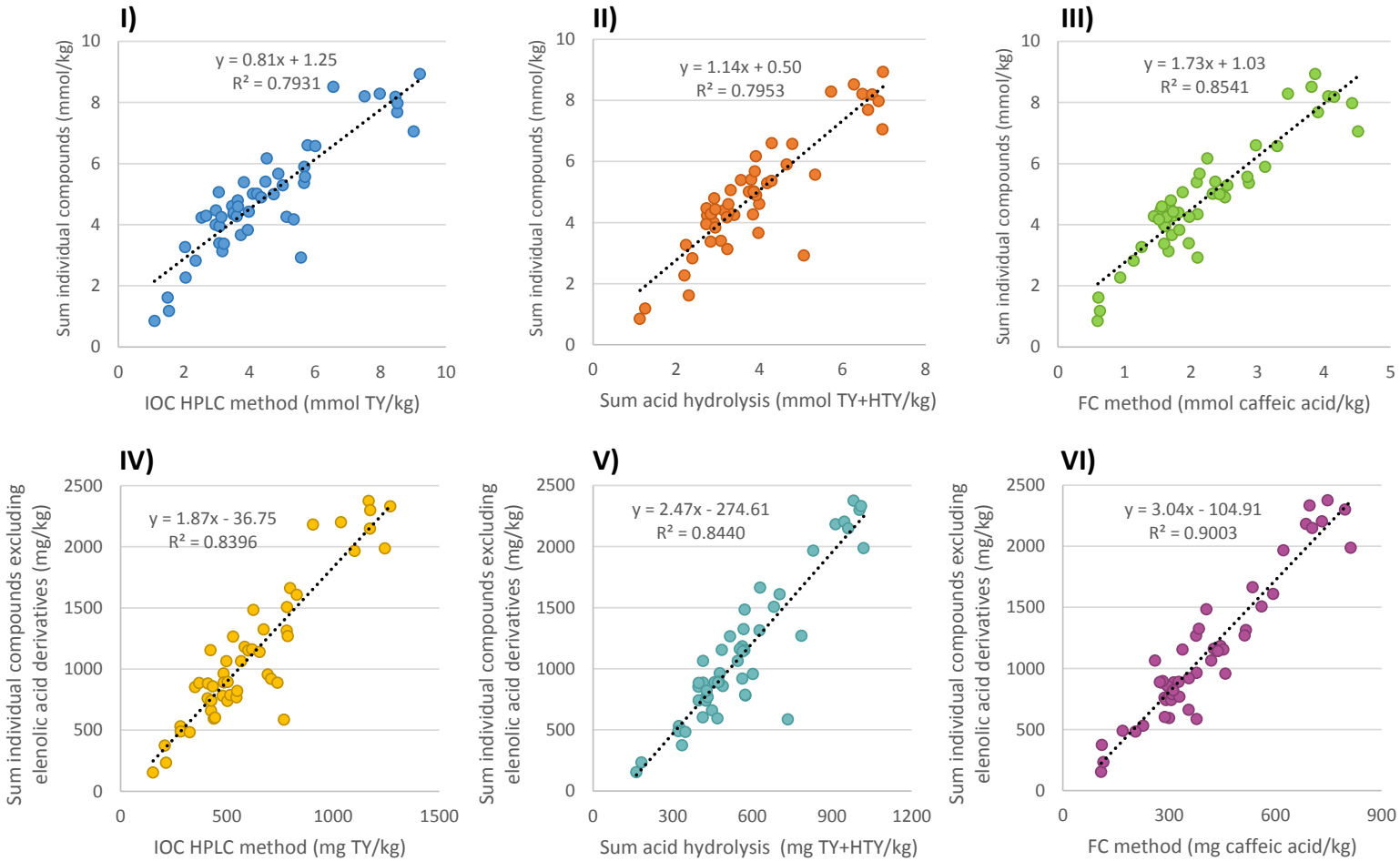

Figure 1 SM. (I, II, III) Correlations between the sum of concentrations found for each determined phenolic compound by the LC-MS profiling method and total phenolic content determined by the three different non-specific methods for the 50 EVOO samples under evaluation (results expressed in molar basis). (IV, V, IV) Same correlations as above, excluding elenolic acid and derivatives from the sum of individual compounds determined by LC-MS (results expressed in mg/kg).

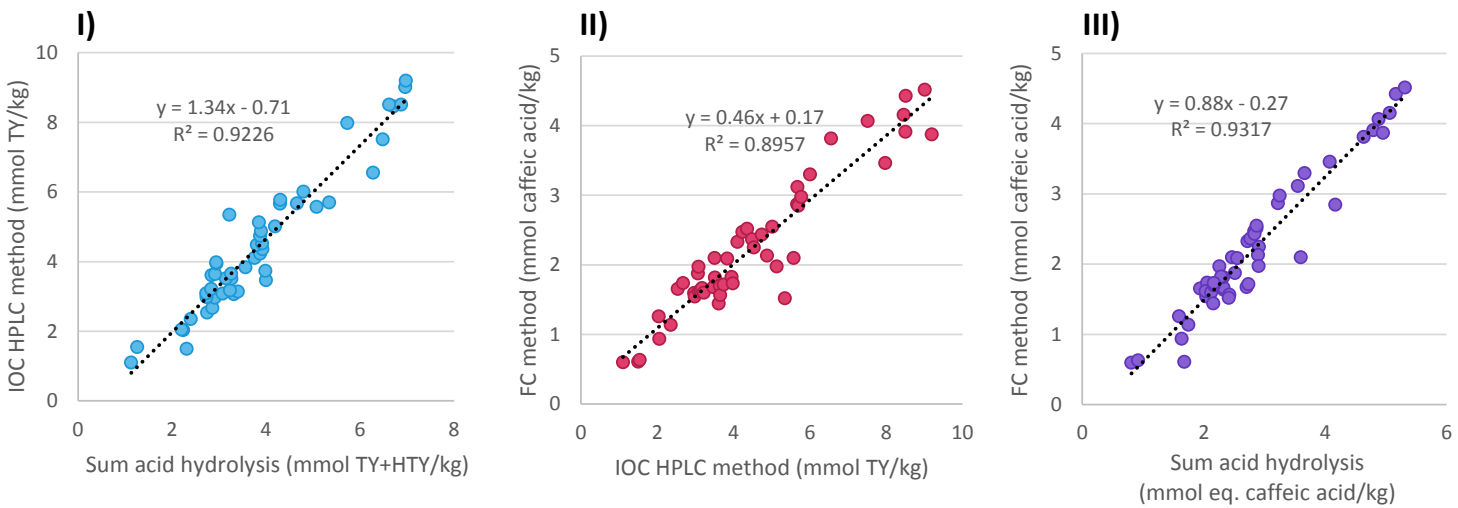

Figure 2 SM. Correlations between the total phenolic content determined by the three different non-specific methods for the 50 EVOO samples under evaluation. Results expressed in molar basis.

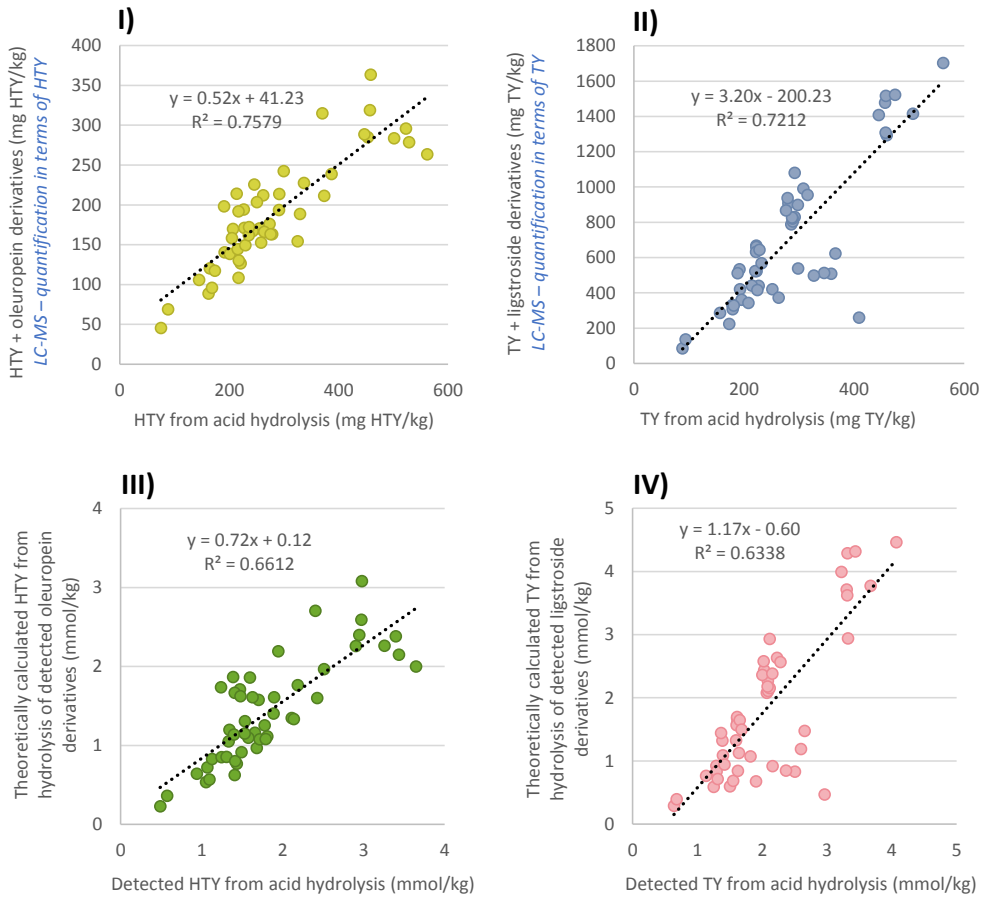

Figure 3 SM. Correlations between HTY (I) and TY (II) related compounds determined after acid hydrolysis and by the LC-MS method, expressed in terms of the corresponding phenolic alcohol. Correlation between the theoretically calculated and the actual molar concentration of HTY (III) and TY (IV) after hydrolysis.

Chapter 5

Phenolic compounds profiling of virgin olive oils from different varieties cultivated in Mendoza, Argentina, by using liquid chromatography-mass spectrometry

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Abstract: The aim of this work was to achieve a preliminary characterization of the profile of the phenolic fraction of virgin olive oils (VOOs) from Maipú (Mendoza, Argentina). Thus, 25 commercial VOO samples from Arauco, Arbequina, Picual, Frantoio, Changlot, Empeltre, Nevadillo, Manzanilla and Coratina (both monovarietals and blends) were analyzed using LC-ESI-QTOF MS and LC-ESI-IT MS for identification and quantification purposes, respectively. A rapid LC method (15 min) accomplished quantitative information about a total of 40 phenolic compounds, including secoiridoid derivatives, which have not been evaluated before in samples coming from the sub-region so-called Maipú (Mendoza province, Argentina). The results make evident that olive oils coming from Mendoza can be considered as important sources of phenolic bioactive compounds, exhibiting similar phenolic compounds levels to those shown by oils from other typical world production regions. Moreover, some distinctive features of Arauco variety (Argentinean autochthonous variety) were pointed out; indeed, a correlation between flavonoids content and botanical variety was established herewith.

Keywords: food metabolomics; phenolic compounds; Argentinean olive oil; Arauco olive variety.

1. INTRODUCTION

Virgin olive oil (VOO) is a valuable vegetable oil which contains minor biomolecules of outstanding importance, such as vitamins, carotenoids, tocopherols, phenolic compounds, and other natural antioxidants [1]. Among these minor constituents, the relevance of phenolic compounds is irrefutable, since they contribute to the stability of VOO against auto-oxidation, are intimately associated to VOO taste, exhibit anti-inflammatory and antimicrobial activities (among others), and could prevent certain diseases linked with the oxidative damage [2,3]. The just mentioned phenolic fraction is composed by a heterogeneous mixture of analytes (phenolic acids, simple phenolic alcohols, flavonoids, secoiridoids, and lignans) [3,4], what explains the difficulties to achieve their accurate determination. This task has been tackled developing different methodologies [3,5-7]. Separative techniques coupled to different detectors have been used when the individual determination of these compounds is aimed, being liquid chromatography-mass spectrometry (LC-MS) one of the most popular and extensively used couplings nowadays [8-11]; this platform is indeed very appreciated in the field of Food Metabolomics.

Studies about phenolic compounds present in VOO have been performed pursuing diverse objectives, as for instance, to observe their link with agronomical factors and technological conditions of production [12-15] assessing the influence of climate and soil, olive cultivar, extraction system, processing conditions, etc [6,16-20]. The samples selected in most of this kind of investigations are olive oils coming from the main producing areas of the world (Spain, Italy, Greece, Morocco, among others) [13,17,21,22]; however, oils originating from other production regions, such as Argentina, lack this valuable information.

Argentina, located in the South of the American continent, has greatly extended the country olive oil production zones over the last years. Its domestic production has several remarkable advantages: the strategic location of the country (being able to market fresh oils when Mediterranean producers cannot supply them); the ability to produce increasing volumes of high quality VOOs [23] and the possibility of producing olive oil with remarkable differences on their characteristics (due to the diverse cultivars grown in Argentina and the very heterogeneous soils and microclimate conditions of the producing areas). Within the country, there is a typical production area, central-west located, so-called Mendoza province, which has a long tradition of olive growing characterized for planted trees of about 100 years old. Inside of Mendoza, the sub-region called Maipú is extensively planted with a botanical variety identify as *Arauco*, typically cultivated for producing table olives, mainly due to its good size and high flesh-to-pit ratio [15,24]. However, over the last years, it has been demonstrated that this cultivar has profitable characteristics for commercial production of VOO, since it has relatively high oil content, a well-balanced fatty acid composition and a distinctive profile of minor antioxidants [15]. It is the only cultivar recognized from Argentina in the World Catalogue of Olive Varieties since 1995 [25]. Some other varieties grown in Argentina are Arbequina, Manzanilla, Picual and Frantoio, among others [15,26].

As previously stated, very few reports have been published including information about the phenolic composition of Argentinean olive oils [7,27,28]. For example, one of these studies carried out a characterization of monovarietal Argentinean olive oils from 4 provinces, accomplishing the determination of the phenolic compounds by using a spectrophotometric method (total content) based on Folin-Ciocalteu reactive [28]. Another contribution described the characterization of the phenolic composition of commercial extra-VOOs from different countries (including just few samples from Argentina) [7]. Later on, the phenolic compounds and antioxidant capacity of monovarietal olive oils produced in Argentina were evaluated by capillary zone electrophoresis, but the analytes under study did not include secoiridoids and its derivatives (main group of phenolic compounds from VOO, which represents a high percentage of the total phenolic fraction and is exclusive of plants belonging to the family *Oleaceae*) [27]. Finally, another stimulating work focused on physiological aspects and minor antioxidant compounds from Arauco *cv.* during fruit ontogeny should be mentioned, since included very interesting results about the optimum maturity index of this cultivar [15].

The aim of our work was to undertake a comprehensive characterization of the phenolic fraction of commercial VOOs from different varieties cultivated in the confines of the geographical zone of Mendoza province (Argentina) by LC-MS. A liquid chromatography-electrospray ionization-quadrupole-time of flight mass spectrometry (LC-ESI-QTOF MS) was used to characterize the phenolic profiles and, afterwards, liquid chromatography-electrospray ionization-ion trap mass spectrometry (LC-ESI-IT MS) was used to carry out the quantification. This is the first

time that VOOs from this territory have been studied by using this technology, making possible to describe in depth the composition of the phenolic fraction.

2. MATERIALS AND METHODS

2.1. Reagents and materials.

All reagents were of analytical grade and were used as received. Methanol and *n*-hexane of HPLC grade were supplied from Panreac (Barcelona, Spain); they were used for the extraction of the phenolic compounds from the olive oil samples. Mobile phases were prepared by using Acetonitrile (ACN) from Lab-Scan (Dublin, Ireland) and acetic acid from Panreac. Doubly deionised water with a conductivity of 18.2 MΩ cm was obtained by using a Milli-Q-system (Millipore, Bedford, MA, USA). Standards of caffeic, *p*-coumaric, quinic and ferulic acids, as well as hydroxytyrosol (HTY), tyrosol, luteolin, apigenin, and 3,4-dihydroxyphenylacetic acid (DOPAC) (internal standard (IS)) were purchased from Sigma-Aldrich (St. Louis, MO, USA). (+)-Pinoresinol (Pin) was acquired from Arbo Nova (Turku, Finland) and oleuropein (Ole) was purchased from Extrasynthese (Lyon, France). Stock solutions were prepared by dissolving the appropriate amount of the compound in methanol at a concentration of 500 μg mL^{-1} for each phenolic compound. Afterwards, they were serially diluted to working concentrations (within the range 0.5 - 250 μg mL^{-1}). Both the samples and stock solutions were stored in dark flasks at -20 °C and, before being injected into the instrument, they were filtered through a Clarinert™ 0.22 μm nylon syringe filter from Agela Technologies (Wilmington, DE, USA).

2.2. Samples

The VOOs studied in this work were commercial samples, acquired from Argentinean companies. The selection included monovarietal olive oils from the following varieties: Arbequina (1 sample), Manzanilla (3 samples), Frantoio (2), Empeltre (1), Nevadillo (1), Arauco (6), Picual (1), Coratina (2) and Changlot (named as Genovesa by some authors in Spain) (1); and different blends (7). Composition of Blends was the following: Blend 2 and 3: 60 % Arbequina, 30 % Frantoio, 3-4 % Arauco, 7-6 % Unknown; Blend 4: 70 % Arauco, 30 %, Arbequina; Blend 5: 70 % Arbequina, 30 % Arauco; and Blends 1, 6 and 7: unknown. The oils were extracted on season 2014 (just one sample was from the end of season 2013 (Arauco number 1)) by two phases continuous centrifuge and were obtained from olives with a maturity index of around 3 (ripening index facilitated by the technical department of the factories). All samples were kept refrigerated in appropriate containers until their analysis. Stability tests were applied to different aliquots of the samples as well as to the achieved extracts in order to assure their proper storage until the analysis. These tests were based on the comparison of the peak areas obtained from the LC-MS analysis of fresh extracts prepared from the properly stored samples with those peak area values of the extracts which had been stored for a certain period of time (max. storage time tested was 4 months), not detecting statistically significant differences.

An important characteristic of this sample-set is that all the different steps of the elaboration process were performed in Maipú (a sub-region of Mendoza province of 617 km^2); the coordinates of the studied zone are 32° 58′ 0″ S, 68° 46′ 0″ W, and their altitudes above the sea level are 804 m (arid temperate and precipitations about 200 mm annual)..

2.3. Extraction of phenolic compounds

The phenolic compounds were isolated by using a liquid-liquid extraction according to a previously reported procedure [3], which can be briefly described as follows: 2.0±0.1 g of olive oil were weighed in a test tube with a screw cap. A volume of 0.025 mL of a solution of the compound selected as IS (at a concentration of 500 mg L^{-1}) was added (to have an internal reference within the samples which could give us the chance to assure that the extraction protocol was carried out properly and the system was operating correctly). The solvent of the IS solution (MeOH) was evaporated (using N_2), 1 mL of n-hexane was added and the tube was shaken in a vortex during 30 s. The phenolic compounds under study were extracted three times, by adding 2 mL of methanol/water (60:40, v/v), shaking over 2 minutes and centrifuging at 3500 rpm for 6 minutes (each time). The supernatants were combined and evaporated to dryness using a rotary evaporator. The residue was redissolved in 1 mL of methanol and filtered through a 0.22 µm membrane filter.

2.4. LC-MS analysis: chromatographic and MS detection conditions

Two LC-MS platforms were used within this study. One of them was a Waters Acquity UPLC™ H–Class system (Waters, Manchester, UK) coupled to a micrOTOF-Q II™ mass spectrometer (Bruker Daltonics) by means of an ESI source. The second one was an Agilent 1260-LC system (Agilent Technologies, Waldbronn, Germany), which was coupled to a Bruker Daltonic Esquire 2000™ IT MS (Bruker Daltonics, Bremen, Germany) with an ESI interface. The first platform was used with qualitative purposes and the second one was employed to carry out the quantification experiments.

The separation of the target compounds was performed using a Zorbax Eclipse Plus C_{18} analytical column (4.6 x 150 mm, 1.8 µm particle size) protected by a guard cartridge of the same packing. The temperature of the column oven was set at 35 °C and a flow rate of 1.2 mL min^{-1} was selected. A volume of 10 µL of the olive oil extracts, pure standards and standard mixtures was injected in each case. The mobile phases used were water with acetic acid (0.5 % v/v) (Phase A) and ACN (Phase B), and the solvent changed as follows: 0 to 10 min, 10-50 % B; 10 to 12 min, 50-100 % B; 12 to 13 min, 100-10 % B. Finally, the column was re-equilibrated for 1.5 min.

With the aim of avoiding the introduction of humidity into the system and achieving stable electrospray ionization and reproducible results, the flow delivered into the MS detectors from LC was reduced to approx. 0.3 mL min^{-1} using a proper split.

The QTOF MS system was operating in negative and positive mode (to increase the information achieved about the VOO samples) within the range of 50-1200 m/z, at a scan speed of 240 ms. A drying gas (N_2) temperature of 300 ºC and a flow of 9.0 L min^{-1} were selected as optimum. The capillary voltage was set at 4500 V and the end plate offset at -500 V. Internal calibration was performed using sodium formate clusters and using similar strategies to those described in previous works [3,16].

The IT MS was operated in negative ion mode and the capillary voltage was set at +3200 V. Acquisition was made in full scan mode within the range of 50-1000 m/z. The nebulizer gas was set at 30 psi, dry gas at 9 L min^{-1}, and drying gas (N_2) temperature at 300 ºC.

The data resulting from both MS systems were processed through Data Analysis 4.0 software (Bruker Daltonics). In the case of accurate mass data of the molecular ions, the software provided a list of possible elemental formulas, giving a parameter (Sigma value) which shows the prediction confidence. MS/MS experiments were conducted with the use of AutoMS data acquisition mode, which is based on the fragmentation of the most abundant precursor ions per scan. For certain masses of interest, if the intensity of the *m/z* was low, a second analysis -including the list of the selected precursor ions- was performed in multiple reaction monitoring mode.

2.5. Statistical data analysis

The Unscrambler® v9.7 (CAMO software, Inc., Aspen, New Jersey, USA) was the software employed for data treatment. First, we carried out one-way analysis of variance (ANOVA) to determine the significance of the differences among the phenolic compounds concentration levels of the diverse cultivars. Afterwards, principal component analysis (PCA) was performed using the LC-MS data. The PCA matrix was composed by 40 variables (the number of phenolic compounds that were quantified in the VOO samples) and 25 samples (average value of the 4 analyzed replicates). Apart from it, we built a series of 2D plots where the samples were modelled considering the total values of the determined chemical classes (one-to-one).

3. RESULTS AND DISCUSSION

3.1. Optimization of the chromatographic conditions

One of the objectives of this study was to obtain a rapid and efficient chromatographic method (if possible, shorter than those previously reported), which could allow the separation of the phenolic compounds under study. To achieve the formulated purpose, the work started with the search of the most convenient chromatographic conditions and the optimization was carried out taking into account separation, selectivity, sensitivity, peak shape and analysis time. Different gradients were tested, together with other variables, such as flow rate and column temperature. Fig. S1 (supplementary materials) shows the base peak chromatogram (BPC) obtained by using the optimum conditions; the gradient employed is also illustrated in the figure. It can be observed that

good resolution and peak shape were achieved by using a flow rate of 1.2 mL min^{-1} at 35 °C, in particular within the analytical window comprised from 10 to 15 min, where achieving a proper resolution between Pin and acetoxypinoresinol (AcPin), apigenin and diosmetin, as well as some secoiridoids was not trivial (the separation between the mentioned compounds can be properly observed in Fig. 1, which is presented in the next section).

3.2. Phenolic compounds determination

Peak identification was done bearing in mind the previously reported information [3-5], retention time (R_t) and ESI-IT MS and ESI QTOF MS and MS/MS information obtained from pure standards and olive oil samples. Fig. 1 includes the extracted ion chromatograms (EICs) of the 40 analytes determined. The compounds have been separated into 4 groups to make easier to the reader its visual inspection. As can be seen, phenolic acids are eluted in the time window from 1 to 7 min approximately, needing relatively low percentages of ACN and sharing the analytical window with simple phenolic alcohols. Flavonoids and lignans are at close proximity in the chromatogram; they have been depicted together with simple phenols. Some of the compounds belonging to secoiridoid class, exhibit lower polarities and, therefore, need higher percentages of ACN. Elenolic and ligstroside derivatives have been represented together, including in the last chromatogram of the figure, the oleuropein derivatives. As mentioned above, 40 compounds could be determined with this method in less than 15 min, demonstrating its great potential for VOO phenolic compounds analysis.

After characterizing the profiles, the analytical parameters of the method were evaluated. The linearity of the detector response was verified with standard solutions at 11 different concentration levels over the range defined from the quantification limit to 250 mg L^{-1} (0.5; 1; 5; 12.5; 25; 35; 50; 100; 150; 200 and 250 mg L^{-1}). Lower concentrations values were injected when necessary to properly estimate the limits of detection and quantification for the analytes under study or to quantify correctly the compounds found at very low concentration levels. Each point of the external calibration curve (no significant matrix effect was observed) was evaluated in triplicate. Calibration curves were built for each standard by plotting the standard concentration as a function of the peak area obtained from LC-ESI-IT MS analyses (using the *m/z* signal considered to quantify). The following equations were obtained: quinic acid ($[M-H]^-$=191; y =43784x-2158; r^2=0.995); HTY ($[M-H]^-$=153; y=33174x+21145; r^2=0.981); tyrosol ($[M-H]^-$=137; y=13415x-4269; r^2=0.985); caffeic acid ($[M-H]^-$=179; y=43411x-19251; r^2=0.986); *p*-coumaric acid ($[M-H]^-$=163; y=21198x-969; r^2=0.994); ferulic acid ($[M-H]^-$=193; y=24317x+714; r^2=0.996); Ole ($[M-H]^-$=539; y=3459x+8238; r^2=0.98); luteolin ($[M-H]^-$=285; y=92191x+30091; r^2=0.986); Pin ($[M-H]^-$=357; y=35311x+147; r^2=0.986); and apigenin ([M-H]=269; y=87233x+157257; r^2=0.985). The compounds which were not available as commercial standards were quantified on the basis of other analytes with similar chemical structures. In particular, lignans hydroxypinoresinol (HPin) and AcPin were quantified in terms of Pin, diosmetin was quantified using the calibration curve of luteolin, and secoiridoids and HTY derivatives were quantified by comparison with HTY or tyrosol. Specifically, elenolic acid (EA),

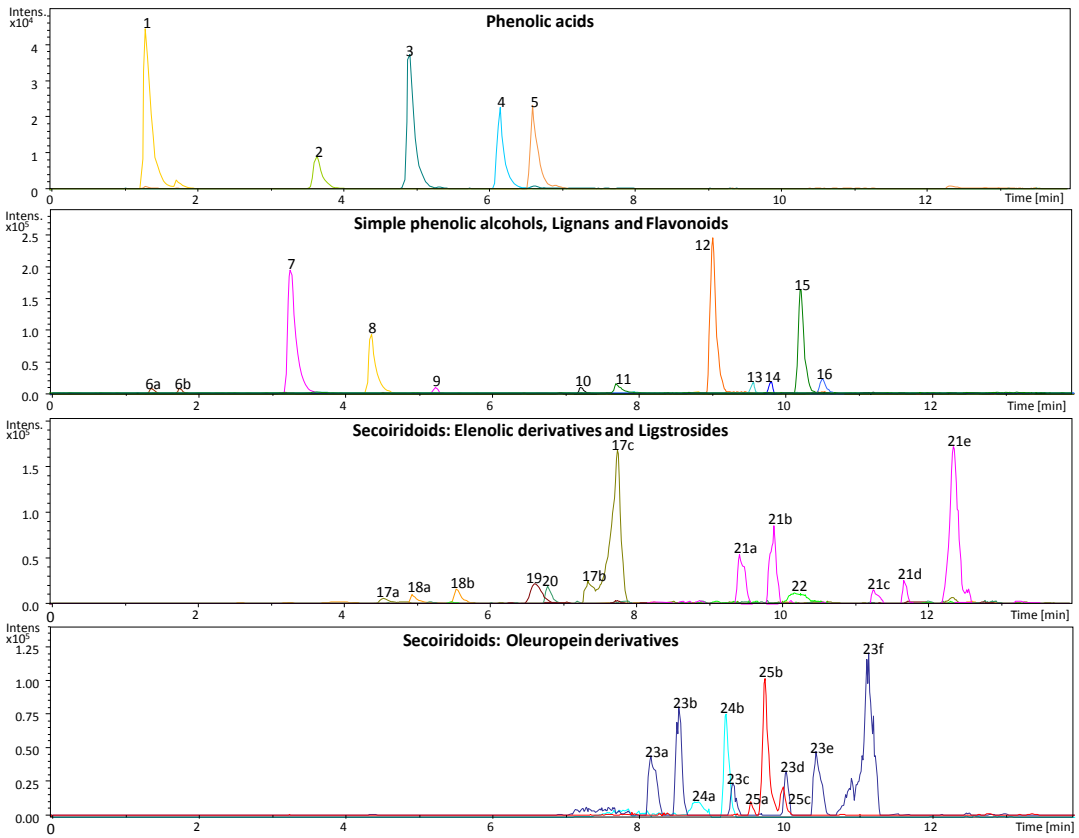

Figure 1. EICs of the 40 analytes quantified in this work. (1) quinic acid; (2) DOPAC; (3) caffeic acid; (4) p-coumaric acid; (5) ferulic acid; (6) OxHTY and isomer; (7) HTY; (8) tyrosol (9) HTY isomer; (10) AcHTY; (11) HPin; (12) luteolin; (13) Pin; (14) AcPin; (15) apigenin; (16) diosmetin; (17) EA and isomers; (18) DEA and isomer; (19) DesoxyEA; (20) HEA; (21) Lig Agly and isomers; (22) DLA; (23) Ole Agly and isomers; (24) DOA and isomers; and (25) 10-H Ole Agly and isomers. The isomers are identified by adding a letter (a, b, c, d, e, f) to the number assigned for the main isomer.

decarboxymethyl elenolic acid (DEA), hydroxyelenolic acid (HEA), desoxy elenolic acid (DesoxyEA), ligstroside aglycone (Lig Agly) and decarboxymethyl ligstroside aglycone (DLA) were quantified in terms of tyrosol; whilst, oxidized hydroxytyrosol (OxHTY), hydroxytyrosol acetate (AcHTY), decarboxymethyl oleuropein aglycone (DOA), 10-hydroxy oleuropein aglycone (10-H Ole Agly) and oleuropein aglycone (Ole Agly) were quantified by comparison with HTY pure standard. Limits of detection (LOD) and quantification (LOQ) (considering S/N equal to 3 and 10, respectively), as well as repeatability (*intra-day* and *inter-day* in terms of relative standard deviation (%RSD) of peak area and retention time) were calculated; these results are included in Table S1. LODs were found between 6.2 and 72.5 μg L^{-1}; %RSD for *inter-day* repeatability was between 1.92 and 7.52% for peak area, not exceeding 1.24% for retention time. Once that the analytical parameters of the method were established, the next step was the determination of the phenolic compounds in the entire sample set. As already stated, the whole idea behind collecting this sample set (including both monovarietal oils and blends) was to get an overall view of the composition (in terms of phenolic compounds) of the VOOs available in the local market at that time. The main requirement that the samples had to fulfill was that they were cultivated and produced in the sub-region of Maipú, being, logically, suitable for consumption. At this point is possible to say that our contribution had a multiple intention: to explore the potential of several varieties grown in Maipú to obtain high-quality olive oils (information missing so far); to expand the knowledge about the phenolic profile of Argentinean commercial oils; and, to a certain extent, to allow the long-term improvement of their international market positioning.

Table 1 shows the results for the individual phenols, which has been divided in Table 1a and 1b in order to include all the samples and facilitate the visual inspection. Results of ANOVA test revealed that statistically significant differences (95%; $p < 0.05$) were observed for the quantified phenolic compounds according to the cultivar (data not shown to contain the size of Tables 1a and 1b and facilitate its visualization). Fig. 2 shows the total phenolic content of each sample, value which has been obtained through the sum of the concentrations of the 40 quantified analytes. In the figure, each bar includes information about the concentration levels of phenolic acids, simple phenolic alcohols, lignans, secoiridoids and flavonoids.

The phenolic profile of all the samples was dominated by the presence of secoiridoid derivatives, being the sample with the highest levels of total phenolic compounds Arauco 5 with 404.09 mg kg^{-1}; Blend 7 was, on the contrary, the sample with the lowest concentrations (91.55 mg kg^{-1}). The found levels are comparable with previously published results obtained from commercial samples coming Argentina [7,15,28] and other production areas, such as Spain [29-31], Italy [7] and Morocco [3]. However, remarkable differences can be observed when the comparison is made with other works where the samples were prepared specifically for the study, using pilot scale; in those cases, the found levels are usually higher [32,33].

Table 1a. Quantitative results expressed in mg kg^{-1}, achieved by using the LC-ESI-IT MS developed method applied of total sample set. The results are given by the mean value (n=4; four independent determinations, including extraction and subsequent injection) ±standard deviation.

Compounds	R_t (min)	m/z	Arauco 1	Arauco 2	Arauco 3	Arauco 4	Arauco 5	Arauco 6	Manzanilla 1	Manzanilla 2	Manzanilla 3	Frantoio 1	Frantoio 2	Coratina 1	Coratina 2
Phenolic acids															
Quinic acid	1.3	191	0.19±0.02	0.07±0.03	n.d.	3.37±0.17	0.05±0.01	0.11±0.02	1.03±0.08	0.03±(<0.01)	0.49±0.05	0.48±0.05	0.11±0.03	0.07±0.03	1.92±0.36
Caffeic acid	4.9	179	0.21±0.08	0.19±0.05	0.08±0.03	n.d.	n.d.	0.09±0.04	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.17±0.07
p-Coumaric acid	6.2	163	1.20±0.13	0.77±0.06	0.52±0.01	0.75±0.10	0.49±0.01	0.76±0.05	0.27±0.02	0.32±0.02	0.22±0.02	0.26±0.04	0.13±0.03	0.22±0.05	0.20±0.04
Ferulic acid	6.6	193	0.16±0.02	0.12±0.02	0.13±0.01	0.12±0.02	0.07±0.04	0.18±0.03	0.12±0.02	0.10±0.04	0.09±0.03	0.09±0.02	n.d.	0.06±0.02	0.03±0.01
Simple phenolic alcohols															
OxHTY	1.4	151	0.23±0.05	0.16±0.04	0.25±0.02	0.34±0.03	0.04±0.01	0.17±0.01	0.12±0.03	0.04±0.01	0.04±0.02	0.27±0.01	0.21±0.04	0.17±0.02	0.28±0.02
OxHTY	1.8	151	0.28±0.07	0.17±0.04	0.32±0.05	0.33±0.04	0.05±0.01	0.21±0.02	0.13±0.02	0.02±(<0.01)	0.06±0.02	0.31±0.05	0.25±0.02	0.20±0.03	0.26±0.01
HTY	3.3	153	40.7±8.9	18.4±1.3	7.05±0.85	4.80±0.21	2.50±0.42	21.0±4.8	22.8±2.0	3.92±0.24	4.29±0.28	29.71±0.65	6.83±0.39	5.65±0.21	29.43±3.04
HTY Isomer	5.2	153	1.90±0.63	1.48±0.33	0.74±0.11	n.d.	4.5±1.5	0.41±0.02	1.49±0.06	1.00±0.10	0.98±0.05	0.32±0.09	0.53±0.09	1.36±0.20	0.73±0.04
Tyrosol	4.4	137	27.9±2.4	10.86±0.54	7.50±0.31	11.40±0.40	8.47±0.98	23.9±2.4	8.85±0.50	7.21±0.27	5.28±0.57	18.1±1.7	5.80±0.28	6.80±0.46	19.62±0.59
AcHTY	7.2	195	0.83±0.05	2.80±0.10	1.32±0.20	0.78±0.09	0.27±0.02	1.18±0.14	0.56±0.05	n.d.	0.62±0.06	3.25±0.31	5.67±0.25	0.51±0.05	0.19±0.02
Secoiridoids															
EA I 1	4.7	241	4.42±0.78	0.84±0.22	1.09±0.21	0.78±0.06	1.35±0.31	0.81±0.17	0.74±0.10	1.49±0.33	1.04±0.17	1.68±0.05	2.60±0.27	1.80±0.17	1.84±0.14
EA I 2	7.3	241	8.33±0.18	10.36±0.35	12.6±3.0	16.3±1.4	14.36±0.58	9.21±0.08	14.14±0.99	4.43±0.54	10.7±1.4	22.18±0.41	40.2±1.6	19.35±0.26	21.0±1.2
EA Ppal	7.7	241	78.6±8.6	22.49±0.60	27.5±2.6	55.0±2.4	33.38±0.41	32.0±2.9	27.6±1.1	17.81±0.68	15.04±0.87	48.0±3.6	57.6±2.3	56.9±2.6	64.7±3.1
DEA	4.9	183	0.36±0.07	0.18±0.02	0.14±0.03	n.d.	n.d.	n.d.	n.d.	0.11±0.01	n.d.	n.d.	n.d.	n.d.	n.d.
DEA Ppal	5.6	183	3.58±0.01	2.95±0.26	0.87±0.05	0.40±0.05	n.d.	6.39±0.55	5.97±0.53	1.52±0.24	n.d.	14.11±0.10	n.d.	0.44±0.05	4.42±0.35
HEA	6.8	257	4.26±0.50	3.54±0.29	1.99±0.25	1.97±0.10	0.37±0.04	1.01±0.10	0.56±0.11	0.51±0.08	0.43±0.04	3.66±0.07	1.56±0.15	0.75±0.11	0.84±0.06
DesoxyEA	6.6	225	5.62±0.61	6.10±0.24	4.28±0.26	8.2±1.0	6.6±1.5	5.48±0.17	7.35±0.74	0.42±0.13	21.3±1.8	3.00±0.20	1.60±0.21	15.56±0.72	19.16±0.94
DOA Ppal	8.8	319	15.21±0.71	24.9±3.2	17.21±0.36	3.67±0.08	11.92±0.55	10.94±0.69	14.7±1.2	4.18±0.27	38.54±0.58	18.3±1.5	26.2±4.7	21.1±5.3	19.8±2.3
DOA	9.2	319	4.57±0.29	8.24±0.66	6.5±1.3	1.34±0.04	0.63±0.04	3.36±0.36	0.87±0.12	3.00±0.01	7.9±8.0	5.68±0.38	3.81±0.35	1.84±0.66	1.25±0.15
Ole Agly I 1	8.1	377	7.70±0.48	1.70±0.17	1.28±0.08	1.87±0.13	2.10±0.27	2.35±0.52	2.43±0.24	0.15±0.03	0.63±0.10	0.44±0.04	0.93±0.06	5.6±1.3	4.76±0.03
Ole Agly I 2	8.5	377	6.35±0.45	3.44±0.46	2.93±0.45	6.51±0.50	4.89±0.19	3.75±0.07	5.55±0.68	0.40±0.05	1.45±0.24	2.89±0.34	3.08±0.30	7.53±0.06	8.9±1.1
Ole Agly I 3	9.3	377	1.44±0.37	1.02±0.18	0.84±0.21	1.93±0.01	1.76±0.52	1.58±0.12	1.16±0.17	0.15±0.04	2.97±0.13	0.46±(<0.01)	1.39±0.20	2.34±0.28	2.34±0.08
Ole Agly I 4	10.0	377	1.90±0.34	1.64±0.72	1.20±0.29	2.20±0.29	2.84±0.14	1.20±0.15	1.34±0.17	0.51±0.07	3.39±0.20	0.38±0.04	3.03±0.02	2.39±0.14	2.37±0.08
Ole Agly I 5	10.4	377	3.59±0.65	3.35±0.25	3.50±0.38	2.22±0.57	4.6±1.2	2.46±0.46	4.22±0.13	1.42±0.31	8.3±3.3	1.15±0.15	6.48±0.29	3.34±0.29	3.23±0.40
Ole Agly Ppal	11.1	377	14.00±0.42	10.99±0.72	9.05±0.51	12.5±4.0	26.77±0.98	14.89±0.96	16.08±0.88	7.3±1.0	21.39±0.91	6.41±0.41	16.37±0.08	9.8±1.0	8.80±0.26
Total Ole Agly			***35.0±1.1***	***22.1±1.2***	***18.80±0.86***	***27.2±4.1***	***43.0±1.7***	***26.2±1.2***	***30.8±1.2***	***10.0±1.1***	***38.1±3.5***	***11.73±0.56***	***31.28±0.47***	***31.0±1.7***	***30.4±1.2***
10-H Ole Agly I 2	9.5	393	0.61±0.08	0.60±0.15	0.24±0.01	0.18±0.02	0.09±0.02	0.19±0.03	0.12±0.03	0.15±0.06	0.09±0.01	0.33±0.02	0.14±0.01	1.26±0.08	n.d.
10-H Ole Agly I 3	9.7	393	7.02±0.12	4.80±0.50	2.69±0.36	2.69±0.44	1.43±0.16	2.42±0.14	1.13±0.08	0.08±0.06	0.45±0.05	1.97±0.14	2.56±0.07	0.23±0.05	2.16±0.14
10-H Ole Agly Ppal	9.9	393	1.28±0.25	1.40±0.29	0.63±0.08	0.80±0.05	0.19±0.02	0.38±0.04	0.27±0.04	0.17±0.03	0.19±0.07	0.70±0.06	0.44±0.03	0.26±0.02	0.27±0.03
Lig Agly I 1	9.4	361	16.0±1.0	10.7±1.7	8.3±1.8	23.11±0.74	30.5±1.2	7.4±1.9	3.20±0.36	0.74±0.11	3.23±0.85	3.61±0.05	11.9±3.6	24.04±0.29	21.11±0.15
Lig Agly I 2	9.9	361	23.0±1.9	25.0±2.8	25.0±1.6	21.8±0.5	35.1±3.2	11.54±0.04	8.15±0.54	2.19±0.09	10.76±0.77	7.70±0.09	18.05±0.60	30.7±1.5	25.2±0.7

Compounds	R_t (min)	m/z	Arauco 1	Arauco 2	Arauco 3	Arauco 4	Arauco 5	Arauco 6	Manzanilla 1	Manzanilla 2	Manzanilla 3	Frantoio 1	Frantoio 2	Coratina 1	Coratina 2
Lig Agly I 3	11.2	361	4.08±0.27	2.09±0.05	2.69±0.18	1.72±0.09	9.03±0.44	1.75±0.17	0.92±0.05	0.62±0.12	1.57±0.26	1.33±0.08	4.41±0.30	2.47±0.88	3.51±0.20
Lig Agly I 4	11.6	361	4.55±0.13	2.18±0.60	5.02±0.78	1.49±0.59	27.5±2.7	3.77±0.61	2.61±0.02	1.78±0.29	4.53±0.75	1.21±0.26	10.2±2.7	5.40±0.44	6.0±3.5
Lig Agly Ppal	12.3	361	67.6±1.5	53.6±13.7	49.6±4.5	28.2±1.9	149.5±5.3	47.3±5.0	32.9±1.2	24.4±1.5	42.3±7.2	24.4±2.2	78.6±9.2	81.8±1.3	86.1±3.2
Total Lig Agly			***115.3±2.6***	***94±14***	***90.6±5.2***	***76.4±2.2***	***251.5±6.9***	***71.8±5.4***	***47.8±1.4***	***29.8±1.6***	***62.3±7.4***	***38.3±2.2***	***123.1±10.3***	***144.4±18.0***	***141.9±4.8***
DLA	10.3	303	4.627.13±0.14	28.0±1.5	18.07±0.93	0.80±0.06	8.68±0.72	10.3±1.5	20.5±1.7	11.96±0.48	33.7±1.8	11.35±0.75	38.9±2.7	18.53±0.07	9.56±0.55
Lignans															
HPin	7.7	373	0.03±0.01	0.03±0.01	0.04±0.02	0.03±0.01	0.03±0.02	0.02±0.01	0.07±0.01	0.28±0.05	0.15±0.02	n.d.	n.d.	n.d.	n.d.
Pin	9.5	357	0.19±0.04	0.53±0.04	0.91±0.10	0.47±0.04	0.26±0.05	0.44±0.02	1.63±0.23	2.53±0.18	1.49±0.16	1.08±0.10	0.56±(<0.01)	0.44±0.01	0.47±0.04
AcPin	9.8	415	0.10±0.07	0.71±0.12	1.51±0.03	0.20±0.01	n.d.	0.15±0.03	1.23±0.11	1.60±0.14	1.12±0.03	3.99±0.27	2.67±0.28	0.78±0.16	0.63±0.05
Flavonoids															
Luteolin	9.0	285	8.42±0.74	6.94±0.57	6.45±0.21	9.52±0.44	7.20±0.22	6.92±0.23	4.11±0.46	3.12±0.04	4.64±0.15	5.27±0.35	5.84±0.20	4.44±0.15	5.11±0.10
Apigenin	10.2	269	3.99±0.19	2.85±0.08	2.07±0.12	4.25±0.05	4.44±0.36	3.57±0.23	0.89±0.17	1.48±0.22	1.07±0.07	1.48±0.04	0.66±0.07	0.96±0.02	1.04±0.13
Diosmetin	10.5	299	0.61±0.02	0.98±0.10	1.13±0.15	0.98±0.07	1.41±0.06	0.74±0.05	0.39±0.02	0.73±0.09	1.02±0.12	0.72±0.05	0.91±0.14	0.32±0.02	0.27±0.04
TOTAL LEVELS			**378.2±13.1**	**278±15**	**233.2±6.9**	**233.1±5.5**	**404.1±7.7**	**240.4±8.5**	**216.22 ±4.0**	**108.0±2.5**	**251.4±34.2**	**246.3±5.1**	**360.2±12.1**	**335.5±19.2**	**377.6±7.3**

n.d.: non-detectable

Table 1b. Quantitative results expressed in mg kg-1, achieved by using the LC-ESI-IT MS developed method applied of total sample set. The results are given by the mean value (n=4; four independent determinations, including extraction and subsequent injection) ±standard deviation.

Compounds	R_t (min)	m/z	Arbequina	Picual	Empeltre	Changlot	Nevadillo	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6	Blend 7
Phenolic acids														
Quinic acid	1.3	191	0.75±0.10	0.43±0.08	0.17±0.01	0.11±0.02	0.21±0.03	0.04±(<0.01)	0.17±0.04	0.16±0.02	n.d.	0.08±0.03	0.15±0.02	0.07±0.03
Caffeic acid	4.9	179	0.10±0.01	n.d.	n.d.	n.d.	0.15±0.06	0.09±0.04	n.d.	0.10±0.01	0.12±(<0.01)	0.15±0.04	0.21±(<0.01)	n.d.
p-Coumaric acid	6.2	163	0.25±0.03	0.27±0.05	0.11±0.03	0.32±0.01	0.14±0.03	0.22±0.05	0.27±0.05	0.30±0.05	0.56±0.02	0.57±0.09	0.30±0.05	0.25±0.01
Ferulic acid	6.6	193	0.08±0.03	0.08±0.03	0.03±0.03	0.13±0.02	0.04±0.03	0.09±0.02	0.11±0.04	0.13±0.02	0.12±0.02	0.18±0.01	0.05±0.02	0.10±0.02
Simple phenolic alcohols														
OxHTY	1.4	151	0.35±0.08	0.05±0.02	0.07±0.02	0.08±0.01	0.28±0.07	0.08±0.02	0.21±0.05	0.18±0.05	0.34±0.08	0.25±0.05	0.18±0.01	0.11±0.02
OxHTY	1.8	151	0.37±0.03	0.05±0.02	0.08±0.01	0.09±0.01	0.32±0.07	0.11±0.03	0.22±0.03	0.23±0.03	0.35±0.08	0.31±0.08	0.22±0.02	0.16±0.03
HTY	3.3	153	10.89±0.04	2.61±0.50	0.51±0.03	4.44±0.29	18.5±7.5	6.5±1.1	9.6±1.2	8.94±0.34	15.0±1.0	8.91±0.61	16.9±1.5	7.0±1.9
HTY isomer	5.2	153	0.44±0.01	4.20±0.33	0.31±0.03	1.42±0.11	0.62±0.38	0.84±0.74	0.81±0.26	0.47±0.01	0.81±0.03	0.45±0.10	0.87±0.64	0.32±0.01
Tyrosol	4.4	137	4.30±0.50	5.85±0.66	1.93±0.04	5.73±0.21	12.56±0.74	5.59±0.31	7.48±0.79	7.79±0.68	8.70±0.70	6.72±0.47	12.95±0.34	4.52±0.26
AcHTY	7.2	195	3.54±0.11	1.76±0.03	0.44±0.03	n.d.	0.21±0.01	0.67±0.05	2.41±0.31	2.26±0.18	0.71±0.09	0.89±0.07	0.31±0.04	1.27±0.04
Secoiridoids														
EA I 1	4.7	241	0.74±0.04	0.26±0.02	0.67±0.01	3.34±0.22	0.20±0.08	0.44±0.31	0.13±(<0.01)	1.12±0.16	2.59±0.21	1.99±0.11	0.84±0.17	0.14±0.04
EA I 2	7.3	241	2.36±0.29	5.74±0.14	10.19±0.09	46.7±4.1	3.0±1.2	2.42±0.40	3.75±0.02	4.14±0.66	11.3±1.0	8.43±0.86	8.34±0.95	3.45±0.65

Compounds	R_t (min)	m/z	Arbequina	Picual	Empeltre	Changlot	Nevadillo	Blend 1	Blend 2	Blend 3	Blend 4	Blend 5	Blend 6	Blend 7
EA Ppal	7.7	241	10.2±1.7	14.51±0.28	26.3±1.2	96.3±6.5	10.40±0.47	6.88±0.91	12.42±0.47	14.67±0.47	29.06±0.34	21.7±1.1	23.89±0.77	9.70±0.11
DEA	4.9	183	0.13±0.03	n.d.	n.d.	n.d.	0.13±0.02	0.17±0.02	0.15±0.03	n.d.	0.15±0.01	0.17±0.02	0.12±0.02	0.13±0.02
DEA Ppal	5.6	183	3.56±0.10	n.d.	n.d.	n.d.	10.9±1.1	0.79±0.18	9.59±0.10	16.38±0.32	1.05±0.08	3.29±0.19	9.33±0.33	0.59±0.04
HEA	6.8	257	2.54±0.19	0.57±0.04	2.37±0.06	1.59±0.32	1.33±0.24	2.14±0.36	2.53±0.22	1.53±0.32	3.39±0.29	3.38±0.32	1.43±0.10	1.17±0.12
DesoxyEA	6.6	225	1.25±0.01	2.45±0.28	1.23±0.02	1.30±0.27	0.73±0.09	0.77±0.16	1.38±0.07	1.09±0.18	4.49±0.42	4.19±0.38	1.52±0.12	0.65±0.01
DOA Ppal	8.8	319	9.00±0.06	4.81±0.33	6.37±0.09	3.66±0.21	11.3±1.2	8.64±0.98	10.11±0.14	9.64±0.46	19.38±0.65	11.8±1.0	8.62±0.26	9.50±0.03
DOA	9.2	319	5.44±0.07	0.25±0.02	2.75±0.20	0.34±0.02	6.15±0.40	8.28±0.34	4.77±0.25	3.35±0.24	5.94±0.51	6.81±0.93	4.25±0.59	5.27±0.24
Ole Agly I 1	8.1	377	n.d.	2.17±0.19	n.d.	1.50±0.20	0.13±0.04	n.d.	n.d.	n.d.	0.73±0.06	0.23±0.03	1.44±0.07	n.d.
Ole Agly I 2	8.5	377	0.26±0.04	4.67±0.58	0.18±0.04	4.36±0.89	0.41±0.11	0.17±0.04	0.22±0.04	0.23±0.04	2.35±0.08	0.73±0.11	3.54±0.18	0.29±0.06
Ole Agly I 3	9.3	377	0.14±0.02	1.39±0.14	0.22±0.08	1.12±0.09	0.26±0.05	0.12±0.02	0.25±0.02	0.19±0.03	0.54±0.09	0.28±0.02	0.42±0.09	n.d.
Ole Agly I 4	10.0	377	0.18±0.05	1.84±0.20	0.14±0.05	2.14±0.15	0.43±0.04	0.45±0.02	0.15±(<0.01)	0.30±0.08	1.37±0.20	0.47±0.02	0.74±0.10	0.31±0.06
Ole Agly I 5	10.4	377	0.72±0.16	3.06±0.26	0.69±0.03	3.13±0.11	1.08±0.21	0.70±0.22	1.56±0.02	1.05±0.19	2.71±0.39	1.88±0.39	2.26±0.24	0.45±0.02
Ole Agly Ppal	11.1	377	5.37±0.84	20.7±1.9	5.16±0.21	16.81±0.57	4.80±0.40	6.03±0.92	6.49±0.77	5.76±0.28	20.0±4.9	9.42±0.60	10.65±0.51	4.34±0.37
Total Ole Agly			***6.67±0.86***	***33.8±2.0***	***6.40±0.31***	***29.1±1.1***	***7.11±0.47***	***7.61±0.95***	***8.67±0.78***	***7.53±0.72***	***27.7±4.9***	***12.99±0.72***	***19.03±0.61***	***5.38±0.38***
10-H Ole Agly I 2	9.5	393	0.23±0.04	0.25±0.16	0.06±0.02	0.14±0.09	0.25±0.05	0.26±0.07	0.27±0.06	0.24±0.04	0.31±0.03	0.27±0.01	0.16±0.04	0.24±0.05
10-H Ole Agly I 3	9.7	393	0.56±0.03	0.72±0.06	0.16±0.01	1.36±0.21	0.57±0.05	0.84±0.16	0.64±0.04	0.62±0.22	3.91±0.57	1.73±0.29	1.66±0.23	0.65±0.14
10-H Ole Agly Ppal	9.9	393	0.38±0.03	0.91±0.41	0.05±0.04	1.06±0.04	0.30±0.08	0.39±0.04	0.29±0.01	0.36±0.08	0.67±0.09	0.66±0.10	0.31±(<0.01)	0.18±0.01
Lig Agly I 1	9.4	361	1.20±0.06	7.35±0.22	3.95±0.14	6.38±0.43	1.22±0.22	0.59±0.12	1.08±0.06	1.38±0.28	7.16±0.33	4.65±0.76	7.27±0.03	0.73±0.15
Lig Agly I 2	9.9	361	3.57±0.13	10.0±1.2	1.78±0.81	25.6±3.4	3.27±0.02	2.58±0.27	2.36±0.34	3.44±0.29	22.91±0.78	12.3±2.5	9.79±0.86	2.54±0.37
Lig Agly I 3	11.2	361	0.82±0.24	2.54±0.88	0.88±0.20	3.37±0.60	0.57±0.06	1.22±0.12	1.85±0.30	0.91±0.21	4.30±0.35	1.57±0.17	1.31±(<0.01)	0.83±0.03
Lig Agly I 4	11.6	361	0.81±0.20	7.6±1.2	2.4±1.2	8.17±0.44	0.67±0.06	1.38±0.31	1.51±0.62	1.62±0.46	5.87±0.21	3.38±0.29	1.47±0.41	1.96±0.09
Lig Agly Ppal	12.3	361	13.3±2.4	56.8±9.5	23.2±2.1	60.08±0.62	18.57±0.17	22.75±0.86	17.4±2.2	21.1±3.4	78.0±6.8	33.0±1.7	46.84±0.30	14.88±0.19
Total Lig Agly			***19.7±2.4***	***84.2±9.7***	***32.2±5.1***	***103.6±3.6***	***24.30±0.29***	***28.52±0.97***	***24.2±2.4***	***28.4±5.4***	***118.2±6.9***	***54.9±3.1***	***66.7±1.0***	***20.94±0.45***
DLA	10.3	303	9.36±0.70	3.17±0.94	21.44±0.70	2.03±0.78	20.16±0.16	23.6±4.2	15.7±1.1	18.6±1.6	17.0±2.5	16.15±0.53	9.16±0.80	11.3±1.9
Lignans														
HPin	7.7	373	0.14±0.01	0.09±0.02	n.d.	n.d.	0.18±0.02	0.15±0.02	0.19±0.04	0.17±0.02	0.03±0.01	0.07±0.02	0.09±0.03	0.09±0.02
Pin	9.5	357	2.35±0.13	6.67±0.46	1.86±0.01	1.61±0.26	2.51±0.24	1.77±0.15	1.59±0.18	1.74±0.18	0.69±0.03	1.25±0.09	1.54±0.21	1.54±0.20
AcePin	9.8	415	4.28±0.32	0.24±0.04	2.52±0.17	2.74±0.12	0.55±0.07	2.78±0.11	4.57±0.21	5.65±0.76	1.65±0.26	2.95±0.19	0.29±0.07	2.64±0.02
Flavonoids														
Luteolin	9.0	285	4.99±0.34	3.37±0.56	3.17±0.15	3.17±0.06	2.30±0.12	2.24±0.27	2.61±0.31	3.86±0.05	6.34±0.75	7.25±0.19	3.11±0.21	2.23±0.06
Apigenin	10.2	269	1.47±0.09	0.64±0.13	0.98±0.02	0.95±0.03	0.99±0.17	1.24±0.13	1.11±0.45	1.32±0.11	2.53±0.24	2.41±0.37	1.53±0.05	0.91±0.11
Diosmetin	10.5	299	1.52±0.11	0.46±0.09	0.82±0.10	0.37±0.02	0.55±0.09	1.26±0.12	1.11±0.19	1.10±0.11	1.16±0.10	1.77±0.20	0.55±0.07	1.01±0.10
TOTAL LEVELS			**108.0±3.3**	**178.4±10.0**	**123.2±9.2**	**311.7±8.6**	**136.9±8.1**	**115.4±5.1**	**127.1±3.3**	**142.1±4.2**	**284.2±9.1**	**182.6±4.4**	**194.6±2.7**	**91.6±2.8**

n.d.: non-detectable

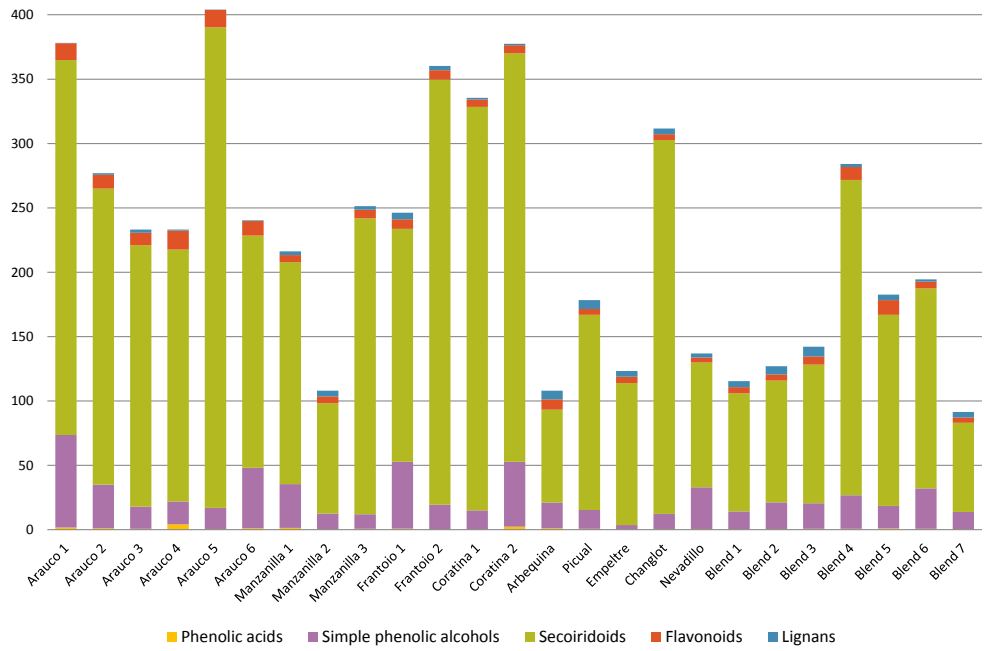

Figure 2. Total concentration of phenolic compounds found in each sample under study; each bar is indicating the overall concentration (expressed in mg kg^{-1}) of the five main classes determined (phenolic acids, simple phenols, secoiridoids, flavonoids and lignans).

Evaluating the quantitative results accordingly to each family, Arauco 4 was the richest sample in terms of phenolic acids (4.24 mg kg^{-1}), being quinic acid the acid found at highest concentration levels (3.37 mg kg^{-1}).

Other important group of phenolic compounds in olive oil is composed by simple phenolic alcohols; group which is principally form by HTY and tyrosol. In this case, the sample with major levels was Arauco 1 (71.85 mg kg^{-1}), which had 40.70 mg kg^{-1} of HTY, 1.90 mg kg^{-1} of a HTY isomer, and 27.91 mg kg^{-1} of tyrosol, apart from other simple phenol-derivatives (OxHTY and AcHTY). This behaviour is in good agreement with the data previously reported by Brenes *et al.* [34], who observed that the main changes in the phenolic compounds were associated with the hydrolysis of the secoiridoid aglycons, increasing the concentration of HTY and tyrosol; Arauco 1 is indeed the only sample coming from season 2013.

With respect to lignans, Arbequina and Picual were the monovarietal oils with the highest concentrations (6.77 and 7.00 mg kg^{-1}, respectively); however, the most remarkable levels of the whole sample-set were found for Blend 3 and Blend 2. This fact could be understood considering that these blends were prepared containing 60 % of olive oil from Arbequina variety. The high concentration of lignans in Arbequina oils (or in oils with strong presence of Arbequina variety) has been previously observed by other authors [35,36].

The flavonoids quantified in this work were diosmetin, apigenin and luteolin (all flavones) and their highest levels were found in Arauco variety samples (samples Arauco 4, 5 and 1 with 14.76, 13.48 and 13.03 mg kg^{-1}, respectively). A remarkable feature of these samples analyzed here is their very high content of flavonoids, if compared to previously reported studies [3,7,30,33]. In some of the samples, the total flavonoid concentration resulted to be three times higher than previously reported values; an hypothesis explaining this fact is the extensive culture and sunny climatic conditions in Maipú department, since these compounds are related to greater exposures to solar radiation [37].

As described above, to facilitate the evaluation of the results, secoiridoid derivatives have been divided in ligstroside-related compounds (aldehydic derivatives of EA with tyrosol) and oleuropein-related compounds (aldehydic derivatives of EA with HTY). We also include in this chemical class, EA and related compounds. As far as oleuropein-derivatives are concerned, the most important detected compounds were DOA (or oleacein) and Ole Agly. The highest concentration of DOA was observed in Manzanilla 3 sample with 46.44 mg kg^{-1} (considering the two DOA isomers), whereas the lowest level was detected in Changlot sample, with 4.0 mg kg^{-1}. Regarding Ole Agly, Blend 7 showed the lowest value (5.38 mg kg^{-1} -total value combining the amount determined for the 6 isomers-) and Arauco 5 exhibited the highest one (42.96 mg kg^{-1}). When we pay attention to ligstroside-derivatives, it is necessary to say that, in the present work, 5 isomers of Lig Agly and two of DLA (or oleocanthal) were quantified, finding total Lig Agly´s maximum and minimum values in Arauco 5 (251.47 mg kg^{-1}) and Arbequina (19.73 mg kg^{-1}), respectively. Arauco 4 (0.80 mg kg^{-1}) and Frantoio 2 (38.94 mg kg^{-1}), respectively, defined the extreme values of the found amounts range of DLA isomers. Apart from these analytes, other secoiridoids were identified: 3 isomers of EA, DEA (two isomers), HEA and DesoxyEA; the maximum EA´s concentration was found in Changlot sample, with 149.16 mg kg^{-1} (taking into account all the isomers).

3.3. Principal Components Analysis and 2D plots

To evaluate the structure of the data, a principal component analysis (PCA) was applied. In Fig. 3a, the score and loading plots of PC1 vs. PC2 are shown for the matrix composed by 40 variables and 25 samples. The first two PCs explained 96% of total variance in raw data; PC1 and PC2 accounted for 92% and 4%, respectively. In the figure, it can be observed that the samples Arauco 5 and Changlot are quite separated from the rest (in particular Arauco 5), fact which can be justified having a look at the loading plots and bearing in mind their high concentrations of Lig Agly (isomer designated as principal one in the current study (12.3 min)) and EA (main isomer at 7.7 min). Fig. 3a (score plot) also shows a grouping of Coratina 1 and 2, Arauco 1 and Frantoio 2 samples; this arrangement could be explained because of their levels of HTY isomer, together with their concentrations regarding the principal isomers of Lig Agly and EA.

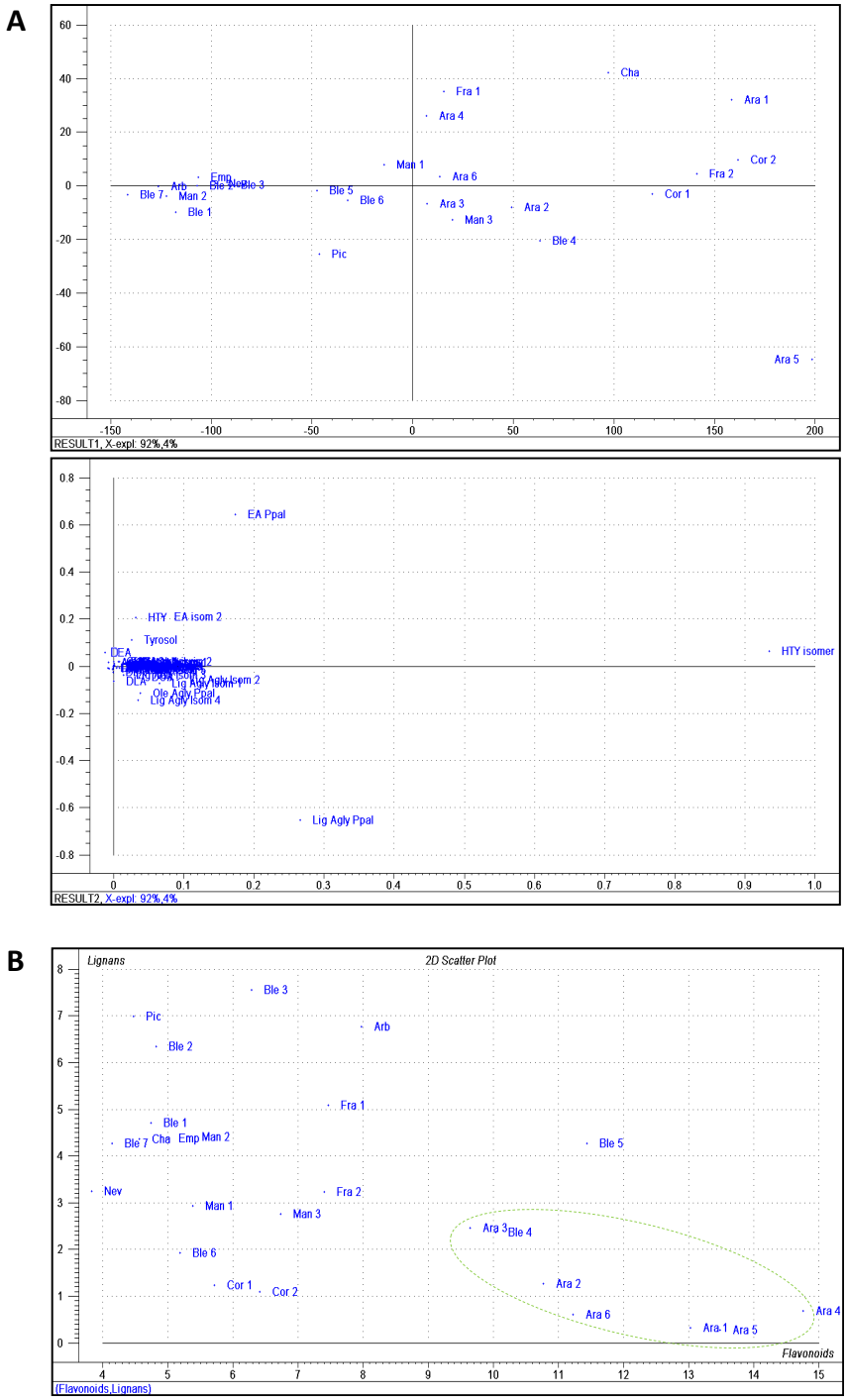

Figure 3. (a) Score and loading plots of PCA considering the concentration of each quantified phenolic compound (average of 4 replicates). (b) 2D scatter plot of lignans versus flavonoids. Arb: Arbequina; Arc: Arauco; Ble: blend; Cha: Changlot; Cor: Coratina; Emp: Empeltre; Fra: Frantoio; Man: Manzanilla; Pic: Picual; Nev: Nevadillo.

With the aim of evaluating further a possible discrimination among the samples based on the cultivar, we built a series of 2D plots; the samples were modelled taking into account the total values of the determined chemical classes (one-to-one), trying to establish existing correlations. Fig. 3b illustrates the 2D graphic of lignans *vs* flavonoids. Interestingly, the *Arauco* samples are clearly separated from the rest of the olive oils, indicating their very high flavonoids content and the relatively low lignans levels. Two blends (blends 4 and 5) appeared quite close in the graphic to *Arauco* samples; this circumstance can be certainly explained observing that those blends contained 70 and 30%, respectively, of Arauco *cv.*, while the rest of the blends only had 3-4% of this variety. A greater number of samples are undoubtedly needed to get a more comprehensive insight into the complete phenolic pattern of these varieties and highlight the main differences among them.

3.3.1. Typical Arauco variety´s features

In the introductory section, we made an allusion to the point that Arauco variety is the only Argentinean autochthonous cultivar recognized by International Olive Council, for this reason, a brief paragraph trying to delineate its most relevant features seems required. The six Arauco samples evaluated in this study possessed important levels of total phenolic compounds, being two of them the richest of the whole sample-set. It is also appealing to note that in the case of Blend 4, which has a 70 % of Arauco variety, the levels of total phenolic compounds are markedly higher than in the other blends. In previously reported works, where other methodologies for determining the phenolic compounds were utilized, the high phenolic contents of Arauco oils (when compared with other varieties) were already observed; several authors have attributed this point to a matter of inappropriate adaptation of diverse varieties to the climatic conditions [28,38]. Indeed, Ceci *et al.* [28]. suggested that the national productive sector should recommend the selection of the cultivars which show a best adaptation to the agronomical media, being the analysis and the implementation of the most advisable cultural and processing conditions absolutely necessary.

3.4. Identification of phenolic compounds scarcely reported in VOO

As comment above, the identification of phenolic compounds barely reported in this matrix was also intended using high resolution MS (QTOF MS). Besides the accurate MS information, we obviously took into account the previously reported knowledge about the composition of olive oil-related samples (fruits, leaves and by-products of the olive oil industry). A peak with experimental *m/z* 199.0620 and R_t of 3.6 min was found in 21 samples (it was not detected in Manzanilla 3, Picual, Coratina and Arauco 5). Its predicted molecular formula ($[M-H]^-$) was $C_9H_{11}H_5$ and their in-source fragments were 155 and 111 *m/z*. These fragments were corroborated by MS/MS experiments. The structure of the compound is included in Fig. 4a and it was tentatively assigned to one analyte related to EA, more precisely, the hydroxylated product of the dialdehydic form of DEA. This compound has been already reported in wastes generated during storage of

VOO [39], as well as in drupes and paste [40]. As can be seen in the figure, the fragments of *m/z* 155 and 111 correspond to the molecular formulae $C_8H_{11}O_3$ and $C_7H_{11}O$, respectively, being the first one the loss of a carboxylic group from the original structure. The *m/z* 111 seems a typical feature of some EA derivatives, as stated by Kanakis *et al.* [40].

Fig. 4b shows the MS spectrum of the compound with *m/z* 213.0771 (with a R_t of 6.4 min). This substance was found in 14 samples: Arauco 1, 2, 3 and 6, Nevadillo, Frantoio 1, Arbequina and all Blends) and its predicted molecular formula was $C_{10}H_{13}O_5$ ($[M-H]^{-1}$). According to previously reported information, this peak could be identified as another EA derivate, more specifically, the decarboxylated form of hydroxyelenolic acid; compound which has been reported in the wastes generated during the storage of VOO [39], drupes and paste [40]. In-source fragments were 181, 169 and 111 being 169 and 111 consistently observed when MS/MS analyses were done. The fragment with *m/z* 181 could be attributed to the loss of CH_4O. The fragment of *m/z* 169 corresponded with the loss of a carboxylic group; and the *m/z* 111 could be explained as the consecutive loss of a carboxylic group and the group $COOCH_2$. The possible fragmentation patterns of this compound have been indicated within Fig. 4b. These two EA-related compounds have been hardly described in VOO; it could be very interesting including them in future studies and establishing what their usual concentration ranges are.

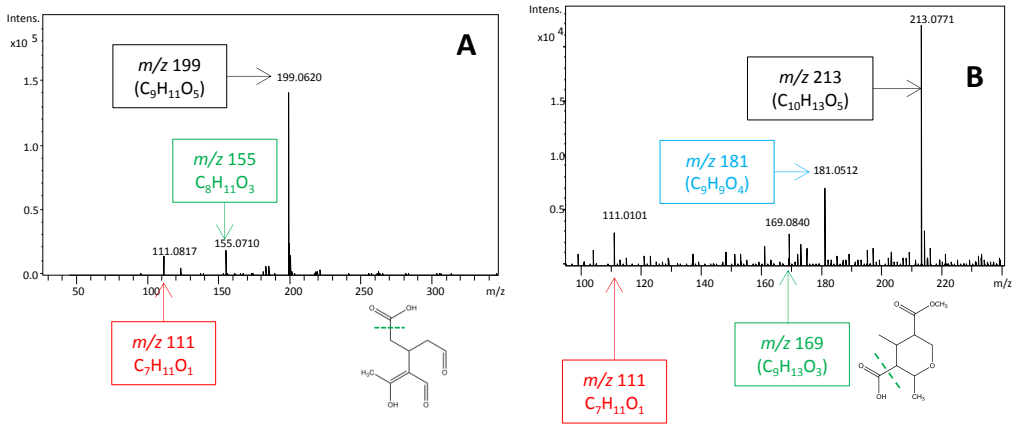

Figure 4. Fragmentation patterns of two phenolic compounds scarcely explored in VOO. a) MS spectrum of *m/z* 199.0620 (Rt of 3.6 min). b) MS spectrum of the peak with *m/z* 213.0771 (with a Rt of 6.4 min).

Summing up, this is the first time in which a deep characterization of the phenolic composition of Maipú VOOs is carried out, getting quantitative information about 40 phenolic compounds of samples of different botanical varieties. The use of LC-ESI-QTOF MS and LC-ESI-IT MS allowed the accurate and reliable determination of a great number of analytes, including the secoiridoid derivatives (not evaluated before in samples coming from this geographical area). The results make evident that olive oils coming from Mendoza can be considered as important sources of phenolic bioactive compounds, exhibiting similar phenolic compounds levels to those shown by oils from other typical world production regions. Moreover, this study has evinced some peculiarities in the

composition of Arauco olive oils; indeed, a correlation between flavonoids and botanical variety was established herewith. Even though this contribution could have some limitations related to the relatively low number of samples and the variety of influencing variables, the results could represent a milestone for the producers, enlarging their knowledge about the composition of their oils and making them aware about its commercial value.

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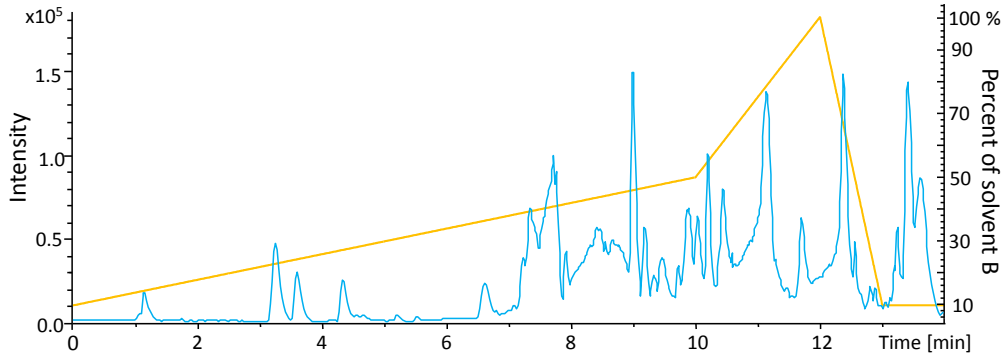

Figure S1. BPC of some of the phenolic profiles obtained by injecting 10 mL of the pool VOO sample using the optimal chromatographic conditions and optimal gradient based on the use of acidic water (acetic acid 0.5 % v/v) as Phase A and ACN as Phase B (see section 2).

Table 1S. Analytical parameters related to detection and quantification limits of the described method and intra-day and inter-day repeatability.

Compound	LOD (μg L^{-1})	LOQ (μg L^{-1})	*Intra-day* Repeatability (%RSD)[a]		*Inter-day* Repeatability (%RSD)[b]	
			Area	R_t	*Area*	R_t
Quinic acid	13.6	45.2	2.17	<0.01	2.70	<0.01
Hydroxytytrosol	6.2	20.6	3.12	0.04	5.71	1.24
Tyrosol	41.1	137.0	3.66	0.04	5.79	0.93
Caffeic acid	11.3	37.8	4.35	<0.01	2.83	<0.01
Homovanillic acid	11.5	38.3	1.44	<0.01	3.64	<0.01
***p*-Coumaric acid**	72.5	241.5	1.03	0.05	7.01	0.84
Ferulic acid	6.3	21.1	1.62	<0.01	4.73	<0.01
Oleuropein	17.3	57.7	3.13	0.05	7.52	0.68
Luteolin	51.4	171.2	3.17	0.04	2.97	0.45
Pinoresinol	9.2	30.6	1.20	<0.01	1.92	<0.01
Apigenin	10.0	33.3	3.13	0.04	4.79	0.40

[a] %RSD values for peak areas and retention times (expressed in min) of the analytes under study measured from 5 injections carried out within the same day.
[b] %RSD values for peak areas and retention times (expressed in min) of the analytes under study measured from 5 injections carried out in five different days.

Chapter 6

Establishing the phenolic composition of *Olea europaea* L. leaves from cultivars grown in Morocco as a crucial step towards their subsequent exploitation

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Molecules **(under review)**

Abstract: In Morocco, the recovery of olive agro-industrial by-products as potential sources of high-added value substances has been underestimated so far. A comprehensive quantitative characterization of olive leaves bioactive compounds is crucial when trying to contribute to change this situation and to implement the valorization concept in emerging countries. Thus, the phenolic fraction of olive leaves of 11 varieties ('Arbequina', 'Hojiblanca', 'Frantoio', 'Koroneiki', 'Lechín', 'Lucque', 'Manzanilla', 'Picholine de Languedoc', 'Picholine Marocaine', 'Picual' and 'Verdal'), cultivated in Meknès Moroccan region, was investigated. 38 phenolic compounds (including 16 secoiridoids, 9 flavonoids in their aglycon form, 7 flavonoids in glycosylated form, 4 simple phenols, 1 phenolic acid and 1 lignan) were determined in a total of 55 samples by using ultrasonic-assisted extraction and liquid chromatography coupled to electrospray ionization-ion trap mass spectrometry (LC-ESI-IT MS). Very remarkable quantitative differences were observed among the profiles of the studied cultivars. 'Picholine Marocaine' variety exhibited the highest total phenolic content (around 44 g/kg dry weight (DW)), and logically showed the highest concentration in terms of various individual compounds. In addition, chemometrics (Principal Components Analysis (PCA) and Stepwise-Linear Discriminant Analysis (s-LDA)) were performed on phenolic compounds quantitative data, allowing good discrimination of the selected samples according to their varietal origin.

Keywords: olive leaves; Moroccan region; phenolic compounds; liquid chromatography-mass spectrometry; chemometrics; metabolic profiling.

1. INTRODUCTION

Global production of virgin olive oil has steadily increased over the past decades, reaching 3.1 million tons during the crop season 2017/2018 [1,2], which makes of olive tree the sixth most relevant oil crop in the world [3]. Furthermore, its undeniable economic importance has induced the expansion of the virgin olive oil agro-industry, but at the same time, has led to the generation (often in geographically concentrated locations) of huge amounts of wastes, so-called olive by-products. Despite the technological efforts, the generation of these residues is ineluctable. The olive oil agro-industry produces large amounts of solid waste (known as olive pomace or olive cake) and high volumes of effluents (known as olive mill wastewater) per year; the amount depends on the olive oil extraction system used [4]. In addition, as a result of olive trees pruning and the washing of harvested olive fruits, considerable amounts of olive leaves (approximately 25 kg per pruned tree and 5% of the total weight of the harvested olive fruits) are accumulated [5].

Consumer awareness of sustainability and new strict environmental regulations (in various Mediterranean countries) are the most important drivers in both the development of strategies for an adequate management of olive by-products and the progress regarding recycling and valorization [6,7]. One of these trends is the recovery of functional components or molecules with

interesting (bio)activity (health-promoting, therapeutic or cosmetic properties) to be further re-utilized in areas such as food, pharmaceutical and cosmetic industries [8–10].

Phenolic compounds are among those bioactive substances occurring at high concentrations in olive by-products. Especially, olive leaves represent an important resource of these components whose bioactivity, anti-oxidant, anti-microbial and anti-inflammatory properties have been extensively demonstrated [11,12]. Several conventional (solvent-based) and more modern extraction techniques (ultrasounds, microwaves, sub- and supercritical fluid extractions, pressurized liquid extraction, pulsed electric fields and high voltage electrical discharges, among others) have been tested for their recovery [13–18]. As stated before, the obtained extracts might have many applications in different fields, including, for instance, food additives and preservatives [19–21], cosmetics [22], as well as nutraceuticals and pharmaceuticals [23]. As a consequence, over the last years, characterizing olive leaves phenolic profile have become a challenging and important analytical task in order to provide comprehensive qualitative and quantitative information regarding the occurrence of these compounds. It is quite evident that their reliable analytical determination is an absolutely pivotal and necessary step preceding (and widely conditioning) the potential subsequent recovery. In this regard, very interesting reports dealing with the identification and quantification of phenolic compounds from olive leaves have been published, including the use of gas chromatography (GC), nuclear magnetic resonance spectroscopy (NMR), high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) and/or mass spectrometry (MS), etc. [24].

The present work was conceived as a first step to develop a thoroughgoing recovery approach of phenolic compounds from olive leaves in Morocco, which ranks sixth in the global production of virgin olive oil. Data from 2015, indicate that the Moroccan olive growing area was approximately 998 mille hectares, yielding 1.15 million tons of olive fruits and 120 mille tons of virgin olive oil [25]. Thus, the olive oil agro-industry certainly stands out as one of the driving sectors of the economy of this country. The recovery of bioactive compounds from olive oil by-products might bring additional benefits to the sector, increasing the profitability and adding value to the supply chain. However, there is a gap regarding olive by-products composition since, to the best of our knowledge, the phenolic profile of leaves from olive trees planted in Morocco has not been studied so far. Therefore, one of the main practical objectives of this study was to deeply investigate the phenolic composition of olive leaves obtained from both autochthonous and recently introduced olive cultivars in this country. To better assess the potential of these compounds as varietal markers, the inter-variety phenolic composition variability was checked. Moreover, chemometric tools were employed to discriminate among the studied cultivars based on the phenolic composition of their leaves.

2. RESULTS AND DISCUSSION

2.1. Profiling and qualitative characterization of the phenolic fraction of olive leaves from the selected eleven cultivars

The first stage of this work was designed to carry out the comprehensive characterization of the phenolic profiles of the leaves from different olive varieties, trying to identify as many compounds as possible. Tentative identifications were achieved by considering the information provided by the two detectors (DAD (UV-*vis* spectra) and MS (*m/z* spectral data)), the data achieved for the commercial standards (when available), as well as by comparing the information regarding retention time (Rt) and elution order with the previously published reports [26–30]. Accurate mass data obtained in full-scan mode in a Q-TOF MS was processed with the SmartFormula™ Editor tool included in DataAnalysis 4.0, which provides a list of possible elemental formulas. Table 1 lists (according to their elution order) the 38 phenolic compounds tentatively identified in the studied leaves samples and presents the calculated molecular formula for each compound, together with the error (difference between experimental and theoretical *m/z* of the detected $[M-H]^-$ ion) and mSigma™ (value showing the concordance with the theoretical isotopic pattern of the compound). Fig. 1 shows the Extracted Ion Chromatograms (EICs) of the main identified phenolic compounds found in a sample of 'Picholine Marocaine' leaves.

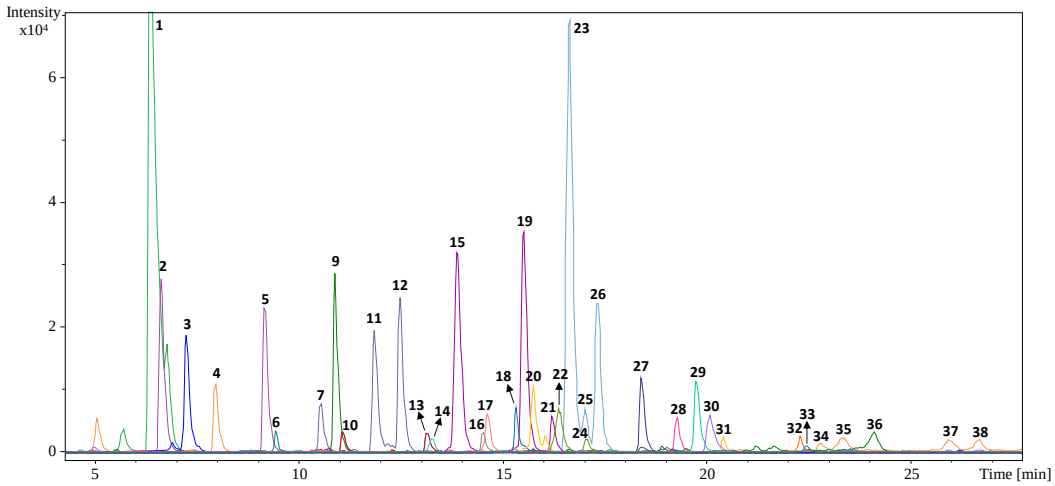

Figure 1. Extracted ion chromatograms (EICs) of the main phenolic compounds identified in a 'Picholine Marocaine' olive leaves sample. Numbers correspond with those included in Table 1.

In general, the phenolic composition of all the investigated samples was dominated by the presence of a high number of different secoiridoids (16 compounds in total) including (in order of elution): secologanoside isomers 1 (peak 2) and 2 (peak 5), elenolic acid glucoside isomers 1, 2 and 3 (peaks 7, 11 and 12 respectively), oleuropein aglycon isomers 1 and 2 (peaks 9 and 36, respectively), hydroxyoleuropein (peak 14), oleuropein diglucoside (peak 17), 2"-methoxyoleouropein isomers 1 and 2 (peaks 22 and 24 respectively), oleuropein isomers 1 (peak 23), 2 (peak 25) and 3 (peak 26), ligstroside (peak 27), and ligstroside aglycon (peak 28).

Table 1. Main phenolic compounds tentatively identified in the olive leaves from the 11 different selected varieties using the optimized LC-ESI-Q-TOF MS profiling approach.

Peak	Rt (min)	Molecular formula	experimental *m/z*	calculated *m/z*	Error (ppm)	mSigma	Suggested compound
1	6.4	$C_{14}H_{20}O_8$	315.1083	315.1085	0.8	6.2	Hydroxytyrosol glucoside
2	6.7	$C_{16}H_{22}O_{11}$	389.1086	389.1089	1.0	5.2	Secologanoside is. 1
3	7.3	$C_8H_{10}O_3$	153.0557	153.0557	0.1	8.7	Hydroxytyrosol
4	8.1	$C_{14}H_{20}O_7$	299.1131	299.1136	1.8	2.3	Tyrosol glucoside
5	9.2	$C_{16}H_{22}O_{11}$	389.1088	389.1089	0.3	18	Secologanoside is. 2
6	9.4	$C_8H_{10}O_2$	137.0607	137.0608	1.0	8.2	Tyrosol
7	10.6	$C_{17}H_{24}O_{11}$	403.1247	403.1246	-0.2	6.3	Elenolic acid glucoside is. 1
8	10.8	$C_8H_8O_4$	167.0348	167.035	1.1	3.1	Vanillic acid
9	10.9	$C_{19}H_{22}O_8$	377.1446	377.1453	2.0	6.9	Oleuropein aglycon is. 1
10	11.1	$C_{27}H_{30}O_{16}$	609.1468	609.1461	-1.2	21.4	Luteolin diglucoside
11	11.9	$C_{17}H_{24}O_{11}$	403.1246	403.1246	0	15.5	Elenolic acid glucoside is. 2
12	12.5	$C_{17}H_{24}O_{11}$	403.1239	403.1246	1.8	10.2	Elenolic acid glucoside is. 3
13	13.2	$C_{27}H_{30}O_{16}$	609.146	609.1461	0.1	3.1	Rutin
14	13.3	$C_{25}H_{32}O_{14}$	555.1707	555.1719	2.2	6.2	Hydroxyoleuropein
15	13.9	$C_{21}H_{20}O_{11}$	447.0934	447.0933	-0.3	11	Luteolin-7-glucoside
16	14.5	$C_{27}H_{30}O_{14}$	577.157	577.1563	-1.3	19.3	Apigenin rutinoside
17	14.7	$C_{31}H_{42}O_{18}$	701.2299	701.2298	0	5.5	Oleuropein diglucoside
18	15. 5	$C_{21}H_{20}O_{10}$	431.0983	431.0984	0.2	4.9	Apigenin-7-glucoside
19	15.6	$C_{21}H_{20}O_{11}$	447.0938	447.0933	-1.1	8.4	Luteolin-glucoside is. 1
20	15.7	$C_{22}H_{22}O_{11}$	461.1086	461.1089	0.7	13.9	Chrysoeriol-7-glucoside
21	16.3	$C_{21}H_{20}O_{11}$	447.0941	447.0933	-1.8	8.1	Luteolin-glucoside is. 2
22	16.3	$C_{26}H_{34}O_{14}$	569.1869	569.1876	1.3	24.2	2"-methoxyoleuropein is. 1
23	16.7	$C_{25}H_{32}O_{13}$	539.1769	539.1770	0.2	12.6	Oleuropein is. 1
24	17.0	$C_{26}H_{34}O_{14}$	569.1875	569.1876	0.1	2.6	2"-methoxyoleuropein is. 2
25	17.1	$C_{25}H_{32}O_{13}$	539.1766	539.1771	0.9	8.3	Oleuropein is. 2
26	17.4	$C_{25}H_{32}O_{13}$	539.1765	539.1769	0.7	4.7	Oleuropein is. 3
27	18.5	$C_{25}H_{32}O_{12}$	523.1812	523.1821	1.8	21.5	Ligstroside
28	19.3	$C_{19}H_{22}O_7$	361.1287	361.1293	1.5	2.7	Ligtroside aglycone
29	19.8	$C_{15}H_{10}O_6$	285.0399	285.0405	2.0	16.3	Luteolin
30	20.1	$C_{15}H_{10}O_7$	301.0354	301.0354	0	7.7	Quercetin
31	20.5	$C_{20}H_{22}O_6$	357.1355	357.1344	-3.2	3	Pinoresinol
32	22.3	$C_{15}H_{10}O_5$	269.0456	269.0455	-0.3	7.4	Apigenin
33	22.5	$C_{15}H_{12}O_5$	271.0612	271.0612	-0.1	13.6	Naringenin
34	22.8	$C_{16}H_{12}O_6$	299.0564	299.0561	-1.0	16.2	Diosmetin
35	23.3	$C_{15}H_8O_7$	299.0202	299.0197	-1.4	12.3	Uk is. 1
36	24.1	$C_{19}H_{22}O_8$	377.1242	377.1242	-0.1	17.1	Oleuropein aglycon is. 2
37	26.0	$C_{15}H_8O_7$	299.0196	299.0197	0.4	6.1	Uk is. 2
38	26.7	$C_{15}H_8O_7$	299.0200	299.0197	-0.9	13.9	Uk is. 3

is.: isomer; Uk: unknown.

Furthermore, the chromatographic profile of the studied samples showed other 16 peaks corresponding to flavonoids (in aglycon or in their glycosylated form). As far as flavonoids in aglycon form are concerned, the group was composed by (in elution order): rutin (peak 13), luteolin (peak 29), quercetin (peak 30), apigenin (peak 32), naringenin (peak 33), diosmetin (peak 34), and three isomers of an unknown compound with calculated molecular formula $C_{15}H_8O_7$ (peaks 35, 37 and 38). In the current report we have decided to include them in this category and quantify them in terms of luteolin (because of their similarity regarding polarity and molecular weight). We logically wanted to compare the concentration levels found in the different cultivars, rather than achieving very accurate quantitative results in absolute terms. Within the group of flavonoids in glycosylated form, we found the following ones: luteolin diglucoside (peak 10), luteolin-7-glucoside (peak 15) and other two luteolin-glucoside isomers (peaks 19 and 21), apigenin rutinoside (peak 16), apigenin-7-glucoside (peak 18), and chrysoeriol-7-glucoside (peak 20). Lastly, it was also possible to find four simple phenols (hydroxytyrosol glucoside (peak 1), hydroxytyrosol (peak 3), tyrosol glucoside (peak 4), and tyrosol (peak 6)), one phenolic acid (vanillic acid (peak 8)) and one lignan (pinoresinol (peak 31)).

It should be emphasized that almost all the phenolic compounds identified in the selected samples had been previously reported in interesting papers about the characterization of olive leaves extracts [26–30]. However, two aspects make this work dissimilar to the others: the number of determined compounds is wider in comparison, and it represents the first report including the comprehensive profiling of olive leaves from the varieties 'Lechín', 'Lucque', 'Picholine de Languedoc', 'Picholine Marocaine' and 'Verdal'.

2.2. Phenolic contents in different olive leaves cultivars

Prior to quantifying the identified phenolic compounds, the analytical method was properly validated in terms of linearity, precision (*intra*- and *inter*-day repeatability), limit of detection (LOD) and limit of quantification (LOQ). Thus, as reported in section 3.2.1, dilutions of the standard solution mixture were prepared and injected into the LC-IT MS system (which was the instrument used for quantifying). Method linearity was evaluated by plotting the peak areas versus the corresponding concentrations (mg/L) of each standard analyte using the least squares method. Calibration curves were built using the values from three replicates of each concentration level analyzed within the same day (n = 3). LODs and LOQs of the individual compounds in the standard solutions were calculated as the lowest concentration at which a signal-to-noise (S/N) ratio was greater than 3 and 10, respectively. *Intra*- and *inter*-day repeatability were also estimated; to do it so, we calculated the relative standard deviation (%RSD) of peak area for 4 injections of 4 different extracts of the quality control (QC) sample carried out within the same sequence (*intra*-day) or over 4 days (*inter*-day). Obtained results for the evaluated analytical parameters are summarized in Table S1 (Supplementary materials).

As shown in the table, linearity of the method was satisfactory over the assayed range with correlation coefficient (r^2) higher than 0.9918 in all cases. The LODs ranged from 3 to 97 µg/L and the LOQs ranged from 11 to 325 µg/L, for apigenin and rutin, apiece. The method led to excellent precision values (%RSD) always lower than 9.4% (values ranged from 1.8% to 7.5 % for the *intra*-day repeatability and from 2.1% to 9.4% for the *inter*-day repeatability). Consequently, the proposed analytical method could be successfully applied for the determination of 38 phenolic compounds in the selected 55 olive leaves samples.

Quantification in MS was done using external calibration curves of the corresponding pure standard analytes for: oleuropein, apigenin, apigenin-7-glucoside, hydroxytyrosol, luteolin, luteolin-7-glucoside, pinoresinol, rutin, tyrosol and vanillic acid; whereas for those identified compounds that reference pure standards were not available, a calibration curve from structurally related substances was used. Thus, tyrosol glucoside, elenolic acid glucoside isomers (1, 2 and 3), secologanoside isomers (1 and 2) and ligstroside aglycon were quantified using tyrosol calibration curve; hydroxytyrosol glucoside and oleuropein aglycon isomers (1 and 2) were quantified in terms of hydroxytyrosol; apigenin rutinoside and luteolin diglucoside in terms of rutin; chrysoeriol-7-glucoside and luteolin-glucoside isomers (1 and 2) by using luteolin-7-glucoside calibration curve; to quantify oleuropein diglucoside, 2"-methoxyoleoropein isomers (1 and 2), hydroxyoleuropein and ligstroside, the standard of oleuropein was employed; naringenin was determined in terms of apigenin; and finally, quercetin, diosmetin, and the unknown isomers of $C_{15}H_8O_7$ were quantified by using luteolin as reference standard. It is important to bear in mind that the response of the standards can differ from the response of the analytes present in the olive leave extract samples, and consequently, the quantification of these compounds (both in terms of total amount and individual contents) is only an estimation of their occurrence in the analyzed samples.

The total phenolic compounds content (sum of the content of individual phenolic compounds determined) and the total phenolic content per chemical class (sum of the content of individual phenolic compounds belonging to the same chemical family) of the olive leaves from the different studied cultivars are given in Fig.2. Results are expressed as mean ± standard deviation. As can be seen, on average terms, total phenolic content ranged from around 11.4 g/kg DW to 44.4 g/kg DW; 'Picual' was the poorest variety of the studied selection and 'Picholine Marocaine' was the richest one. Secoiridoids were by far the most abundant group of phenols in all the analyzed samples regardless of the variety, excepting 'Arbequina' and 'Picual' samples for which flavonoids (in glycosylated form) were predominant.

Among the studied cultivars, the highest secoiridoids content (34.5 g/kg DW) was found in 'Picholine Marocaine' leaves extracts, whilst 'Picual' samples presented the lowest concentration level (4.6 g/kg DW). The highest level of total flavonoids in glycosylated form was observed in 'Picholine de Languedoc' samples (9.9 g/kg DW) and the lowest one (5.9 g/kg DW) in 'Verdal' leaves; however, regarding this group of analytes, the differences found among the cultivars were not as noticeable as for others. As far as the other sub-category of flavonoids is concerned, it is

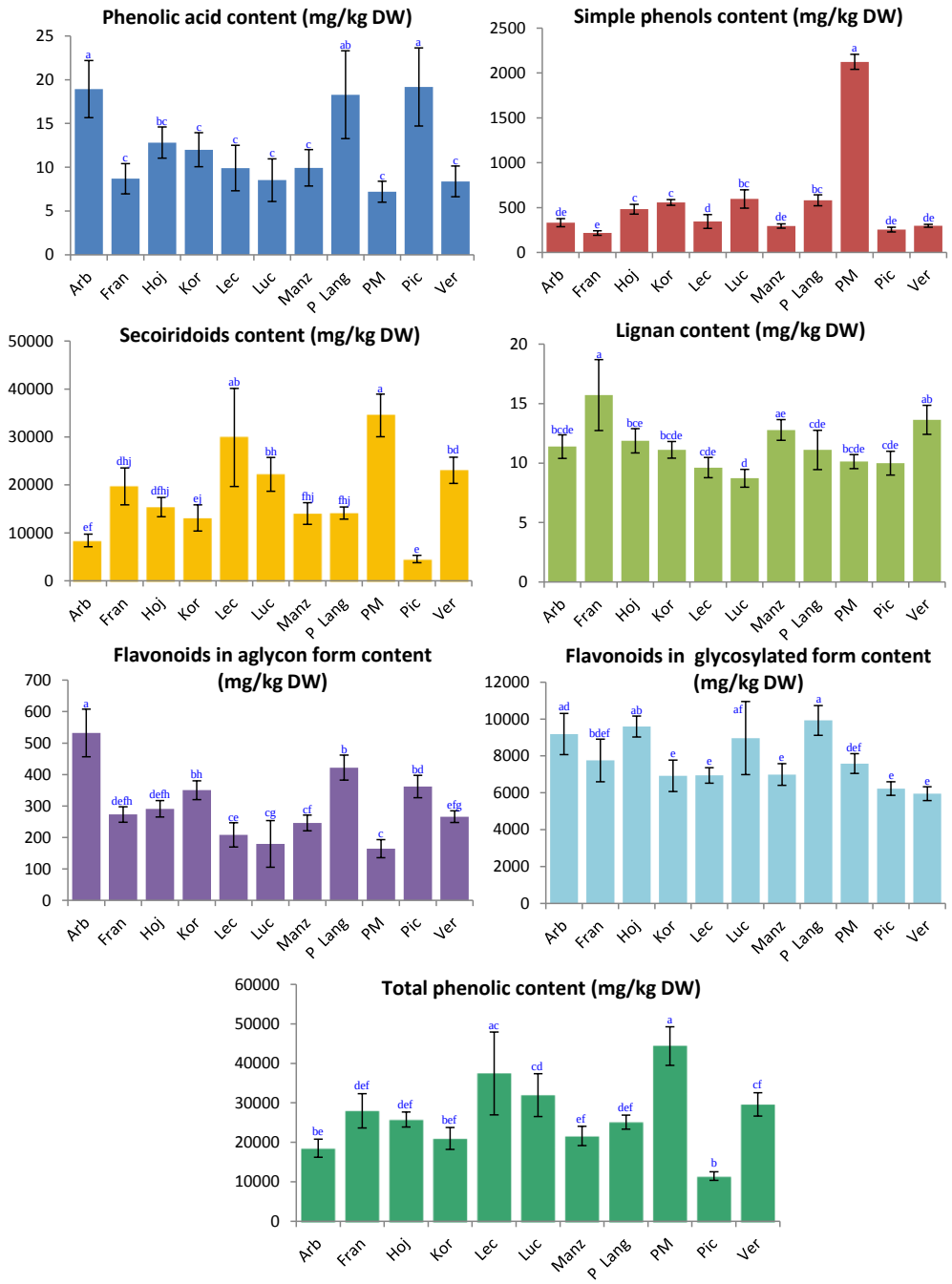

Figure 2. Total phenolic content and content in terms of the different chemical classes (content of secoiridoids, flavonoids in aglycon form, flavonoids in glycosylated form, simple phenols, one phenolic acid and one lignan) of the studied olive leaves samples, expressed in mg/kg DW. Different letters above the bars indicate significant differences at $p < 0.05$, Turkey's test (comparison among the 11 cultivars investigated in this study). Abbreviations meaning: Arb: 'Arbequina'; Fran: 'Frantoio'; Hoj: 'Hojiblanca'; Kor: 'Koroneiki'; Lech: 'Lechín'; Luc: 'Lucque'; Manz: 'Manzanilla'; P Lang: 'Picholine de Languedoc'; PM: 'Picholine Marocaine'; Pic: 'Picual'; and Verd: 'Verdal'.

possible to highlight that flavonoids in aglycon form were found within the range 164.6-532.5 mg/kg DW, defined by 'Picholine Marocaine' and 'Arbequina', respectively. The content in terms of simple phenols and, in particular, the amounts of vanillic acid and pinoresinol were negligible – in all the cultivars– when compared with secoiridoids levels. In this regard, the concentrations of simple phenols ranged between 217.8 mg/kg DW and 2124.1 mg/kg DW, for 'Frantoio' and 'Picholine Marocaine' leaves extracts, respectively. The content of the quantified lignan was found between 8.7 mg/kg DW (Lucque) and 15.7 mg/kg DW ('Frantoio'). Finally, the amount of the phenolic acid fluctuated from 7.2 mg/kg DW to 19.2 mg/kg DW; 'Picholine Marocaine' and 'Picual' exhibited the extreme concentration levels.

After getting the quantitative results, the existence of significant variations (both regarding total phenolic content and chemical class content) was investigated. One-way ANOVA revealed statistically significant differences among the concentration of phenolic compounds in leaves from different cultivars. Our results support those found in literature with regard to the *inter*-variety variability of the total phenolic content in olive leaves [26,27,30,31]. In general, our quantitative data are also similar to those included in previous reports, even though the comparison in this regard is not very straightforward; it is necessary to check whether the results from other authors are given as DW (or maybe without drying), and also to have a look at the compounds used as pure standards for the quantification and the methodology applied (extraction protocol and determination conditions). In addition, there are other obvious factors influencing the possible quantitative results, such as the cultivar, the pedoclimatic conditions, the harvesting time, etc.

In this work, for instance, the adaptability of an olive variety to the pedoclimatic conditions of the site of cultivation could largely condition its leaves metabolites. That could explain the divergence between our results regarding 'Picual' and 'Arbequina' *cv.* and those achieved by Talhaoui *et al.* [26,27]; generally the concentration levels found for some phenolic compounds were higher for the varieties which were cultivated in their country of origin (Spain, in this case). The same is applicable to underline that 'Picholine Marocaine' proved to be the cultivar (from the 11 selected herewith) with the highest quantity of phenolic compounds, possibly due to the fact that it is a Moroccan autochthonous variety with verified high adaptability to Moroccan environmental conditions.

When exploring the profile of phenolic compounds present in the studied samples (Tables 2a, 2b and 2c) to get an idea about their individual (or class) distribution, oleuropein isomer 1 was the prevalent substance in all the analyzed samples regardless of the variety, except for 'Picual', in which luteolin-7-glucoside was predominant. Oleuropein, which has been widely investigated for its functional properties as well as its possible recovery and reutilization in various fields [13,32], was the main olive leaf secoiridoid. Oleuropein isomer 1 concentration levels varied from 1632.0 to 23962.7 mg/kg DW, for 'Picual' and 'Picholine Marocaine' leaves, respectively. Additionally, 2"-methoxyoleuropein isomer 1 was also detected at remarkable levels, fluctuating from 572.5 (in 'Picholine Marocaine') to 2328.8 mg/kg DW (in 'Frantoio'). The concentration of some of the other

Table 2a. Found content (average values and standard deviation, mg/kg DW) of the determined phenolic compounds in the evaluated olive leaves cultivars. ANOVA results are included; significant differences in the same row are indicated with different superscript letters (comparison among the 11 cultivars investigated in this study, $p < 0.05$).

	'Arbequina'	'Frantoio'	'Hojiblanca'	'Koroneiki'
Hydroxytyrosol glucoside	$9.7^{a}\pm5.4$	$22.2^{a}\pm6.8$	$185.4^{b}\pm32.6$	$202.6^{b}\pm16.3$
Secologanoside is. 1	$333.2^{ab}\pm28.5$	$754.3^{e}\pm119.5$	$844.4^{ef}\pm79.6$	$642.7^{de}\pm42.0$
Hydroxytyrosol	$209.2^{a}\pm38.8$	$119.1^{b}\pm13.6$	$147.2^{ab}\pm23.1$	$135.6^{b}\pm7.2$
Tyrosol glucoside	$60.7^{f}\pm5.1$	$47.9^{ef}\pm6.4$	$119.8^{d}\pm13.4$	$178.5^{b}\pm19.5$
Secologanoside is. 2	$482.9^{ac}\pm67.4$	$1311.5^{bd}\pm79.8$	$1330.3^{bd}\pm69.1$	$769.3^{ce}\pm184.4$
Tyrosol	$52.8^{ab}\pm10.0$	$28.6^{cd}\pm5.2$	$30.9^{cd}\pm2.7$	$41.0^{bd}\pm7.1$
Elenolic acid glucoside is. 1	$484.5^{d}\pm43.3$	$849.7^{c}\pm62.6$	$742.4^{b}\pm33.8$	$576.4^{d}\pm37.8$
Vanillic acid	$18.9^{a}\pm3.3$	$8.7^{c}\pm1.7$	$12.82^{bc}\pm1.8$	$12.0^{c}\pm1.9$
Oleuropein aglycon is. 1	$48.1^{a}\pm11.3$	$421.9^{b}\pm59.5$	$206.3^{cd}\pm19.8$	$397.2^{b}\pm38.3$
Luteolin diglucoside	$625.9^{a}\pm79.2$	$420.6^{c}\pm66.0$	$240.0^{b}\pm25.8$	$354.7^{bc}\pm35.8$
Elenolic acid glucoside is. 2	$95.2^{b}\pm13.4$	$467.2^{ef}\pm104.5$	$430.8^{def}\pm15.0$	$369.8^{def}\pm27.2$
Elenolic acid glucoside is. 3	$73.3^{c}\pm4.4$	$174.4^{e}\pm13.5$	$139.7^{de}\pm17.1$	$134.8^{de}\pm19.5$
Rutin	$410.7^{de}\pm34.4$	$542.0^{ce}\pm113.0$	$489.2^{ce}\pm52.9$	$1099.0^{a}\pm223.3$
Hydroxyoleuropein	$524.7^{cf}\pm53.5$	$758.4^{de}\pm89.6$	$757.0^{de}\pm47.9$	$843.2^{d}\pm43.1$
Luteolin-7-glucoside	$3323.5^{ab}\pm374.6$	$2526.6^{c}\pm408.1$	$3708.0^{a}\pm321.8$	$2631.8^{c}\pm190.8$
Apigenin rutinoside	$430.7^{def}\pm59.8$	$353.6^{bdf}\pm36.5$	$541.9^{a}\pm64.8$	$312.3^{bcf}\pm46.3$
Oleuropein diglucoside	$94.4^{c}\pm19.4$	$248.9^{efh}\pm36.4$	$457.8^{a}\pm24.5$	$300.8^{df}\pm38.2$
Apigenin-7-glucoside	$65.3^{bc}\pm11.6$	$64.7^{bc}\pm5.7$	$245.6^{ad}\pm12.7$	$157.6^{c}\pm22.6$
Luteolin-glucoside is. 1	$3428.0^{ac}\pm542.1$	$3013.4^{abc}\pm555.1$	$3584.3^{c}\pm172.1$	$1630.2^{de}\pm512.7$
Chrysoeriol-7-glucoside	$605.9^{b}\pm43.8$	$495.7^{cd}\pm27.3$	$551.9^{bc}\pm23.9$	$387.4^{a}\pm40.6$
Luteolin-glucoside is. 2	$295.3^{cdfgh}\pm31.8$	$340.7^{dgh}\pm49.2$	$230.5^{cbf}\pm12.4$	$346.6^{degh}\pm50.2$
2"-methoxyoleuropein is.1	$1499.0^{bd}\pm194.0$	$2328.8^{a}\pm230.6$	$2062.8^{ad}\pm158.6$	$1642.5^{bd}\pm510.5$
Oleuropein is. 1	$3465.0^{e}\pm959.7$	$10959.1^{cdf}\pm3283.3$	$6923.5^{def}\pm1813.4$	$6023.1^{def}\pm1679.0$
2"-methoxyoleuropein is. 2	$129.5^{e}\pm31.4$	$99.5^{de}\pm16.5$	$176.3^{a}\pm21.5$	$128.4^{e}\pm30.8$
Oleuropein is. 2	$56.9^{ce}\pm20.8$	$159.1^{def}\pm51.0$	$129.8^{cf}\pm53.8$	$139.3^{cf}\pm60.2$
Oleuropein is. 3	$233.5^{cf}\pm49.9$	$335.9^{cf}\pm105.6$	$440.4^{df}\pm115.6$	$375.0^{ef}\pm95.7$
Ligstroside	$504.8^{df}\pm92.5$	$343.0^{cd}\pm51.4$	$406.0^{cd}\pm36.6$	$495.9^{de}\pm130.3$
Ligstroside aglycon	$334.4^{bc}\pm92.7$	$141.7^{c}\pm103.9$	$312.1^{c}\pm41.1$	$277.7^{c}\pm33.4$
Luteolin	$372.9^{a}\pm63.2$	$189.1^{e}\pm23.2$	$175.2^{de}\pm19.8$	$278.8^{b}\pm34.2$
Quercetin	$40.6^{a}\pm10.8$	$13.5^{b}\pm2.8$	$14.1^{b}\pm1.0$	$9.4^{b}\pm5.2$
Pinoresinol	$11.4^{bcde}\pm1.0$	$15.7^{a}\pm3.0$	$11.9^{bce}\pm1.0$	$11.1^{bcde}\pm0.7$
Apigenin	$20.8^{bc}\pm5.6$	$12.3^{acdf}\pm2.2$	$17.2^{bde}\pm2.1$	$24.3^{b}\pm10.9$
Naringenin	$7.4^{ac}\pm1.0$	$5.4^{c}\pm0.7$	$6.2^{bc}\pm1.0$	$5.3^{c}\pm0.4$
Diosmetin	$27.3^{a}\pm6.8$	$14.2^{cd}\pm2.1$	$6.2^{b}\pm0.7$	$15.0^{cd}\pm2.3$
Unknown is. 1	$13.4^{efg}\pm2.8$	$12.8^{ef}\pm1.6$	$20.8^{d}\pm1.9$	$2.9^{b}\pm2.9$
Oleuropein aglycon is. 2	$60.3^{e}\pm36.4$	$359.0^{bc}\pm65.9$	$17.8^{e}\pm5.2$	$13.2^{e}\pm4.8$
Unknown is. 2	$35.8^{a}\pm6.4$	$14.1^{cd}\pm2.3$	$28.0^{a}\pm2.3$	$8.4^{bc}\pm4.0$
Unknown is. 3	$14.4^{a}\pm3.8$	$11.9^{a}\pm2.2$	$23.5^{a}\pm3.4$	$6.5^{bc}\pm1.3$

Table 2b. Found content (average values and standard deviation, mg/kg DW) of the determined phenolic compounds in the evaluated olive leaves cultivars. ANOVA results are included; significant differences in the same row are indicated with different superscript letters (comparison among the 11 cultivars investigated in this study, $p < 0.05$).

	'Lechin'	'Lucque'	'Manzanilla'	'Picholine de Languedoc'
Hydroxytyrosol glucoside	$38.9^{a}\pm19.2$	$316.3^{c}\pm64.0$	$48.0^{a}\pm7.3$	$186.1^{b}\pm18.5$
Secologanoside is. 1	$876.3^{cef}\pm197.9$	$1018.4^{cf}\pm91.2$	$506.8^{bd}\pm73.7$	$607.6^{de}\pm48.7$
Hydroxytyrosol	$147.1^{ab}\pm41.5$	$143.2^{ab}\pm57.7$	$143.7^{ab}\pm19.9$	$202.3^{a}\pm46.2$
Tyrosol glucoside	$122.5^{cd}\pm27.6$	$114.2^{cd}\pm22.2$	$60.4^{efg}\pm2.3$	$159.9^{b}\pm18.9$
Secologanoside is. 2	$1455.1^{b}\pm297.7$	$854.5^{e}\pm131.9$	$745.6^{ce}\pm125.4$	$571.8^{ace}\pm61.2$
Tyrosol	$37.6^{d}\pm4.2$	$23.0^{c}\pm6.4$	$43.5^{bd}\pm4.2$	$33.4^{cd}\pm4.4$
Elenolic acid glucoside is. 1	$799.3^{bc}\pm98.9$	$507.4^{d}\pm47.7$	$512.6^{d}\pm15.3$	$494.0^{d}\pm24.2$
Vanillic acid	$9.9^{c}\pm2.6$	$8.5^{c}\pm2.4$	$9.9^{c}\pm2.1$	$18.3^{ab}\pm5.0$
Oleuropein aglycon is. 1	$142.9^{de}\pm37.3$	$244.5^{c}\pm56.9$	$202.3^{cd}\pm11.7$	$164.3^{de}\pm25.3$
Luteolin diglucoside	$392.7^{c}\pm48.2$	$302.1^{bc}\pm103.9$	$344.2^{bc}\pm31.1$	$606.9^{a}\pm101.7$
Elenolic acid glucoside is. 2	$323.2^{df}\pm94.9$	$345.7^{cdef}\pm48.8$	$226.1^{cd}\pm28.7$	$426.3^{f}\pm26.8$
Elenolic acid glucoside is. 3	$92.9^{cd}\pm12.3$	$153.2^{de}\pm22.3$	$155.7^{e}\pm18.0$	$264.5^{a}\pm32.6$
Rutin	$293.8^{de}\pm25.6$	$2436.3^{b}\pm319.6$	$383.7^{de}\pm66.8$	$689.1^{c}\pm82.0$
Hydroxyoleuropein	$576.6^{fg}\pm40.9$	$551.2^{cf}\pm83.4$	$642.6^{ef}\pm16.9$	$490.0^{cg}\pm33.1$
Luteolin-7-glucoside	$2715.1^{bc}\pm101.1$	$2257.5^{c}\pm561.0$	$2561.5^{c}\pm223.1$	$3547.8^{a}\pm357.5$
Apigenin rutinoside	$275.3^{b}\pm15.1$	$384.8^{cdef}\pm56.1$	$470.9^{aef}\pm32.4$	$395.8^{cdef}\pm38.9$
Oleuropein diglucoside	$163.6^{cg}\pm37.6$	$311.6^{dfh}\pm60.4$	$219.1^{eg}\pm27.0$	$354.0^{d}\pm20.0$
Apigenin-7-glucoside	$93.4^{f}\pm3.2$	$220.7^{ae}\pm41.5$	$157.2^{d}\pm5.3$	$134.9^{df}\pm5.6$
Luteolin-glucoside is. 1	$2288.6^{bde}\pm249.6$	$2598.5^{ab}\pm965.2$	$2425.1^{bde}\pm270.8$	$3687.1^{c}\pm265.9$
Chrysoeriol-7-glucoside	$532.2^{bc}\pm30.1$	$498.2^{cd}\pm54.0$	$436.9^{ad}\pm83.1$	$547.1^{bc}\pm22.6$
Luteolin-glucoside is. 2	$350.2^{degh}\pm42.9$	$266.6^{fg}\pm20.7$	$208.6^{bf}\pm23.9$	$317.2^{gh}\pm39.9$
2"-methoxyoleuropein is.1	$1588.5^{bd}\pm255.2$	$760.8^{ce}\pm314.5$	$1161.7^{bc}\pm213.2$	$928.1^{ce}\pm86.1$
Oleuropein is. 1	$20645.4^{ab}\pm8348.4$	$15351.2^{bc}\pm2708.2$	$7696.0^{def}\pm1583.4$	$8176.4^{def}\pm895.2$
2"-methoxyoleuropein is. 2	$67.5^{bd}\pm7.6$	$54.4^{bc}\pm14.2$	$100.0^{de}\pm21.2$	$132.7^{e}\pm10.3$
Oleuropein is. 2	$247.3^{bd}\pm85.3$	$301.0^{b}\pm54.2$	$114.8^{cf}\pm41.6$	$174.3^{df}\pm22.0$
Oleuropein is. 3	$637.7^{bd}\pm197.3$	$873.0^{b}\pm206.9$	$396.9^{df}\pm106.9$	$597.3^{de}\pm67.1$
Ligstroside	$652.6^{d}\pm147.4$	$424.6^{cd}\pm28.0$	$575.2^{d}\pm31.4$	$184.7^{cef}\pm21.8$
Ligstroside aglycon	$979.4^{a}\pm494.0$	$400.1^{bc}\pm112.7$	$526.4^{bc}\pm185.4$	$446.8^{bc}\pm103.7$
Luteolin	$168.8^{de}\pm34.8$	$112.6^{cd}\pm56.6$	$157.1^{de}\pm17.6$	$276.4^{b}\pm31.6$
Quercetin	$3.9^{b}\pm0.6$	$6.6^{b}\pm4.3$	$9.6^{b}\pm1.6$	$18.7^{b}\pm2.6$
Pinoresinol	$9.6^{cde}\pm0.8$	$8.7^{d}\pm0.7$	$12.8^{ae}\pm0.9$	$11.1^{cde}\pm1.6$
Apigenin	$11.3^{aef}\pm1.7$	$11.4^{aef}\pm1.8$	$15.9^{ab}\pm1.5$	$16.8^{bf}\pm2.8$
Naringenin	$6.8^{ac}\pm1.3$	$5.0^{c}\pm0.5$	$8.0^{ab}\pm2.2$	$8.7^{a}\pm0.8$
Diosmetin	$6.2^{b}\pm2.4$	$6.0^{b}\pm3.6$	$6.3^{b}\pm2.2$	$20.4^{d}\pm3.7$
Unknown is. 1	$3.8^{bc}\pm2.3$	$8.7^{ce}\pm3.4$	$16.3^{df}\pm2.4$	$28.9^{a}\pm2.3$
Oleuropein aglycon is. 2	$665.5^{a}\pm260.0$	$53.3^{e}\pm16.7$	$262.0^{cd}\pm100.5$	$132.5^{de}\pm18.7$
Unknown is. 2	$4.1^{b}\pm1.1$	$18.5^{d}\pm6.0$	$18.5^{d}\pm2.4$	$29.5^{a}\pm2.9$
Unknown is. 3	$3.4^{b}\pm2.2$	$10.6^{cd}\pm3.1$	$14.4^{d}\pm2.3$	$22.6^{a}\pm0.7$

Table 2c. Found content (average values and standard deviation, mg/kg DW) of the determined phenolic compounds in the evaluated olive leaves cultivars. ANOVA results are included; significant differences in the same row are indicated with different superscript letters (comparison among the 11 cultivars investigated in this study, $p < 0.05$).

	'Picholine Marocaine'	'Picual'	'Verdal'
Hydroxytyrosol glucoside	1510.2^{d}±66.6	10.6^{a}±6.5	14.8^{a}±8.6
Secologanoside is. 1	1058.7^{c}±50.5	181.8^{a}±37.6	1005.3^{cf}±111.6
Hydroxytyrosol	322.8^{c}±21.8	155.0^{ab}±14.5	139.7^{b}±10.6
Tyrosol glucoside	237.4^{a}±13.4	61.9^{efg}±10.1	82.0^{cefg}±5.6
Secologanoside is. 2	1199.3^{bd}±225.6	375.6^{a}±94.4	1100.2^{de}±144.0
Tyrosol	53.7^{ab}±9.9	28.2^{cd}±4.4	61.0^{a}±7.9
Elenolic acid glucoside is. 1	342.3^{a}±29.1	265.6^{a}±41.7	786.9^{bc}±57.8
Vanillic acid	7.2^{c}±1.2	19.2^{a}±4.5	8.4^{c}±1.8
Oleuropein aglycon is. 1	437.3^{b}±37.0	104.9^{ae}±41.2	173.2^{cde}±22.7
Luteolin diglucoside	394.9^{c}±30.6	353.4^{bc}±37.6	294.2^{bc}±27.1
Elenolic acid glucoside is. 2	887.1^{a}±95.2	85.0^{b}±19.3	401.7^{cdef}±64.7
Elenolic acid glucoside is. 3	989.3^{b}±74.9	114.2^{ce}±11.1	126.7^{ce}±17.1
Rutin	554.4^{ce}±45.3	161.2^{d}±28.0	361.8^{de}±16.8
Hydroxyoleuropein	146.6^{a}±16.3	419.7^{c}±69.7	1027.0^{b}±140.4
Luteolin-7-glucoside	2800.3^{bc}±232.4	2283.8^{c}±151.8	2661.8^{bc}±291.8
Apigenin rutinoside	456.5^{ade}±31.5	394.6^{cdef}±80.5	326.8^{f}±45.0
Oleuropein diglucoside	623.4^{b}±47.2	94.0^{c}±38.5	243.3^{efg}±28.3
Apigenin-7-glucoside	147.9^{df}±18.4	113.73^{f}±7.04	201.8^{e}±11.3
Luteolin-glucoside is. 1	2471.2^{bd}±228.3	2131.6^{de}±130.5	1493.9^{e}±115.1
Chrysoeriol-7-glucoside	480.3^{cd}±26.2	424.2^{ad}±13.6	495.3^{ad}±12.3
Luteolin-glucoside is. 2	277.4^{befgh}±35.9	363.9^{h}±57.7	116.4^{a}±7.0
2"-methoxyoleuropein is.1	572.5^{e}±47.7	611.2^{ce}±188.5	2241.2^{a}±384.1
Oleuropein is. 1	23962.7^{a}±3512.8	1632.0^{e}±437.0	12443.3^{cf}±2402.7
2"-methoxyoleuropein is. 2	127.0^{e}±9.6	52.2^{b}±18.6	95.4^{cde}±16.2
Oleuropein is. 2	433.5^{a}±46.7	41.7^{c}±14.8	193.0^{df}±53.6
Oleuropein is. 3	2248.8^{a}±125.8	114.5^{c}±40.4	419.3^{df}±86.6
Ligstroside	1118.2^{a}±357.9	129.4^{c}±54.9	1607.8^{b}±259.6
Ligstroside aglycon	209.3^{c}±20.4	297.8^{c}±38.8	729.8^{ab}±299.8
Luteolin	49.2^{c}±8.5	264.7^{b}±28.4	184.0^{de}±15.6
Quercetin	50.0±14.1	6.7^{b}±2.1	7.1^{b}±1.7
Pinoresinol	10.1^{bcde}±0.6	10.0^{cde}±1.0	13.6^{ab}±1.2
Apigenin	7.5^{ac}±0.7	21.1^{b}±1.6	18.6^{bf}±2.6
Naringenin	5.2^{c}±0.5	6.6^{ac}±0.5	6.3^{ac}±0.3
Diosmetin	4.2^{b}±1.1	15.6^{cd}±2.4	13.0^{c}±1.9
Unknown is. 1	18.8^{dg}±3.6	16.4^{df}±2.2	9.5^{e}±1.8
Oleuropein aglycon is. 2	125.3^{de}±17.5	31.6^{e}±11.6	464.8^{b}±90.9
Unknown is. 2	15.2^{cd}±2.8	17.8^{d}±2.9	16.2^{d}±2.1
Unknown is. 3	14.6^{d}±2.4	12.8^{d}±1.4	11.5^{cd}±2.3

secoiridoids was as follows: secologanoside isomer 1 (181.8-1058.7 mg/kg DW); secologanoside isomer 2 (375.6-1455.1 mg/kg DW); elenolic acid glucoside isomer 1 (265.6-849.7 mg/kg DW); oleuropein aglycon isomer 1 (48.1-437.3 mg/kg DW); elenolic acid glucoside isomer 2 (85.0-887.1 mg/kg DW); elenolic acid glucoside isomer 3 (73.3-989.3 mg/kg DW); hydroxyoleuropein (146.6-1027.0 mg/kg DW) and oleuropein diglucoside (94.4-623.4 mg/kg DW). The latter was the minor compound found in samples of 7 varieties ('Arbequina', 'Frantoio', 'Lechín', 'Manzanilla', 'Picholine de Languedoc', 'Picual' and 'Verdal'), whereas oleuropein aglycon isomer 2 showed the lowest content in leaves from 'Hojiblanca', 'Koroneiki', 'Lucque' and 'Picholine Marocaine'. It is necessary to emphasize that large standard deviations were obtained for most of the characterized secoiridoids (Tables 2a, 2b and 2c); that reflects the considerable variability among samples from the same variety. In any case, these *intra*-cultivar differences remain rather small when compared with those observed among the studied cultivars.

A great variability was also observed with regard to flavonoids content. According to Tables 2, glycosylated flavonoids were much more abundant than aglycon ones. Luteolin-7-glucoside was the major flavonoid compound in the leaves samples of eight varieties ('Hojiblanca', 'Koroneiki', 'Lechín', 'Lucque', 'Manzanilla', 'Picholine Marocaine', 'Picual' and 'Verdal'), with a total concentration range defined by 'Hojiblanca' and 'Lucque' with values from 2257.5 to 3708.0 mg/kg DW. However, luteolin-glucoside isomer 1 was the predominant glycosylated flavonoid for 'Arbequina', 'Frantoio' and 'Picholine de Languedoc' cultivars, being found within the overall range 1493.9-3687.9 mg/kg DW, defined by 'Verdal' and 'Picholine de Languedoc' *cv.* In addition, leaves from 'Arbequina' cultivar were characterized by the highest content of luteolin diglucoside (625.9 mg/kg DW) and chrysoeriol-7-glucoside (605.9 mg/kg DW), whereas 'Hojiblanca' samples exhibited the highest amounts of apigenin rutinoside (541.9 mg/kg DW) and apigenin-7-glucoside (245.6 mg/kg DW). Finally, rutin and luteolin-glucoside isomer 2 were prevailing in 'Lucque' (2,436.3 mg/kg DW) and 'Picual' (363.9 mg/kg DW) leaves, respectively. In fact, leaves from 'Lucque' were outstandingly richest on rutin if compared with samples from the other varieties.

In the sub-category of flavonoids in not-glycosylated form, luteolin was the dominant compound in every case. 'Arbequina' leaves showed the highest levels of luteolin (372.9 mg/kg DW), diosmetin (27.3 mg/kg DW) and unknown isomer 2 (35.8 mg/kg DW). 'Picholine Marocaine' samples contained the highest amount of quercetin (50.0 mg/kg DW) and 'Picholine de Languedoc' leaves were the richest ones in terms of naringenin (8.7 mg/kg DW) and unknown isomer 1 (28.9 mg/kg DW). 'Koroneiki' and 'Hojiblanca' samples showed the highest content of apigenin (24.3 mg/kg DW) and unknown isomer 3 (23.5 mg/kg DW), respectively (Tables 2). At this point, it is worthy to highlight that this is the first time that the quantification of so many flavonoids derivatives has been performed in olive leaves.

Considering the simple phenols content, the selected varieties could be clustered in two groups: those with hydroxytyrosol as the most abundant simple phenol ('Arbequina', 'Frantoio', 'Lucque', 'Manzanilla', 'Picholine de Languedoc', 'Picual' and 'Verdal'), and those cultivars with

hydroxytyrosol glucoside as the predominant substance within this category ('Hojiblanca', 'Koroneiki', 'Lucque', and 'Picholine Marocaine'). Hydroxytyrosol levels varied from 119.1 to 322.8 mg/kg DW, in 'Frantoio' and 'Picholine Marocaine', respectively. The latter variety was also the richest regarding hydroxytyrosol glucoside (1510.2 mg/kg DW), whilst 'Arbequina' was the poorest one (9.7 mg/kg DW). Tyrosol (23.0-61.0 mg/kg DW) and tyrosol glucoside (47.9-237.4 mg/kg DW) were also found in the samples under study. Vanillic acid and pinoresinol were quantified in the studied olive leaves too. Their concentration levels were relatively low in every sample (< 19.2 mg/kg DW for vanillic acid, and < 15.3 mg/kg DW for pinoresinol) (Tables 2).

The results of the current study demonstrate that content of individual phenolic compounds in olive leaves is, as expected, closely related to the variety. Indeed, when compared by one-way ANOVA, the contents of the determined compounds were significantly different among the cultivars. Since all the varieties investigated in the current work were grown in the same experimental field using similar agronomic practices, the observed differences regarding the biosynthesis of secondary metabolites can be attributed to the genetic variability. These findings are in good agreement with those reported in literature, as reviewed in detail by Talhaoui and co-workers [24].

Besides, the results of Tukey's test indicated that individual contents of olive leaves from different cultivars had their own features. Focusing, for instance, on 'Picholine Marocaine' traits (Table 2c), some specific characteristics can be pointed out. These leaves showed, on average, the highest total phenolic compounds content. This variety is the richest one in terms of secoiridoids (presenting the highest amount of various of these compounds); it presents low concentrations levels of flavonoids in aglycon form, lignans and phenolic acids; however, it contains considerable amounts of simple phenols (in particular, hydroxytyrosol glucoside) and flavonoids in glycosylated form. Thus, it appears that this variety presents, among the other studied cultivars, the greatest potential to be used as plausible source of bioactive compounds, what means that it could be a very promising choice in a future strategy of recycling and valorization of olive leaves from Moroccan olive agro-industry.

2.3. Varietal discrimination

Tremendous efforts have been made to explore the genetic diversity of olive trees cultivated all around the world and to identify their varietal origin. Discrimination of the varietal origin of olive trees based on their leaves traits is frequently carried out studying morphological characteristics and genetic markers. Certainly, great advances have been made to explore and prove the usefulness of various olive leaf's molecular markers, such as amplified fragment length polymorphism, random amplified polymorphic DNA and genomic simple sequence repeat, as reliable tools to differentiate and characterize the genetic diversity of olive cultivars [33,34]. Although these techniques are very valuable, they also have some drawbacks such as complicated pretreatment and DNA extraction procedures, high cost and special requirements for operators.

Consequently, there is a need to explore the effectiveness of other analytical approaches to deal with these limitations. The combined application of profiling of olive leaves and chemometrics could be an effective alternative. Hence, in this study, beyond our interest on evaluating the phenolic composition of leaves from different cultivars, we also explored the ability of these compounds to trace the samples varietal origin.

A first attempt to differentiate among the studied varieties was carried out by applying Principal Components Analysis (PCA) to a standardized and centered matrix data, which was constructed with the 38 measured variables (phenolic compounds) and the 55 leaves samples (three extraction replicates). PCA was logically employed as unsupervised method to examine natural grouping of the samples according to their varietal origin in two-dimensional principal components (PCs) plans where each PC is a linear correlation of the original variables (latent variable), and each PC is orthogonal to any other. In this manner, this method studies data structure in a reduced dimension, covering the maximum amount of the information present in the original dataset.

Thus, PCA on leaves phenolic composition resulted in eight PCs with eigenvalues > 1 (PC1 = 10.82; PC2 = 7.61; PC3 = 4.66; PC4 = 3.35; PC5 = 2.47; PC6 = 2.22; PC7 = 1.69 and PC8 = 1.23) that accounted for 89.60% of the total variance of the original result data matrix. Despite the relatively low explained variability retained in the three first PCs (60.77%), the explorative analysis of the projections on the first three PCs (PC1 *vs* PC2 (Fig. 3a) and PC2 *vs* PC3 (Fig. 3b)) was crucial to check possible clustering of the leaves samples according to their varietal origin based on their phenolic composition. The results given in Fig. 3 show that good separation of 6 varieties could be achieved with a simple PCA ('Arbequina', 'Hojiblanca', 'Picholine de Languedoc', 'Picholine Marocaine', 'Picual' and 'Verdal'); the other varieties appeared barely separated in the projections (PC1 *vs* PC2 and PC2 *vs* PC3).

Subsequently, the potential of applying a supervised multivariate method (Stepwise Linear Discriminant Analysis (s-LDA)) was tested. The applicability of the method was cross-validated by using the leave-one-out procedure. The Wilks λ value (0.000) showed that the model was very discriminating, and, in addition, revealed that the probability of correct classification was very high, considering that the p value was very low ($p < 0.0001$). Moreover, the forward stepwise statistics, with F-to-enter equal to 1.0 and F-to-remove equal to 0.5, selected 20 variables to be used in the relevant final models: hydroxytyrosol glucoside, 2"-methoxyoleuropein isomer 2, apigenin-7-glucoside, unknown isomer 1, unknown isomer 2, unknown isomer 3, elenolic acid glucoside isomer 1, elenolic acid glucoside isomer 2, ligstroside, ligstroside aglycon, luteolin, luteolin diglucoside, luteolin-glucoside isomer 1, oleuropein aglycon isomer 1, oleuropein isomer 2, oleuropein isomer 3, rutin, secologanoside isomer 1, secologanoside isomer 2 and tyrosol glucoside.

The results of s-LDA classification and prediction are summarized in the confusion matrices shown in Table 3, displaying re-allocation of samples coming from a given cultivar (corresponding

to a matrix row) into the possible categories (the columns). As can be seen from this table, the s-LDA discriminant functions achieved very satisfactory recognition and prediction abilities, being the overall correct rate in both cases 100%. Accordingly, it is possible to assert that the olive leaves phenolic content could be useful for olive cultivars differentiation.

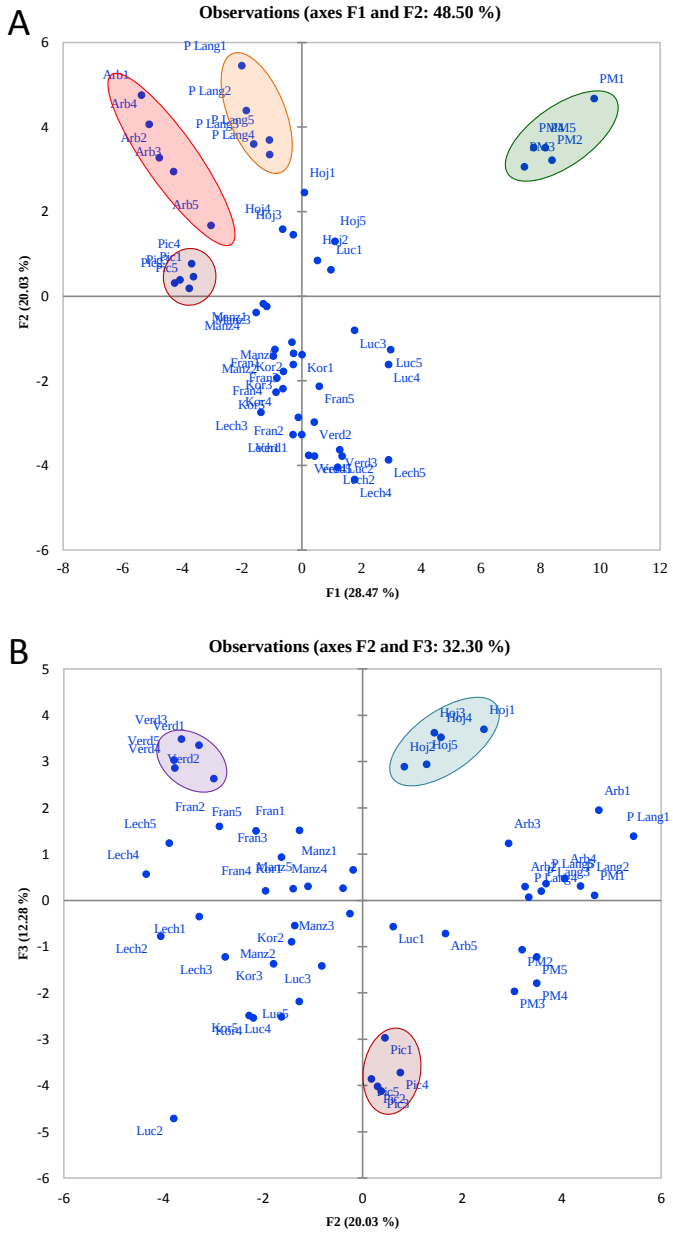

Figure 3. Scatter plot of the PCA scores projected on PC1, PC2 (a) and PC2, PC3 (b). Abbreviations meaning as in Figure 2. (Even though the statistical treatment was carried out considering the independent extracts and injections of each sample, just the mean value was represented here to facilitate the visual inspection of the figure.

Table 3. Classification and Prediction ability results of s-LDA model, based on olive leaves phenolic composition, for achieving varietal origin separation.

Confusion matrix for the training sample													
Variety / classified as	**Arbequina**	**Frantoio**	**Hojiblanca**	**Koroneiki**	**Lechín**	**Lucque**	**Manzanilla**	**Picholine Marocaine**	**Picholine de Languedoc**	**Picual**	**Verdal**	**Total**	**% correct**
Arbequina	5	0	0	0	0	0	0	0	0	0	0	5	100.0
Frantoio	0	5	0	0	0	0	0	0	0	0	0	5	100.0
Hojiblanca	0	0	5	0	0	0	0	0	0	0	0	5	100.0
Koroneiki	0	0	0	5	0	0	0	0	0	0	0	5	100.0
Lechín	0	0	0	0	5	0	0	0	0	0	0	5	100.0
Lucque	0	0	0	0	0	5	0	0	0	0	0	5	100.0
Manzanilla	0	0	0	0	0	0	5	0	0	0	0	5	100.0
Picholine Marocaine	0	0	0	0	0	0	0	5	0	0	0	5	100.0
Picholine de Languedoc	0	0	0	0	0	0	0	0	5	0	0	5	100.0
Picual	0	0	0	0	0	0	0	0	0	5	0	5	100.0
Verdal	0	0	0	0	0	0	0	0	0	0	5	5	100.0
Total	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*55*	*100.0*
Confusion matrix for the cross-validation results													
Variety / classified as	**Arbequina**	**Frantoio**	**Hojiblanca**	**Koroneiki**	**Lechín**	**Lucque**	**Manzanilla**	**Picholine Marocaine**	**Picholine de Languedoc**	**Picual**	**Verdal**	**Total**	**% correct**
Arbequina	5	0	0	0	0	0	0	0	0	0	0	5	100.0
Frantoio	0	5	0	0	0	0	0	0	0	0	0	5	100.0
Hojiblanca	0	0	5	0	0	0	0	0	0	0	0	5	100.0
Koroneiki	0	0	0	5	0	0	0	0	0	0	0	5	100.0
Lechín	0	0	0	0	5	0	0	0	0	0	0	5	100.0
Lucque	0	0	0	0	0	5	0	0	0	0	0	5	100.0
Manzanilla	0	0	0	0	0	0	5	0	0	0	0	5	100.0
Picholine Marocaine	0	0	0	0	0	0	0	5	0	0	0	5	100.0
Picholine de Languedoc	0	0	0	0	0	0	0	0	5	0	0	5	100.0
Picual	0	0	0	0	0	0	0	0	0	5	0	5	100.0
Verdal	0	0	0	0	0	0	0	0	0	0	5	5	100.0
Total	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*5*	*55*	*100.0*

3. MATERIALS AND METHODS

3.1. Olive leaves sampling and preparation

In order to avoid any possible influence of the environmental and agricultural management practices on the obtained results, all olive leaves samples were collected at an experimental orchard in the National School of Agriculture of Meknès in Northern Morocco. Sampling was performed in December 2015, coinciding with the harvesting season in Meknès region, when olive leaves are available as an olive oil processing by-product. This region has a Mediterranean climate type with an average pluviometry of 660 mm/year, and hot and dry summers (maximum temperature up to 40 °C). All necessary agronomic practices (pruning, irrigation, fertilization and pest management) were done according to current olive orchards management standards. Olive trees were vase-trained at a spacing of 7 × 5 m.

Eleven different cultivars were included in this study: a Moroccan autochthonous and predominant variety so-called 'Picholine Marocaine', and ten Mediterranean cultivars recently introduced in Morocco ('Arbequina', 'Hojiblanca', 'Frantoio', 'Koroneiki', 'Lechín', 'Lucque', 'Manzanilla', 'Picholine de Languedoc', 'Picual' and 'Verdal'). Five olive leaves samples per cultivar were randomly collected from cardinally-oriented branches with different directions around the tree's canopy. Accordingly, a total of 55 olive leaves samples were considered in this work. The leaves were dried at room temperature to constant weight during several days. Once their water content was less than 3%, samples were finely ground in a kind of coffee grinder (but controlling the temperature). Average moisture was calculated after drying different samples in a desiccation oven for 12 h at 100 °C (these tests were just valid to assess the olive leaves moisture; the extraction protocol was obviously not applied to the resulting dried olive leaves). Pre-treated samples were stored in sealed containers and kept below -20 °C in the absence of light till analyzed.

A QC sample was prepared by mixing an equivalent amount of each one of the studied samples; it was used for different purposes: to optimize the extraction procedure, to ensure the proper performance of the analytical system, and to evaluate the analytical parameters of the method.

3.2. Phenolic compounds profiling

3.2.1. Chemical and reagents

All the chemicals used in this study were of analytical grade. Water was daily deionized by using a Milli-Q system from Millipore (Bedford, MA, USA). Ethanol was supplied by J.T. Baker (Deventer, The Netherlands). Methanol and acetonitrile, both of LC-MS grade, were purchased from Prolabo (Paris, France). Acetic acid and pure standards of apigenin, apigenin-7-glucoside, hydroxytyrosol, luteolin, luteolin-7-glucoside, pinoresinol, rutin, tyrosol and vanillic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA); whereas oleuropein was purchased from Extrasynthese (Lyon, France).

A stock standard solution was prepared by dissolving the appropriate amount of each compound in methanol. Then, diluted working solutions were obtained at nine different concentrations (0.5 mg/L; 1 mg/L; 2.5 mg/L; 5 mg/L; 12.5 mg/L; 25 mg/L; 50 mg/L; 100 mg/L and 200 mg/L) and were stored at -20 °C. If any other concentration level was required for a particular sample or to establish the analytical parameters of the method, it was logically prepared.

3.2.2. Phenolic compounds extraction

Pre-treated olive leaves were taken from the freezer and sieved through a 0.5 mm metal sieve, to obtain a standard particle size. 0.1 g of each powdered sample were accurately weighed into a centrifuge tube with a screw cap, and 10 mL of ethanol-water (80:20, v/v) were added. Then, the mixture was vortexed for 45 s and sonicated for 30 min in an ultrasonic bath from J.P. Selecta (Barcelona, Spain). The resulting extract was centrifuged for 5 min at 7000 rpm, the supernatant was collected and the residue was re-extracted again following the same procedure as above. Both supernatants were pooled and evaporated to dryness under reduced pressure at 35 °C in a rotavap R-210 (Buchi Labortechnik AG, Flawil, Switzerland). Next, the residue was reconstituted with 5 mL methanol, filtered through a 0.22 µm Nylaflo™ nylon membrane filter from Pall Corporation (Ann Arbor, MI, USA) and subsequently analyzed (or stored in a freezer below -20 °C prior to analysis). Each sample was prepared in triplicate. Every sample was extracted and analyzed by LC-MS on the same day (or within 48-72 hours approx.).

3.2.3. Analytical procedure and MS conditions

For chromatographic analysis, an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA, USA) operated by Windows NT based ChemStation software and equipped with a binary solvent pump, a degasser, an autosampler, a column oven and a diode array detector (DAD) was used. Separation was performed on a Zorbax C18 analytical column (Agilent Technologies) (4.6 x 150 mm, 1.8 µm particle size) protected by a guard cartridge and maintained at 25 °C. Injection volume was set at 5 µL. Phenolic compounds elution was achieved with 0.5% acetic acid in water (Phase A) and acetonitrile (Phase B) at a flow rate of 0.8 mL/min and the following gradient program: 0 to 25 min, 5-50% B; 25 to 27 min, 50-95% B; 27 to 27.5 min, 95-100% B; finally, the B content was decreased to the initial conditions (5%) in 1 min and the column was re-equilibrated for 0.5 min prior to the next injection. Double on-line detection was carried out using a DAD (with 240 nm, 254 nm, 280 nm and 330 nm as selected wavelengths) and a mass spectrometer.

MS analyses were made using two mass spectrometers (both running in negative ionization mode). The first one, a micrOTOF-Q II™ (Bruker Daltonik, Bremen, Germany) equipped with a quadrupole-time-of-flight (Q-TOF) analyzer and an electrospray ionization interface (ESI), was used to investigate the phenolic extracts of the studied olive leaves and to identify as many compounds as possible within the profiles. For this purpose, mixtures of all the extracts coming from the same variety (prepared by mixing an equivalent volume of each one) and the QC sample were analyzed by using this platform. External MS calibration was performed using a 74900-00-05

Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA). The calibration solution (sodium formate cluster containing 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 v/v) was injected at the beginning of the run, and all the spectra were calibrated prior to compound identification. The other MS platform was a Bruker Daltonic Esquire 2000™ Ion Trap (IT) mass spectrometer (Bruker Daltonik), which was also coupled to the LC system through an ESI source. This coupling was used to carry out the quantification of the identified substances in all the samples under study.

For both MS detectors, the flow eluting from the LC column was split using a flow divisor 1:4, so that the flow rate entering into the MS detector was approximately 0.2 mL/min. The following source parameters were adopted for IT MS (and equivalent ones for Q-TOF MS: capillary voltage, 3200 V; drying gas (N_2) flow and temperature, 9 L/min and 300 °C, respectively; nebulizer pressure, 30 psi. In IT MS, Ion Charge Control (ICC) was set at 10000 and 50–1000 *m/z* was the selected scan range. Instrument control and data processing were carried out using the software Esquire Control and Data Analysis 4.0, respectively (Bruker Daltonik).

Quantitative determinations were carried out using the calibration curves obtained from commercially available pure standards. The results were expressed as mg of analyte/kg of olive leaves dry weight (DW).

3.3. Statistical analysis

All data were reported as mean ± standard deviation (n = 5, corresponding to the number of samples per studied cultivar). Comparisons between means were performed by applying One-way Analysis of Variance (ANOVA) with Tukey's *post-hoc* test, using IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL USA). The differences between studied varieties were considered significant with $p < 0.05$. Furthermore, PCA and s-LDA were performed on phenolic compounds quantitative data to assess the potential of these substances to discriminate the studied samples according to their varietal origin. Multivariate data analysis was performed with the Microsoft Office Excel 2016 software (Microsoft Office, USA) and the statistical software XLSTAT version 2015.04.1 (Addinsoft, France).

4. CONCLUSIONS

The achieved results demonstrated -in the Moroccan context- the potential of the olive leaves as an underexploited natural source of interesting substances with inherent applications in different fields; their recovery could be a valuable alternative for the sustainable and environmentally friendly management of olive leaves mills by-products.

In Morocco, olive orchards are predominantly planted by 'Picholine Marocaine' variety. In 2015 about 1.15 million tons of olive fruits were harvested; olive leaves represented on average 6% of

harvested olive fruits, which means about 27.6-34.5 mille tons of dry olive leaves. Considering our results (for the autochthonous Moroccan *cv.* in particular), they could potentially contain around 650-825 tons of oleuropein, which are actually wasted. It is time to establish an integrated approach for the sustainable extraction of high value-added molecules from olive leaves in Morocco.

Apart from the clear future practical application of this work, it is important to highlight that the comprehensive methodology used, combining LC-MS data on phenolic compounds with chemometrics, resulted to be a very effective tool for achieving an adequate discrimination among the olive leaves from different cultivars.

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Table S1. Analytical parameters of the developed LC-MS method, including calibration curves equations and r^2, LOD and LOQ, linear ranges and repeatability (expressed as %RSD).

Compound	Rt (min)	Calibration curves	r^2	LOD (µg/L)	LOQ (µg/L)	Linear range[a] (mg/L)	Repeatability[b]	
							$\%RSD_{intra\text{-}day}$	$\%RSD_{inter\text{-}day}$
Hydroxytyrosol	7.3	y = 29083x + 7653.4	0.9985	14	45	60	2.1	5.6
Tyrosol	9.4	y = 11972x - 1367.2	0.9999	42	141	70	5.1	7.1
Vanillic acid	10.8	y = 13497x - 636.8	0.9984	36	120	5	4.3	5.5
Rutin	13.2	y = 3420x - 902.06	0.9957	97	325	12.5	4.4	9.4
Luteolin-7-glucoside	13.9	y = 9917x + 6620.8	0.9951	41	136	12.5	7.5	8.6
Apigenin-7-glucoside	15.5	y = 19166x + 15207	0.9918	15	51	12.5	5.8	6.7
Oleuropein	16.7	y = 8630.3x + 509.45	0.9981	40	132	12.5	3.8	3.9
Luteolin	19.8	y = 127143x + 7126.3	0.9984	5	18	12.5	3.4	3.8
Pinoresinol	20.5	y = 48060x - 5210.3	0.9996	16	53	12.5	5.1	5.7
Apigenin	22.3	y = 190558x - 6919.3	0.9971	3	11	12.5	1.8	2.1

[a]Linear ranges were established from LOQ to the indicated value.
[b]Repeatability is expressed as %RSD of peak area for 4 injections of 4 different extracts of the QC carried out within the same sequence (*intra*-day) or over 4 days (*inter*-day).

SECTION II

"Multi-class" Methodologies

This section encompasses the development of LC-MS and GC-MS "multi-class" methodologies for the determination of minor compounds belonging to several chemical classes from olive oil or related matrices (**Chapter 7**) and their application in different metabolomic studies: varietal or geographical authentication of olive oils (**Chapters 8** and **10**), quantitative characterization of new olive fruit derived products (**Chapter 9**) and semi-quantitative study of diverse *Olea europaea* L. matrices (oils and tissues) using complementary analytical platforms (**Chapter 11**).

Chapter 7

Deep insight into the minor fraction of virgin olive oil by using LC-MS and GC-MS multi-class methodologies

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Abstract: Several analytical methods are available to evaluate virgin olive oil (VOO) minor compounds; however, multi-class methodologies are yet rarely studied. Herewith, LC-MS and GC-MS platforms were used to develop two methods capable of simultaneously determine more than 40 compounds belonging to different VOO minor chemical classes within a single run. A non-selective and highly efficient liquid-liquid extraction protocol was optimized for VOO minor components isolation. The separation and detection conditions were adjusted for determining phenolic and triterpenic compounds, free fatty acids and tocopherols by LC-MS, plus sterols and hydrocarbons by GC-MS. Chromatographic analysis times were 31 and 50 min, respectively. A comparative assessment of both methods in terms of analytical performance, easiness, cost and adequacy to the analysis of each class was carried out. The emergence of this kind of multi-class analytical methodology greatly increases throughput and reduces cost, while avoiding the complexity and redundancy of single-chemical class determinations.

Keywords: virgin olive oil; liquid chromatography; gas chromatography; mass spectrometry; multi-class methodologies; phenolic compounds; pentacyclic triterpenes; tocopherols, sterols, fatty acids.

1. INTRODUCTION

Apart from being one of the three main macronutrients (together with carbohydrates and proteins) with structural and metabolic functions, fat plays an important role in cooking, since it has different culinary uses (emulsifiers, texturizers, flavorings…) and improves food appearance and acceptability. Virgin olive oil (VOO) has been the main source of lipids in the Mediterranean diet for thousands of years. Over the last decades, its consumption has increased in popularity outside the Mediterranean basin due to its unique sensory characteristics and the health benefits associated with its intake [1].

VOO has a plethora of minor components of undeniable significance that remain in the oil due to the lack of chemical refining. This minor fraction (2–5%) includes phenolic and triterpenic compounds, tocopherols, sterols, hydrocarbons and pigments (chlorophylls and carotenoids), among others [2]. Their concentration in VOO is strongly affected by different agro-technological parameters (pedoclimatic conditions, cultivar, fruits' maturity, extraction procedures, etc.), which determine their biosynthesis and degradation [3]. Therefore, the determination of VOO minor components can be used for the assessment of VOO quality, purity, authenticity and/or typicity [4].

Over the last 20 years, extensive studies have been conducted to elucidate the causes of greater longevity and low-incidence of nutrition related-diseases in Mediterranean countries [5]. It has been shown that several health-promoting effects of VOO are linked to its fatty acids profile and minor components [1,6]. Regarding the latter ones, some reviews have compiled all the

available scientific evidences concerning the biological activities of phenolic compounds [7–10], tocopherols [11], triterpenic compounds [12,13] and phytosterols [14], predominantly. As a result, and promoted by health claims regulations, producers and consumers have shown great interest in knowing the content of the main bioactive compounds in VOO.

The determination of VOO minor compounds has undoubtedly been a real challenge in the field of food analysis over the last years. Technological advances have led to the proliferation of analytical methods based on advanced instrumental techniques capable of (qualitatively and quantitatively) characterize the formerly unknown analytes of this VOO fraction. Each family of metabolites has been traditionally studied separately because of their chemical heterogeneity (*i.e.* using specific methods for phenolic compounds, triterpenic substances, sterols, fatty acids, etc., respectively). Some spectroscopic methods of analysis can be found in literature [15–17], although the complexity of the matrix requires the use of separative techniques (such as liquid/gas chromatography (LC/GC) or capillary electrophoresis) to facilitate the subsequent determination of the individual components [18–21]. Table 1a SM (supplementary materials) provides a general overview of the available methods for the determination of the main families of VOO minor compounds; five examples per family have been selected among all the published protocols in order to show different sample treatments, separation techniques and detection systems.

In contrast to the great number of published methodologies for specific and independent determinations, robust and high-throughput multi-class methodologies capable of monitoring compounds from different chemical classes within a single run (and using just one sample treatment) are very difficult to find. Several researchers have put great efforts trying to develop multi-class methods, being aware of their potential and looking for robust, powerful and high-throughput alternatives [22,23]. Table 1b SM includes some attempts to simultaneously determine compounds belonging to different chemical classes of VOO minor fraction. It is also worth mentioning that different non-targeted metabolomic approaches have been reported too. In this regard, Purcaro *et al.* [24] showed the potential of a multidimensional GC×GC–FID/MS (flame ionization and mass spectrometry detectors) method for the generation of a VOO chemical fingerprint, including sterols, terpenic alcohols, tocopherols, fatty acids and waxes. In another interesting work, a LC-MS method was used to monitor some sterols, triterpenic compounds, tocopherols, carotenoids and fatty acid derivatives, when comparing four saponification methods for the characterization of the VOO unsaponifiable fraction [25]. Another methodology capable of determining several minor components in edible oils (involving solid phase micro extraction and GC-MS) was recently reported [26]. Although the method was not applied to VOO samples, it showed its capability to monitor sterols and derivatives, tocols, hydrocarbons, aromatic esters, lactones, monoglycerides and fatty amides in a single run without using solvents or reagents for sample preparation. ^{1}H NMR spectra obtained directly from VOO samples (suppressing the main lipid signals) can also be considered as a very useful approach to characterize several VOO minor

components (acyl groups, squalene, sterols, triterpenes, fatty alcohols, wax esters and phenols) [27].

In the current work, LC-MS and GC-MS platforms were used to develop two multi-class methodologies. As working in the context of non-targeted approaches, a non-selective and highly reproducible and effective extraction protocol was adequately optimized. The chromatographic and detection conditions were assessed for LC-MS and GC-MS to achieve a larger number of analytes within a shorter run as well as appropriate analytical performance. This was a challenging task bearing in mind the heterogeneity regarding the physicochemical properties of the analytes under study. These methods represent tangible alternatives to traditional single-class methods and definitely stand for interesting additions to the non-targeted protocols of any laboratory working in the evaluation of oil quality, purity and/or typicity.

2. MATERIALS AND METHODS

2.1. Reagents and standards

Methanol (MeOH) and acetonitrile (ACN) LC-MS grade and ethanol (EtOH) 98%, v/v were purchased from Prolabo (Paris, France). Water was daily deionized by using a Milli-Q system from Millipore (Bedford, MA, USA). Acetic acid (AcH) for acidification of mobile phases in LC, and the derivatization reagent for GC (N,O-bis(trimethylsilyl)trifluoroacetamide plus 1% of trimethylchlorosilane, (BSTFA+TMCS, 99:1, v/v)), were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Pure standards of phenolic compounds (vanillin, *p*-coumaric, quinic and ferulic acids, hydroxytyrosol, tyrosol, oleuropein, luteolin, apigenin and pinoresinol); tocopherols (α-, β-, γ- and δ-tocopherols); sterols (β-sitosterol, campesterol and stigmasterol); pentacyclic triterpenes (maslinic, betulinic and oleanolic acids; erythrodiol and uvaol); and fatty acids (palmitoleic, oleic, linoleic and linolenic acids) were all supplied by Sigma-Aldrich. Stock solutions for each analyte were prepared by dissolving the appropriate amount of each chemical standard in EtOH/H_2O (80:20, v/v) and then, they were serially diluted to working concentrations. All the samples and stock solutions were filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela Technologies (Wilmington, DE, USA) and stored at −20 °C.

2.2. Samples and sample treatment

Monovarietal olive oil samples (*cv.* Carolea, Casaliva, Cayon, Frantoio, Kalamon, Maurino, Moraiolo and Taggiasca) produced at laboratory scale at the UC Davis Olive Center (Davis, CA, USA) by means of an Abencor® laboratory oil mill (MC2 Ingeniería y Sistemas, Seville, Spain) were used in this study. A mixture of equivalent volumes of each sample (multi-varietal VOO blend) was used for sample treatment optimization and chromatographic methods development.

A sample treatment pursuing the extraction of maximum number of compounds (belonging to different chemical classes) from the VOO matrix was carried out by using LLE. A portion of 1 (± 0.01) g of VOO was weighed in a conical centrifuge tube and vortexed for 4 min with 6 mL of an EtOH/H_2O mixture. Then, the tube was centrifuged at 7500 rpm for 6 min. These steps were repeated four times, the first three stages with EtOH/H_2O (80:20, v/v) as extractant agent, and the last one with EtOH/H_2O (60:40, v/v). All supernatants were combined, the solvent was evaporated to dryness under reduced pressure at 35ºC and the obtained residue was reconstituted in 1 mL of EtOH/H_2O (80:20, v/v). During the extraction protocol optimization, different solvents, volumes and cycles number were tested as described in section 3.1. (Sample treatment optimization).

For GC analyses, aliquots of the extracts were preconcentrated and derivatized, following a protocol previously described in some of our reports [28,29]. Briefly, 200 µL of the extracts were dried under N_2 flow, redissolved with 50 µL of BSTFA+TMCS (99:1, v/v) and kept at room temperature for about 1 h to ensure the complete trimethylsilylation reaction before GC injection. The same derivatization procedure was applied to the standards solutions.

2.3. Separation and detection conditions

2.3.1. LC-MS methodology

The LC-MS analyses were performed on an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker Daltonics Esquire 2000™ ion trap (IT) mass spectrometer (Bruker Daltonik, Bremen, Germany) by an electrospray ionization (ESI) source. An Acquity UPLC™ H-Class system (Waters, Manchester, UK) coupled to a high resolution mass spectrometer (micrOTOF-QII™ (Bruker Daltonik)) by an ESI source was also used for obtaining the accurate *m/z* signals of the compounds being studied.

The separation was carried out in a Zorbax Extend C18 column (4.6 × 100 mm, 1.8 µm particle size) (Agilent Technologies), operating at 40 ºC. Analytes were eluted with acidified water (0.5% of AcH) (Phase A) and acidified ACN (0.5% of AcH) (Phase B) as mobile phases, with the following gradient: 0 to 2 min, 10%–25% B; 2 to 16 min, 25%–60% B; 16 to 18 min, 60%–80% B; 18 to 23 min, 80%–100% B (kept for 6.5 min); and finally, 29.5 to 31 min, 100%–10% B. Total run time was 31 min with a post-run time for column equilibration between each run. The flow rate was set at 1 mL/min from 0 to 23 min, increasing it to 1.5 mL/min during the isocratic part of the gradient, and setting it again to the initial value afterwards (from 29.5 to 31 min). The injection volume was 10 µL.

The MS detection conditions were selected in accordance with previous works of our research group involving the determination of analytes belonging to different chemical classes (phenolic compounds, triterpenoids, tocopherols, sterols, etc.) [29–31]. A flow divisor (1:4) was used to reduce the flow delivered into the MS and ESI parameters were accordingly chosen: nebulizer pressure was set at 30 psi, drying gas temperature at 300 ºC and drying gas flow at 9 L/min. Acquisition of the spectra in the IT MS detector were made in full scan (50-1000 *m/z*) using three different segments: 1-17 min, capillary voltage of +3200 V in negative polarity; 17-22.5 min, capillary voltage

set at +3500 V in negative ion mode; and 22.5-31 min, capillary voltage of -3500 V in positive ion mode. The skimmers, octopoles and lenses voltages were tuned considering the average mass which was set as target mass value for each segment.

Lastly, these voltages were transferred to the Q-TOF MS detector. Since switching polarity during a run is not recommended in this system, two injections per sample (one for each polarity) were needed. Data Analysis 4.0 (Bruker Daltonik) was used for LC-MS data treatment.

2.3.2. GC-MS methodology

GC-MS analyses were carried out on a Varian 450-GC coupled to a Varian 220-MS IT (Agilent Technologies) through an electron impact (EI) ion source. A 5%-phenyl-methyl polysiloxane (HP-5MS) capillary column (30 m × 0.25 mm i.d., 0.25 µm) (Agilent Technologies) was used to separate the analytes, with He as a carrier gas at a flow rate of 1 mL/min. Oven temperature was initially kept at 140 ºC for 5 min, ramped at 4 ºC/min to 310 ºC and held for 2.5 min. A sample volume of 1 µL was injected at a split ratio of 1:25. Injector and transfer line temperatures were 240 ºC and 290 ºC, respectively. Spectra were recorded in full scan (from 50 to 600 *m/z)*, with the EI source operating at a potential of 70 eV in positive ion mode, and a source temperature of 200 ºC. Instrument control and data processing for GC-MS analyses were done with MS Workstation v6.9.3 (Agilent Technologies).

2.4. Method characterization

The main analytical parameters of the developed LC-MS and GC-MS methods, were evaluated and compared in a subsequent stage of the project. Both the multi-varietal VOO blend and solutions containing standards belonging to different VOO minor chemical classes identified in the extracts were used for this purpose.

First, external calibration curves for each individual standard were established to check the linearity of the proposed methods. To that end, standard solutions at 8 concentration levels (using the appropriate ranges for each compound considering the system response and the expected concentration levels in VOO samples) were analyzed in triplicates and the resulting peak areas were plotted as a function of their concentrations, performing a linear regression by the least-squares method. The signal to noise (S/N) ratio of the analytes at the lowest concentration level was used for the theoretical estimation of detection (LOD) and quantification (LOQ) limits, which were calculated as the concentrations that generate an S/N ratio equal to 3 and 10, respectively. Afterwards, the presence/absence of matrix effect was assessed in both platforms comparing the slope of two calibration curves (the external one, prepared in EtOH/H_20 (80:20 v/v), and another one resulting from the standard addition of each compound (at 3 concentration levels) to an extract of the multi-varietal VOO blend. Finally, accuracy was evaluated in terms of precision (*intra*- and *inter*-day repeatability) and trueness. *Intra*-day repeatability was expressed as the relative standard deviation (%RSD) of peak area and retention time (Rt) of the targeted compounds for 4 injections of the standard mixture carried out within the same sequence, and *inter*-day

repeatability, as the %RSD of 4 injections (4 different sequences carried out over 4 days) of the same standard mixture. Trueness was calculated based on the difference between the concentration of each analyte in the sample extracted before and after the standard addition (at 3 concentration levels) and was expressed as the found percentage of the spiked amount.

3. RESULTS AND DISCUSSION

3.1. Sample treatment optimization

The isolation of the targeted compounds is a key step in any analytical determination, so we paid special attention to the optimization of the sample treatment in order to obtain extracts with the best achievable recoveries containing as many compounds as possible. Considering the fact that a non-targeted approach was selected, an unselective extraction protocol should be followed. Saponification, SPE and LLE were considered as possible strategies to be used in the preliminary tests, but after those assays, LLE was pointed out as the most suitable method taking into account the following aspects: saponification was a tedious (and dispensable) process, SPE led to selective extracts, and both strategies resulted to be more expensive (reagents and SPE cartridges) than LLE.

First of all, several pure solvents and mixtures of solvents -covering a broad range of polarities (some of them traditionally used for the isolation of individual families)- were tested, intending to extract as many compounds as possible. Therefore, 1 g of multi-varietal VOO blend was mixed in a vortex with 10 mL of MeOH, ACN, EtOH, ACN/EtOH (50:50, v/v), MeOH/H_2O (60:40, v/v), ACN/H_2O (60:40, v/v) and EtOH/H_2O (80:20, v/v) for 4 min; after centrifugation, evaporation of the supernatants and reconstitution in 1 mL of the proper solvent, the obtained extracts were analyzed by LC-MS. To facilitate the fair comparison among the different sample preparations, EtOH/H_2O (80:20, v/v) was selected for redissolving the dried extracts (after corroborating in the preliminary studies that it was the best possible option in terms of).

Fig. 1 SM shows the normalized areas of the peaks (grouped by chemical class) obtained after using each tested extractant (solvent or mixture of solvents). When comparing the total area for each family of compounds, the mixture of EtOH/H_2O (80:20, v/v) was noted as the best extractant agent for tocopherols, triterpenic compounds and the less polar phenolic compounds (flavonoids and lignans). This mixture was also the second best option for fatty acids extraction and gave high recovery for secoiridoid derivatives (achieving the 95% of the total area accomplished when using ACN, which was found as the optimal choice for complex phenols). With regard to simple phenols, ACN/H_2O (60:40, v/v) gave the maximum recovery. Organic solvents without water gave, in general, worse results for simple phenols and better recoveries for the less polar families.

Being EtOH/H_2O (80:20, v/v) the most promising mixture for the isolation of most families and taking into account that the mixtures organic solvent/water (60:40, v/v) gave the best recoveries

for polar phenols, a new strategy implying the use of two mixtures of diverse polarity was designed searching for a compromise solution. To that end, a first step with EtOH/H_2O (80:20, v/v) as extractant mixture was followed by a second one with a more polar combination of solvents (EtOH/H_2O (60:40, v/v)). The increase of the water percentage led to a remarkable improvement of the recovery of the most polar phenols (achieving a value very close to 75% in the normalized area axis).

After choosing the optimized combination of the extractant solvents, the potential of ultrasound-assisted extraction was tested, aiming to facilitate the removal of the targeted compounds from the VOO matrix. However, the use of UAE was discarded in the end, since it resulted in highly emulsified and hardly separable solvent-oil mixtures. As a consequence, vortex shaking was maintained for the last stage of the optimization process, where the extractant agent volume and number of iterations with each ethanolic mixture was adjusted. 4, 6, 8 and 10 mL were the evaluated volumes to be used merging cycles of EtOH/H_2O (80:20, v/v) (cycle/s a) and cycles of EtOH/H_2O (60:40, v/v) (cycle/s b). The alternate use of both solvent polarities that we tried can be summarized as follows: 1 cycle a + 1 cycle b, 2 cycles a + 1 cycle b, and 3 cycles a + 1 cycle b. As a result of this study, the protocol including 3 cycles a + 1 cycle b with 6 mL (per cycle) was selected. To estimate the yield of the optimized protocol, the samples' remnants after the 4-cycles extraction, were subjected to two further consecutive extraction stages (consisting of 1 cycle a + 1 cycle b). Table 2 SM shows the amount of each compound extracted by using the optimized protocol (recovery (%) of 4-cycles entire protocol, which is named in the table as 1st stage) and after applying the two additional stages (2nd stage and 3rd stage). The results are expressed as a percentage of the total amount extracted in all the stages. Keeping in mind the concentration ranges of the analytes in VOO, and the fitness for purpose and detection limits of each methodology, we decided that the recovery of phenolic and triterpenic compounds could be better studied using the LC-IT MS platform, whilst the recovery of fatty acids, tocopherols and sterols could be properly assessed with GC-MS. The percentage of the total amount extracted with the optimized protocol was higher than 75% for most of the compounds except for two sterols (β–sitosterol and methylencycloartanol), which exhibited a extraction yield of about 70% and are found at very high concentration levels in VOO. The repeatability of the extraction was also checked, finding %RSD values lower than 9.8 % in every case.

3.2. Chromatographic methods optimization

Due to the chemical complexity of the obtained extracts, chromatographic conditions (for both LC and GC) where optimized to cover a wide range of polarities and volatilities, respectively, and to monitor as many compounds as possible in a reasonable run time. Since commercially available standards do not include some of the most abundant phenolic compounds in olive oil, several VOO and multi-varietal VOO blend extracts were used for optimization purposes in both platforms. In LC, a linear gradient ramp from 5% to 100% ACN (and 95% to 0% of acidified water) in 60 min was firstly designed, adjusting the flow and temperature to 1 mL/min and 40 ºC,

respectively (in order to work under moderate pressure conditions). 10 min of extra time at 100% ACN (lengthening the run time over 70 min) were needed to elute α-tocopherol, which was considered as the less polar compound to be determined by using a RP-LC methodology. In order to reduce analysis time; different solvents (MeOH, 2-propanol and tetrahydrofuran) were added to the organic mobile phase to promote the elution of tocopherols. When the mixtures ACN/MeOH, ACN/2-propanol and ACN/tetrahydrofuran (80:20, v/v; same proportion in the three cases) were used as Phase B, there was a reduction in the α-tocopherol Rt of about 3%, 15% and 30%, respectively. Nevertheless, the addition of these solvents presented a negative influence in peak resolution and shape for most of the other analytes. Thus, the ACN/acidic water gradient was modified to decrease the run time. A 6 steps-gradient together with a flow gradient was designed giving rise to a 31 min total run time (flow rate was set at 1.5 mL/min when pumping 100% ACN to speed up the elution of tocopherols).

Mobile phase composition influenced the analyte's response in the MS detector, which decreased when pumping high percentages of Phase B (coinciding with tocopherols elution). MS signal reduction coexists with the fact that tocopherols are *per se* hardly ionizable analytes in ESI, since they lack strong protonation sites [32]. When testing different solvent mixtures in Phase B, the intensity of the tocopherols MS signal decreased in the following order: ACN/MeOH (80:20, v/v) > ACN/2-propanol (80:20, v/v) > ACN/tetrahydrofuran (80:20, v/v) > ACN. Therefore, achieving an enhancement of tocopherols' ionization in a mobile phase composed by 100% ACN was required. To that end, the strategy of adding an organic acid to the mobile phase, proposed by other authors [32], was tested with good results. The acidification of ACN with 0.5% of AcH produced a more efficient ionization, leading to an increment in the signals of more than a 50% when compared with the responses obtained with ACN/MeOH (80:20, v/v).

The chromatogram resulting from the final optimized conditions is shown in Fig. 2 SM (part A) together with the flow and mobile phase gradients. The visual inspection of this illustration drives us to observe that the steepest ramps in the Phase B gradient correspond to the less crowded parts of the chromatogram. In those parts, a faster elution was logically pursued. In contrast, a slower increment of Phase B percentage was needed for the appropriate separation within the crowded chromatographic area of phenolic compounds. Moreover, 6 min of isocratic pumping of Phase B at a higher flow (1.5 mL/min instead of 1 mL/min) were needed to elute the last compound of interest (α-tocopherol).

In GC, a temperature ramp from 120 ºC to 320 ºC at 3 ºC/min was initially tested. Good peak resolution was found by using these conditions, but the potential for shortening the chromatographic run was evident. After testing different possibilities, a 4 ºC/min ramp from 140 ºC to 310 ºC resulted in chromatograms with the best resolution/analysis time ratio, as shown in the part B of Fig. 2 SM. Injection volume and split ratio were two crucial parameters when looking for a compromise solution between desirable sensibility and low background noise. Injections of 0.1, 0.5 and 1 µL of sample, in both splitless and split modes (using 1:10, 1:25 1:50 and 1:75 as split

ratios) were carried out in the last stage of the optimization process. 1 µL was the optimum injection volume with a split of 1:25; this decision was made considering that the selected combination of volume and split ratio drove to appropriate S/N values for most of the compounds under study (and therefore, proper LODs), preventing at the same time column contamination. A reduction of the split ratio (1:10) caused a drastic soiling of the column after the injection of 15-20 extracts, producing a broad solvent front.

3.3. Compounds identification

Preliminary studies in LC-IT MS and GC-MS showed the presence of compounds belonging to 6 VOO minor chemical classes in the extracts. A mixture of 26 pure standards as well as 8 different monovarietal VOO extracts were analyzed under the optimal conditions. The use of pure standards was logically very useful to assign the identity of some of the analytes under study in both platforms (on the basis of their Rt and MS signals). Moreover, relative Rt and MS data, together with databases and previously published reports were thoughtfully studied in order to identify some other compounds within the detected profiles.

At this point, LC-MS analyses were carried out using a QTOF platform, which allowed the prediction of the molecular formula for the compounds under study from their exact mass. Table 1a shows Rt of the identified compounds, their high resolution MS data including experimental and theoretical (calculated by the software) *m/z* values, error (difference between both values), mSigma™ (value which indicates the similarity between the measured and the theoretical isotopic pattern of the compound) and the predicted molecular formula of the *pseudo*-molecular ion in negative or positive ion mode ($[M-H]^-$ or $[M+H]^+$, respectively) depending on the compound. Tocopherols -detected in positive mode- produced MS signals not corresponding with the expected $[M+H]^+$; this fact had been previously observed by other authors [32,33].

Table 1a. Compounds identified using LC-QTOF MS.

Rt (min)	Peak	Experimental *m/z*	Calculated *m/z*	Calculated formula	Error (ppm)	mSigma	MS Polarity	Compound
0.9	1	191.0563	191.0561	$C_7H_{11}O_6$	1.0	6.6	-	quinic acid
1.1		169.0505	169.0506	$C_8H_9O_4$	0.9	15.1	-	3,4-dihydroxyphenylglycol
1.8	2	153.0554	153.0557	$C_8H_9O_3$	2.1	4.7	-	hydroxytyrosol
2.7	3	137.0604	137.0608	$C_8H_9O_2$	3.1	11.5	-	tyrosol
3.1	4	167.0339	167.0344	$C_8H_7O_4$	3.2	9.6	-	vanillic acid
3.7	5	163.0400	163.0401	$C_9H_7O_3$	0.5	6.6	-	*p*-coumaric acid
3.9	6	151.0401	151.0401	$C_8H_7O_3$	0.1	17.3	-	vanillin
4.0	7	193.0506	193.0501	$C_{10}H_9O_4$	1.1	16.2	-	ferulic acid
4.1	8	225.0766	225.0768	$C_{11}H_{13}O_5$	1.2	4.0	-	desoxy elenolic acid
4.2	9	257.0668	257.0667	$C_{11}H_{13}O_7$	0.4	12.4	-	hydroxy elenolic acid
4.5	10	195.0665	195.0663	$C_{10}H_{11}O_4$	1.1	8.1	-	hydroxytyrosol acetate
4.7	11	381.1546	381.1555	$C_{19}H_{25}O_8$	2.4	7.4	-	hydroxytytosol acyclodihydroelenolate
4.8		539.1765	539.1770	$C_{25}H_{31}O_{13}$	1.0	19.1	-	oleuropein

Table 1a. Cont.

Rt (min)	Peak	Experimental *m/z*	Calculated *m/z*	Calculated formula	Error (ppm)	mSigma	MS Polarity	Compound
4.8	12	377.1214	377.1242	$C_{19}H_{21}O_8$	0.3	1.7	-	oleuropein aglycone I
4.9	13	241.0717	241.0718	$C_{11}H_{13}O_6$	0.1	9.4	-	elenolic acid
5.0	14	335.1132	335.1136	$C_{17}H_{19}O_7$	1.4	26.3	-	hydroxy decarboxymethyl oleuropein aglycone
5.5	15	319.1188	319.1187	$C_{17}H_{19}O_6$	0.3	6.4	-	decarboxymethyl oleuropein aglycone
5.7	16	285.0412	285.0405	$C_{15}H_9O_6$	2.7	9.1	-	luteolin
6.2	17	417.1548	417.1555	$C_{22}H_{25}O_8$	1.7	2.3	-	syringaresinol
6.5	18	377.1255	377.1242	$C_{19}H_{21}O_8$	3.6	2.7	-	oleuropein aglycone II
6.6	19	357.1346	357.1344	$C_{20}H_{21}O_6$	0.5	1.6	-	pinoresinol
6.8		393.1203	393.1191	$C_{19}H_{21}O_9$	2.9	22.2	-	hydroxy oleuropein aglycone
6.9	20	415.1406	415.1398	$C_{22}H_{23}O_8$	2	12.2	-	acetoxypinoresinol
7.1	21	269.0460	269.0455	$C_{15}H_9O_5$	1.5	23.9	-	apigenin
7.2	22	303.1239	303.1238	$C_{17}H_{19}O_5$	0.4	18.8	-	decarboxymethyl ligstroside aglycone
7.4	23	199.0614	199.0612	$C_9H_7O_3$	1.2	1.4	-	hydroxy decarboxymethyl elenolic acid
7.4	24	299.0556	299.0561	$C_{16}H_{11}O_6$	1.7	27.8	-	diosmetin
7.9	25	361.1296	361.1293	$C_{19}H_{21}O_7$	1.0	3.6	-	ligstroside aglycone I
8.3	26	361.1293	361.1293	$C_{19}H_{21}O_7$	0.0	2.4	-	ligstroside aglycone II
8.4	27	375.1094	375.1085	$C_{19}H_{19}O_8$	2.3	18.5	-	dehydro oleuropein aglycone
8.6	28	377.1251	377.1242	$C_{19}H_{21}O_8$	2.5	11.5	-	oleuropein aglycone III
9.8	29	391.1398	391.1402	$C_{20}H_{23}O_8$	1.0	30.0	-	methyl oleuropein aglycone
9.9	30	359.1118	359.1131	$C_{19}H_{19}O_7$	3.6	26.9	-	dehydro ligstroside aglycone
10.3	31	361.1294	361.1293	$C_{19}H_{21}O_7$	0.4	7.3	-	ligstroside aglycone III
11.3	32	377.1245	377.1242	$C_{19}H_{21}O_8$	0.7	1.2	-	oleuropein aglycone IV
13.3	33	361.1294	361.1293	$C_{19}H_{21}O_7$	0.2	4.8	-	ligstroside aglycone IV
18.5	34	471.3484	471.3480	$C_{30}H_{47}O_4$	1.0	2.2	-	maslinic acid
21.2	35	455.3533	455.3531	$C_{30}H_{47}O_3$	0.4	16.1	-	betulinic acid
21.3	36	277.2159	277.2167	$C_{18}H_{29}O_2$	3.1	17.7	-	linolenic acid
21.5	37	455.3537	455.3531	$C_{30}H_{47}O_3$	1.4	26.7	-	oleanolic acid
22.3	38	279.2334	279.2324	$C_{18}H_{31}O_2$	3.6	22.1	-	linoleic acid
22.9	39	443.3879	443.3889	$C_{30}H_{51}O_2$	2.3	24.4	+	erythrodiol
23.0	40	443.3876	443.3889	$C_{30}H_{51}O_2$	2.9	21.2	+	uvaol
23.0	41	281.2484	281.2486	$C_{18}H_{33}O_2$	0.7	24.6	-	oleic acid
27.2	42	415.3574	415.3571	$C_{28}H_{47}O_2$	0.8	35.3	+	β+γ-tocopherol
28.2	43	429.3740	429.3727	$C_{29}H_{49}O_2$	3.0	37.7	+	α-tocopherol

The compounds which have no number peak assigned were not found in the VOO sample chosen to illustrate Fig. 1 and Fig. 2 (SM). Table 1a only includes the compounds recurrently found in most of the oils.

9 simple phenols, 15 secoiridoids (with 4 isomers for each one of the two aglycone derivatives), 3 flavonoids, 3 lignans, 5 triterpenic compounds, 3 free fatty acids and 3 tocopherols (46 peaks in total) were identified in, at least, one of the studied VOOs; the identity of 22 of them was corroborated with their pure standards. Part A of Fig. 1 shows the LC-IT MS Extracted Ion Chromatograms (EICs) of the identified compounds in a Cayon VOO extract (same sample in Fig.

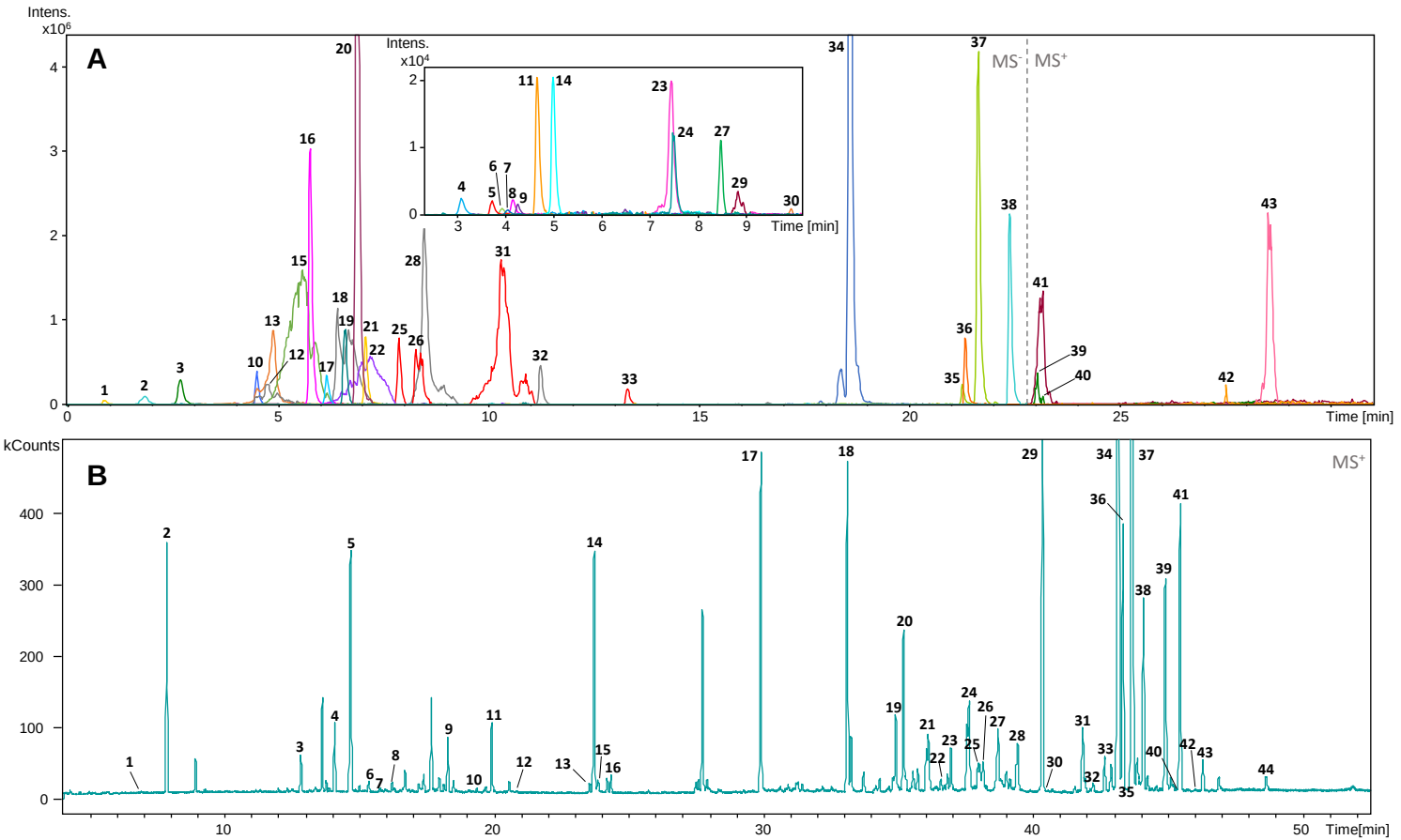

Figure 1. A) LC-MS extracted ion chromatograms (EICs) of the identified compounds in a Cayon monovarietal oil extract. Peak number identification can be found in Table 1a. A vertical line demarcates the time in which the MS system changed from negative polarity to positive mode. B) GC-MS base peak chromatogram (BPC) of the same Cayon extract. Peak identification numbers as in Table 2a.

2 SM). The positive ion mode was preferred for triterpenic alcohols and tocopherols detection. As a result, oleic acid (peak number 41 at 23.0 min) is shown in this polarity in the chromatogram; however, it was significantly better detected in negative ion mode.

Some unknown or tentatively identified compounds were also detected in the extracts by using the LC-MS optimized method; they are reported in Table 1b. For instance, *m/z* 405.1555 and 389.1696 could correspond to the dimethyl oleuropein aglycone and dimethyl ligstroside aglycone, respectively. The compounds at Rt 19.9 and 21.0 min were proposed as terpene-glucosides (arjulonic acid-glucoside and maslinic acid-glucoside, respectively). Ongoing experiments are being conducted in our lab to corroborate the identity of the compounds included in Table 1b.

Table 1b. Compounds tentatively identified using LC-QTOF MS.

Rt (min)	Experimental *m/z*	Calculated *m/z*	Calculated formula	Error (ppm)	mSigma	MS/MS fragmentation pattern	Compound	Chemical class / Other comments
12.8	405.1552	405.1555	$C_{21}H_{25}O_8$	0.7	5.2	377.1312 (100)	dimethyl oleuropein aglycone	secoiridoid
13.3/ 13.6	451.1976	451.1974	$C_{23}H_{31}O_9$	0.5	6.1	147.0435 (100); 149.0989 (96); 121.0255 (60); 119.0506 (56); 223.0601 (53)	oleuropein aglycone-related compound	secoiridoid
14.9	389.1596	389.1606	$C_{21}H_{25}O_7$	2.4	3.6	269.1049 (100); 361.1295 (12)	dimethyl ligstroside aglycone	secoiridoid
15.2	567.3179	567.3175	$C_{30}H_{47}O_{10}$	0.8	25.4	368.2435 (100); 308.2233 (32)	di-O-acetyldarutoside	terpene glycoside
15.4/ 15.7	435.2026	435.2024	$C_{23}H_{31}O_8$	0.4	21.0	315.1455 (100)	ligstroside aglycone-related compound	secoiridoid
16.2	529.2352	529.2384	$C_{36}H_{33}O_4$	6.2	20.5	469.2153 (100)	-	
17.8	547.3633	547.3640	$C_{32}H_{51}O_7$	1.3	9.3	296.0723 (100); 180.0657 (87)	methoxyl-passifloic acid	cycloartane triterpenoid
19.4	563.3226	563.3226	$C_{31}H_{47}O_9$	0.1	14.7	223.1329 (100)	-	
19.9	649.3924	649.3957	$C_{36}H_{57}O_{10}$	5.2	20.6	205.1220 (100); 306.1612 (72)	arjulonic acid-glucoside	terpene-glucoside / triterpene saponin
21.0	633.3982	633.4008	$C_{36}H_{57}O_9$	4.1	24.2	285.0478 (100); 392.0990 (83)	maslinic acid-glucoside	terpene-glucoside / triterpene saponin

MS negative polarity was used to achieve the data included in this table.
In the MS/MS fragmentation pattern-column the relative intensity of each fragment is indicated between brackets.

In GC-MS, the identification of the peaks corresponding to compounds whose standard was not commercially available was not as straightforward as in the case of LC-ESI-QTOF MS due to

two main reasons. On the one hand, most of the compounds under study were not in the GC-EI MS databases. On the other hand, as EI is a harsh ionization method (*i.e.* which produces high fragmentation in-source), the *m/z* signal of the molecular ion (or *pseudo*-molecular ion) was not found in the MS spectra of some peaks (when the molecular ion signal was found, the intensity was very low). As a consequence, in this platform, peak identification was mainly achieved bearing in mind the relative Rt of the analytes, studying the fragmentation patterns and taking into account the previously published results [18,28,34,35]. In some of the mentioned contributions, isolated pure standards were used to corroborate the identity of some of the compounds under study, what means that even if the GC-EI-MS identification was more intricate than in LC-MS, the final result was very reliable too. 47 peaks in total were identified: 9 simple phenols, 5 secoiridoids (with 3 isomers of oleuropein and ligstroside aglycones and elenolic acid), 2 flavonoids, 2 lignans, 5 triterpenic compounds, 5 free fatty acids (with two isomers of oleic acid), 4 tocopherols, 7 sterols and a hydrocarbon (squalene). Table 2a shows Rt, the most relevant MS signals found in the spectrum of each peak (the relative intensity of each one is written between brackets) and the formula assigned to the signal used for identification purposes (MS signal which is presented in bold letters).

Table 2a. Identified compounds in GC-MS

Rt (min)	Peak	MS Signals (Relative abundance)	Identifier	Compound
6.93	1	194 (100)/209 (65)/**224** (38)/45 (11)	M-H+TMS	vanillin
7.84	2	179 (100)/267 (34)/**282** (31)/180 (29)	M-2H+2TMS	tyrosol
9.26		267 (100)/73 (62)/223 (60)/**282** (5)	M-2H+2TMS	4-hydroxybenzoic acid
12.83	3	267 (100)/**370** (85)/73 (28)/193 (14)	M-3H+3TMS	hydroxytyrosol
14.07	4	280 (100)/73 (55)/193 (20)/**340** (1)	M-2H+2TMS	hydroxytyrosol acetate
14.66	5	153 (100)/299 (76)/196 (74)/**314** (2)	M-H+TMS	elenolic acid I
15.34	6	**346** (100)/256 (41)/73 (39)/419 (8)	M-5H+5TMS-2TMSO-CO	quinic acid
16.26	7	73 (100)/178 (77)/165 (59)/**314** (2)	M-H+TMS	elenolic acid II
17.20	8	294 (100)/73 (40)/**308** (38)/250 (29)	M-2H+2TMS	*p*-coumaric acid
18.29	9	73 (100)/249 (36)/193 (30)/**355** (15)	M-2H+2TMS-OCH_3	elenolic acid III
19.30	10	312 (100)/129 (66)/117 (54)/**326** (7)	M-H+TMS	palmitoleic acid
19.92	11	117 (100)/314 (86)/129 (50)/**328** (14)	M-H+TMS	palmitic acid
20.83	12	**338** (100)/324 (31)/294 (16)/73 (12)	M-2H+2TMS	ferulic acid
21.94		**396** (100)/220 (36)/73 (17)/382 (8)	M-3H+3TMS	caffeic acid
23.54	13	338 (100)/73 (60)/129 (52)/**352** (8)	M-H+TMS	linoleic acid
23.76	14	**354** (100)/117 (79)/129 (76)/340 (22)	M-H+TMS	oleic acid I
23.87	15	340 (100)/129 (71)/117 (64)/**354** (11)	M-H+TMS	oleic acid II
24.34	16	342 (100)/117 (78)/129 (44)/**356** (13)	M-H+TMS	stearic acid
29.91	17	192 (100)/177 (6)/73 (4)/**361** (1)	M-H+TMS-CH_3	decarboxymethyl ligstroside aglycone
33.12	18	280 (100)/73 (14)/193 (8)/**464** (1)	M-2H+2TMS	decarboxymethyl oleuropein aglycone
34.89	19	**192** (100)/73 (11)/280 (11)/**177** (8)	*	ligstroside aglycone I
35.17	20	69 (100)/81 (85)/41 (66)/95 (32)	NIST Ref. spectrum	squalene
36.15	21	**192** (100)/73 (19)/**177** (10)/297 (8)	*	ligstroside aglycone II

Table 2a. Cont.

Rt (min)	Peak	MS Signals (Relative abundance)	Identifier	Compound
36.56	22	**475** (100)/209 (6)/73 (6)/249 (3)	M-H+TMS	δ-tocopherol
36.98	23	193 (100)/73 (22)/**417** (20)/177 (10)	M-2H+2TMS-TMSO	ligstroside aglycone III
37.62	24	280 (100)/73 (16)/193 (8)/**522** (3)	M-2H+2TMS	oleuropein aglycone I
37.95	25	**489** (100)/223 (10)/73 (6)/41 (3)	M-H+TMS	β-tocopherol
38.13	26	**489** (100)/223 (13)/73 (6)/43 (3)	M-H+TMS	γ-tocopherol
38.67	27	280 (100)/73 (18)/45 (4)/**505** (4)	M-3H+3TMS-TMSO	oleuropein aglycone II
39.46	28	280 (100)/73 (14)/193 (6)/**594** (1)	M-3H+3TMS	oleuropein aglycone III
40.33	29	**503** (100)/238 (10)/73 (9)/43 (4)	M-H+TMS	α-tocopherol
40.35	30	472 (100)/45 (4)/399 (3)/**486** (2)	M-3H+3TMS	apigenin
41.82	31	503 (100)/**472** (70)/73 (68)/383 (55)	M-H+TMS	campesterol
42.21	32	395 (100)/**485** (74)/83 (63)/256 (62)	M-H+TMS	stigmasterol
42.61	33	560 (100)/45 (3)/472 (3)/**574** (1)	M-4H+4TMS	luteolin
43.16	34	397 (100)/358 (41)/**486** (40)/381 (28)	M-H+TMS	β-sitosterol
43.20	35	**502** (100)/223 (69)/235 (33)/488 (24)	M-2H+2TMS	pinoresinol
43.34	36	386 (100)/297 (75)/282 (49)/**484**(10)	M-H+TMS	Δ^5-avenasterol
43.67	37	276 (100)/246 (40)/546 (14)/**560** (2)	M-2H+2TMS	acetoxy pinoresinol
44.07	38	393 (100)/366 (53)/**408** (37)/69 (28)	M-H+TMS-TMSOH	cycloartenol
44.89	39	408 (100)/380 (96)/422 (45)/**512** (1)	M-H+TMS	methylencycloartanol
45.41	40	**497** (100)/216 (68)/73 (38)/203 (25)	M-2H+2TMS-TMSO	eythrodiol
45.44	41	358 (100)/400 (33)//268 (17)/**394** (8)	M-H+TMS-TMSOH-CH_2	citrostadienol
45.96	42	**497** (100)/73 (38)/216 (32)/203 (23)	M-2H+2TMS-TMSO	uvaol
46.29	43	203 (100)/**483** (53)/73 (43)/320 (40)	M-2H+2TMS-TMSO-CO	oleanolic acid
46.58		189 (100)/73 (90)/203 (40)/**483** (21)	M-2H+2TMS-TMSO-CO	betulinic acid
48.63	44	203 (100)/73 (62)/**571** (50)/320 (42)	M-3H+3TMS-TMSO-CO	maslinic acid

*Fragments previously reported in literature

The compounds which have no number peak assigned were not found in the VOO sample chosen to illustrate Fig. 1 and Fig. 2 (SM).

As shown in the table, most of the substances under study showed a MS signal (with low intensity in some cases) which corresponded to the completely trimethylsilylated molecule (total substitution of active hydrogen by TMS groups). Nevertheless, some exceptions to that rule can be listed. For example, in the case of quinic acid (Rt of 15.34 min), the *pseudo*-molecular ion of the complete silylated structure (with 5 positions to be replaced by TMS groups, giving a *m/z* signal of 552 (M-5H+5TMS)) was not observed. The main MS signal was 346, which corresponds to the loss of TMSO (*m/z* 89) together with the loss of TMSO-CO (*m/z* 117). The first loss is characteristic of hydroxyl group and the second is typical of the carboxylic group. The absence of the MS signal corresponding to the completely trimethylsilylated molecule was also observed for the third isomer of elenolic acid (Rt of 18.29). This substance generates a MS signal (with very low intensity, but perfectly observable) which can be assigned to M-2H+2TMS-OCH_3 (*m/z* 355) as previously reported [28]. As far as decarboxymethyl ligstroside aglycone (Rt of 29.91 min) is concerned, it gave the characteristic signal of ligstroside aglycone derivatives (*m/z* 192), resulting from the McLafferty rearrangement [35], apart from a signal of very low intensity with *m/z* 361, which was

assigned to M-H+TMS-CH_3. Ligstroside aglycone presented different isomers (Rt of 34.89, 36.15 and 36.98 min). None of them exhibited as main MS signal the complete silylated molecule, being the fragment 192 the most intense one in every case. Moreover, the *m/z* signal 177 was detected for the three isomers (as reported by other authors [28,34]); additionally, for the third one, we found *m/z* 417 corresponding to M-2H+2TMS-TMSO. Concerning the three oleuropein aglycone isomers, all of them presented the *m/z* 280 as major feature in their spectra. This fragment is the main product of the above mentioned McLafferty rearrangement of secoiridoid aglycons containing a hydroxytyrosol moiety in their structures. The 2nd isomer (Rt of 38.67) did not show either the *m/z* signals 522 or 594 (molecule with 2 or 3 hydrogens substituted by TMS groups) but the loss of TMSO from the complete silylated chemical entity (*m/z* 505). Squalene is suitable for being analyzed by GC without the need of derivatization (indeed, it is a dehydrotriterpenic hydrocarbon which has no active hydrogens to be replaced by TMS groups); its identification was done by comparison with its reference NIST spectrum. It is worth noting that all fatty acids, apart from the trimethylsilylated molecule, showed the cluster series with consecutive losses of CH_2 (14 *m/z)* [36]. Sterols exhibited some peculiarities too: cycloartenol did not present the *pseudo*-molecular ion but the loss of the trimethylsilanol group (90 *m/z)*, which is common to most sterols. Methylencycloartanol showed the silylated *pseudo*-molecular ion (with low intensity), but also the M-H+TMS-TMSOH (*m/z* 422) and the further loss of CH_2 (*m/z* 408). Citrostadienol´s spectrum had *m/z* 358 and 400 as distinctive signals, which have been also reported by other authors [37]. The *m/z* signal 394 was also detectable in its spectrum, corresponding to M-H+TMS-TMSOH-CH_2. Triterpenic dialcohols instead of producing the MS signal corresponding to the totally trimethylsilylated molecule had a predominant fragment (*m/z* 497) coming from the loss of one TMSO. All pentacyclic triterpenoids that contain a C-12–C-13 double bond undergo a retro-Diels-Alder cleavage of the C-ring into the EI source, leading to dienophile and diene fragments [38]. Accordingly, fragmentation of trimethylsilylated oleanolic acid, for instance, led to an ion of *m/z* 320 (with relatively high intensity in the spectra). This ion underwent a subsequent fragmentation, losing its TMSO-CO, and leading to a signal of *m/z* 203 [39]. The double silylated $C_{30}H_{48}O_3$ mass isomers also suffered a loss of 117 Da, which, as stated above, is characteristic of the carboxylic groups (*m/z* 483). Betulinic acid was the only triterpenoid which showed the *m/z* 189 in MS (indeed, it was the major feature in its spectrum); that is a fragmentation pattern typical of a saturated lupane skeleton, involving ring C cleavage [39]. Maslinic acid had a MS spectrum defined by *m/z* signals at 203, 73, 571 and 320 (in decreasing order of intensity). *m/z* 571 could be assigned to the completed silylated molecule after losing TMSO-CO.

Table 2b shows the *m/z* signals of the major unknown peaks detected in GC-MS. Two ligstroside derivatives were detected with Rt of 16.68 and 17.66 min, respectively. Moreover, the MS signals detected at 27.72 min were assigned to *cis*-vaccenic acid, which is a positional isomer of oleic acid. Apart from them, hydroxy decarboxymethyl ligstroside aglycone appeared in the profile (Rt 33.23 min) and one oleuropein derivative was detected at 33.69 min. Some other

unknown peaks were detected with a considerable intensity; however, with the EI-MS signals, it was not possible to find a plausible identity for them.

Table 2b. Major unknown peaks in GC-MS

Rt (min)	MS Signals (Relative abundance)	Compound
8.91	192 (100)/178 (25)/43 (12)/73 (10)/117 (3)/151 (3)/237 (1)/297 (1)	-
13.60	153 (100)/225 (91)/196 (62)/167(50)/239(45)/135(42)/163 (40)/270 (5)	-
16.68	73 (100)/192 (62)/45(41)/267 (39)/297 (32)/165 (31)/267 (30)/327 (30)	ligstroside aglycone-related compound
17.66	73 (100)/192 (56)/165 (38)/297 (37)/267 (35)/253 (27)/119 (26)/311 (25)	ligstroside aglycone-related compound
27.72	338 (100)/128 (78)/116 (75)/75 (74)/131 (66)/144 (59)/198 (46)/354 (30)	*cis*-vaccenic acid
33.23	192 (100)/73(12)/177 (8)/280 (5)/45 (3)/299 (1)/151 (1)/255 (1)/359 (1)	hydroxy decarboxymethyl ligstroside aglycone
33.69	73 (100)/280 (76)/129 (57)/147 (32)/103 (28)/257 (23)/203 (23)/339 (22)	oleuropein aglycone-related compound
46.86	563 (100)/147 (36)/240 (15)/73 (14)/253 (11)/266 (8)/225 (8)/45 (7)	-

3.4. Methods characterization and comparison

The performance of both LC-MS and GC-MS methods was compared considering some illustrative analytical parameters. The main results of the characterization study are shown in Table 3; the pure standards of some relevant compounds (which belong to different chemical categories and can be easily found in VOO extracts) were considered.

Good linearity was achieved for all the calibration curves within the working concentration ranges, with correlation coefficients (r^2) higher than 0.9927 (for maslinic acid) in LC-MS and 0.9926 (for β-tocopherol) in GC-MS, respectively. LOD and LOQ in LC-MS were lower than those achieved by GC-MS in every case. Regarding repeatability, Rt %RSD was lower than 2.7 and 3.1% for *intra*- and *inter*-day, respectively, in LC-MS, and lower than 0.03% and 0.05% for *intra*- and *inter*-day, respectively, in GC-MS (data not included to contain the size of the table). These values were logically higher for peak area repeatability (they can be seen in the table), but not exceeding 12% in any case. Trueness, expressed as recovery (%), presented values within the range from 75.1 to 113.4% in LC-MS, and between 81.0 and 108.3% in the case of GC-MS. Matrix effect was also evaluated in both platforms, calculating the corresponding coefficients. Most of them were between −20% to +20%, considered as the range in which there is a mild signal suppression or enhancement effect. Only two analytes in LC-MS (pinoresinol and uvaol) and luteolin in GC-MS showed a slightly more significant matrix effect.

To go even further into the comparison, we decided to consider some other aspects apart from those clearly stated in Table 3, such as analysis time, number of determined compounds, easiness, relative cost and facilities and reagents needed in the lab. We have also tried to point out the chemical classes which are better covered by LC-MS or GC-MS, respectively. Table 4 shows

Table 3. Analytical parameters of representative analytes belonging to different VOO families that can be determined by both LC-MS and GC-MS methodologies.

Compound	LC-MS						GC-MS					
	LOD (µg/L)	LOQ (µg/L)	Matrix Effect Coef. (%)[a]	Accuracy			LOD (µg/L)	LOQ (µg/L)	Matrix Effect Coef. (%)[a]	Accuracy		
				Repeatability (area %RSD)[b]		Trueness[c]				Repeatability (area %RSD)[b]		Trueness[c]
				Intra-day	*Inter-day*					*Intra-day*	*Inter-day*	
quinic acid	22	75	4.3	4.3	5.7	99.2	1000	3333	-2.7	1.1	5.2	98.2
hydroxytyrosol	40	133	-0.9	3.1	4.8	84.7	167	556	3.5	1.9	8.7	81.0
tyrosol	47	156	-3.8	5.3	5.8	100.6	167	556	6.1	0.7	5.2	99.0
***p*-coumaric acid**	32	106	-1.9	5.5	6.9	80.6	446	1486	-2.1	0.9	5.9	99.0
vanillin	6	21	-7.4	6.3	7.6	107.9	94	313	-2.7	1.8	5.9	105.7
ferulic acid	36	118	-7.6	5.5	8.8	96.7	120	400	0.5	1.0	7.3	102.5
luteolin	1	3	2.9	1.8	3.8	84.4	323	1076	21.3	1.2	11.9	98.4
apigenin	2	7	4.9	3.0	5.3	86.7	443	1477	-11.3	3.8	8.8	99.5
pinoresinol	4	14	24.7	1.8	9.8	89.5	255	850	-0.3	6.6	8.0	111.0
maslinic acid	2	8	-3.5	5.9	7.5	100.6	1731	5769	-11.0	2.6	8.5	102.7
betulinic acid	2	6	0.9	4.3	8.7	91.2	833	2778	-14.2	1.8	7.9	108.3
oleanolic acid	2	7	3.4	3.3	4.3	83.5	1667	5556	-2.4	5.7	7.4	104.1
erythrodiol	109	362	2.3	8.4	10.6	75.1	594	1980	4.8	3.2	5.6	101.0
uvaol	113	377	23.3	9.7	11.1	107.8	750	2500	7.8	2.4	7.4	93.7
linoleic acid	1	3	15.3	3.2	4.7	113.4	195	650	-0.9	2.4	6.8	96.4
***α*-tocopherol**	27	90	4.6	0.9	3.4	101.8	54	179	-4.5	1.7	7.1	103.1
***β*-tocopherol**	87	289	9.5	0.7	8.1	110.4	100	333	5.9	1.3	5.2	96.4
***γ*-tocopherol**							56	185	4.9	0.6	8.9	91.1

[a]Matrix effect coefficient (%)=(1−(slope matrix/slope solvent))×100.

[b]Repeatability is expressed as the %RSD of peak area for 4 injections (of the standard mixture at an intermediate concentration level) carried out within the same sequence (*intra*-day) or for 4 injections from different sequences carried out over 4 days (*inter*-day).

[c]Trueness is expressed as recovery (%), which was estimated by analyzing the multi-varietal VOO blend extracted before and after the standard addition and calculating the difference between the obtained results afterwards. The values included in this table are those achieved for the intermediate concentration level.

this critical comparison in view of different aspects other than those strictly related to the analytical performance of the methods. In an attempt to summarize the info from the table in few sentences, it is possible to say that the LC-MS methodology is more convenient in terms of analysis time, sensitivity, and simplicity for identifying the analytes under study. GC-MS requires cheaper instrumentation and allows the determination of sterols and squalene; but its main drawbacks are the necessity of derivatization and the intricacy of identification. Considering the number of compounds covered by each method, the two options were quite similar, although GC-MS fit better for fatty acids, hydrocarbon, tocopherols, sterols and triterpenic dialcohols, whereas LC-MS was more suitable for phenolic compounds and triterpenic acids.

Table 4. Comparison between the two developed methodologies taking into account different aspects other than those strictly analytical.

	LC-MS	GC-MS
Relative cost. Facilites, materials and reagents needed	· LC-MS instrumentation more expensive than GC-MS · Higher cost in terms of mobile phases	· Need of derivation reagents
Easiness	· No need of any derivatization reaction · More straightforward identification	· Limited stability of extracts after being derivatized · Difficult identification of analytes when they are not available as pure standards or present in commercial databases · Harsh ionization sources in most of commercial equipments (pseudo-molecular ions not detected sometimes)
Analysis time	31 min	50 min
Analytical performance	· Better LODs · 2 analytes showing a slight matrix effect	· More robust methodology · 1 analyte showing a slight matrix effect
Number of compounds	46 peaks in total: *9 simple phenols, 15 secoiridoids (with 4 isomers for each one of the two aglycone derivatives), 3 flavonoids, 3 lignans, 5 triterpenic compounds, 3 free fatty acids and 3 tocopherols (two coeluting)*	47 peaks in total: *9 simple phenols, 5 secoiridoids (with 3 isomers of elenolic acid and the aglycones), 2 flavonoids, 2 lignans, 5 triterpenic compounds, 5 free fatty acids (with two isomers of oleic acid), 4 tocopherols, 7 sterols and 1 hydrocarbon*
Chemical classes more suitable to be determined	· Simple phenols · Secoiridoids · Flavonoids and lignans · Triterpenic acids	· Fatty acids · Hydrocarbon · Tocopherols · Sterols · Triterpenic alcohols

4. CONCLUSIONS

Two multi-class methodologies -LC-MS and GC-MS- were developed in an attempt to simultaneously determine relevant minor components of VOO (different subclasses of phenolic compounds, triterpenoids, free fatty acids, tocopherols, sterols and one hydrocarbon) within a single run.

Few previous reports have dealt with the development of multi-class methods with application in the field of olive oil, but to the best of our knowledge, the methodologies presented herewith cover a significant number of analytes. These methodologies could represent a good chance to evaluate (including but not limited to): the effect of technological parameters on the final composition of olive oil minor fraction; the typicity and genuineness of different olive oil samples; the potential healthful properties of an oil; and the profiling of olive oil-related matrices to get a comprehensive characterization of their minor components.

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Table 1a SM. Main characteristics of diverse methodologies, selected as example, for the specific determination of different minor VOO chemical classes: Single-class methodologies. (The official method, when available, is described in the shaded row).

Sample treatment	Separation conditions	Detection conditions	Number of Analytes	Ref.
	Phenolic compounds			
UAE (15 min), 2 g sample + 5 mL MeOH/H_2O (80:20, v/v)	**RP-LC**, C18 (250 × 4.6 mm, 5 µm) Gradient: H_2O (0.2% H_3PO_4)/MeOH/ACN (82 min)	**UV** 280 nm	27 (total referred to TY)	[1]
LLE, 2g sample + 6 mL MeOH/H_2O (60:40, v/v) → 1 mL MeOH	**RP-LC**, C18 (150 × 4.6 mm, 1.8 µm) Gradient: H_2O (0.5% AcH)/ACN (25 min)	**MS** (ESI-IT/TOF) Negative polarity	20	[2]
LLE, 1 g sample + 2 mL MeOH → 850 µL MeOH/H_2O (80:20, v/v)	**RP-LC**, C18 (100 × 2.1 mm, 1.8 µm) Gradient: H2O (0.1% CO_2H_2)/ACN (0.1% CO_2H_2) (30 min)	**MS** (ESI-QTOF) Negative polarity	34 (no quantification)	[3]
SPE (diol-bonded phase cartdridge), 2.5 g sample (10 mL MeOH) → 100 µL derivatization reagent	**GC**, ZB-5MS (30 m × 0.25 mm, 0.25 µm) T: 150-295 ºC (71 min)	**MS** (EI-IT) Positive polarity	21 (no quantification)	[4]
SPE (diol-bonded phase cartdridge), 60 g sample (40 mL MeOH) → 2 mL MeOH/H_2O (50:50, v/v)	**CE**, Fused silica capillary (400 × 0.375 mm, 50 µm) Running buffer: (45 mM sodium tetraborate, pH 9.3) (7 min)	**DAD** 200, 240, 280, 340 nm	16	[5]
	Triterpenic compounds			
Saponification, **TLC**, 5 g sample → 50 µL silylation reagent/mg analyte	**GC**, SE-52 or SE-54 (20-30 m × 0.25-0.32 mm, 0.10-0.30 µm) T: 260 ºC (30-60 min)	**FID**	2 (+ 15 sterols)	[6]
SPE (bonded aminopropyl phase cartdridge), 0.2 g sample (6 mL diethyl ether/acetic acid (98:2, v/v)) → 200 µL silylation reagent	**GC**, SGL-5 (25 m × 0.25 mm, 0.25 µm) T: 260-320 ºC (34 min)	**FID**	3	[7]
SPE (bonded aminopropyl phase cartdridge), 0.2 g sample (6 mL diethyl ether/acetic acid (98:2, v/v)) → 200 µL silylation reagent	**GC**, Rtx-65TG (30 m × 0.25 mm, 0.10 µm) T: 260-320 ºC	**MS** (EI-IT MS) Positive polarity	1	[8]
LLE, 0.8 g sample (9.6 mL MeOH/EtOH (50:50, v/v)) → 2.4 mL MeOH	**RP-LC**, C18 (250 × 4.6 mm, 5 µm) Isocratic: 8% H_2O (H_3PO_4)/92% MeOH (20 min)	**UV** 210 nm	2 (+ 2 fatty acids)	[9]
UAE, 0.2 g sample (10 mL MeOH) → 1 mL MeOH	**RP-LC**, C18 (100 × 4.6 mm, 1.8 µm) Isocratic: 10% ammonium buffer (pH 9.6)/54% ACN/36% MeOH (13 min)	**MS** (ESI-IT) Negative/positive polarities	6	[10]
	Tocopherols			
Dilution, 2 g sample + 25 mL hexane	**NP-LC**, Si60 (250 × 4.0 mm, 5 µm) Isocratic: 0.5% 2-propanol/99.5% hexane (30 min)	**FLD** λ_{ex} 290 nm, λ_{em} 330 nm **UV** 290 nm	4	[11]
Dilution, 2.5 g sample + 5 mL hexane	**NP-LC**, Si60 (250 × 4.6 mm, 5 µm) Isocratic: 1% 2-propanol/98.5% hexane/0.5% EtOH	**FLD** λ_{ex} 290 nm, λ_{em} 330 nm	4	[12]
LLE, 4 g sample + 20 mL MeOH + 10 mL MeOH/2-propanol (80:20 (v/v)) → 1 mL MeOH	**Nano-RP-LC**, Monolithic C18 (250 × 0.1 mm) Isocratic: 75% ACN/8% MeOH/17% H_2O (0.2% AcH) (20 min)	**UV** 295 nm	4	[13]
Dilution, (1:10, v/v) with isopropanol	**RP-LC**, C18 (150 × 2.1 mm, 5 µm) Isocratic: 50% MeOH/50% ACN (12 min)	**FLD** λ_{ex} 290 nm, λ_{em} 330 nm	4	[14]
Saponification, 0.025 g sample → 1 mL MeOH	**RP-LC**, FPF (200 × 4.6 mm, 5 µm) Isocratic: 95% MeOH/5% H_2O (15 min)	**MS** (APCI/ESI-Q) Negative polarity	4	[15]

		Sterols		
Saponification, **TLC**, 5 g sample → 50 µL silylation reagent/mg analyte	**GC,** SE-52 or SE-54 (20-30 m × 0.25-0.32 mm, 0.10-0.30 µm) T: 260 ºC (30-60 min)	**FID**	15 (+ 2 triterpenic alcohols)	[6]
Saponification, **solid supported liquid extraction**, **SPE** (base-activated silica cartdridge), 0.2 g sample (60 mL diethyl ether), (10 mL hexane/diethyl ether (60:40, v/v)) → 250 µL silylation reagent	**GC**, HP-5MS (30 m × 0.25 mm, 0.25 µm) Isothermal T: 260 ºC (55 min)	**FID**	15 (+ 2 triterpenic alcohols)	[16]
Saponification, **TLC**, 5 g sample → 50 µL silylation reagent/mg analyte	**RP-LC**, C18 (150 × 2.1 mm, 5 µm) Gradient: H_2O (0.01% AcH)/ACN (20 min)	**MS** (APCI-Q) Positive polarity	5 (+ 2 triterpenic alcohols)	[17]
Saponification, **SPE** (silica-based octadecyl bonded phase) 1 g sample (15 mL 5% MeOH in chloroform (v/v) → 1 mL MeOH (dilution 1:10, v/v)	**Nano-RP-LC**, C18 (150 × 0.1 mm, sub-2 µm) Isocratic: MeOH (22 min)	**DAD** 195 nm	5	[18]
Saponification, **TLC**, 5 g sample → 50 µL silylation reagent/mg analyte	**CEC**, Methacrylate ester-based monolithic column (8.5 cm) Mobile phase: 85% ACN/10% 2-propanol/5% aqueous Tris buffer (5 mM, pH 8.0) (8 min)	**DAD** 210 nm	5	[19]
		Fatty acids		
***Trans*-esterification**, 0.1 g → 2 mL heptane	GC, Polar polysiloxane column (60 m × 0.20-0.32 mm, 0.10-0.20 µm) T: 165-210 ºC (37 min)	**FID**	23	[6]
Saponification, **trans-esterification**, 0.1 g → H_2SO_4 0.5 M in MeOH	GC, Supelcowax 10 (30 m × 0.25 mm, 0.25 µm) T: 180- 240 ºC (15 min)	**FID**	6	[20]
***Trans*-esterification**, 0.1 g → 2 mL hexane (dilution 1:50, v/v)	GC, SPTM-2380 (60 m × 0.25 mm, 0.20 µm) T: 120-250 ºC (36 min)	**FID**	10	[21]
***Trans*-esterification**, 0.1 g → 5 mL hexane	GC, DB-1 (30 m × 0.25 mm × 0.10 µm) T: 90-250 ºC (9 min)	**MS** (EI-Q) Positive polarity	18	[22]
Dilution, (1:20, v/v) with isopropanol	RP-LC, C18 (50 × 2.1 mm, 1.9 µm) Gradient: ACN/H_2O (8 min)	**MS** (ESI-QqQ) Negative polarity	12	[23]

Table 1b SM. Main characteristics of different methodologies for simultaneous determination of two (or more) minor VOO chemical classes: Multi-class approaches.

Sample treatment	Separation conditions	Detection conditions	Number of Analytes	Ref
LLE, 10 g sample + 50 mL MeOH + 25 mL MeOH/2-propanol (80:20 (v/v)) → 5 mL MeOH/2-propanol/hexane (1:3:1, v/v/v)	**RP-LC**, C18 (250 × 4 mm, 5 µm) Gradient: H_2O (2% AcH)/MeOH/ACN/2-propanol (70 min)	**DAD** 280 nm	10 phenolic compounds, 2 tocopherols	[24]
LLE, 1 g sample + 15 mL MeOH → 1 mL MeOH	**GC**, HP-5 MS (30 m × 0.25 mm, 0.25 µm) T: 70-300 ºC (60.5 min)	**MS** (EI-Q) Positive polarity	13 phenolic compounds, 3 triterpenic compounds	[25]
Saponification, 5 g sample → 0.5 mL ACN	**RP-LC,** C18 (250 × 3 mm, 5 µm) Gradient: H_2O (0.01% AcH)/ACN (65 min)	**MS** (APCI-IT) Positive polarity	9 sterols, 3 tocopherols, 2 triterpenic dialcohols	[26]
Saponification, 1 g sample → 130 µL silylation reagent/mg analyte	**GC**, SPB-5 (30 m × 0.53 mm, 0.5 µm) T: 180-270 ºC (76 min)	**FID**	4 sterols, α-tocopherol, squalene, 7 aliphatic alcohols	[27]
Dilution, 0.5 g sample → 10 mL 2-propanol/ hexane (1.5:98.5, v/v)	**NP-LC**, Si60 (250 × 4.6 mm, 5 µm) Isocratic: 1.5% 2-propanol/98.5% hexane (30 min)	**DAD** 409, 430, 433, 452 nm **FLD** λ_{ex} 295 nm, λ_{em} 330 nm	3 tocopherols, 3 tocotrienols, 2 chlorophylls, 2 pheophytins, *β*-carotene	[28]

The symbol "→" means evaporation and reconstitution.
For solid supported liquid extraction and SPE, only the extraction or elution solvent (not considering cleaning steps) is indicated between brackets.

Abbreviations (used in Tables 1a and b SM)

methanol (MeOH), ethanol (EtOH), liquid-liquid extraction (LLE), solid phase extraction (SPE), ultrasounds assisted extraction (UAE), liquid/gas chromatography (LC/GC), capillary electrophoresis (CE), diode array detector (DAD), fluorescence detector (FLD), flame ionization detector (FID), mass spectrometry (MS), electron impact (EI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), ion trap (IT), time of light (TOF), quadrupole (Q), triple quadrupole (QqQ).

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Table 2 SM. Evaluation of the performance (efficiency) of the extraction protocol considering the percentage of different target compounds obtained in each successive extraction stage as well as the extraction repeatability.

Chemical class	Compound	1st%	2nd%	3rd%	Repeatability (%RSD)	
Simple phenols	quinic acid	99.5	0.5	-	**9.7**	LC-MS
	hydroxytyrosol	75.6	24.1	0.3	**3.6**	
	tyrosol	93.6	6.4	-	**6.4**	
	p-coumaric acid	100.0	-	-	**1.1**	
	vanillin	100.0	-	-	**0.7**	
	ferulic acid	100.0	-	-	**8.8**	
Secoiridoids	desoxy elenolic acid	99.9	0.1	-	**0.6**	
	elenolic acid	93.7	5.2	1.1	**3.3**	
	decarboxymethyl oleuropein aglycone	76.8	14.8	8.4	**7.8**	
	oleuropein aglycone	84.7	11.9	3.4	**7.9**	
	decarboxymethyl ligstroside aglycone	77.9	11.2	10.9	**3.0**	
	ligstroside aglycone	89.7	5.5	4.7	**9.8**	
Flavonoids	luteolin	99.8	0.2	-	**0.9**	
	apigenin	99.2	0.8	-	**6.5**	
Lignans	pinoresinol	98.7	1.3	-	**5.0**	
	acetoxy pinoresinol	97.9	2.1	-	**5.4**	
Triterpenic compounds	maslinic acid	96.4	2.8	0.7	**2.7**	
	betulinic acid	99.8	0.2	-	**4.3**	
	oleanolic acid	95.7	2.4	2.0	**7.4**	
	erythrodiol	75.5	24.5	-	**8.0**	
Fatty Acids	linolenic acid	97.5	2.3	0.2	**2.8**	GC-MS
	linoleic acid	98.4	1.5	0.2	**3.5**	
	oleic acid	94.5	5.0	0.5	**5.9**	
	palmitic acid	95.2	3.7	1.1	**1.5**	
	palmitoleic acid	98.9	1.1	0.0	**1.7**	
	stearic acid	88.3	7.5	4.1	**1.5**	
Tocopherols	α-tocopherol	78.1	15.9	5.9	**1.3**	
	β-tocopherol	86.6	10.3	3.0	**2.5**	
	γ-tocopherol	82.9	13.1	4.0	**0.1**	
	δ-tocopherol	90.5	7.3	2.2	**4.0**	
Sterols	β-sitosterol	70.6	22.4	7.0	**4.4**	
	campesterol	78.6	17.2	4.2	**5.4**	
	stigmasterol	80.7	15.4	3.9	**0.7**	
	Δ^5-avenasterol	76.1	18.6	5.2	**7.2**	
	cycloartenol	75.3	19.2	5.6	**0.5**	
	methylencycloartanol	70.6	22.7	6.6	**5.0**	

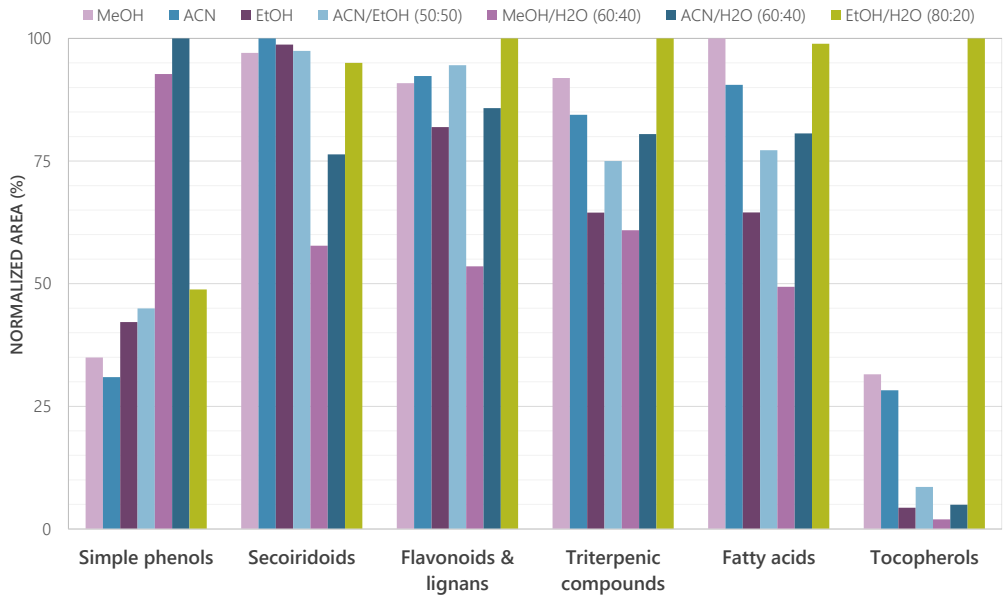

Figure 1 SM. Total (normalized) peak areas of the different VOO minor chemical classes (simple phenols, secoiridoids, flavonoids and lignans, triterpenic compounds, fatty acids, and tocopherols) found in the extracts obtained with the seven extractant agents selected in the first stage of the sample treatment optimization (MeOH, ACN, EtOH, ACN/EtOH (50:50, v/v), MeOH/H2O (60:40, v/v), ACN/H2O (60:40, v/v) and EtOH/H2O (80:20, v/v)). Analyses were made in the LC-IT MS system. Normalization was made considering as 100% the total area value of the most effective extractant agent (for each chemical class) and referring the rest to that value.

The compounds considered as members of each chemical class are those included in Table 2 SM.

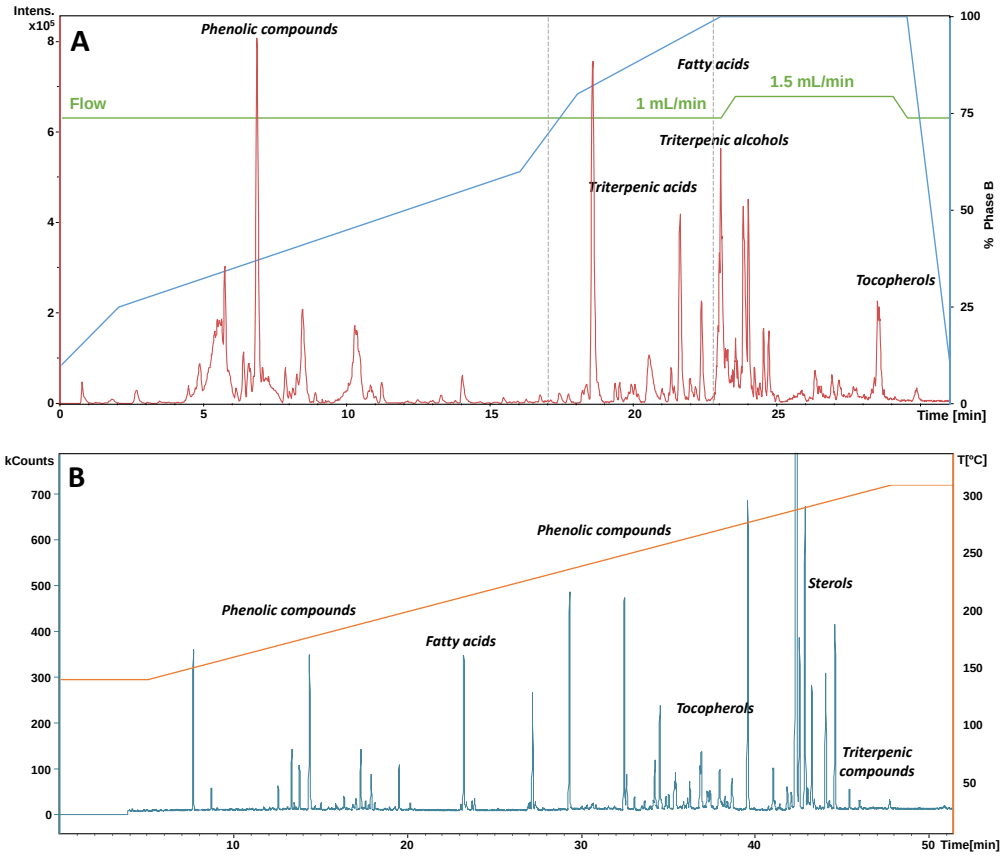

Figure 2 SM. A) LC-MS Base Peak Chromatogram (BPC) of a VOO extract of Cayon variety obtained under the optimum conditions. The optimized gradient composition of mobile phases and flow are also shown in the figure, as well as the elution areas of each one of the determined families. B) GC-MS BPC of the same VOO extract together with the optimized temperature ramp gradient and elution areas of each chemical class

Chapter 8

Study of the minor fraction of virgin olive oil by a multi-class GC-MS approach: comprehensive quantitative characterization and varietal discrimination potential

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Abstract: For the first time, a multi-class GC-MS method was applied to identify and quantify more than 40 compounds from the virgin olive oil (VOO) minor fraction in a single run. This innovative methodology has demonstrated a comprehensive profiling ability on five groups of compounds (phenolic and triterpenic compounds, tocopherols, sterols and free fatty acids) with wide range of polarities/volatilities and chemical entities. First, highly satisfactory results were achieved regarding linearity, sensitivity, accuracy and matrix effect during method validation. Second, 32 VOO samples from eight different cultivars (some of them very scarcely studied before) were analyzed by applying the proposed methodology and the quantitative results were subjected to chemometrics. Both non-supervised and supervised multivariate statistical tools were used for testing the capability of the determined VOO minor compounds to discriminate the varietal origin of the samples, pointing out potential chemical markers of each cultivar.

Keywords: virgin olive oil; cultivar; minor fraction; gas chromatography; mass spectrometry; multi-class methodology.

1. INTRODUCTION

The health benefits associated with virgin olive oil (VOO) intake and its unique sensory properties are the main reasons of increased olive oil consumption and production during the last decades [1,2].

Different olive oil categories can be found in the markets, but only VOO -obtained exclusively by mechanical means without any refining steps- preserves its minor compounds that are responsible for the taste and nutritional value. The VOO minor fraction comprises a heterogeneous mix of molecules, including phenolic compounds (simple phenols, phenolic acids, secoiridoids, flavonoids and lignans), triterpenic compounds (acids and dialcohols), tocopherols and sterols [3]. In any VOO the concentration of these minor compounds is highly influenced by agro-technological parameters such as cultivar, pedoclimatic conditions, irrigation methods, extraction procedures and storage practices [4]. Acceptable concentration ranges for some of these compounds have been included in several legal frames settled to protect consumers from product mislabeling and adulteration [5–7] as well as to promote health claims related to VOO biomolecules [8]. Therefore, the determination of these compounds is of great interest to both VOO producers and regulatory bodies, who are continuously challenging the analytical community to offer rapid and accurate testing methods [9–11].

Gas chromatography (GC) is a very common separative technique used by routine laboratories. It is also the technique of choice in several official methods for the analysis of different VOO components such as waxes, fatty acid methyl esters, fatty acid ethyl esters, aliphatic alcohols, sterols and triterpenic dialcohols, among others [10]. The use of mass spectrometry (MS) as detection system coupled to the unbeatable resolving power of GC has also become

commonplace. As a result, GC-MS seems to be a promising coupling for characterizing the complex VOO minor fraction.

Multi-class methodologies, which exhibit the ability to monitor analytes belonging to diverse chemical classes in one single analysis, bring out a remarkable progression of the traditional single-class methods in terms of throughput and cost. At the same time, they enlarge the information achievable by the analyst and provide enhanced possibilities to take advantage of the results. In other words, those comprehensive profiling methods allow comparing samples from a deeper perspective, providing quantitative data on a great number of substances and facilitating the extraction of relevant information through the use of chemometrics. When applied to VOO, multi-class methodologies can be used, for instance, to correlate the healthy properties of an oil with its minor fraction composition, to investigate the influence of different agro-technological parameters on the quality of the obtained oils, or to look for distinctive features to classify samples regarding their botanical or geographical origin [12].

The main goal of this study was to demonstrate the suitability of a GC-MS multi-class methodology for the determination of VOO minor compounds of different chemical nature (phenolic and triterpenic compounds, tocopherols, sterols and free fatty acids) in a single run. We also had the intention of checking the capability of the determined compounds to trace the varietal origin of VOO samples, in the same way as single-class approaches have previously demonstrated for compounds such as triacylglycerols [13], phenolic compounds [14] and sterols [15]. The analytical performance of the method was assessed and then, it was applied to the analysis of VOO samples from different cultivars grown under the same conditions in the same orchard in California. The quantitative characterization of the selected oils is considered to be very relevant, as the information about the chemical composition of some of the cultivars included in this study is quite scarce in literature. Apart from that, the use of chemometrics expedited the study of the results and made possible the establishment of statistical models to differentiate samples with distinctive botanical provenance.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Deionized water generated by a MilliQ system (Millipore, Bedford, MA) and ethanol (EtOH) 95% from Koptec (King of Prussia, PA, USA) were used for the extraction of the VOO minor components. The derivatization reagent (N,O-bis(trimethylsilyl)trifluoroacetamide plus 1% of trimethylchlorosilane (BSTFA+1% TMCS)) as well as commercial standards of palmitoleic (C16:1), oleic (C18:1) and linoleic (C18:2) acids, α-, β-, γ- and δ-tocopherols (α-, β-, γ- and δ-Toc), oleanolic (OA), ursolic (UA), betulinic (BA) and maslinic (MA) acids, erythrodiol (ER), uvaol (UV), stigmasterol (Sti), campesterol (Cam), β-sitosterol (β-Sit), luteolin (Lut), apigenin (Api), pinoresinol (Pin), vanillin (Van), *p*-coumaric (*p*-Cou), quinic (Qui) and ferulic (Fer) acids, tyrosol (TY) and hydroxytyrosol

(HTY) were purchased from Sigma Aldrich (St. Louis, MO, USA). All the stock solutions and extracts were filtered through 0.4 µm nylon syringe filters (Thermo Scientific, Santa Clara, CA, USA) and stored in dark glass vials at −20 ºC.

2.2. Samples

Olive fruit sampling was performed in October 2016 on eight different cultivars (cv. Carolea, Casaliva, Cayon, Frantoio, Kalamon, Maurino, Moraiolo and Taggiasca) grown in an experimental orchard of the UC Davis Olive Center (Davis, CA, USA) under controlled agronomical conditions. The geographical coordinates of olive grove are 38°32'10"N 121°47'42"W and the altitude is around 16 m. The area has a Csa climate type according to the Köppen-Geiger climate classification [16], with average temperatures of 16.2ºC (8.7 and 23.7°C, for minimum and maximum means, respectively), and annual rainfalls of around 500 mm for the year of 2016. Four batches of fruit samples from each cultivar (32 samples in total), with ripening indices between 2.3 and 2.9, were hand-picked from randomly selected olive trees. Those relatively low ripening index values considering the European standards are quite common taking into account the location of the olive grove and the Californian practices. VOOs from each sample were obtained within the next 3 h by means of an Abencor® laboratory oil mill (MC2 Ingeniería y Sistemas, Seville, Spain) and stored at -20ºC until further analysis. A quality control (QC) sample was prepared by mixing equivalent amounts of individual VOO samples.

2.3. Extraction and GC-MS determination of minor compounds

The extraction of the minor components from VOO samples was performed by applying a previously published liquid-liquid extraction (LLE) protocol [12]. In short, 1.00 ± 0.01 g of VOO was successively extracted (by using vortex followed by centrifugation and collection of the supernatants) with three 6 mL portions of EtOH/H_2O (80:20, v/v) and one portion of EtOH/H_2O (60:40, v/v). After solvent evaporation, the residue was reconstituted in 1 mL of EtOH/H_2O (80:20, v/v). An aliquot of 200 µL of the extract (or the appropriate amount of standards mixture) was derivatized by adding 50 µL BSTFA+1% TMCS to the dried residue and kept at room temperature for 1 h before injecting into the GC.

The analysis of the prepared extracts was performed as described in a previous work [12] on a Varian 450 gas chromatograph coupled with a Varian 220 mass spectrometer equipped with an Ion Trap (IT) analyzer (Agilent Technologies, Santa Clara, CA, USA). The separation of the analytes was carried out using a (5%-phenyl)-methyl polysiloxane (HP-5MS) capillary column (30 m x 0.25 mm i.d., 0.25 µm) (Agilent Technologies) with He as carrier gas at 1 mL/min. A 52.5 min temperature gradient was used: the temperature was initially held at 140ºC for 5 min, followed by a 4°C/min ramp to 310°C (held for 5 min). A sample volume of 1 µL was injected at a split ratio of 1:25. The injector and transfer line temperatures were 240ºC and 290ºC, respectively. Spectra (in a range from 50 to 600 *m/z*) were recorded with the electron impact ion source operating in positive mode at 70 eV and 200ºC.

2.4. Method characterization

Both the QC sample and a standards mixture containing 25 VOO minor compounds were used for method validation.

Method linearity was checked by establishing external calibration curves for each individual compound. For this purpose, a stock solution of the standards mixture was prepared in EtOH/H_2O (80:20, v/v) and serially diluted to eight different concentration levels (ranging from 0.5 mg/L to a maximum level that depended on the compound); each level was analyzed in triplicate. Signal to noise ratio (S/N) was determined for each analyte at the lowest concentration level and limits of detection (LOD) and quantification (LOQ) were estimated by calculating the concentration that generates a S/N equals to 3 and 10, respectively.

Intra and *inter*-day repeatability for peak area and retention time (Rt) were expressed as the relative standard deviation (%RSD) obtained for the values from four injections of four different extracts of the QC, which were carried out within the same day and over four different days, respectively. Trueness was estimated by analyzing the QC extracted before and after fortification with the mixture of standards at three distinct concentration levels (low, intermediate and high); the recovery for each single pure standard was estimated afterwards.

Additionally, the matrix effect was evaluated by comparing the slope of the external calibration curve (prepared in solvent) and the slope resulting from the standard addition (at three concentration levels) to the QC extract. A matrix effect coefficient was calculated (in percentage) for each analyte: the more similarity between the values of two slopes, the less significant the magnitude of the matrix effect.

2.5. Data treatment

Instrument control and data processing were performed with MS Workstation v. 6.9.3 (Agilent Technologies). External calibration curves were used to convert automatically integrated peak areas into concentrations. Good linearity was achieved for all the standards (except for flavonoids) based on least-squares regression. Quadratic calibration curves -which are also common models for calibration in GC-IT MS- were generated for Lut and Api [17]. Analytes lacking pure standards were quantified in terms of a structure-related compound (commercially available): HTY calibration curve was used for quantification of acetylated HTY (AcHTY), oleuropein aglycone isomers (OleAgly) and decarboxymethyl oleuropein aglycone (DOA); TY calibration curve was applied for ligstroside aglycone isomers (LigAgly) and decarboxymethyl ligstroside aglycone (DLA); Pin calibration curve was used for acetoxypinoresinol (AcPin); the relative response of C16:1 standard was used to quantify palmitic acid (C16:0); C18:1 for stearic acid (C18:0); and β-Sit for Δ^5-avenasterol (Δ^5-Ave), cycloartenol (CyArten), methylencycloartanol (MeCyArtan) and citrostadienol (Cit). Elenolic acid (EA) does not have a commercially available standard, but since it is considered as a highly related compound to secoiridoids, it has been frequently quantified in terms of oleuropein in LC-MS [14]. In this study, the *m/z* of the derivatized oleuropein pure standard was out of the

selected working mass range and, therefore, it could not be properly detected. Thus, in the absence of a suitable standard to accurately carry out EA quantification, its area was directly used for statistics after the required pre-treatment.

Statistical analysis was performed with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA) and The Unscrambler v9.7 (CAMO Software, Inc., Woodbridge, NJ, USA). In a first stage, one-way analysis of variance (ANOVA) was carried out to determine the significant difference(s) regarding the concentration of the targeted analytes among different cultivars. Afterwards, the natural clustering of the samples was studied by conducting Principal Component Analysis (PCA). The PCA matrix was composed by 40 variables (chemical compounds) and 32 samples (average value of triplicates). Apart from it, Partial Least Squares-Discriminant Analysis (PLS-DA) was performed to build two-class models by confronting the samples of each cultivar against the rest of the samples (which composed one global group in each case). Data normalization was carried out (for both PCA and PLS-DA) to reduce experimental variance and all variables were weighted by 1/standard deviation (1/SD) for PLS-DA to allow all of them to contribute to the model, regardless of whether the quantitative value had a small or large standard deviation (SD) from the outset. Full cross-validation was applied to evaluate the prediction power of the obtained models.

3. RESULTS AND DISCUSSION

3.1. Analytical parameters of the method

To evaluate the adequacy of the quantification method on 40 minor compounds of different chemical classes in VOO samples, the method validation was conducted.

Table 1 summarizes the main analytical parameters of the method, which give a profound idea of its linearity, sensitivity, accuracy and the presence/absence of matrix effect. The table also contains information about the *m/z* signals used for the quantification of each substance; they were selected considering specificity, relative response and S/N (seeking the achievement of the most favorable LOD and LOQ). All the external calibration curves showed good linearity within the evaluated concentration range, with regression coefficients (R^2) higher than 0.988. The different concentration ranges selected for each analyte were chosen considering the concentration levels found in the VOO samples. The lowest LOD and LOQ were found for *β*-Sit (40 μg/L and 140 μg/L, respectively), while the highest ones were found in MA (LOD of 1.7 mg/L and LOQ of 5.8 mg/L).

As far as precision is concerned, %RSD values for *intra* and *inter*-day repeatability, in terms of area, were lower than 5.9% (Pin) and 9.2% (*β*-Sit), respectively. In general, the *intra* and *inter*-day repeatability in terms of Rt exhibited very low values; Pin was the compound which presented the highest *inter*-day %RSD value (0.05%). In addition, good recoveries were found for most of the analytes with values ranging from 80.7 to 105.7%, which are within the limits proposed by the AOAC for a truthful method [18]. Only two sterols (Cam and *β*-Sit) presented recoveries slightly

Table 1. Analytical parameters of the GC-MS method.

Rt (min)	Compound	*m/z* signal used for quantification	Calibration function	R^2	LOD (mg/L)	LOQ (mg/L)	Accuracy					Matrix Effect Coef. (%)[c]
							Intra-day Repeatability[a]		*Inter-day* Repeatability[a]		Trueness (% recovery)[b]	
							Area	*Rt*	*Area*	*Rt*		
6.9	Van	194	y = 2178 x -924	0.998	0.09	0.31	2.73	<0.01	4.92	0.03	105.7	-2.7
7.8	TY	179	y = 8751 x -4173	0.999	0.17	0.56	2.66	0.02	6.66	0.04	93.1	6.1
12.8	HTY	267	y = 13800 x -13681	0.998	0.17	0.56	2.11	0.02	4.01	0.02	91.7	3.5
15.3	Qui	346	y = 2605 x -1528	0.997	1.0	3.33	4.15	0.03	4.56	0.03	98.2	-2.7
17.2	*p*-Cou	294	y = 6686 x -8579	0.999	0.45	1.49	5.78	0.01	7.51	0.02	99.0	-2.1
19.3	C16:1	312	y = 6473 x -5741	0.996	0.08	0.27	2.11	0.01	5.26	0.02	98.7	-6.6
20.8	Fer	338	y = 5294 x -10189	0.997	0.12	0.40	2.70	0.01	4.85	0.02	102.5	0.5
23.5	C18:2	338	y = 4551 x -5846	0.996	0.19	0.65	3.96	<0.01	5.54	0.02	93.8	-0.9
23.8	C18:1	354	y = 7990 x -3218	0.995	0.08	0.27	5.36	<0.01	8.03	<0.01	90.2	16.3
36.6	*δ*-Toc	475	y = 6512 x -2429	1.000	0.04	0.15	2.04	<0.01	5.70	0.01	90.5	10.3
37.9	*β*-Toc	489	y = 5633 x -4043	1.000	0.10	0.33	4.49	0.01	7.63	0.01	86.6	5.9
38.1	*γ*-Toc	489	y = 7217 x -6405	0.999	0.06	0.18	5.09	0.01	6.62	0.01	82.9	4.9
40.3	*α*-Toc	503	y = 7644 x -22011	0.999	0.05	0.18	2.68	0.02	5.10	0.02	81.3	-4.5
40.4	Api	472	$y = 37 x^2 - 653 x + 3796$	1.000	0.44	1.48	4.88	0.03	5.86	0.03	99.5	-11.3
41.8	Cam	503	y = 7026 x -16668	0.996	0.05	0.17	5.22	0.01	7.82	0.01	78.6	-36.2
42.2	Sti	395	y = 1094 x -2228	0.996	0.59	1.96	3.01	0.03	5.43	0.03	80.7	1.0
42.6	Lut	560	$y = 46 x^2 - 963 x + 5731$	1.000	0.32	1.08	1.25	<0.01	5.01	0.04	82.4	21.3
43.1	*β*-Sit	397	y = 21289 x -39661	0.998	0.04	0.14	5.40	0.01	9.19	0.01	75.1	-3.7
43.2	Pin	502	y = 1332 x -406	1.000	0.26	0.85	5.86	0.01	6.44	0.05	102.3	-0.3
45.4	ER	497	y = 2916 x -8770	0.998	0.59	1.98	4.41	0.01	5.29	0.02	97.3	4.8
45.9	UV	497	y = 2410 x -6474	0.993	0.75	2.50	3.66	0.01	7.33	0.01	98.5	7.8
46.3	OA	203	y = 2648 x -4981	0.994	1.67	5.56	5.76	0.02	7.49	0.02	99.2	-2.4
46.6	BA	189	y = 1308 x -2443	0.993	0.83	2.78	2.11	0.01	6.75	0.01	101.3	-14.2
47.0	UA	320	y = 2595 x -5711	0.988	0.79	2.63	3.66	0.03	7.33	0.03	102.5	0.6
48.6	MA	203	y = 1198 x -3360	0.991	1.73	5.77	4.67	0.02	8.40	0.03	99.8	-11.0

[a] Repeatability is expressed as the %RSD of peak area values for four injections of four different extracts of the QC carried out within the same sequence (*intra*-day) or over four days (*inter*-day).
[b] Trueness, expressed as recovery (%), was estimated by analyzing the QC extracted before and after the standard addition and calculating the difference between the obtained results. The values included in this table are those achieved for the intermediate concentration level to contain the size of the table.
[c] Matrix effect coefficient (%) = [1−(slope matrix/slope solvent)]×100.

lower than 80% (78.6 and 75.1%, respectively); in spite of it, those values were reasonably good and, most importantly, the repeatability of the overall process including both the sample extraction and instrumental analysis was outstanding (<10% of RSD measured in terms of area).

Finally, matrix effect was assessed as described in Section 2.4. For 23 out of 25 standards, the calculated coefficients were in the range between -14.2% and +16.3%, indicating a mild signal suppression or enhancement effect (from −20% to +20%). Nevertheless, Lut presented a slight enhancement effect (21.3 %) and Cam was suppressed to some extent (-36.2%). External calibration equations were used for targeted analytes quantification based on the following assumptions: i) standard addition calibration implies the construction of a calibration curve for each sample; and ii) the matrix effect was firstly evaluated using a QC sample which was a mixture of equivalent volumes of all the VOOs under study. Afterwards, the matrix effect of each cultivar was checked individually and the slight enhancement/suppression observed for Lut and Camp was not found as noticeable as within the QC sample. Thus, reliable quantitative results could be obtained for the 32 VOO samples by employing the external calibration approach.

3.2. Application of the method to the analysis of the selected samples

Extracts of 32 VOO samples from eight different cultivars were analyzed in the current study by using the described GC-MS methodology. Fig. 1 shows the complexity of the chromatograms obtained from three oils (Frantoio, Kalamon, and Cayon) which were selected as example. The applied methodology was useful to get information about five different chemical classes of VOO minor compounds. In particular, 41 compounds were quantified in the studied samples: 19 phenolic compounds (seven simple phenols, eight secoiridoid derivatives, two flavonoids and two lignans), four tocopherols, six triterpenic compounds (two triterpenic alcohols and four acids), seven sterols and five free fatty acids. Moreover, two EA peaks were identified and integrated in the chromatograms; and their reported area (normalized) was also included in the statistical analysis.

Table 1 SM (supplementary materials) shows Rt, name of the analyte, *m/z* signals, and the formula of the feature detected in the MS spectra which allowed the identification for each compound.

The quantitative data obtained for the 41 selected analytes are presented in Table 2. The given number is the average of the four VOO samples from each cultivar, which were obtained from olives harvested from different olive trees and processed independently. For each sample, we calculated the mean of three extraction and injection replicates. Later on, further calculations were made to achieve the global value (on average) for each cultivar, combining all the results from the different samples belonging to each variety. Some compounds presented high variability within samples from the same cultivar, whilst the levels of some others remained constant. For example, ER levels were very similar in all the samples with the same varietal origin (less than 11% of variability) conversely to Qui, whose *intra*-cultivar fluctuation was substantially higher in Casaliva samples. Some analytes belonging to phenolic compounds-chemical class (in particular,

secoiridoids) usually show several isomeric forms in the profiles, as it has been extensively discussed in literature [19,20]. In Table 2, we have denoted the different isomers by adding a Roman numeral after the name of the compound. *Intra*-cultivar variations were also found, for instance, for LigAgly I and OleAgly III concentrations; they varied more than 30% in the samples of five (Taggiasca, Moraiolo, Frantoio, Cayon and Carolea) and four cultivars (Taggiasca, Moraiolo, Frantoio, Cayon), respectively. However, when the total concentration of secoiridoid aglycones is considered, the overall *intra*-cultivar variability remarkably decreased, suggesting that the distribution of the isomers varied more than their global levels in the samples.

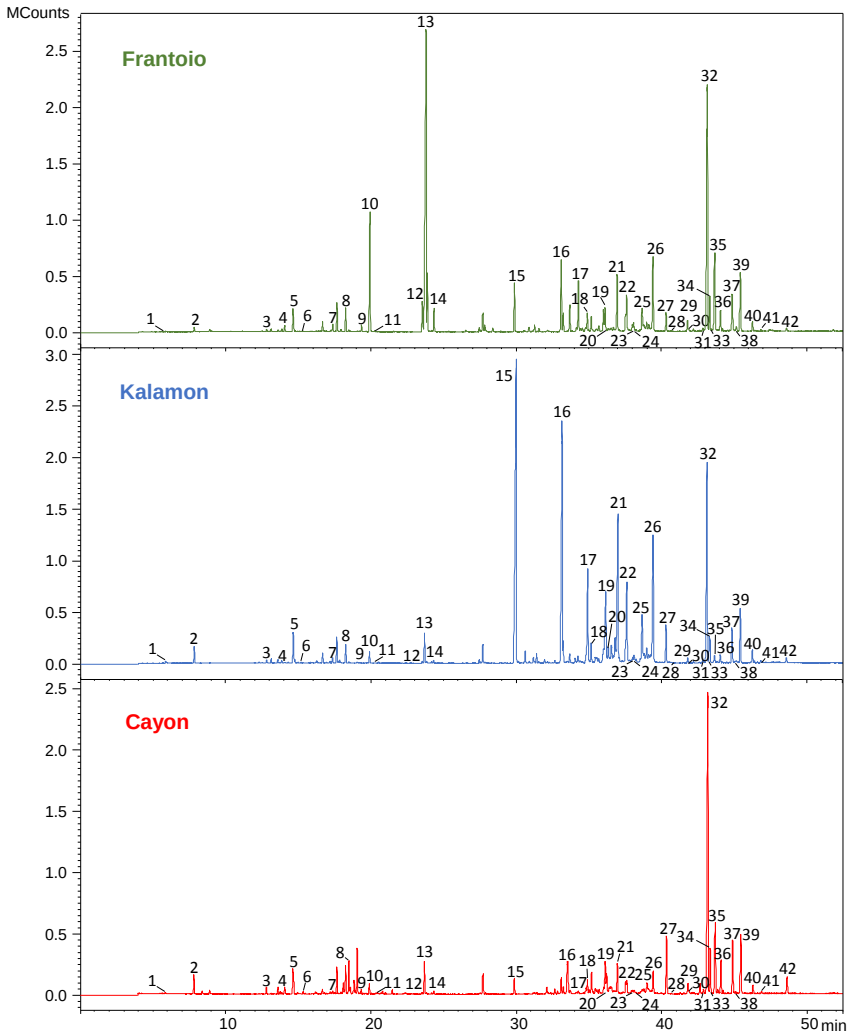

Figure. 1. Total ion chromatograms (TICs) of three extracts of monovarietal VOO showing great variability within the profiles. Peak identification numbers: 1, Van; 2,TY; 3, HTY; 4, AcHTY; 5, EA I; 6, Qui; 7, *p*-Cou; 8, EA II; 9, C16:1; 10, C16:0; 11, Fer; 12, C18:2; 13, C18:1; 14, C18:0; 15, DLA; 16, DOA; 17, LigAgly I; 18, squalene; 19, LigAgly II; 20, δ-Toc; 21, LigAgly III; 22, OleAgly I; 23, β-Toc; 24, γ-Toc; 25, OleAgly II; 26, OleAgly III; 27, α-Toc; 28, Api; 29, Cam; 30, Sti; 31, Lut; 32, β-Sit; 33, Pin; 34, Δ^5-Ave; 35, AcPin; 36, CyArten; 37, MeCyArtan; 38, ER; 39, Cit; 40, OA; 41, BA; 42, MA.

Table 2. Average concentration of the 41 determined compounds (mg/kg of VOO) in four samples of each cultivar. Results are given in mean value ± SD; SD expresses the *intra*-cultivar variability.

		Carolea	Casaliva	Cayon	Frantoio	Kalamon	Maurino	Moraiolo	Taggiasca
Phenolic compounds	Van	0.41 ± 0.03	0.42 ± 0.04	0.46 ± 0.09	0.89 ± 0.08	0.45 ± 0.01	0.69 ± 0.02	0.52 ± 0.03	0.69 ± 0.14
	TY	1.8 ± 0.2	3.8 ± 0.7	11 ± 5	2.7 ± 0.5	9 ± 1	1.1 ± 0.6	1.0 ± 0.1	2.1 ± 0.2
	HTY	1.28 ± 0.05	2.7 ± 0.5	1.8 ± 0.5	1.7 ± 0.3	2.0 ± 0.1	1.5 ± 0.5	1.7 ± 0.1	1.7 ± 0.2
	AcHTY	0.41 ± 0.02	0.63 ± 0.09	3 ± 1	2.7 ± 0.7	0.51 ± 0.03	0.16 ± 0.03	0.44 ± 0.07	3 ± 1
	Qui	0.8 ± 0.1	2 ± 2	1.5 ± 0.2	0.9 ± 0.1	0.89 ± 0.04	0.60 ± 0.03	0.44 ± 0.02	1.2 ± 0.4
	p-Cou	7.6 ± 0.5	0.49 ± 0.02	1.00 ± 0.04	0.54 ± 0.01	1.2 ± 0.1	0.28 ± 0.03	0.6 ± 0.1	0.17 ± 0.01
	Fer	0.10 ± <0.01	0.11 ± 0.01	nq	0.11 ± 0.01	0.12 ± 0.01	nq	nq	nd
	DLA	4 ± 1	126 ± 25	29 ± 4	22 ± 2	356 ± 27	1.6 ± 0.1	22 ± 9	22 ± 4
	DOA	3.9 ± 0.6	48 ± 7	16 ± 5	21 ± 3	90 ± 5	3.8 ± 0.4	38 ± 12	23 ± 3
	LigAgly I	15 ± 6	62 ± 6	6 ± 3	6 ± 2	55 ± 9	5.2 ± 0.8	2.3 ± 0.8	8 ± 3
	LigAgly II	11 ± 2	24 ± 2	1.6 ± 0.3	7.4 ± 0.9	33 ± 3	7 ± 3	2.5 ± 0.3	4 ± 2
	LigAgly III	27 ± 5	53 ± 2	5 ± 4	26 ± 10	74 ± 5	19 ± 5	10 ± 1	15 ± 6
	OleAgly I	28 ± 4	39 ± 4	6 ± 1	11 ± 2	41 ± 7	37 ± 6	14 ± 6	15 ± 4
	OleAgly II	9 ± 2	10 ± 1	0.8 ± 0.2	6 ± 3	11.7 ± 0.9	19 ± 4	2.9 ± 0.8	3 ± 2
	OleAgly III	41 ± 6	41 ± 3	3 ± 2	29 ± 12	39 ± 9	73 ± 17	20 ± 6	21 ± 7
	Api	12 ± 2	4.9 ± 0.2	9 ± 2	4.3 ± 0.2	4.4 ± 0.3	2.2 ± 0.6	4.1 ± 0.5	2.8 ± 0.4
	Lut	11 ± 2	5.6 ± 0.9	15 ± 4	6.6 ± 0.6	7.3 ± 0.7	5 ± 2	6 ± 1	5.8 ± 0.8
	Pin	9 ± 1	7.9 ± 0.5	14.3 ± 0.7	8.1 ± 0.2	9.2 ± 0.4	11 ± 2	4.3 ± 0.3	6.4 ± 0.4
	AcPin	4.1 ± 0.2	4.0 ± 0.2	6.5± 0.4	7.4 ± 0.3	2.62 ± 0.04	5.5 ± 0.9	1.8 ± 0.5	5.4 ± 0.9
Tocopherols	*δ*-Toc	nq	10.4 ± 0.4	11 ± 1	7.73 ± 0.02	8.0 ± 0.1	1.8 ± 0.2	1.9 ± 0.1	1.46 ± 0.06
	β-Toc	11.5 ± 0.4	15.2 ± 0.9	45 ± 8	12.7 ± 0.2	17.6 ± 0.7	6.7 ± 0.7	5.0 ± 0.5	6.5 ± 0.6
	γ-Toc	8.5 ± 0.4	42 ± 6	19 ± 5	9.6 ± 0.4	11.0 ± 0.6	15 ± 5	29 ± 4	5.9 ± 0.4
	α-Toc	112 ± 13	213 ± 32	460 ± 53	128 ± 19	324 ± 21	181 ± 49	96 ± 17	93 ± 21
Triterpenic compounds	ER	4.2 ± 0.1	3.5 ± 0.1	4.2 ± 0.4	5.11 ± 0.08	5.1 ± 0.2	2.9 ± 0.3	3.59 ± 0.08	5.1 ± 0.1
	UV	nd	nd	nd	nd	nd	nd	nd	nd
	OA	4.6 ± 0.7	7.2 ± 0.4	7.7 ± 0.8	14 ± 2	19 ± 4	9 ± 2	11 ± 1	11 ± 2
	BA	nd	nd	nd	nd	nq	nd	nq	nd
	UA	nd	nd	nd	nd	nd	nd	nd	nd
	MA	20 ± 2	24 ± 2	24 ± 6	35.9 ± 0.4	48 ± 1	23.8 ± 0.7	33.9 ± 0.9	28 ± 2
Sterols	Cam	12 ± 1	17 ± 2	22 ± 2	22 ± 3	11.0 ± 0.6	18 ± 2	18 ± 2	21 ± 2
	Sti	3.8 ± 0.5	2.8 ± 0.2	3.6 ± 0.5	4.6 ± 0.5	2.8 ± 0.2	2.17 ± 0.09	3.6 ± 0.1	4.4 ± 0.6
	β-Sit	280 ± 21	314 ± 40	452 ± 28	362 ± 66	287 ± 29	286 ± 48	330 ± 13	350 ± 22
	Δ^5-Ave	7.7 ± 0.5	37 ± 2	10 ± 1	15 ± 1	9 ± 1	20 ± 8	11 ± 4	15 ± 2
	CyArten	25 ± 4	20 ± 1	35 ± 7	25 ± 4	13 ± 2	9.9 ± 0.2	32 ± 10	32 ± 2
	MeCyArtan	60 ± 10	51 ± 6	61 ± 13	50 ± 5	69 ± 7	35 ± 4	38 ± 13	59 ± 7
	Cit	32 ± 1	25 ± 2	38 ± 8	34 ± 2	37 ± 3	17 ± 2	18 ± 3	39 ± 3
Free fatty acids	C16:1	3.1 ± 0.5	nq	1.9 ± 0.1	6 ± 1	nq	2.4 ± 0.3	4 ± 2	4.7 ± 0.7
	C16:0	355 ± 80	228 ± 35	28 ± 2	520 ± 108	49 ± 10	124 ± 22	498 ± 241	326 ± 52
	C18:2	40 ± 10	21 ± 3	1.9 ± 0.2	43 ± 6	3.1 ± 0.4	9 ± 2	28 ± 12	27 ± 4
	C18:1	364 ± 67	243 ± 33	26 ± 6	611 ± 67	64 ± 24	92 ± 20	411 ± 197	410 ± 57
	C18:0	63 ± 9	37 ± 4	8.4 ± 0.8	64 ± 7	11.2 ± 0.9	12 ± 2	55 ± 26	42 ± 7

nd: non detected / nq: non quantifiable

The level of different minor compounds greatly differed from one cultivar to the other (as illustrated in Fig. 1), which is in agreement with previous reports which state that the cultivar is one of the most influential factors affecting VOO composition [4,14,21-23]. Regarding *inter*-cultivar variability, *p*-Cou and DLA were the compounds presenting the greatest variances, with concentration ranges from around 0.2 to 7.6 mg/kg and 1.6 to 356 mg/kg, respectively. Consequently, it might be expected that they will have a significant impact on the statistical modeling to discriminate the varietal origin of the samples; this will be explained by the chemometric studies.

Even though the *inter*-cultivar differences were not as drastic as for the two mentioned compounds, significant disparities were found in other substances. For instance, the concentration of LigAgly I and OleAgly III considerably varied among the cultivars; indeed, LigAgly I levels varied from 2.3 to 62 mg/kg in Moraiolo and Casaliva, respectively, and the found amounts of OleAgly III ranged from 3 to 73 mg/kg for Cayon and Maurino, apiece. Something similar was observed in other isomeric forms of these secoiridoid aglycons. Regarding fatty acids, C16:0 levels varied substantially in the studied oils, showing averaged values of 28 mg/kg for Cayon *cv.* and of 520 mg/kg for Frantoio oils. Another example to be cited is α-Toc, which showed concentrations within the range 96-460 mg/kg, defined by the mean values of Moraiolo and Cayon, respectively.

Fig. 2 illustrates the differences in the VOO minor compounds content in the tested oils, grouped by chemical class. A normalized scale is used to facilitate the proper evaluation of the results. Kalamon was considered the "richer" cultivar in terms of phenolic and triterpenic compounds. This cultivar is characterized by very high levels of DLA as well as by a remarkable content of OA and MA. Cayon samples presented the highest levels of tocopherols and sterols, whereas Frantoio VOOs had the major content of free fatty acids. Sterols turned out to be the chemical class presenting the lowest variation among the eight tested cultivars.

A reasonable comparison with previously published results regarding concentration values can just be made when the same pure standard is used for the quantification of a given compound. Moreover, information about the chemical composition of VOO from some of the cultivars studied herein is barely reported. Nevertheless, a global comparison of the concentration ranges found in VOO minor compounds reveals that the applied methodology gives comparable results to previous studies. Levels found in phenolic compounds are in agreement with results obtained by applying LC-FLD and LC-MS methodologies [14,24], except for lignans, which are closer to the levels reported by Brenes *et al.* [25]. As far as tocopherols are concerned, concentrations found in this study are similar to those described by different authors for VOOs of different varietal origins [26,27]. Triterpenic compounds content is also within the range previously found in other VOOs [22]. Sterols and, particularly, β-Sit concentration seems to be lower than the results obtained by Cañabate-Díaz *et al.* [28], where only one VOO was included in the sample selection. Moreover, another factor to take into account is that sterols are generally quantified using an internal standard [29] instead of the corresponding response of each pure standard (which would make

possible to achieve an absolute quantification). Regarding free fatty acids, C18:2 content is generally below the concentration range reported by Wabaidur and coworkers [30]; the same is observed for the C18:1 content in Cayon, Kalamon and Maurino VOOs.

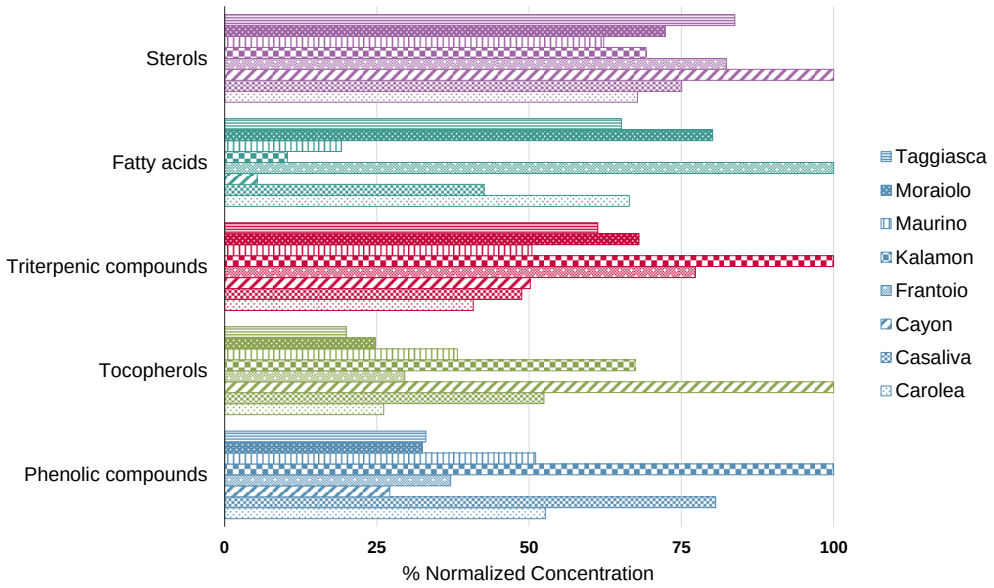

Figure 2. Bars diagram comparing the average VOO minor compounds content in the eight different cultivars tested within this study, grouped by chemical class. Normalization was used considering the concentration level of the "richest" cultivar (for each chemical class) as 100% and referring the rest to that value. The compounds considered as members of each chemical class are detailed in Table 2.

3.3. Statistical analysis

The visual inspection of chromatograms and quantitative data showed the significant compositional heterogeneity among the samples obtained from different cultivars. This was confirmed by ANOVA, finding significant differences ($p < 0.05$) for all the tested variables (determined chemical compounds) except for Qui. Multivariate analyses were consequently carried out to evaluate the whole data set-structure and test the discriminant power of the identified compounds to distinguish the varietal origin of the oils under evaluation.

Firstly, non-supervised PCA was performed as an exploratory approach to study data structure over a reduced dimension. Among the 20 identified principal components (PCs), the first five components explained 99.06 % of the total variance. The obtained PCA score plots and loadings plots for the first four PCs are shown in Fig. 1 SM. The first two PCs, which covered 76.46 % and 16.98 % of the variance, respectively, exhibited good discrimination capability among Kalamon, Cayon, Casaliva and Maurino (to a less extent, as one of the Moraiolo samples was quite close to Maurino´s) VOOs (Fig. 1 SM A1). Another grouping can be perceived in the PC1 *vs.* PC2 scores plot which encompasses Carolea, Moraiolo, Taggiasca and Frantoio samples together. The third and fourth PCs simply covered 2.98 % and 1.53 % of the variance, respectively. Improved separation of

Taggiasca, Frantoio and Carolea samples could be barely found in the PC3 *vs.* PC4 scores plot (Fig. 1 SM B1). Both PC1 *vs.* PC2 (Fig. 1 SM A2) and PC3 *vs.* PC4 (Fig. 1 SM B2) loadings plots, revealed the importance of the following variables for the clustering of the samples: DLA, α-Toc, β-Sit, C16:0, C18:1 and OleAgly III.

Next, supervised chemometrics were applied to build two-class discrimination models through PLS-DA; the resulting PC1 *vs.* PC2 scores plots are presented in Fig. 3. The worse class separation was found for the models built to discriminate Frantoio (d), Moraiolo (g) or Taggiasca (h) samples from the rest. That can be corroborated looking at Table 3, which presents the key parameters used to assess the quality of the models, such as R (or correlation), which measures the linear relationship between the predicted and measured values; R-Square; Root Mean Square Error of Prediction (RMSEP), which can be interpreted as the average prediction error; Standard Error of Performance (SEP), which is the standard deviation of the prediction residuals; and Bias, which is calculated as the average value of the residuals. Table 3 also includes the possible "varietal" markers which are useful to distinguish the VOO samples belonging to each cultivar from the rest.

Moraiolo and Taggiasca models had the lowest correlation and R-square parameters and the model for discriminating Frantoio from the rest needed the highest number of components to achieve reasonable quality parameters. The best quality parameters were found for Cayon and Maurino models. In Table 3, distinctive features are presented with their estimated regression coefficients (between brackets), value which points out the cumulative importance of each variable (chemical compound) to identify the varietal origin. Some compounds were common possible markers for different cultivars, such as MA, γ-Toc and Δ^5-Ave, which were significant variables for three models, or δ-Toc which was influential for four. Negative coefficients imply a negative contribution; for example, ER and AcHTY were two of the most distinctive features for Moraiolo and Taggiasca, but it was due to their low levels in the first case and because of their high content in the second one. As revealed in Table 3, and trying to underline specific varietal-features, high levels of p-Cou, EA isomers, C18:2 and Api, together with low levels of MA were typical for Carolea oils. The model to distinguish Casaliva *cv.* from the rest was mainly defined by the influence of Δ^5-Ave, two tocopherols, LigAgly I and the low concentrations of C16:1 and MA. The relative levels of tocopherols (β-,α-, and δ-Toc) together with characteristic concentrations of Lut, β-Sit and TY were the most specific features of Cayon VOOs. Fer and p-Cou, two phenolic acids, seemed to be particularly relevant in Frantoio. The role of δ-Toc and AcPin, among other substances (two free fatty acids), was also remarkable in the same model. Moreover, as stated above, Kalamon VOOs showed the highest levels of triterpenic compounds (MA and OA in particular) and DLA and DOA, with contrasting low levels of Δ^5-Ave and γ-Toc. The three determined isomers of OleAgly, as well as Pin and Van were found at considerably high levels in Maurino oils, for which the comparatively moderate concentrations of DOA could be noted as a typical feature for this cultivar. Moraiolo was

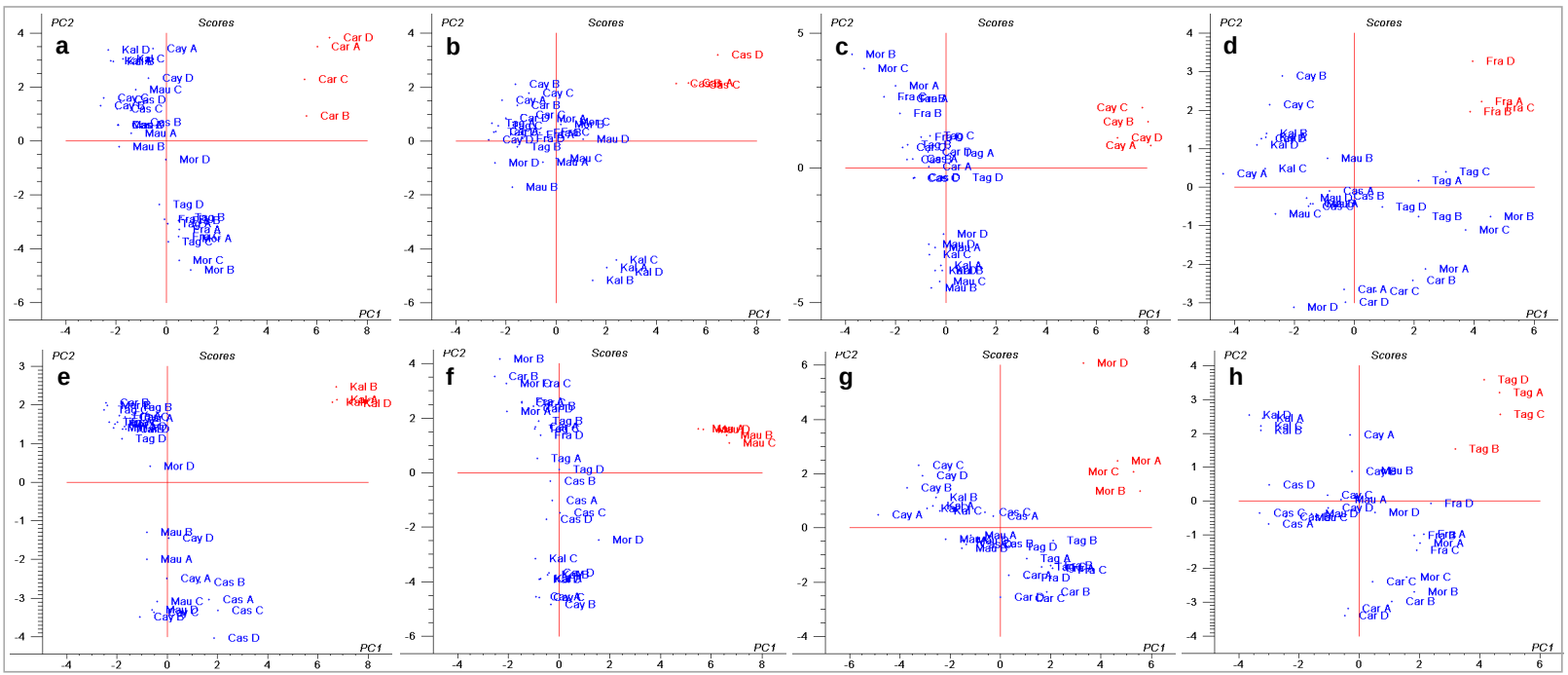

Figure 3. Scores plot (PC1 *vs.* PC2) for the eight two-class models obtained applying PLS-DA to discriminate Carolea (a), Casaliva (b), Cayon (c), Frantoio (d), Kalamon (e), Maurino (f), Moraiolo (g) and Taggiasca (h) samples from the rest of the sample set. Abbreviations: Car (Carolea), Cas (Casaliva), Cay (Cayon), Fra (Frantoio), Kal (Kalamon), Mau (Maurino), Mor (Moraiolo) and Tag (Taggiasca).

Table 3. Quality parameters of the two-class PLS-DA models (each cultivar compared with the rest) and most relevant distinctive features of each model.

	Carolea	Casaliva	Cayon	Frantoio	Kalamon	Maurino	Moraiolo	Taggiasca
PC number	5	5	4	10	3	6	4	5
Slope	0.947	0.882	0.944	0.955	0.945	0.955	0.787	0.780
Offset	0.013	0.007	0.005	0.006	0.012	0.004	0.017	0.047
R	0.968	0.939	0.984	0.977	0.970	0.983	0.903	0.825
R-Square	0.941	0.888	0.970	0.955	0.945	0.969	0.826	0.684
RMSEP	0.083	0.114	0.059	0.070	0.080	0.060	0.142	0.192
SEP	0.084	0.116	0.060	0.071	0.081	0.061	0.144	0.194
Bias	0.007	-0.007	-0.002	0.000	0.005	-0.001	-0.010	0.020
Distinctive features (regression coefficients)	*p*-Cou (0.282) EA I (0.152) C18:2 (0.146) Api (0.140) EA II (0.110) MA (-0.103)	Δ^5-Ave (0.277) γ-Toc (0.237) LigAgly I (0.225) C16:1(-0.181) δ-Toc (0.130) MA (-0.130)	β-Toc (0.136) α-Toc (0.115) Lut (0.113) δ-Toc (0.095) β-Sit (0.090) TY (0.088)	Fer (0.784) δ-Toc (0.512) AcPin (0.354) C18:1 (0.337) *p*-Cou (-0.335) C16:1 (0.240)	DLA (0.147) MA (0.125) Δ^5-Ave (-0.118) DOA (0.117) γ-Toc (-0.117) OA (0.114)	OleAgly II (0.195) OleAgly III (0.191) Pin (0.170) OleAgly I (0.141) Van (0.114) DOA (-0.107)	γ-Toc (0.238) AcPin (-0.237) Cit (-0.220) Fer (-0.207) ER (-0.179) AcHTY (-0.172)	Fer (-0.350) ER (0.273) Cit (0.263) δ-Toc (-0.201) Δ^5-Ave (0.179) AcHTY (0.145)

- Offset: point where a regression line crosses the ordinate (Y-axis).
- R: covariance between the two variables divided by the square root of the product of their variances.
- R-Square: square of the correlation coefficient between predicted and measured values.
- RMSEP (Root Mean Square Error of Prediction): measurement of the average difference between predicted and measured response values, at the prediction or validation stage.
- SEP (Standard Error of Performance): standard deviation of the prediction residuals.
- Bias: average value of the residuals.

one of the cultivars presenting the highest amounts of γ-Toc; on the contrary, its overall profile was unusual considering its low concentrations of AcPin, Cit, Fer, ER and AcHTY. The variables with higher absolute values of regression coefficients to characterize Taggiasca VOOs were Fer, ER and Cit.

Nonetheless, the potential markers designated in the current study have to be further tested and validated with a more comprehensive sample-set (covering different seasons). However, we believe that the importance of the findings presented herewith is undeniable. The latter together with the development and fully analytical validation of an innovative and powerful GC-MS multi-class methodology and its application to the analysis of VOO minor compounds are, from our point of view, the most valuable achievements of this work.

4. CONCLUSIONS

The effectiveness of a multi-class GC-MS methodology to carry out the minor fraction profiling of VOOs has been evaluated using 32 samples coming from eight different cultivars. Promising results have been achieved as: 1) a satisfactory analytical performance has been exhibited by the proposed method; 2) a comprehensive quantitative characterization of eight cultivars has been accomplished, successfully determining more than 40 compounds (phenolic and triterpenic compounds, tocopherols, sterols and free fatty acids); and 3) PLS-DA models have been established to discriminate among the eight selected cultivars and, most importantly, to identify varietal potential markers. Innovative tools and methods providing extensive information in just one run are absolutely in great demand when demonstrating the typicity and genuineness of an olive oil. Future studies could apply the proposed analytical methodology and statistical models; indeed, the new methodology represents a very useful implement for the "tool-box" of a wide number of laboratories worldwide.

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Table 1 SM. Identified compounds in GC-MS

Rt (min)	Compound	*m/z* characteristic signals	Assigned formula to the *m/z* signals in bold letters
6.9	Van	194/209/**224**/45	M-H+TMS
7.8	TY	179/267/**282**/180	M-2H+2TMS
12.8	HTY	267/**370**/73/193	M-3H+3TMS
14.1	AcHTY	280/73/193/**340**	M-2H+2TMS
14.7	EA I	153/299/196/**314**	M-H+TMS
15.3	Qui	**346**/256/73/419	M-5H+5TMS-2TMSO-CO
16.3	EA II	73/178/165/**314**	M-H+TMS
17.2	*p*-Cou	294/73/**308**/250	M-2H+2TMS
19.3	C16:1	312/129/117/**326**	M-H+TMS
19.9	C16:0	117/314/129/**328**	M-H+TMS
20.8	Fer	**338**/324/294/73	M-2H+2TMS
23.5	C18:2	338/73/129/**352**	M-H+TMS
23.8	C18:1	**354**/117/129/340	M-H+TMS
24.3	C18:0	342/117/129/**356**	M-H+TMS
29.9	DLA	192/177/73/**361**	M-H+TMS-CH_3
33.1	DOA	280/73/193/**464**	M-2H+2TMS
34.9	LigAgly I	**192**/73/280/**177**	(Olmo-García *et al.*, 2018)
36.1	LigAgly II	**192**/73/**177**/297	(Olmo-García *et al.*, 2018)
36.6	*δ*-Toc	**475**/209/73/249	M-H+TMS
37.0	LigAgly III	193/73/**417**/177	M-2H+2TMS-TMSO
37.6	OleAgly I	280/73/193/**522**	M-2H+2TMS
37.9	*β*-Toc	**489**/223/73/41	M-H+TMS
38.1	*γ*-Toc	**489**/223/73/43	M-H+TMS
38.7	OleAgly II	280/73/45/**505**	M-3H+3TMS-TMSO
39.5	OleAgly III	280/73/193/**594**	M-3H+3TMS
40.3	*α*-Toc	**503**/238/73/43	M-H+TMS
40.4	Api	472/45/399/**486**	M-3H+3TMS
41.8	Cam	503/**472**/73/383	M-H+TMS
42.2	Sti	395/**485**/83/256	M-H+TMS
42.6	Lut	560/45/472/**574**	M-4H+4TMS
43.1	*β*-Sit	397/358/**486**/381	M-H+TMS
43.2	Pin	**502**/223/235/488	M-2H+2TMS
43.3	Δ^5-Ave	386/297/282/**484**	M-H+TMS
43.7	AcPin	276/246/546/**560**	M-2H+2TMS
44.1	CyArten	393/366/**408**/69	M-H+TMS-TMSOH
44.9	MeCyArtan	408/380/422/**512**	M-H+TMS
45.4	ER	**497**/216/73/203	M-2H+2TMS-TMSO
45.4	Cit	358/400/268/**394**	M-H+TMS-TMSOH-CH_2
45.9	UV	**497**/73/216/203	M-2H+2TMS-TMSO
46.3	OA	203/**483**/73/320	M-2H+2TMS-TMSO-CO
46.6	BA	189/73/203/**483**	M-2H+2TMS-TMSO-CO
47.0	UA	320/203/73/**483**	M-2H+2TMS-TMSO-CO
48.6	MA	203/73/**571**/320	M-3H+3TMS-TMSO-CO

Olmo-García, L.; Polari, J. J.; Li, X.; Bajoub, A.; Fernández-Gutiérrez, A.; Wang, S. C.; Carrasco-Pancorbo, A. (2018). Deep insight into the minor fraction of virgin olive oil by using LC-MS and GC-MS multi-class methodologies. *Food Chemistry,* 261, 184-193. https://doi.org/10.1016/j.foodchem.2018.04.006.

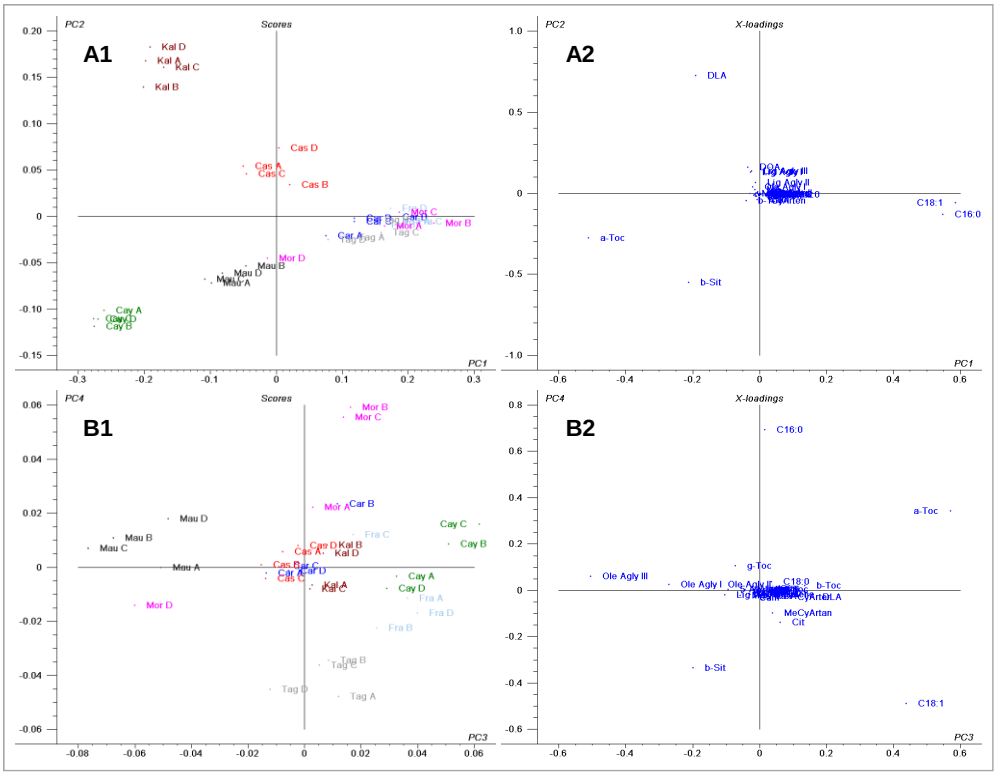

Fig. 1 SM. Scores plot (1) and loadings plot (2) for PC1 *vs.* PC2 (A) and PC3 *vs.* PC4 (B) charts of the PCA model. *Abbreviations: Car (Carolea), Cas (Casaliva), Cay (Cayon), Fra (Frantoio), Kal (Kalamon), Mau (Maurino), Mor (Moraiolo) and Tag (Taggiasca).*

Chapter 9

New olive fruit processing method involving stone removal and dehydration: towards obtaining highly valuable products with zero waste generation

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Abstract: An alternative olive fruit processing method has been proposed in an attempt to avoid the huge waste generation of the traditional virgin olive oil (VOO) production systems. It consists in performing the stoning of the olives, followed by a dehydration step and a cold press of the dry pulp. As a result, two new products are generated: olive oil and defatted pulp, which after grinding, is designated as 'olive flour'. The main objective of this work has been to accomplish the comprehensive characterization of these new products, which could help to enhance the profitability of the olive sector, in response to the increasing demand for functional foods. To this end, olive oil and flour samples from 15 different cultivars obtained through the described methodology (including also 'conventional' VOOs to facilitate the comparison with the new ones) have been analyzed by LC-MS. The applied analytical method has allowed the determination of around 60 metabolites (in a total of 135 samples) belonging to different chemical classes (phenolic compounds, pentacyclic trirterpenes and tocopherols). Qualitative and quantitative differences were found among VOOs and oils produced using different dehydration temperatures (35, 55, 75 and 100 ºC) probably due to the inhibition of some enzymes (*e.g.* β-glucosidase and polyphenoloxidase) caused by the temperature increase or the absence of water during the processing. Thus, both the flours and the new oils presented considerable amounts of olive fruit metabolites that are usually absent from VOOs. In general, products obtained from fruits dehydrated at 100ºC were the richest samples in terms of most of the evaluated families of compounds.

Keywords: olive oil; olive-by products; phenolic compounds; triterpenic compounds; tocopherols; LC-MS.

1. INTRODUCTION

Olive tree (*Olea europaea L.*) is the sixth most relevant oil crop in the world. Currently, more than 11 million hectares of olive groves are cultivated in around 60 countries and its economic importance is unquestionable, with an estimated annual turnover of 9.5-13.5 million euros. Almost 90% of the olive fruits globally produced are processed to obtain virgin olive oil (VOO), while the remaining 10% is consumed as table olives [1,2].

Since ancient times, VOO production has been traditionally based on the entire olive fruits crushing followed by paste pressing and decantation to separate the oil. It was not until the 20th century when some technological developments took place, including the introduction of electric crushers and continuous malaxation and paste centrifugation systems [3]. Nevertheless, VOO is still produced using, essentially, the same principle implemented by Romans, which involves huge simultaneous waste generation (mainly fruit skin, pulp, seeds, pieces of stone and water). There are two main kinds of olive by-products: olive pomace (solid or semi-solid wastes) and mill wastewater (liquid effluents); their amount, composition and environmental impact depend on the extraction system of choice (*i.e.* two or three-phase systems) [4].

Over the last years, the interest in looking for a cost-efficient, technically feasible and environmentally sound solution for the residues generated from the VOO industry has drastically increased. Different management strategies for the recovery, recycling and upgrading of VOO by-products have been suggested (mainly using them as renewable fuel or fertilizers [5,6]). They have been also recognized as valuable sources of bioactive compounds [7,8] although the scaling up of the extraction processes to the industrial level has not been successfully achieved in many cases. In addition, new approaches involving the separate use of different olive fruit fractions (pulp and stones) [9] or new processing methods pursuing the reduction of waste generation, such as solvent extraction of the oil from dehydrated pulp [10], have been proposed. The latter does not only avoids the production of pomace and wastewater but also originates a multifunctional ingredient consisting of stoned, dehydrated and defatted olive pulp.

A promising alternative to this new methodology, replaces the solvent extraction step by cold pressing with a screw press to obtain olive oil and pulp pellet that can be converted into 'olive flour' by grinding [11,12]. This powder, which is expected to contain high levels of fiber and bioactive compounds (tocopherols, phenolic compounds and pentacyclic triterpenes, among others) [13,14], could fulfill the criteria to act as a potential ingredient in functional food. Moreover, the olive oil obtained in this way, could have higher content of health promoting phytochemicals due to two main facts: on the one hand, stoning of fruits prevents the contact of the oil with seed endogenous peroxidases that may catalyze biomolecules oxidation during the traditional processing [15,16]; and on the other hand, the removal of water from the pulp could avoid the loss of the most hydrophilic metabolites through migration to the vegetation water during malaxation [17]. Thus, the proposed methodology provides a way to achieve the full exploitation of olive fruits, which, at the same time, could overcome the waste generation issue and boost the economic outcomes of the olive grove. Firstly, the resulting olive oil may meet the increasing demand for high-quality oils (with the highest possible content of bioactive compounds) [18]; and secondly, the novel and, *a priori*, highly functional olive flour may represent a very worthwhile new output for the diversification of olive sector.

Carrying out the chemical characterization of both the olive oil and olive flour resulting from applying the described novel olive fruit processing methodology (stone removal, pulp dehydration and cold pressing) is essential to estimate its viability and to check the advantages that it could bring to the VOO industry. Moreover, some technological aspects such as the influence of the dehydration temperature in the obtained products must be evaluated. VOO composition has been extensively investigated and the modulation of its minor compounds has been achieved by studying the influence of different technical aspects related to its conventional production [19–21]. However, as far as we know, the oil obtained from stoned and dehydrated olive fruits has not been studied so far. In the same way, some stimulating reports have been published with regard to the composition of olive fruit [22–24], but, to the best of our knowledge, there is no single report including information about olive flour's composition. Thus, the main objective of the present work

has been to accomplish the comprehensive qualitative and quantitative characterization of olive oil and olive flour from 15 different cultivars obtained through the described novel methodology (performing the dehydration step at four different temperatures: 35, 55, 75 and 100 ºC). To this end, a total of 75 olive oil samples and 60 olive flour samples have been analyzed by applying a powerful LC-MS method capable of determining a wide number of molecules belonging to three different chemical classes (phenolic compounds, pentacyclic trirterpenes and tocopherols).

2. MATERIALS AND METHODS

2.1. Chemicals and standards

Absolute ethanol and LC-MS grade acetonitrile were purchased from Prolabo (Paris, France). Water was daily deionized with a Milli-Q system (Millipore, Bedford, MA, USA). Acetic acid together with pure standards of phenolic compounds (quinic, p-coumaric and ferulic acids, vanillin, hydroxytyrosol, tyrosol, oleuropein, rutin, luteolin, luteolin 7-O-glucoside, apigenin and pinoresinol), triterpenic compounds (maslinic, betulinic and oleanolic acids) and tocopherols (α-, β- and γ- tocopherols) were all supplied by Sigma-Aldrich (St. Louis, MO, USA). A stock solution containing all the standards was prepared in ethanol/water (80:20, v/v) and serially diluted to working concentrations over the range 0.1-500 mg/L.

2.2. Samples

Olive fruit samples from 15 different cultivars were kindly donated by Acer Campestres S.L. (Castillo de Locubín, Jaén, Spain). The varieties under study were: 'Arbequina', 'Brillante', 'Chorreao de Montefrío', 'Gordal', 'Hojiblanca', 'Lechín de Granada', 'Loaime de Alhama', 'Loaime de Tiena', 'Lucio', 'Manzanilla', 'Nevadillo de Alhama', 'Ombliguillo', 'Picual', 'Picual de Huétor Tájar' and 'Picudo'. Olive fruits were harvested in December 2015 and processed within six hours from the time they were gathered from the olive trees.

Fruits were firstly conditioned (washing and size-sorting) and subsequently, they were stoned by means of a pitting machine from the table olive industry. Thereupon, water removal from the pulp was conducted in a lab-scale dehydrator (Memmert, Schwabach, Germany) at four different temperatures (35, 55, 75 and 100ºC) for an average of 50, 18, 9 and 4.5 hours, respectively. The weight loss resulting from the dehydration process was found between 55 and 65%, depending on the variety. Afterwards, dry pulp was pressed with a screw press to obtain olive oil and defatted pulp separately. Finally, the obtained oils were filtered through a paper filter to remove solid particles and the stoned, dehydrated and defatted pulp was grinded to obtain 'olive flour'. Additionally, monovarietal VOOs from each cultivar were obtained in the traditional way (two-phase system). To do this, entire fresh fruits were processed with an Abencor® laboratory oil mill (MC2 Ingeniería y Sistemas, Seville, Spain) equipped with a hammer crusher, malaxer and centrifuge.

2.3. Sample treatment

The isolation of the minor compounds from the oils was achieved by applying the liquid-liquid extraction protocol described in a recent publication [25]. Briefly, 1 (± 0.01) g of olive oil were extracted three times with ethanol/water mixtures by vortex shaking followed by centrifugation to separate the aqueous phase from the oil. The first extraction step was done with 6 mL of ethanol/water (60:40, v/v) and the next two steps with 6 mL of ethanol/water (80:20, v/v). Olive flours were subjected to a homologous solid-liquid extraction procedure, using ultrasounds to assist the release of the targeted metabolites from the fruit tissues. Therefore, 0.25 (± 0.01) g of sample were extracted in an ultrasonic bath for 30 min in three consecutive steps with 10 mL of the same ethanol/water mixtures used for the oils. For both kinds of samples, the three supernatants were collected together and after solvent evaporation, the residue was redissolved in the adequate volume of ethanol/water (80:20, v/v) (1 mL for the olive oils and 5 mL for the flour samples). The prepared extracts were filtered through 0.22 µm nylon syringe filters from Agela Technologies (Wilmington, DE, USA) before their analysis.

2.4. LC-MS analysis

Olive oil and olive flour extracts were analyzed according to a previously reported LC-MS methodology [25] on an Agilent 1260 LC system (Agilent Technologies, Waldbronn, Germany) coupled to a Bruker Daltonics Esquire 2000™ ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) through an electrospray ionization source. A Zorbax Extend C18 column (4.6 × 100 mm, 1.8 µm particle size) (Agilent Technologies) was used for compound separation. The elution of the analytes was carried out at 40 °C with a mobile phase gradient of acidified water and acetonitrile (0.5% acetic acid) and a flow rate of 1 mL/min (increasing it at 1.5 mL/min during part of the run). MS spectra were acquired in full scan (50-1000 *m/z*), in negative ion mode from the beginning to min 22.5 and in positive polarity from that point until the end of the run.

3. RESULTS AND DISCUSSION

3.1. Qualitative characterization of oils and flours obtained from stoned and dehydrated olive fruits

The qualitative characterization of the samples under study was addressed in a first stage of this work. The applied LC-MS methodology allowed the determination of 57 metabolites belonging to three different chemical classes (phenolic compounds, pentacyclic triterpenes and tocopherols). Some examples of the typical chromatograms acquired for VOO, the olive oil obtained from stoned and dehydrated olives and its homologous olive flour, are shown in Fig. 1. It presents the extracted ion chromatograms of three 'Manzanilla' samples; in the example, 100ºC was the dehydration temperature applied for the samples obtained through the novel olive fruit

processing method. Even though it was an extreme temperature value, it led to samples exhibiting the highest content of a wide number of the compounds under study.

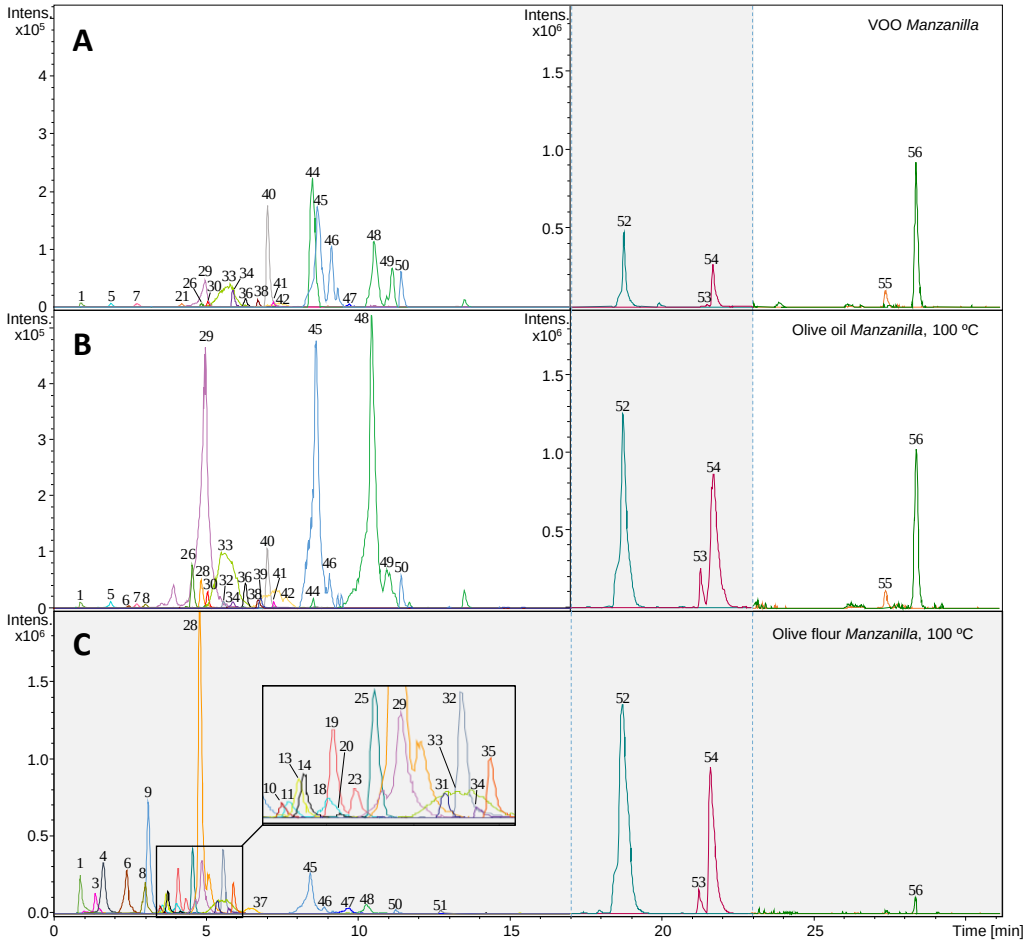

Figure 1. Extracted ion chromatograms of samples from 'Manzanilla' variety: (A) VOO obtained by the conventional two-phase system; and (B) olive oil and (C) flour obtained from stoned and dehydrated (at 100 ºC) olive fruits. Peak identification numbers as in Table 1. In order to facilitate the visual comparison of samples, chromatograms are shown at two different scales: 0-5.2×10^5 intensity units (white background), 0-2.0×10^6 intensity units (shaded background).

The determined peaks are listed in Table 1, which includes retention time (Rt), *m/z* of the *pseudo*-molecular ion, molecular formula of the assigned compound, name, chemical family and analytical standard used for its quantification. Peak identification was achieved by comparing relative Rts and *m/z* of the available pure standards, as well as using information from previous reports [22,26–28]. The last column of Table 1 indicates the type of matrix where each substance was detected. 45 and 37 compounds were determined in oils and flour samples, respectively. Just 25 out of the 57 total determined metabolites were found in both kind of matrices: quinic acid, hydroxytyrosol glucoside, acyclodihydroelenolic acid hexoside, a compound with *m/z* 389 (Rt 2.4

Table 1. Main metabolites found in olive oil and olive flour samples obtained by the novel fruit processing method involving stone removal, dehydration and cold pressing.

Peak number	Rt (min)	*m/z*	Molecular formula	Name	Chemical family	Standard for quantification	Matrix	
							Olive oil	*Olive flour*
1	0.9	191	$C_7H_{12}O_6$	Quinic acid	Organic acids	Quinic acid	x	x
2	1.0	151	$C_8H_8O_3$	Oxidized hydroxytyrosol	Simple phenols	Hydroxytyrosol	x	
3	1.4	315	$C_{14}H_{20}O_8$	Hydroxytyrosol glucoside	Simple phenols	Hydroxytyrosol	x	x
4	1.7	407	$C_{17}H_{28}O_{11}$	Acyclodihydroelenolic acid hexoside	Secoiridoids	Oleuropein	x	x
5	1.9	153	$C_8H_{10}O_3$	Hydroxytyrosol	Simple phenols	Hydroxytyrosol	x	
6	2.4	389	$C_{16}H_{22}O_{11}$	Oleoside/Secologanoside	Secoiridoids	Oleuropein	x	x
7	2.7	137	$C_8H_{10}O_2$	Tyrosol	Simple phenols	Tyrosol	x	
8	3.0	403	$C_{17}H_{24}O_{11}$	Elenolic acid glucoside	Secoiridoids	Oleuropein	x	x
9	3.1	377	$C_{16}H_{26}O_{10}$	Unknown 1	Unknown	Oleuropein		x
10	3.5	609	$C_{27}H_{30}O_{16}$	Rutin	Flavonoids	Rutin		x
11	3.6	701	$C_{31}H_{42}O_{18}$	Neo-nuzhenide	Secoiridoids	Oleuropein		x
12	3.7	163	$C_9H_8O_3$	*p*-coumaric acid	Phenolic acids and derivatives	*p*-coumaric acid	x	
13	3.7	555	$C_{25}H_{32}O_{14}$	Hydroxyoleuropein	Secoiridoids	Oleuropein		x
14	3.8	447	$C_{21}H_{20}O_{11}$	Luteolin 7-O-glucoside	Flavonoids	Luteolin 7-O-glucoside	x	x
15	3.9	151	$C_8H_8O_3$	Vanilllin	Phenolic acids and derivatives	Vanilllin	x	
16	4.0	193	$C_{10}H_{10}O_4$	Ferulic acid	Phenolic acids and derivatives	Ferulic acid	x	
17	4.0	623	$C_{29}H_{36}O_{15}$	Verbascoside	Secoiridoids	Oleuropein		x
18	4.1	701	$C_{31}H_{42}O_{18}$	Oleuropein glucoside	Secoiridoids	Oleuropein		x
19	4.1	551	$C_{25}H_{28}O_{14}$	Caffeoyl 6-oleoside	Secoiridoids	Oleuropein		x
20	4.2	447	$C_{21}H_{20}O_{11}$	Luteolin glucoside (isomer I)	Flavonoids	Luteolin 7-O-glucoside		x
21	4.3	225	$C_{11}H_{14}O_5$	Desoxy elenoic acid	Secoiridoids	Oleuropein	x	
22	4.3	257	$C_{11}H_{14}O_7$	Hydroxy elenolic acid	Secoiridoids	Oleuropein	x	
23	4.4	551	$C_{25}H_{28}O_{14}$	Caffeoyl 6-secologanoside	Secoiridoids	Oleuropein		x
24	4.4	447	$C_{21}H_{20}O_{11}$	Luteolin glucoside (isomer II)	Flavonoids	Luteolin 7-O-glucoside		x
25	4.6	535	$C_{25}H_{28}O_{13}$	Comselogoside	Secoiridoids	Oleuropein	x	x
26	4.6	195	$C_{10}H_{12}O_4$	Hydroxytyrosol acetate	Secoiridoids	Hydroxytyrosol	x	
27	4.7	381	$C_{19}H_{26}O_8$	Hydroxytyrosol acyclodihydroelenolate	Secoiridoids	Oleuropein	x	
28	4.8	539	$C_{25}H_{32}O_{13}$	Oleuropein	Secoiridoids	Oleuropein	x	x
29	5.0	241	$C_{11}H_{14}O_6$	Elenolic acid	Secoiridoids	Oleuropein	x	x
30	5.1	335	$C_{17}H_{20}O_7$	Hydroxydecarboxymethyl oleuropein aglycone	Secoiridoids	Oleuropein	x	

Peak number	Rt (min)	*m/z*	Molecular formula	Name	Chemical family	Standard for quantification	Matrix	
							Olive oil	*Olive flour*
31	5.4	583	$C_{27}H_{36}O_{14}$	Lucidumoside C	Secoiridoids	Oleuropein		x
32	5.6	523	$C_{25}H_{32}O_{12}$	Ligstroside	Secoiridoids	Oleuropein	x	x
33	5.7	319	$C_{17}H_{20}O_6$	Decarboxymethyl oleuropein aglycone (oleacein)	Secoiridoids	Oleuropein	x	x
34	5.8	285	$C_{15}H_{10}O_6$	Luteolin	Flavonoids	Luteolin	x	x
35	6.0	557	$C_{26}H_{38}O_{13}$	6-O-[(2E)-2,6-Dimethyl-8-hydroxy-2-octenoyloxy] secologanoside	Secoiridoids	Oleuropein		x
36	6.3	417	$C_{22}H_{26}O_8$	Syringaresinol	Lignans	Pinoresinol	x	
37	6.6	363	-	Unknown 2	Unknown	Oleuropein	x	x
38	6.7	357	$C_{20}H_{22}O_6$	Pinoresinol	Lignans	Pinoresinol	x	
39	6.8	393	$C_{19}H_{22}O_9$	10-hydroxy oleuropein aglycone	Secoiridoids	Oleuropein	x	
40	7.0	415	$C_{22}H_{24}O_8$	Acetoxypinoresinol	Lignans	Pinoresinol	x	
41	7.2	269	$C_{15}H_{10}O_5$	Apigenin	Flavonoids	Apigenin	x	
42	7.3	303	$C_{17}H_{20}O_5$	Decarboxymethyl ligstroside aglycone (oleocanthal)	Secoiridoids	Oleuropein	x	
43	7.6	299	$C_{16}H_{12}O_6$	Methyl luteolin	Flavonoids	Luteolin	x	
44	8.5	361	$C_{19}H_{22}O_7$	Ligstroside aglycone (isomer I)	Secoiridoids	Oleuropein	x	
45	8.6	377	$C_{19}H_{22}O_8$	Oleuropein aglycone (isomer I)	Secoiridoids	Oleuropein	x	x
46	9.2	377	$C_{19}H_{22}O_8$	Oleuropein aglycone isomer (isomer II)	Secoiridoids	Oleuropein	x	x
47	9.7	421	$C_{21}H_{26}O_9$	Unknown 3	Unknown	Oleuropein	x	x
48	10.5	361	$C_{19}H_{22}O_7$	Ligstroside aglycone (isomer II)	Secoiridoids	Oleuropein	x	x
49	11.1	361	$C_{19}H_{22}O_7$	Ligstroside aglycone (isomer III)	Secoiridoids	Oleuropein	x	
50	11.4	377	$C_{19}H_{22}O_8$	Oleuropein aglycone(isomer III)	Secoiridoids	Oleuropein	x	x
51	12.8	487	$C_{30}H_{48}O_5$	Monohydroxylated derivative of maslinic acid	Triterpenic compounds	Maslinic acid	x	x
52	18.6	471	$C_{30}H_{48}O_4$	Maslinic acid	Triterpenic compounds	Maslinic acid	x	x
53	21.3	455	$C_{30}H_{48}O_3$	Betulinic acid	Triterpenic compounds	Betulinic acid	x	x
54	21.5	455	$C_{30}H_{48}O_3$	Oleanolic acid	Triterpenic compounds	Oleanolic acid	x	x
55	27.4[a]	415[b]	$C_{28}H_{48}O_2$	β– and γ–tocopherols	Tocopherols	β– and γ–tocopherols	x	x
56	28.3	429[b]	$C_{29}H_{50}O_2$	α– tocopherols	Tocopherols	α– tocopherol	x	x

[a] Analytes coeluting in reverse-phase LC

[b] Analytes detected in positive polarity; *m/z* corresponding to $[M+H-H_2]^-$

min) which could correspond to either oleoside or secologanoside, elenolic acid and its glucoside, comselogoside, oleuropein, ligstroside, some isomers of oleuropein and ligstroside aglycones, decarboxymethyl oleuropein aglycone (also designated as oleacein), luteolin and luteolin 7-O-glucoside, four triterpenic compounds (maslinic, betulinic and oleanolic acids and a monohydroxylated derivative of maslinic acid), three tocopherols (α, β and γ-tocopherols) and two unknown compounds with *m/z* 363 (Rt 6.6 min) and 421 (Rt 9.7 min). Peak assignment could not been achieved for these two compounds, although the latter one had been already found by our research team in several 'Picudo' olive tree derived matrices (leaves, stems, seed, fruit skin and pulp)[27]. Its reported molecular formula (calculated from the exact mass measured with a QTOF MS analyzer) was $C_{21}H_{26}O_9$.

Although the just mentioned metabolites were found in both oils and flours, some of them were absent from specific samples, depending on the cultivar and processing conditions. For example, oleuropein, ligstroside and luteolin 7-O-glucoside were detected at very low concentrations in all the analyzed VOOs. This finding was in agreement with previous reports describing the presence of an endogenous enzyme so-called β-glucosidase in the olive fruit, that catalyzes the enzymatic hydrolysis of glucosidic bounds during the conventional oil extraction procedure [21,22]. As a result, glucosilated phenolic compounds (mainly secoiridoids and flavonoids), which usually appear in olive leaves and fruits, are just found in aglycone forms in VOO. However, these glucosidic forms were found in relative abundance in the oils obtained with the new olive fruit processing method that includes the dehydration step. This may be caused by the absence of water during the oil extraction, which could hinder β-glucosidase action to a certain extent. Nevertheless, the general trend was that aglycone forms were found at higher relative concentrations in all the oil samples (as it will be detailed below).

As far as simple phenols are concerned, while tyrosol, hydroxytyrosol and three derivatives (the oxidized, the glucosilated and the acetylated forms) were detected in the oils, only the glucosidic form of hydroxytyrosol was found in olive flours. Regarding flavonoids, besides luteolin and luteolin 7-O-glucoside, three glycosilated flavonoids (rutin and two luteolin glucoside isomers) were detected in dehydrated and deffated pulp, whilst two additional non-glycosilated flavonoids (apigenin and methyl luteolin) were found in the oils. Two phenolic acids (p-coumaric and ferulic) and an aldehyde (vanillin), as well as three lignans (syringaresinol, pinoresinol and acetoxypinoresinol) were determined in the oils, but none of them was found in olive flours. Apart from the previously mentioned secoiridoids, which were found in both kind of matrices, great differences were found between the rest of the members of this chemical family. As already exposed, VOO mainly presented aglycone forms: desoxy and hydroxy elenoic acid, hydroxytyrosol acyclodihydroelenolate, 10-hydroxy oleuropein aglycone, hydroxy decarboxymethyl oleuropein aglycone, decarboxymethyl ligstroside aglycone (also known as oleocanthal) and two extra ligstroside aglycone isomers. Conversely, olive flours were dominated by glycosylated secoiridoids. Those solely detected in defatted and grinded pulp samples were tentatively identified as neo-

nuzhenide, hydroxyoleuropein, verbascoside, oleuropein glucoside, caffeoyl 6-oleoside, caffeoyl 6-secologanoside, 6-O-[(2E)-2,6-dimethyl-8-hydroxy-2-octenoyloxy] secologanoside and lucidumoside C. Furthermore, another unknown peak with relatively high intensity was detected in olive flours at Rt 3.1 min (*m/z* 377). This analyte had been also reported in a previous publication, where $C_{16}H_{26}O_{10}$ was assigned as its calculated molecular formula [27].

3.2. Quantitative analysis of the new olive derived matrices

The quantitative analysis of all the samples under study was carried out in another stage of the project. All the metabolites described in section 3.1. were quantified in terms of their pure standard or on the basis of a compound presenting a related chemical structure, as indicated in Table 1. Quantification of unknown peaks was carried out in terms of oleuropein. It is important to bear in mind that the obtained quantitative data for the compounds lacking their corresponding pure standard are just an estimation of the real concentration, even though they are perfectly valid to compare the occurrence of those metabolites in the studied matrices.

Table 1 SM (Supplementary Materials) and Table 2 SM present the results of the quantitative analysis of 75 oil samples (VOO and oils obtained from stoned and dehydrated fruits at 35, 55, 75 and 100 ºC) and 60 olive flours (dehydrated at the same four temperatures), respectively. Apart from the clear differences found between samples obtained using different processing conditions, most of the analytes covered very wide ranges of concentration when samples obtained by applying the same settings were observed, pointing out a strong dependence with the olive cultivar from which they were produced. Table 2 provides an overview of the concentration ranges found for each chemical class in every kind of matrix (VOO, and oils and flours obtained using different dehydration temperatures). The given concentration values are the sum of all the metabolites belonging to each chemical family of compounds. Cultivars presenting the concentrations at the lower and upper ends of the range are also displayed below in the table. As clearly seen, not all the varieties were proportionally affected by the dehydration temperature, *i.e.* the cultivar presenting the highest concentration of a family of compounds in an oil obtained at a given temperature may not be the richest one at a different temperature or in the homologous flour. Nevertheless, some general trends can be inferred from the table. As far as "acids and derivatives" class is concerned, great variability was found in oil samples; Arbequina presented the lowest content of quinic acid in the flours obtained with three dehydration temperatures, and Picual de Huétor was the richest variety regardless of the processing conditions. In respect of simple phenols, Picudo and Picual de Huétor presented the lowest and highest concentrations in oil samples, at two and three dehydration temperatures, respectively; in the flours, Ombliguillo was the poorest variety at every tested temperature, whilst Lucio presented the highest content of simple phenols at 35, 75 and 100ºC. Arbequina stood out for its low content in terms of secoiridoids in oils and flours obtained at the four different tested dehydration temperatures, whereas Picual de Huétor and Ombliguillo (in oils) and Lechín and Gordal (in flours) were the richest cultivars (each one at two different temperatures). Concerning flavonoids, Loaime de Alhama and

Table 2. Establishment of the found concentration ranges of each chemical family in all the evaluated kinds of sample, from the 15 selected cultivars. For each type of sample, minimum and maximum levels are given together with the name of the variety presenting that value.

	Concentration ranges (mg/kg) in olive oils					Concentration ranges (mg/g) in olive flours			
Dehydration T (ºC)	**VOO**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**
Acids and derivatives	1.05 - 6.04 OM/PD	0.36 - 2.52 LA/OM	0.35 - 2.27 LE/BR	0.35 - 5.49 LE/HO	0.52 - 19.06 PI/LE	4.07 - 23.88 GO/PH	3.15 - 23.01 AR/PH	3.95 -22.98 AR/PH	6.48 - 26.19 AR/PH
Simple phenols	3.40 - 25.70 AR/PI	1.81 - 22.05 LA/PH	2.40 - 29.64 OM/PH	8.68 - 56.37 PD/AR	21.61 - 103.88 PD/PH	0.10 - 1.63 OM/LU	0.07 - 2.05 OM/HO	0.10 -3.06 OM/LU	0.12 - 3.19 OM/LU
Secoiridoids	18.72 - 203.80 AR/CH	23.10 - 257.03 PD/PH	8.91 - 306.92 GO/PH	18.44 - 672.80 PI/OM	45.23 - 1574.96 AR/OM	2.66 - 20.01 LT/LE	2.56 - 25.75 AR/LE	2.83 -33.12 AR/GO	3.08 - 27.91 PD/GO
Flavonoids	0.88 - 4.10 LA/LE	0.03 - 3.39 LA/HO	0.10 - 0.72 LA/NE	0.05 - 1.45 CH/HO	0.09 - 3.57 LU/LE	0.13 - 0.93 MA/LE	0.10 - 1.02 MA/HO	0.12 -1.01 MA/HO	0.12 - 0.78 MA/LE
Lignans	0.59 - 8.70 LE/MA	0.67 - 8.97 LE/AR	0.58 - 13.73 LE/AR	0.92 - 12.79 LE/PI	1.12 - 13.10 PD/AR	n.d.	n.d.	n.d.	n.d.
Triterpenic compounds	9.92 - 98.97 MA/HO	53.13 - 159.24 HO/PD	92.29 - 149.87 AR/NE	97.15 - 159.79 PD/NE	106.42 - 161.49 AR/NE	8.42 – 20.41 PI/OM	3.45 - 18.99 PH/OM	3.95 -20.51 PH/LA	5.45 - 20.38 PI/BR
Tocopherols	83.61 - 447.30 AR/LU	77.54 - 419.45 MA/LU	118.69 - 461.54 MA/LU	125.59 - 439.95 MA/PH	162.29 - 573.01 MA/LU	0.02 - 0.57 CH/PI	0.02 - 0.59 CH/PI	0.01 -0.75 PD/PI	0.05 - 0.41 PD/PH
Unknown	0.22 - 2.63 LE/CH	0.20 - 5.39 AR/BR	<0.01 - 1.77 AR/OM	<0.01 - 1.21 PI/OM	0.10 - 1.29 AR/BR	0.62 - 2.84 PH/BR	0.52 - 1.95 AR/OM	0.35 -1.44 AR/LA	0.38 - 1.51 LT/GO

AR, Arbequina; BR, Brillante; CH, Chorreao; GO, Gordal; HO, Hojiblanca; LE, Lechín; LA, Loaime de Alhama; LT, Loaime de Tiena; LU, Lucio; MA, Manzanilla; NE, Nevadillo; OM, Ombliguillo; PI, Picual; PH, Picual de Huétor; PD, Picudo; n.d., non detected.

Manzanilla were among the poorest varieties in oils and flours, respectively; Lechín and Hojiblanca presented the highest flavonoids' content in flour matrices. With regard to lignans, which were just determined in oil samples, a typical feature of Lechín variety was its low contents; while in contrast, Arbequina presented the highest concentrations at three diverse temperatures. With respect to triterpenic compounds, it is worth mentioning that the lowest average content was found in oils from Hojiblanca (at 35ºC), Arbequina (obtained at 55 and 100ºC) and Picudo (75ºC) varieties and Picual and Picual de Huétor flours obtained at two temperatures each; in contrast, Nevadillo and Ombliguillo were pointed out among the richest cultivars in three oils and two flours, respectively. Manzanilla and Lucio stood out for their low and high tocopherols' content in oils, apiece; Chorreao and Picudo were the poorest varieties in terms of tocopherols in the flours, whilst Picual was one of the richest cultivars. In general terms, Arbequina presented reduced amounts of the unknowns peaks in both kind of matrices (oils and flours), whereas Ombliguillo and Brillante could be underlined among the richest cultivars.

Fig. 1 SM illustrates the described differences among samples obtained from different olive varieties using the same processing conditions. Sum concentrations of all the metabolites belonging to each chemical class are displayed at the same scale in the Y axis to facilitate the visual comparison of the bars.

3.3. Evaluation of the impact of the dehydration temperature in the obtained olive oils and flours

Once the exhaustive characterization of the previously unexplored matrices obtained by the new olive fruit processing method was carried out, the influence of the dehydration temperature on the composition of the new products was thoroughly evaluated. The high number of analytes determined in the 135 evaluated samples made difficult the visualization and trends assessment in the obtained quantitative data. Thus, average concentrations for the determined compounds in each kind of matrix (VOO extracted in the conventional way, as well as oils and flours produced through the novel methodology, using four different dehydration temperatures) were calculated in order to facilitate the inspection of the data and the finding of common tendencies in all the samples obtained in the same way. Nevertheless, these mean values should be taken cautiously, bearing in mind the differences among cultivars and the wide concentration ranges for each chemical family established in section 3.2.

Table 3 includes the calculated mean values for each metabolite in the 15 tested cultivars, together with the sum concentrations (global concentration levels) of the analytes belonging to each chemical family, and Fig. 2 depicts the general trends followed by each family of compounds in oils and flours as a function of the dehydration temperature (including VOO obtained in the traditional way).

As shown in Fig. 2 (I-V), in general, the higher the selected dehydration temperature, the greater the phenolic compounds content in both the oils and flours obtained through the novel

Table 3. Average concentrations for the oils and flours obtained at each temperature (or processing method, in the case of VOO) from the 15 evaluated olive varieties.

	Olive oils' mean values (mg/kg)						Olive flours' mean values (mg/g)			
Dehydration T (ºC)	**VOO**	**35**	**55**	**75**	**100**	**Dehydration T (ºC)**	**35**	**55**	**75**	**100**
Quinic acid	0.99	0.40	0.36	0.60	2.74	Quinic acid	13.06	13.13	13.99	15.30
p-coumaric acid	0.22	0.11	0.18	0.24	0.19					
Vanillin	0.41	0.29	0.29	0.36	0.37					
Ferulic acid	0.28	0.13	0.14	0.14	0.13					
Total acids and derivatives	*1.91*	*0.93*	*0.97*	*1.34*	*3.42*	*Total acids and derivatives*	*13.06*	*13.13*	*13.99*	*15.30*
Oxidized HTY	0.15	0.11	0.09	0.13	0.14	HTY glucoside	0.69	0.85	1.02	1.08
HTY glucoside	0.36	0.20	0.24	0.60	1.82					
HTY	6.31	1.87	2.16	3.74	6.34					
Tyrosol	6.58	3.07	5.08	6.05	8.60					
HTY acetate	1.21	1.47	4.48	10.17	25.93					
Total simple phenols	*14.59*	*6.72*	*12.05*	*20.69*	*42.83*	*Total Simple phenols*	*0.69*	*0.85*	*1.02*	*1.08*
Acyclodihydro EA hexoside	0.07	0.04	0.03	0.04	0.33	Acyclodihydro EA hexoside	0.37	0.41	0.37	0.32
Oleoside/secologanoside	0.15	0.66	0.62	0.41	1.25	Oleoside/secologanoside	0.62	0.67	0.72	0.68
EA glucoside	0.14	0.20	0.30	0.41	0.65	EA glucoside	0.79	0.84	0.75	0.45
Desoxy EA	0.66	0.05	0.01	0.22	0.36	Neo-nuzhenide	0.02	0.02	0.03	0.02
Comselogoside	0.01	0.01	0.02	0.03	0.09	Hydroxyoleuropein	0.16	0.30	0.14	0.08
HTY acyclodihydroelenolate	1.33	0.32	0.36	0.57	0.27	Verbascoside	0.04	0.06	0.10	0.13
Oleuropein	0.02	0.37	0.59	1.87	9.37	Oleuropein glucoside	0.06	0.06	0.07	0.07
EA	5.11	8.38	12.12	19.20	46.92	Caffeoyl 6-oleoside	0.16	0.18	0.20	0.16
Hydroxy EA	0.03	0.05	0.03	0.03	0.06	Caffeoyl 6-secologanoside	0.03	0.03	0.05	0.03
Hydroxy oleacein	1.45	0.61	0.61	1.02	1.38	Comselogoside	0.19	0.18	0.20	0.20
Ligstroside	<0.01	0.04	0.06	0.10	0.26	Oleuropein	4.89	6.18	12.28	10.50
Oleacein	19.01	11.76	14.10	29.53	37.77	EA	0.09	0.09	0.15	0.30
10-hydroxy OleAgly	1.16	0.59	0.42	0.49	1.72	Lucidumoside C	0.70	0.61	0.12	0.06
Oleocanthal	1.84	5.25	5.81	8.16	7.10	Ligstroside	0.16	0.29	0.40	0.32
OleAgly (isomers I+II+III)	36.71	23.36	32.77	60.21	226.35	Oleacein	0.11	0.14	0.32	0.39
LigAgly (isomers I+II+III)	52.13	33.72	35.03	44.56	132.76	6-O-[...] secologanoside	0.06	0.05	0.06	0.06
						OleAgly (isomers I+II+III)	0.19	0.15	0.32	0.74
						LigAgly (isomer II)	0.03	0.04	0.04	0.09
Total secoiridoids	*119.82*	*85.39*	*102.86*	*166.85*	*466.65*	*Total secoiridoids*	*8.66*	*10.30*	*16.33*	*14.63*
Luteolin 7-O-glucoside	0.01	0.01	0.02	0.10	0.25	Rutin	0.24	0.21	0.23	0.21
Luteolin	1.50	0.38	0.17	0.20	0.14	Luteolin 7-O-glucoside	0.18	0.18	0.19	0.19
Apigenin	0.44	0.11	0.07	0.09	0.11	Luteolin glucoside (is. I)	0.03	0.04	0.03	0.03
Methyl luteolin	0.17	0.07	0.04	0.06	0.06	Luteolin glucoside (is. II)	0.01	0.01	0.01	0.01
						Luteolin	0.06	0.06	0.06	0.06
Total flavonoids	*2.12*	*0.58*	*0.31*	*0.45*	*0.56*	*Total flavonoids*	*0.53*	*0.51*	*0.51*	*0.49*
Syringaresinol	0.42	0.50	0.53	0.70	1.00					
Pinoresinol	0.43	0.66	0.66	0.75	1.04					
Acetoxypinoresinol	1.99	2.15	2.25	2.10	2.48					
Total lignans	*2.84*	*3.30*	*3.44*	*3.56*	*4.53*	*Total lignans*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
Hydroxy maslinic acid	0.09	0.24	0.26	0.25	0.26	Hydroxy maslinic acid	0.02	0.02	0.02	0.02
Maslinic acid	15.14	64.88	71.03	72.69	75.01	Maslinic acid	9.77	9.79	9.82	10.69
Betulinic acid	0.81	1.11	1.59	2.65	3.26	Betulinic acid	0.04	0.03	0.04	0.04
Oleanolic acid	9.99	38.06	48.18	56.80	62.98	Oleanolic acid	3.83	3.74	3.68	3.92
Total triterpenic acids	*26.03*	*104.29*	*121.07*	*132.39*	*141.51*	*Total triterpenic acids*	*13.66*	*13.58*	*13.55*	*14.67*
β+γ-tocopherols	21.42	23.07	24.64	25.04	25.71	*β+γ*-tocopherols	0.01	0.01	0.01	<0.01
α-tocopherol	214.44	228.39	242.54	287.83	323.37	*α*-tocopherol	0.19	0.17	0.20	0.16
Total tocopherols	*235.86*	*251.46*	*267.18*	*312.87*	*349.08*	*Total tocopherols*	*0.20*	*0.18*	*0.21*	*0.16*
Unknown 2	0.42	0.82	0.51	0.42	0.43	Unknown 1	0.49	0.54	0.62	0.64
Unknown 3	1.11	0.87	0.33	0.22	0.18	Unknown 2	0.71	0.28	0.11	0.05
						Unknown 3	0.49	0.26	0.12	0.06
Total unknown	*1.53*	*1.68*	*0.83*	*0.63*	*0.61*	*Total unknown*	*1.68*	*1.08*	*0.85*	*0.75*
Overall concentration	***404.7***	***454.3***	***508.7***	***638.8***	***1009.2***	***Overall concentration***	***38.47***	***39.63***	***46.45***	***47.08***

"acids and derivatives" class includes organic acids, phenolic acids and aldehydes in oil samples.

HTY: hydroxytyrosol; EA: elenolic acid; OleAgly: oleuropein aglycone; LigAgly: ligstroside aglycone; 6-O-[...] secologanoside: 6-O-[(2E)-2,6-Dimethyl-8-hydroxy-2-octenoyloxy] secologanoside; n.d., non detected.

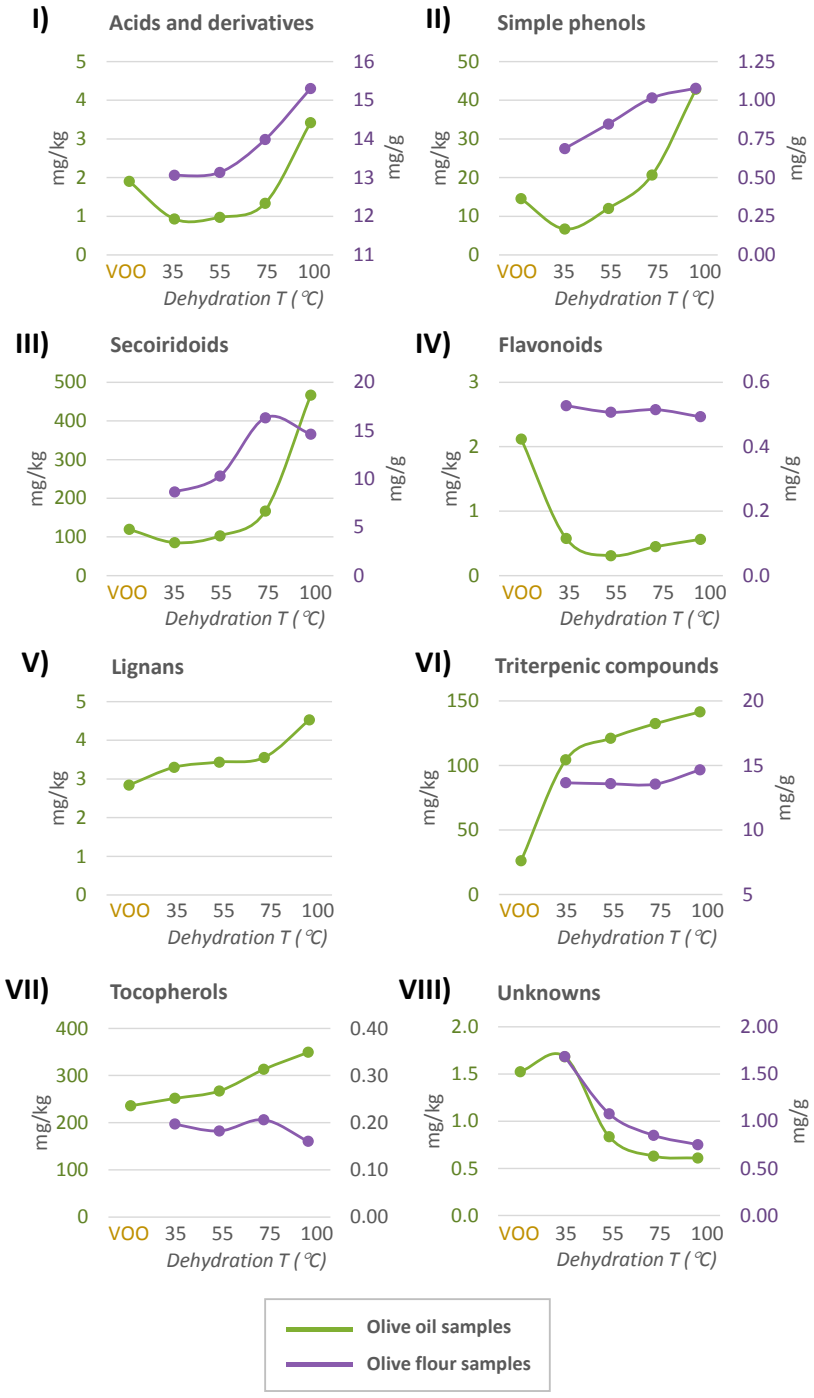

Figure 2. Average concentrations for each family of compounds in every kind of matrix (from the 15 evaluated varieties). Green lines (left axis) correspond to the VOO samples and the oils obtained at each tested dehydration temperature (sum concentrations expressed in mg/kg). Purple lines (right axis) correspond to the four kind of olive flours produced by the novel methodology (sum concentrations expressed in mg/g).

methodology. This finding suggest that the degradative enzyme polyphenoloxidase may be inhibited by the temperature increase, as described in literature [29]. However, trends variations were found among some of the evaluated phenolic subfamilies. In addition, the comparison of the new oils with the VOO obtained in the traditional way, also revealed different responses to the new process depending on the type of phenolic compound.

In order to reduce the number of graphs in Fig. 2 and despite the chemical disparity, the sole representative of organic acids (quinic acid) was grouped together with phenolic acids and aldehydes (in oil samples; they were not found in flours) in the "acids and derivatives" family, as in Table 2. The sum concentrations of this "miscellaneous category" showed an upward trend with increasing dehydration temperature in the oils obtained through the novel methodology, and the same trend was observed for quinic acid in the flours. VOOs presented higher mean contents of "acids and derivatives" than the oils obtained from fruits dehydrated at 35, 55 and 75 ºC. The richest oils in terms of this family of compounds were those obtained using 100ºC as dehydration temperature (this result can be explained considering the much higher relative concentration of quinic acid at this temperature). Nevertheless, *p*-coumaric and ferulic acids, as well as vanillin concentrations were generally higher in VOOs.

A similar general trend was found for simple phenols in both kind of matrices. The concentration of hydroxytyrosol glucoside grew with increasing temperatures in the flours, following the same trend as the five simple phenols determined in the oils. Moreover, for all the members of this chemical class except for hydroxytyrosol acetate, the concentrations found in VOOs were higher than in the new oils produced using 35, 55 and 75 ºC as dehydration temperatures. However, when the temperature was set at 100ºC, similar or even higher concentrations than in VOOs were achieved. Compared with VOOs, the oils obtained from stoned and dehydrated olive fruits (especially at 100ºC) stood out for their notably high content of hydroxytyrosol acetate. This fact is very remarkable, since this simple phenol has an antioxidant capacity similar to that of hydroxytyrosol but presents higher lipophilicity, which may facilitate membrane crossing and cell uptake, and thus, enhanced bioavailability [30,31].

As far as secoiridoids are concerned, the general ascending trend with increasing dehydration temperature was more severe in the oils. The concentration in VOOs of seven secoiridoids (oleoside/secologanoside, comselogoside, elenolic acid and its glucoside, oleuropein, ligstroside and oleacein) was always lower than in the oils obtained by means of the novel methodology. VOOs average content of the other ten secoiridoids (acyclodihydroelenolic acid hexoside, oleacein, oleocanthal, 10-hydroxy oleuropein aglycone, and the sum of all the oleuropein and ligstroside aglycone isomers (3 isomeric forms in each case)) was slightly higher than the concentration levels of the new oils obtained from fruits dehydrated at low and moderate temperatures, but lower than in the oils resulting from fruits dehydrated at 100ºC. Just three secoiridoids (desoxy elenolic acid, hydroxytyrosol acyclodihydroelenolate and hydroxy oleacein) were always more concentrated in VOOs than in the new oils. In olive flour samples, the highest average total secoiridoids content

was found when using 75ºC as dehydration temperature, followed by 100ºC and 55ºC, respectively. This trend was mostly influenced by the high relative content of oleuropein (around 12.3, 10.5 and 6.2 mg/g, at 75, 100 and 50ºC, correspondingly). Great variability was found for the less abundant secoiridoids; for example, 35ºC was the most favorable temperature to obtain flours rich in lucidumoside C, 55ºC for elenolic acid glucoside, and 100ºC for oleuropein aglycone.

Flavonoids, which were among the scarcest determined families, were the most adversely affected by the new processing method. Their content drastically decreased in the oils obtained from stoned and dehydrated fruits (in around a 75%) compared to the VOOs, excluding luteolin 7-O-glucoside, which was almost absent from VOOs and increased its levels with the dehydration temperature. The minimum total flavonoids average concentration in the oils was found for those produced using 55ºC as dehydration temperature. In the case of the flours, flavonoids were the second less abundant family (after tocopherols) and followed a slight downward trend with increasing temperatures.

The opposite trend to the one observed for flavonoids was monitored for lignans. They were systematically found at higher concentrations in the oils produced through the novel methodology and their content augmented as the dehydration temperature increased. As mentioned in section 3.2, they were not found in olive flours.

Triterpenic substances represented one of the most abundant chemical families in both the novel olive oils and flours. They were found at higher concentrations in oils obtained from stoned and dehydrated fruits than in VOOs, and their content grew with temperature increments. Their content in the flours remained almost unaffected by the temperature, although the highest average concentration of the two main triterpenic acids (maslinic and oleanolic) was found in flours obtained at 100ºC.

Tocopherols also showed an ascending tendency in oils, what suggests an improved transfer from the olive cells to the oil at higher temperatures. Their presence in flours was very low and their content did not follow any clear pattern depending on the thermal dehydration conditions. The lowest average tocopherols concentration was found in flours obtained at 100 ºC, although the value (0.16 mg/g) was quite similar to the concentration found at 55ºC (0.18 mg/g).

Lastly, the unknown compounds, generally decreased in oils and flours produced at higher temperatures of dehydration. As a matter of fact, the richest oils in terms of those compounds achieved by applying the new procedure were the ones obtained from olive fruits dehydrated at 35ºC (with concentration levels of 1.68 mg/kg).

4. CONCLUSIONS

Over the last years, the public environmental concern has encouraged researchers to look for industrial processes that follow the “zero waste” philosophy. In this context, a novel methodology

for olive fruit processing, has been proposed as an alternative to the traditional VOO extraction systems. It involves fruit stoning, pulp dehydration and cold press, and generates two new products: olive oil with a distinctive metabolic profile (compared to the conventional VOO) and olive flour, a powder of defatted pulp with a high content in biomolecules usually found in fresh olive fruits. The comprehensive qualitative and quantitative characterization of these new matrices has been addressed by applying a powerful LC-MS method that has allowed the determination of 57 metabolites in total, belonging to different chemical classes: organic acids, phenolic compounds (phenolic acids and aldehydes, simple phenols, secoiridoids, flavonoids and lignans), triterpenic compounds and tocopherols. 135 samples from 15 olive cultivars have been analyzed, including VOOs and the new olive oils and flours obtained at four different dehydration temperatures (35, 55, 75 and 100ºC). Concentration ranges for the determined metabolites were established for the first time in the previously unexplored matrices and the effect of the dehydration temperature in the composition of the resulting products was studied in depth. In general terms, all the evaluated chemical families were found at higher concentration levels in samples produced from fruits dehydrated at 100ºC. The oils obtained in these conditions were also richer than the conventional VOO in terms of most of the determined metabolites except for phenolic acids and aldehydes, three minor secoiridoids and the aglycone flavonoids.

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Figure 1 SM. Sum concentrations of all the metabolites belonging to each chemical family in every evaluated kind of sample, from the 15 evaluated cultivars.

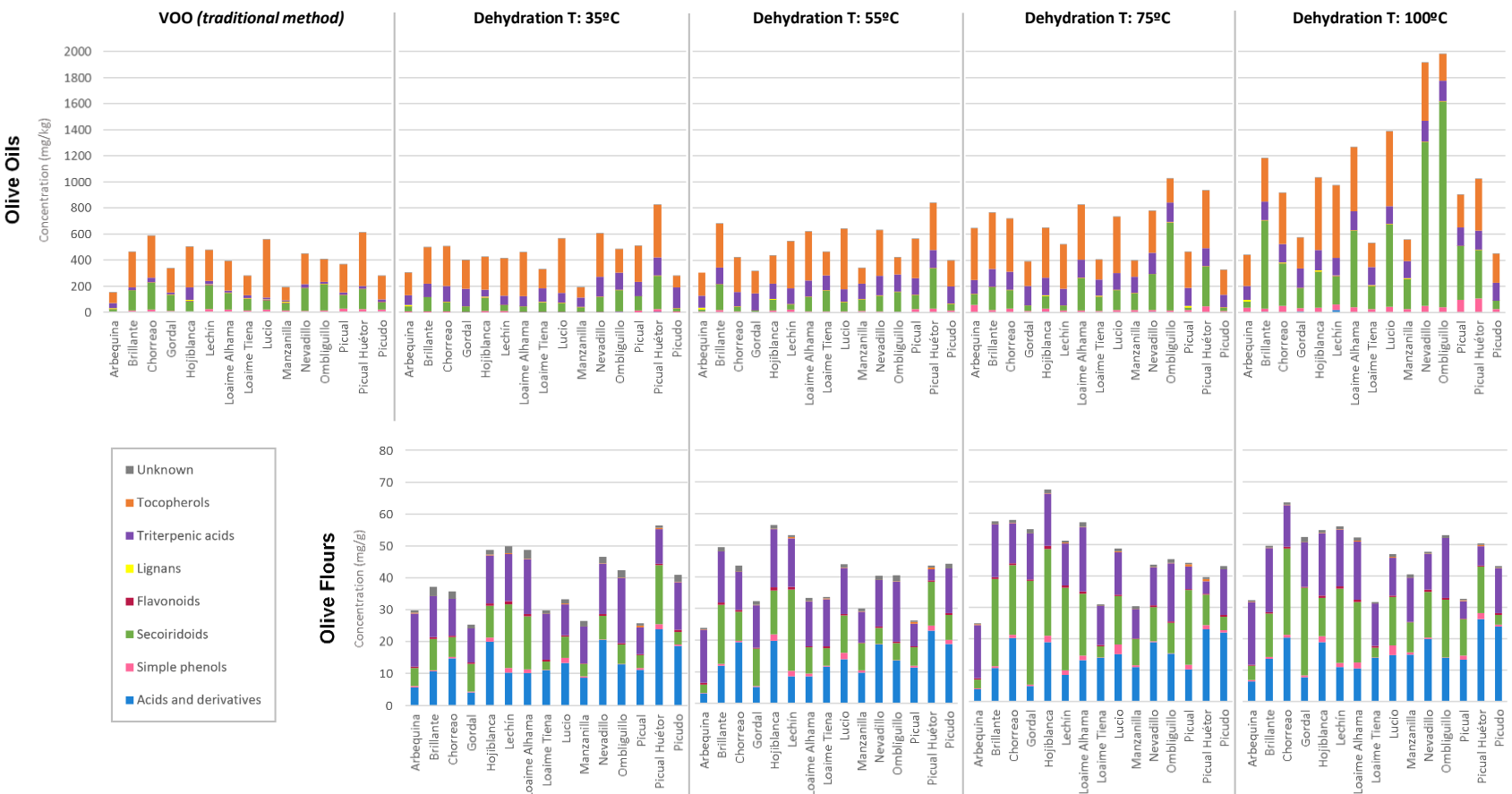

Table 1 SM. Quantitative data obtained for the oils evaluated in this study (VOO and oils produced from stoned and dehydrated olive fruits). Every result (expressed in mg/kg of olive oil) is the mean value of two independent replicates. %RSD values were lower than 9.8% in every case.

	Arbequina					Brillante					Chorreao					Gordal				
Dehydration T (°C)	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**
Quinic acid	0.45	0.01	0.12	0.09	0.07	0.44	0.10	1.80	0.46	4.08	0.69	0.14	0.08	0.01	0.18	0.37	0.14	0.04	0.10	8.68
p-coumaric acid	0.13	0.09	0.07	0.07	0.14	0.11	0.03	0.17	0.09	<0.01	0.10	0.09	0.08	0.13	0.23	0.66	0.08	0.20	0.26	0.26
Vanillin	0.58	0.60	0.56	0.35	0.54	0.53	0.15	0.16	0.24	0.13	0.37	0.36	0.33	0.27	0.48	0.45	0.21	0.25	0.26	0.52
Ferulic acid	0.19	0.11	0.13	0.06	0.04	0.15	0.13	0.15	0.14	0.17	0.15	0.09	0.10	0.10	0.11	0.57	0.14	0.17	0.13	0.07
Total acids and derivatives	*1.36*	*0.82*	*0.88*	*0.58*	*0.78*	*1.23*	*0.41*	*2.27*	*0.92*	*4.39*	*1.32*	*0.68*	*0.59*	*0.51*	*1.00*	*2.05*	*0.58*	*0.66*	*0.75*	*9.52*
Oxidized HTY	0.22	0.14	<0.01	<0.01	0.11	0.01	0.13	0.17	<0.01	<0.01	0.28	0.27	0.36	0.19	0.20	0.01	0.01	0.05	0.01	0.18
HTY glucoside	0.15	<0.01	0.13	0.17	<0.01	0.15	0.15	0.95	0.29	1.37	0.18	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	1.41
HTY	1.01	1.61	1.24	3.94	3.33	5.48	1.72	3.06	2.19	5.07	9.72	2.20	1.68	7.70	5.78	4.20	0.93	0.84	1.86	2.81
Tyrosol	1.70	2.58	1.77	4.97	3.56	4.89	2.44	3.99	3.23	6.23	10.11	4.32	4.98	14.89	14.94	3.48	2.02	2.19	3.27	3.33
HTY acetate	0.32	1.88	2.23	47.30	27.93	0.87	0.91	6.58	13.32	10.62	0.15	0.82	0.17	4.55	28.28	1.08	0.18	1.45	5.89	16.07
Total simple phenols	*3.40*	*6.21*	*5.37*	*56.37*	*34.93*	*11.41*	*5.35*	*14.76*	*19.03*	*23.30*	*20.45*	*7.61*	*7.20*	*27.34*	*49.19*	*8.76*	*3.13*	*4.52*	*11.03*	*23.80*
Acyclodihydro EA hexoside	0.10	<0.01	0.05	0.02	0.05	0.03	0.02	0.18	0.06	0.34	0.09	0.01	<0.01	<0.01	<0.01	0.03	0.01	<0.01	0.03	0.33
Oleoside/secologanoside	0.12	0.25	0.30	0.01	0.67	0.13	0.70	1.03	0.60	0.81	0.14	0.40	0.25	0.10	0.15	<0.01	0.25	0.27	0.14	2.03
EA glucoside	0.67	0.19	0.45	0.13	0.29	0.07	0.08	0.95	0.50	0.97	0.18	0.21	0.28	0.23	<0.01	0.03	0.19	0.21	0.28	0.32
Desoxy EA	0.33	<0.01	<0.01	0.30	0.03	1.42	0.01	0.02	0.82	0.62	1.00	0.06	0.02	0.53	0.30	0.21	0.01	0.01	0.01	0.06
Comselogoside	<0.01	<0.01	0.01	0.01	0.07	<0.01	0.04	0.14	0.05	0.15	<0.01	<0.01	<0.01	0.04	0.15	<0.01	<0.01	<0.01	<0.01	0.04
HTY acyclodihydroelenolate	0.10	0.11	0.14	1.09	0.12	0.94	0.15	0.33	0.33	0.21	0.69	0.40	0.38	0.85	0.41	1.08	0.10	0.10	0.20	0.25
Oleuropein	<0.01	0.16	0.13	0.82	0.03	0.08	0.25	4.45	3.00	9.25	0.03	0.19	0.10	0.61	0.98	<0.01	0.13	0.08	1.03	34.76
EA	0.83	2.55	1.28	8.51	12.17	8.36	14.42	20.06	19.54	63.31	2.25	5.91	3.21	7.37	19.29	4.42	4.91	1.21	8.87	20.57
Hydroxy elenolic acid	<0.01	<0.01	<0.01	0.03	0.05	0.06	0.08	0.07	0.04	0.11	<0.01	<0.01	<0.01	0.02	0.02	0.01	0.02	0.02	0.08	0.08
Hydroxy oleacein	0.41	0.46	0.20	0.32	0.98	1.09	0.63	1.91	1.17	2.45	0.66	0.17	0.05	0.71	0.76	2.12	0.73	0.12	0.54	0.55
Ligstroside	<0.01	0.03	0.02	0.12	0.02	<0.01	<0.01	0.25	0.22	0.42	<0.01	0.02	0.09	0.14	0.12	<0.01	<0.01	<0.01	0.22	<0.01
Oleacein	9.65	14.52	6.81	5.45	22.77	28.52	26.19	68.24	63.31	113.09	5.96	7.58	4.93	32.95	35.01	48.12	7.52	2.21	3.98	12.14
10-hydroxy OleAgly	0.06	0.06	<0.01	0.50	0.02	0.38	0.28	0.21	0.23	0.62	1.88	0.12	0.01	0.11	0.28	0.66	0.36	0.12	0.41	0.49
Oleocanthal	1.67	3.77	1.24	0.76	3.71	2.89	11.99	15.29	13.50	15.44	1.21	5.58	4.87	11.07	10.95	2.98	1.30	0.87	0.98	1.93
OleAgly (isomers I+II+III)	3.66	10.35	2.36	46.28	3.09	48.85	19.35	52.25	38.02	344.97	84.73	15.59	4.41	31.69	137.52	24.15	13.55	1.10	15.14	63.45
LigAgly (isomers I+II+III)	1.11	5.89	1.10	17.58	1.18	63.44	35.14	33.64	34.31	123.20	104.97	31.78	16.75	54.43	119.59	38.31	12.29	2.58	8.79	15.72
Total secoiridoids	*18.72*	*38.35*	*14.09*	*81.93*	*45.23*	*156.26*	*109.35*	*199.02*	*175.70*	*675.96*	*203.80*	*68.01*	*35.35*	*140.86*	*325.53*	*122.13*	*41.39*	*8.91*	*40.69*	*152.73*
Luteolin 7-O-glucoside	0.02	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	0.22	0.05	0.31	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.23
Luteolin	1.24	0.27	0.25	0.06	0.15	1.02	0.06	0.09	0.01	0.09	1.05	0.07	0.11	0.01	0.03	1.99	0.06	0.18	0.19	0.05
Apigenin	0.30	0.10	0.12	0.06	0.09	0.45	0.07	0.05	0.06	0.09	0.17	0.06	0.03	0.03	0.04	0.59	0.03	0.05	0.05	0.04
Methyl luteolin	0.52	0.22	0.23	0.04	0.23	0.04	<0.01	<0.01	<0.01	<0.01	0.07	0.01	<0.01	<0.01	0.06	0.10	0.02	0.01	0.02	0.01
Total flavonoids	*2.07*	*0.59*	*0.62*	*0.16*	*0.47*	*1.51*	*0.14*	*0.36*	*0.12*	*0.50*	*1.29*	*0.13*	*0.14*	*0.05*	*0.14*	*2.68*	*0.12*	*0.24*	*0.27*	*0.32*
Syringaresinol	0.40	0.93	1.08	0.30	1.76	0.32	0.60	0.68	0.61	1.04	0.39	0.50	0.50	0.54	0.85	0.75	0.66	0.80	2.02	1.27
Pinoresinol	0.87	0.90	1.30	0.73	1.17	0.31	0.33	0.40	0.41	0.98	0.25	0.43	0.41	0.48	0.63	0.20	0.27	0.26	0.41	0.47
Acetoxypinoresinol	4.31	7.14	11.36	0.33	10.17	0.03	0.04	0.33	0.07	0.02	2.60	2.75	3.57	3.41	3.55	0.07	0.42	0.08	0.08	0.05
Total lignans	*5.58*	*8.97*	*13.73*	*1.36*	*13.10*	*0.65*	*0.97*	*1.42*	*1.09*	*2.04*	*3.24*	*3.68*	*4.48*	*4.43*	*5.04*	*1.02*	*1.34*	*1.14*	*2.52*	*1.79*
Hydroxy maslinic acid	0.58	0.74	0.67	0.06	0.55	0.03	0.20	0.25	0.21	0.30	0.12	0.01	0.17	0.16	0.21	0.02	0.22	0.16	0.24	0.21
Maslinic acid	19.11	36.48	42.01	48.91	47.13	11.65	69.05	75.31	73.79	74.26	18.60	73.23	69.06	74.78	80.86	9.52	79.93	77.23	72.57	79.18
Betulinic acid	0.25	1.04	1.60	2.33	2.83	0.08	0.67	1.37	1.76	4.96	0.17	1.36	0.52	2.25	2.76	0.08	1.82	1.23	4.06	3.67
Oleanolic acid	18.39	39.00	48.00	55.23	55.90	7.23	33.04	49.11	59.82	64.35	15.70	46.19	38.91	60.20	60.57	6.14	51.18	50.01	68.51	66.48
Total triterpenic acids	*38.32*	*77.25*	*92.29*	*106.53*	*106.42*	*18.98*	*102.95*	*126.04*	*135.57*	*143.87*	*34.58*	*120.78*	*108.66*	*137.38*	*144.40*	*15.76*	*133.15*	*128.62*	*145.37*	*149.54*
β+*γ*-tocopherols	6.12	7.22	7.50	45.91	7.46	20.09	20.09	23.27	27.16	21.15	19.47	14.16	14.16	20.06	17.70	25.40	26.81	24.69	25.04	26.10
α-tocopherol	77.49	163.59	168.66	352.96	231.22	254.05	257.43	314.07	406.22	310.69	303.00	289.00	252.00	388.00	374.00	157.67	194.02	146.68	163.59	210.08
Total tocopherols	*83.61*	*170.80*	*176.16*	*398.87*	*238.68*	*274.14*	*277.52*	*337.34*	*433.38*	*331.84*	*322.47*	*303.16*	*266.16*	*408.06*	*391.70*	*183.06*	*220.83*	*171.37*	*188.63*	*236.19*
Unknown 2	0.05	0.17	<0.01	0.07	0.09	0.16	2.40	0.72	0.38	0.80	1.11	0.16	1.06	0.58	0.48	0.36	0.49	0.59	0.24	0.36
Unknown 3	2.10	0.03	<0.01	0.13	0.01	0.76	2.99	0.82	0.29	0.49	1.52	0.41	0.19	<0.01	0.25	0.90	0.43	0.09	0.04	0.04
Total unknown	*2.15*	*0.20*	*<0.01*	*0.20*	*0.10*	*0.92*	*5.39*	*1.55*	*0.67*	*1.29*	*2.63*	*0.57*	*1.26*	*0.58*	*0.73*	*1.26*	*0.92*	*0.68*	*0.27*	*0.40*
TOTAL	***155.22***	***303.19***	***303.14***	***646.00***	***439.70***	***465.10***	***502.09***	***682.76***	***766.48***	***1183.2***	***589.77***	***504.62***	***423.82***	***719.21***	***917.72***	***336.71***	***401.46***	***316.15***	***389.53***	***574.29***

	Hojiblanca					Lechín					Loaime Alhama					Loaime Tiena				
Dehydration T (°C)	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**
Quinic acid	1.06	0.38	0.05	4.95	1.86	1.03	0.91	0.12	0.06	18.66	1.01	0.08	0.41	1.35	0.54	0.63	0.14	0.04	0.06	0.19
p-coumaric acid	0.23	0.18	0.16	0.12	0.16	0.19	0.07	0.07	0.07	0.06	0.07	<0.01	0.12	0.14	0.12	0.24	0.14	0.37	0.33	0.29
Vanillin	0.19	0.23	0.17	0.33	0.21	0.34	0.14	0.10	0.15	0.31	0.62	0.19	0.27	0.21	0.10	0.33	0.39	0.29	0.40	0.20
Ferulic acid	0.13	0.18	0.19	0.09	0.18	0.10	0.08	0.06	0.07	0.04	0.79	0.08	0.09	0.08	0.09	0.26	0.16	0.22	0.20	0.16
Total acids and derivatives	*1.61*	*0.97*	*0.57*	*5.49*	*2.41*	*1.65*	*1.20*	*0.35*	*0.35*	*19.06*	*2.49*	*0.36*	*0.88*	*1.78*	*0.85*	*1.45*	*0.83*	*0.93*	*0.99*	*0.83*
Oxidized HTY	0.03	0.03	<0.01	0.01	0.03	<0.01	0.14	0.01	0.03	0.10	0.18	0.05	0.10	0.17	0.01	0.32	0.13	0.03	0.17	0.37
HTY glucoside	0.51	0.16	<0.01	3.35	0.79	0.59	1.22	0.43	0.63	19.36	0.91	<0.01	0.92	1.33	1.16	0.16	<0.01	<0.01	0.60	<0.01
HTY	1.67	4.18	3.20	3.13	6.63	8.40	1.65	2.81	1.68	6.32	11.02	0.72	1.99	3.62	7.66	5.45	0.83	1.49	1.73	2.05
Tyrosol	2.59	3.36	4.16	3.11	5.36	13.37	3.32	6.48	3.28	7.55	3.71	1.02	2.40	2.90	5.19	7.18	2.31	6.27	5.76	6.71
HTY acetate	1.39	2.62	8.29	15.35	19.85	1.82	3.72	12.78	8.19	9.76	1.01	0.01	0.29	3.27	25.14	0.48	0.26	0.12	0.89	16.93
Total simple phenols	*6.19*	*10.35*	*15.65*	*24.95*	*32.65*	*24.19*	*10.05*	*22.50*	*13.80*	*43.10*	*16.83*	*1.81*	*5.71*	*11.29*	*39.16*	*13.60*	*3.53*	*7.92*	*9.15*	*26.06*
Acyclodihydro EA hexoside	0.04	<0.01	<0.01	0.17	0.06	0.08	0.20	0.05	0.01	3.69	0.07	0.01	0.03	0.09	0.03	0.09	0.01	<0.01	0.01	0.04
Oleoside/secologanoside	0.65	0.71	0.74	0.02	0.58	0.57	0.32	0.72	1.15	9.85	0.21	0.92	1.16	0.07	0.51	0.05	0.90	0.70	0.73	0.35
EA glucoside	0.15	0.06	0.08	1.86	0.22	0.04	0.13	0.35	0.23	6.32	0.21	0.22	0.43	0.90	0.16	0.10	0.09	0.12	0.12	0.17
Desoxy EA	0.05	0.49	<0.01	0.10	0.01	0.45	0.01	<0.01	<0.01	<0.01	0.24	<0.01	0.02	0.19	0.28	0.10	<0.01	0.01	0.01	0.02
Comselogoside	<0.01	<0.01	<0.01	0.05	0.02	0.01	<0.01	<0.01	<0.01	0.63	0.05	<0.01	0.05	0.16	0.05	<0.01	<0.01	<0.01	<0.01	<0.01
HTY acyclodihydroelenolate	0.62	1.00	0.01	0.43	0.05	3.54	0.34	0.42	0.31	0.55	0.35	0.05	0.20	0.40	0.02	1.08	0.24	1.05	0.63	0.46
Oleuropein	0.03	<0.01	0.16	12.89	1.47	0.01	1.76	0.77	0.62	81.78	0.05	0.09	1.07	5.26	3.18	0.06	0.09	0.16	0.13	0.24
EA	8.02	11.10	11.43	10.99	99.26	0.90	1.67	2.26	6.68	23.02	5.59	4.81	17.41	43.53	86.72	4.93	9.75	19.10	19.71	37.08
Hydroxy elenolic acid	0.07	0.06	0.07	0.05	0.15	0.01	0.01	0.02	<0.01	0.19	0.08	0.09	0.05	0.08	0.19	0.07	0.01	0.01	<0.01	0.01
Hydroxy oleacein	2.20	2.76	2.01	0.70	1.65	5.94	0.90	0.72	0.50	1.09	0.82	0.36	0.65	1.84	2.17	0.17	0.14	0.23	0.26	0.32
Ligstroside	<0.01	<0.01	0.03	0.34	0.05	<0.01	0.14	0.16	0.14	2.84	<0.01	0.01	0.03	0.06	0.04	<0.01	<0.01	0.04	<0.01	0.04
Oleacein	21.11	32.62	14.42	15.60	46.47	65.78	19.07	10.76	13.96	20.65	24.13	7.19	21.33	42.68	40.32	4.49	4.10	9.99	9.60	9.72
10-hydroxy OleAgly	1.38	2.15	1.05	0.48	1.82	1.88	0.15	0.16	0.11	0.32	0.56	0.33	0.34	0.72	3.77	0.25	0.14	0.44	0.19	0.50
Oleocanthal	2.41	2.39	1.75	1.44	2.64	5.01	3.14	2.07	2.74	3.16	1.34	1.50	4.11	4.83	2.27	1.44	4.90	6.65	6.91	6.26
OleAgly (isomers I+II+III)	25.08	12.68	37.47	30.49	75.77	56.08	10.25	9.37	5.72	44.38	57.06	15.15	38.22	98.80	298.61	9.94	8.74	34.97	21.58	23.66
LigAgly (isomers I+II+III)	16.61	31.47	11.85	17.45	47.39	42.81	6.97	11.65	6.59	17.51	38.14	12.87	26.81	49.22	149.60	65.30	41.93	84.66	50.98	96.19
Total secoiridoids	*78.43*	*97.47*	*81.08*	*93.06*	*277.60*	*183.10*	*45.06*	*39.47*	*38.77*	*215.97*	*128.89*	*43.60*	*111.90*	*248.82*	*587.93*	*88.07*	*71.05*	*158.11*	*110.85*	*175.05*
Luteolin 7-O-glucoside	0.02	<0.01	<0.01	1.25	0.05	0.01	0.08	0.01	<0.01	2.91	<0.01	<0.01	0.01	0.01	0.02	0.01	<0.01	<0.01	<0.01	0.01
Luteolin	0.69	2.67	0.26	0.12	0.31	3.27	0.21	0.12	0.25	0.52	0.71	0.01	0.06	0.11	0.01	1.75	0.63	0.36	0.63	0.09
Apigenin	0.29	0.50	0.11	0.08	0.18	0.42	0.07	0.03	0.07	0.07	0.13	0.01	0.02	0.05	0.09	0.53	0.21	0.09	0.18	0.16
Methyl luteolin	0.09	0.21	0.03	<0.01	0.05	0.40	0.06	0.03	0.13	0.07	0.04	<0.01	0.01	<0.01	<0.01	0.35	0.23	0.10	0.17	0.20
Total flavonoids	*1.09*	*3.39*	*0.40*	*1.45*	*0.59*	*4.10*	*0.41*	*0.18*	*0.44*	*3.57*	*0.88*	*0.03*	*0.10*	*0.17*	*0.11*	*2.63*	*1.07*	*0.54*	*0.98*	*0.45*
Syringaresinol	1.01	0.59	1.05	0.91	2.39	0.22	0.35	0.32	0.41	0.88	0.36	0.18	0.32	0.36	0.52	0.44	0.72	0.46	0.68	0.85
Pinoresinol	0.50	0.60	0.62	0.44	1.56	0.22	0.24	0.16	0.37	0.71	0.14	0.23	0.39	0.80	0.59	0.42	0.88	0.50	0.89	1.11
Acetoxypinoresinol	4.54	6.76	4.69	3.65	5.75	0.14	0.08	0.11	0.14	0.19	0.84	0.50	0.89	0.88	1.16	3.91	4.55	3.43	4.00	5.48
Total lignans	*6.05*	*7.95*	*6.35*	*4.99*	*9.69*	*0.59*	*0.67*	*0.58*	*0.92*	*1.78*	*1.34*	*0.91*	*1.60*	*2.04*	*2.27*	*4.78*	*6.16*	*4.39*	*5.57*	*7.44*
Hydroxy maslinic acid	0.33	0.12	0.45	0.34	0.40	0.09	0.55	0.62	0.55	0.59	0.01	0.18	0.15	0.17	0.16	0.02	0.17	0.11	0.15	0.19
Maslinic acid	61.13	23.91	71.10	80.92	84.20	16.79	36.61	70.35	70.89	71.80	10.12	48.52	74.65	73.79	81.81	12.44	61.41	66.81	71.13	73.54
Betulinic acid	10.66	0.43	1.42	1.37	3.32	0.09	0.40	0.72	1.50	3.01	0.07	0.56	1.38	6.83	3.01	0.17	0.92	0.97	1.42	2.11
Oleanolic acid	26.85	28.67	42.58	52.74	65.80	11.07	32.93	48.17	53.90	60.42	5.45	28.40	48.97	58.64	60.26	8.52	37.64	42.01	53.15	62.83
Total triterpenic acids	*98.97*	*53.13*	*115.55*	*135.36*	*153.71*	*28.03*	*70.49*	*119.86*	*126.83*	*135.82*	*15.65*	*77.66*	*125.15*	*139.44*	*145.23*	*21.16*	*100.14*	*109.91*	*125.85*	*138.67*
β+γ-tocopherols	27.87	27.87	29.64	29.99	36.71	23.27	32.12	38.13	34.24	47.68	16.20	19.74	20.80	20.80	22.57	13.02	12.66	13.02	11.96	11.96
α-tocopherol	281.10	224.46	186.41	351.27	520.35	212.62	254.05	324.21	306.46	504.29	209.24	315.76	353.80	398.61	467.93	134.00	134.84	170.35	137.38	171.20
Total tocopherols	*308.97*	*252.33*	*216.05*	*381.26*	*557.06*	*235.89*	*286.16*	*362.34*	*340.70*	*551.96*	*225.44*	*335.50*	*374.60*	*419.41*	*490.50*	*147.01*	*147.51*	*183.37*	*149.34*	*183.15*
Unknown 2	0.25	0.27	0.43	0.33	0.44	0.12	0.65	0.30	0.23	0.39	0.12	0.39	0.49	0.37	0.12	0.69	0.35	0.27	0.76	0.50
Unknown 3	1.00	1.03	0.34	0.12	0.08	0.10	0.23	0.10	0.08	0.12	1.98	0.57	0.39	0.19	0.13	0.89	0.65	0.28	0.13	0.04
Total unknown	*1.25*	*1.29*	*0.78*	*0.45*	*0.52*	*0.22*	*0.88*	*0.39*	*0.32*	*0.50*	*2.10*	*0.96*	*0.88*	*0.56*	*0.25*	*1.58*	*1.00*	*0.56*	*0.89*	*0.54*
TOTAL	***502.55***	***426.89***	***436.43***	***647.01***	***1034.2***	***477.78***	***414.93***	***545.68***	***522.14***	***971.78***	***393.62***	***460.82***	***620.82***	***823.52***	***1266.3***	***280.28***	***331.29***	***465.73***	***403.62***	***532.19***

	Lucio					Manzanilla					Nevadillo					Ombliguillo				
Dehydration T (°C)	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**
Quinic acid	0.45	0.06	0.44	0.59	0.65	1.41	0.45	0.63	0.47	1.71	0.48	0.03	0.02	0.02	0.63	0.39	2.01	0.62	0.30	0.25
p-coumaric acid	0.21	0.11	0.20	0.12	0.18	0.32	0.07	0.11	0.10	0.12	0.56	0.31	0.72	1.69	0.45	0.10	0.09	0.12	0.20	0.41
Vanillin	0.15	0.23	0.25	0.23	0.21	0.38	0.12	0.16	0.09	0.30	0.42	0.69	0.79	0.97	0.87	0.39	0.33	0.42	0.57	0.94
Ferulic acid	0.31	0.14	0.20	0.09	<0.01	0.45	0.11	0.14	0.19	0.15	0.28	0.17	0.19	0.35	0.31	0.17	0.10	0.10	0.15	0.16
Total acids and derivatives	*1.13*	*0.53*	*1.10*	*1.03*	*1.04*	*2.56*	*0.75*	*1.05*	*0.86*	*2.29*	*1.73*	*1.20*	*1.72*	*3.03*	*2.26*	*1.05*	*2.52*	*1.26*	*1.21*	*1.76*
Oxidized HTY	0.40	0.08	0.06	0.19	0.20	0.14	<0.01	0.09	0.13	0.30	0.13	0.01	0.10	0.19	0.20	0.09	0.16	0.02	0.40	0.18
HTY glucoside	0.37	<0.01	0.43	0.79	0.51	0.38	0.73	0.32	0.95	0.75	0.11	<0.01	<0.01	0.29	0.85	0.14	0.18	<0.01	<0.01	<0.01
HTY	11.42	1.19	2.43	5.57	6.22	1.71	0.31	1.18	2.38	2.92	3.28	0.83	0.93	4.48	8.51	5.70	0.73	0.76	4.60	9.01
Tyrosol	7.61	1.77	5.33	8.17	7.09	6.65	1.67	6.55	6.92	6.90	3.92	2.23	3.91	8.94	14.33	3.66	2.35	1.59	5.68	10.73
HTY acetate	1.03	0.05	0.07	3.34	26.51	1.59	0.47	3.64	7.37	12.47	0.72	0.01	0.12	2.35	22.57	1.55	0.10	0.02	3.68	19.13
Total simple phenols	*20.83*	*3.09*	*8.32*	*18.07*	*40.52*	*10.46*	*3.18*	*11.77*	*17.75*	*23.34*	*8.16*	*3.07*	*5.06*	*16.25*	*46.47*	*11.14*	*3.51*	*2.40*	*14.36*	*39.06*
Acyclodihydro EA hexoside	0.02	<0.01	0.02	0.05	0.04	0.11	0.03	0.05	<0.01	0.16	0.04	<0.01	<0.01	<0.01	0.07	0.03	0.18	0.06	0.03	0.01
Oleoside/secologanoside	0.01	0.71	0.48	0.33	0.17	0.04	1.05	0.72	1.08	0.73	0.08	1.72	1.21	0.80	1.31	0.03	0.65	0.76	0.31	0.67
EA glucoside	0.06	0.17	0.31	0.38	0.22	0.08	0.31	0.42	0.30	0.37	0.10	0.08	0.09	0.14	0.16	0.07	0.68	0.22	0.17	0.17
Desoxy EA	0.16	<0.01	<0.01	0.04	0.02	0.28	<0.01	0.01	0.04	0.09	1.97	0.01	0.01	0.87	1.98	0.84	<0.01	<0.01	0.37	0.63
Comselogoside	0.01	<0.01	0.02	0.02	0.03	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.06	0.08	0.01	0.03	0.01	<0.01	0.02
HTY acyclodihydroelenolate	0.54	0.01	0.11	0.81	0.14	0.30	<0.01	0.02	0.20	0.08	0.38	0.12	0.02	0.45	0.29	0.29	<0.01	0.03	0.34	0.03
Oleuropein	0.04	0.10	0.55	1.92	1.22	0.02	0.32	0.55	0.60	1.84	0.01	0.07	0.09	0.13	1.29	0.01	0.31	0.25	0.69	0.83
EA	5.81	11.17	12.18	22.44	62.46	3.09	7.68	16.12	31.89	69.20	8.61	7.38	10.90	19.81	60.52	11.67	17.78	33.64	53.53	60.53
Hydroxy elenolic acid	0.01	0.06	0.03	<0.01	<0.01	0.01	0.07	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.09	0.18	0.05	0.03	0.11
Hydroxy oleacein	0.44	0.36	0.39	0.88	1.20	0.33	0.15	0.75	1.18	0.95	0.51	0.38	0.45	3.89	5.13	0.49	0.14	0.09	1.96	2.27
Ligstroside	<0.01	<0.01	0.05	0.09	0.05	<0.01	0.02	0.10	0.02	0.10	<0.01	<0.01	0.02	<0.01	0.06	<0.01	0.21	0.08	0.04	0.05
Oleacein	5.87	6.71	8.84	15.44	19.93	8.42	2.96	13.00	25.19	23.96	14.65	14.05	20.61	121.78	120.40	10.88	8.61	6.38	63.70	71.26
10-hydroxy OleAgly	1.15	0.60	0.45	0.89	3.00	0.38	0.20	0.53	0.47	0.92	0.42	0.11	0.14	0.26	1.74	0.48	0.29	0.33	1.08	3.97
Oleocanthal	0.50	1.52	1.59	1.94	1.62	1.27	2.66	4.75	7.79	7.82	1.65	23.27	27.20	47.56	32.24	1.48	10.37	9.39	17.24	10.50
OleAgly (isomers I+II+III)	29.16	25.37	25.22	74.56	465.34	26.64	3.38	21.19	25.00	55.97	33.49	12.63	8.72	20.91	547.98	39.00	19.40	13.00	329.03	926.48
LigAgly (isomers I+II+III)	26.90	21.35	19.70	31.28	79.88	17.56	14.94	29.88	31.90	66.14	110.91	52.04	52.59	56.90	483.18	138.05	105.75	87.92	204.29	497.43
Total secoiridoids	*70.66*	*68.13*	*69.93*	*151.07*	*635.33*	*58.54*	*33.76*	*88.09*	*125.66*	*228.34*	*172.81*	*111.86*	*122.03*	*273.55*	*1256.44*	*203.42*	*164.56*	*152.21*	*672.80*	*1574.96*
Luteolin 7-O-glucoside	<0.01	<0.01	0.01	0.02	0.01	0.01	0.01	<0.01	0.02	0.03	0.02	<0.01	<0.01	<0.01	0.04	<0.01	0.07	0.02	0.05	0.04
Luteolin	1.85	0.09	0.09	0.01	0.02	0.91	0.11	0.10	0.12	0.02	2.62	0.53	0.42	0.50	0.16	1.02	0.30	0.18	0.16	0.07
Apigenin	0.45	0.05	0.03	0.03	0.05	0.46	0.05	0.02	0.04	0.05	1.02	0.17	0.21	0.23	0.13	0.35	0.06	0.06	0.08	0.05
Methyl luteolin	0.23	0.08	0.03	0.02	0.01	0.09	0.01	<0.01	0.03	0.01	0.06	0.08	0.09	0.07	0.06	0.08	<0.01	0.05	0.04	0.03
Total flavonoids	*2.53*	*0.22*	*0.16*	*0.08*	*0.09*	*1.47*	*0.17*	*0.13*	*0.21*	*0.10*	*3.72*	*0.79*	*0.72*	*0.79*	*0.39*	*1.46*	*0.44*	*0.31*	*0.32*	*0.19*
Syringaresinol	0.28	0.49	0.48	0.65	0.85	0.83	<0.01	<0.01	<0.01	1.99	0.25	0.55	0.63	0.87	0.87	0.15	0.36	0.42	0.59	0.46
Pinoresinol	0.23	0.30	0.28	0.36	0.48	0.48	0.61	0.45	0.56	0.89	0.35	0.77	0.64	1.00	1.17	0.33	0.79	0.93	1.00	1.43
Acetoxypinoresinol	2.36	1.79	1.95	2.32	2.77	7.39	4.00	3.21	2.97	4.16	0.30	0.28	0.27	0.30	0.29	1.86	2.00	2.70	2.23	2.50
Total lignans	*2.87*	*2.59*	*2.71*	*3.34*	*4.11*	*8.70*	*4.61*	*3.66*	*3.53*	*7.04*	*0.90*	*1.60*	*1.54*	*2.17*	*2.33*	*2.35*	*3.14*	*4.05*	*3.82*	*4.39*
Hydroxy maslinic acid	0.03	0.16	0.25	0.28	0.28	<0.01	0.08	0.19	0.17	0.16	0.01	0.27	0.19	0.21	0.22	0.02	0.32	0.36	0.37	0.27
Maslinic acid	7.95	45.06	62.49	67.54	69.92	6.89	46.18	71.53	66.15	73.37	13.71	89.81	83.24	85.61	87.35	9.17	78.82	73.13	77.39	79.64
Betulinic acid	0.07	0.53	0.99	2.28	2.55	0.03	0.43	1.06	2.05	2.09	0.12	2.16	5.86	4.53	4.06	0.06	1.56	1.81	2.78	3.62
Oleanolic acid	5.65	27.02	32.81	56.50	60.40	3.01	23.52	43.76	56.36	56.06	14.40	59.87	60.58	69.44	69.86	4.92	50.30	56.29	67.68	72.58
Total triterpenic acids	*13.70*	*72.77*	*96.54*	*126.60*	*133.14*	*9.92*	*70.21*	*116.53*	*124.73*	*131.68*	*28.24*	*152.11*	*149.87*	*159.79*	*161.49*	*14.16*	*131.00*	*131.59*	*148.23*	*156.11*
β+γ-tocopherols	25.40	26.46	28.22	23.63	28.22	8.42	9.48	10.90	10.19	10.54	19.38	26.81	26.81	24.33	27.87	19.03	23.63	19.74	22.57	20.09
α-tocopherol	421.90	392.99	433.32	409.73	544.78	89.19	68.06	107.79	115.40	151.75	213.47	307.31	322.52	297.16	418.06	155.13	155.13	108.64	163.59	183.88
Total tocopherols	*447.30*	*419.45*	*461.54*	*433.36*	*573.01*	*97.61*	*77.54*	*118.69*	*125.59*	*162.29*	*232.85*	*334.12*	*349.33*	*321.50*	*445.93*	*174.16*	*178.76*	*128.37*	*186.15*	*203.97*
Unknown 2	0.41	0.79	0.43	0.33	0.18	0.21	0.83	0.01	0.73	0.64	1.46	1.56	0.96	0.82	0.78	0.52	2.38	1.32	0.45	0.55
Unknown 3	0.89	0.92	0.38	0.21	0.28	1.03	0.86	0.36	0.47	0.19	1.13	0.59	0.32	0.12	0.20	1.37	2.03	0.45	0.76	0.18
Total unknown	*1.30*	*1.70*	*0.81*	*0.54*	*0.46*	*1.23*	*1.69*	*0.37*	*1.20*	*0.82*	*2.59*	*2.15*	*1.29*	*0.94*	*0.98*	*1.89*	*4.41*	*1.77*	*1.21*	*0.73*
TOTAL	***560.32***	***568.47***	***641.11***	***734.08***	***1387.7***	***190.50***	***191.91***	***340.28***	***399.54***	***555.91***	***451.00***	***606.90***	***631.55***	***778.02***	***1916.3***	***409.63***	***488.34***	***421.96***	***1028.1***	***1981.2***

	Picual					Picual Huétor					Picudo				
Dehydration T (ºC)	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**	**VOO**	**35**	**55**	**75**	**100**
Quinic acid	0.50	0.54	0.26	0.18	<0.01	0.49	1.02	0.60	0.15	1.61	5.50	<0.01	0.11	0.25	1.94
p-coumaric acid	0.10	0.10	0.09	0.08	0.11	0.21	0.17	0.14	0.14	0.15	0.08	0.08	0.12	0.08	0.11
Vanillin	0.56	0.27	0.26	0.62	0.27	0.66	0.22	0.18	0.19	0.19	0.25	0.28	0.18	0.43	0.26
Ferulic acid	0.26	0.13	0.08	0.13	0.14	0.16	0.10	0.10	0.08	0.23	0.21	0.20	0.21	0.21	0.13
Total acids and derivatives	*1.42*	*1.04*	*0.69*	*1.01*	*0.52*	*1.52*	*1.50*	*1.02*	*0.56*	*2.18*	*6.04*	*0.56*	*0.62*	*0.97*	*2.45*
Oxidized HTY	0.08	0.30	0.08	0.12	0.19	0.12	0.17	0.13	0.17	0.06	0.19	<0.01	0.06	0.17	0.01
HTY glucoside	0.28	0.22	0.13	0.28	<0.01	0.15	0.26	0.19	0.12	0.60	1.26	0.11	0.11	0.15	0.48
HTY	12.19	3.18	2.88	2.63	11.72	10.78	7.48	6.21	8.71	14.88	2.56	0.42	1.74	1.84	2.18
Tyrosol	11.29	6.27	6.69	3.84	14.61	7.48	6.05	7.43	9.39	13.18	10.99	4.43	12.49	6.44	9.36
HTY acetate	1.85	2.76	15.69	9.51	68.91	2.95	8.10	15.68	27.44	75.15	1.29	0.13	0.12	0.07	9.58
Total simple phenols	*25.70*	*12.74*	*25.46*	*16.39*	*95.43*	*21.47*	*22.05*	*29.64*	*45.82*	*103.88*	*16.30*	*5.09*	*14.52*	*8.68*	*21.61*
Acyclodihydro EA hexoside	0.04	0.07	0.03	0.10	<0.01	0.01	0.04	0.01	<0.01	0.07	0.30	<0.01	<0.01	<0.01	0.09
Oleoside/secologanoside	0.01	0.15	0.01	0.51	0.06	0.10	0.20	0.07	0.11	0.15	0.07	0.90	0.84	0.13	0.77
EA glucoside	0.03	0.25	0.16	0.53	0.08	0.06	0.22	0.15	0.19	0.19	0.32	0.14	0.21	0.15	0.14
Desoxy EA	0.93	<0.01	0.02	0.01	0.68	1.73	0.07	<0.01	<0.01	0.61	0.25	<0.01	<0.01	<0.01	0.01
Comselogoside	<0.01	0.01	<0.01	0.04	<0.01	<0.01	0.01	<0.01	0.02	0.01	0.02	0.01	<0.01	<0.01	0.16
HTY acyclodihydroelenolate	3.45	0.31	0.50	0.22	0.79	6.18	1.77	1.89	2.06	0.56	0.41	0.13	0.14	0.16	0.05
Oleuropein	<0.01	0.10	0.13	0.09	0.32	<0.01	1.93	0.30	0.32	3.10	<0.01	0.01	0.11	0.02	0.20
EA	4.60	8.50	5.97	2.76	57.91	5.83	13.99	18.45	25.42	30.53	1.66	4.11	8.55	6.99	1.31
Hydroxy elenolic acid	<0.01	0.01	<0.01	0.01	0.01	<0.01	0.15	0.05	0.04	0.01	0.01	0.03	0.02	0.02	0.05
Hydroxy oleacein	0.30	0.37	0.09	0.26	0.27	6.07	1.43	1.18	0.89	0.42	0.17	0.17	0.29	0.25	0.51
Ligstroside	0.01	<0.01	0.01	0.05	0.06	<0.01	0.15	<0.01	0.02	0.11	<0.01	<0.01	0.03	0.01	<0.01
Oleacein	11.98	7.35	3.49	9.37	11.12	21.25	15.37	13.24	13.76	4.87	4.36	2.52	7.19	6.23	14.82
10-hydroxy OleAgly	0.23	0.41	0.29	<0.01	2.50	7.27	3.54	2.11	1.79	5.67	0.39	0.08	0.12	0.09	0.10
Oleocanthal	1.21	1.78	1.11	2.35	1.08	1.28	1.73	1.40	0.97	0.40	1.25	2.84	4.92	2.35	6.50
OleAgly (isomers I+II+III)	44.14	49.81	43.12	1.41	193.99	59.18	131.80	193.62	161.82	197.47	9.53	2.39	6.49	2.75	16.57
LigAgly (isomers I+II+III)	37.04	39.01	50.90	0.73	144.53	44.75	84.61	74.45	96.81	126.82	35.99	9.77	21.02	7.16	22.99
Total secoiridoids	*103.98*	*108.13*	*105.82*	*18.44*	*413.39*	*153.71*	*257.03*	*306.92*	*304.20*	*371.00*	*54.73*	*23.10*	*49.93*	*26.30*	*64.27*
Luteolin 7-O-glucoside	<0.01	0.01	<0.01	0.03	<0.01	0.01	0.02	<0.01	<0.01	0.03	0.01	<0.01	<0.01	<0.01	0.04
Luteolin	1.38	0.24	0.08	0.18	0.06	1.08	0.08	0.18	0.20	0.12	1.89	0.31	0.14	0.48	0.48
Apigenin	0.28	0.04	0.04	0.08	0.03	0.66	0.04	0.07	0.06	0.37	0.53	0.24	0.13	0.31	0.21
Methyl luteolin	0.11	0.04	<0.01	0.26	<0.01	0.09	0.01	0.04	0.09	0.02	0.25	0.13	0.04	<0.01	0.16
Total flavonoids	*1.77*	*0.32*	*0.12*	*0.56*	*0.09*	*1.84*	*0.14*	*0.29*	*0.35*	*0.55*	*2.69*	*0.68*	*0.32*	*0.79*	*0.90*
Syringaresinol	0.41	0.34	0.24	1.18	0.38	<0.01	0.47	0.38	0.47	0.95	0.49	0.75	0.54	0.90	<0.01
Pinoresinol	1.09	1.64	1.35	1.18	1.29	0.55	1.08	1.56	1.79	2.00	0.47	0.77	0.58	0.88	1.11
Acetoxypinoresinol	1.08	0.75	0.34	10.42	0.31	0.26	0.80	0.84	0.65	0.84	0.22	0.34	0.05	0.08	0.01
Total lignans	*2.57*	*2.73*	*1.93*	*12.79*	*1.98*	*0.81*	*2.35*	*2.77*	*2.92*	*3.80*	*1.18*	*1.86*	*1.18*	*1.85*	*1.12*
Hydroxy maslinic acid	0.01	0.18	0.09	0.55	0.11	0.02	0.18	0.08	0.11	0.16	0.10	0.19	0.15	0.14	0.14
Maslinic acid	8.75	64.14	69.51	75.98	72.37	12.47	76.69	77.46	79.38	82.45	8.86	143.36	81.63	71.51	67.23
Betulinic acid	0.09	1.14	1.80	2.63	3.26	0.13	2.92	1.79	2.03	3.49	0.05	0.76	1.37	1.92	4.12
Oleanolic acid	5.93	42.09	55.85	58.90	65.55	8.49	56.10	56.82	57.38	58.50	8.11	14.93	48.89	23.58	65.17
Total triterpenic acids	*14.78*	*107.55*	*127.25*	*138.05*	*141.29*	*21.12*	*135.88*	*136.15*	*138.90*	*144.61*	*17.12*	*159.24*	*132.04*	*97.15*	*136.66*
β+*γ*-tocopherols	31.41	40.25	43.08	7.85	37.42	38.67	36.17	35.78	37.32	34.82	27.52	22.57	33.88	34.59	35.30
α-tocopherol	184.72	237.14	259.96	267.57	212.62	371.35	367.12	324.43	402.63	363.32	151.75	65.01	165.28	156.82	186.41
Total tocopherols	*216.13*	*277.39*	*303.04*	*275.43*	*250.04*	*410.02*	*403.29*	*360.21*	*439.95*	*398.14*	*179.27*	*87.58*	*199.16*	*191.41*	*221.71*
Unknown 2	0.03	0.49	0.09	<0.01	0.54	0.50	0.64	0.60	0.82	0.30	0.29	0.69	0.31	0.12	0.25
Unknown 3	0.35	0.88	0.43	<0.01	0.34	0.38	0.74	0.58	0.37	0.27	2.22	0.67	0.19	0.31	0.12
Total unknown	*0.38*	*1.37*	*0.52*	*<0.01*	*0.89*	*0.88*	*1.38*	*1.18*	*1.19*	*0.58*	*2.50*	*1.36*	*0.49*	*0.43*	*0.36*
TOTAL	***366.73***	***511.25***	***564.84***	***462.66***	***903.63***	***611.36***	***823.62***	***838.19***	***933.90***	***1024.7***	***279.83***	***279.46***	***398.25***	***327.60***	***449.07***

Abbreviations. HTY: hydroxytyrosol; EA: elenolic acid; OleAgly: oleuropein aglycone; LigAgly: ligstroside aglycone

Table 2 SM. Quantitative data obtained for the flours evaluated in this study (produced from stoned, dehydrated and defatted olive fruits). Every result (expressed in mg/g of olive flour) is the mean value of two independent replicates. %RSD values were lower than 8.7% in every case.

	Arbequina				Brillante				Chorreao				Gordal				Hojiblanca			
Dehydration T (°C)	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**
Quinic acid	5.72	3.15	3.95	6.48	10.70	11.92	10.70	13.60	14.81	19.34	20.22	20.43	4.07	5.14	4.93	7.80	19.97	19.78	18.86	18.86
Total acids and derivatives	*5.72*	*3.15*	*3.95*	*6.48*	*10.70*	*11.92*	*10.70*	*13.60*	*14.81*	*19.34*	*20.22*	*20.43*	*4.07*	*5.14*	*4.93*	*7.80*	*19.97*	*19.78*	*18.86*	*18.86*
HTY glucoside	0.32	0.19	0.28	0.32	0.33	0.51	0.53	0.60	0.46	0.41	0.97	0.87	0.33	0.29	0.41	0.49	1.28	2.05	2.17	1.92
Total simple phenols	*0.32*	*0.19*	*0.28*	*0.32*	*0.33*	*0.51*	*0.53*	*0.60*	*0.46*	*0.41*	*0.97*	*0.87*	*0.33*	*0.29*	*0.41*	*0.49*	*1.28*	*2.05*	*2.17*	*1.92*
Acyclodihydro EA hexoside	0.48	0.43	0.47	0.37	0.24	0.24	0.26	0.11	0.34	0.40	0.31	0.26	0.38	0.41	0.37	0.46	0.13	0.15	0.14	0.08
Oleoside/secologanoside	0.75	0.56	0.65	0.76	0.59	0.98	1.12	0.68	0.88	1.26	1.39	1.40	0.74	0.97	1.23	1.27	0.25	0.34	0.42	0.31
EA glucoside	1.18	0.80	0.78	0.29	0.61	1.04	1.21	0.46	0.41	0.61	0.50	0.37	0.81	0.79	0.51	0.76	1.10	1.37	1.40	0.46
Neo-nuzhenide	0.01	0.01	0.01	0.01	0.03	0.05	0.09	0.04	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.09	0.13	0.18	0.11
Hydroxyoleuropein	0.02	<0.01	<0.01	0.03	0.22	0.21	0.20	0.12	0.16	0.57	0.19	0.13	0.21	0.94	0.13	0.17	0.16	0.20	0.17	0.11
Verbascoside	0.04	0.04	0.14	0.11	<0.01	0.02	0.02	0.02	0.02	0.03	0.20	0.16	<0.01	<0.01	0.01	<0.01	0.06	0.15	0.22	0.16
Oleuropein glucoside	0.03	0.02	0.03	0.04	0.05	0.12	0.14	0.08	0.04	0.05	0.07	0.09	0.06	0.07	0.07	0.11	0.11	0.18	0.23	0.15
Caffeoyl 6-oleoside	0.05	0.05	0.05	0.04	0.06	0.13	0.11	0.07	0.04	0.05	0.08	0.07	0.07	0.05	0.03	0.08	0.16	0.24	0.30	0.18
Caffeoyl 6-secologanoside	0.01	0.01	0.01	0.01	0.02	0.03	0.04	0.02	0.01	0.01	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.03	0.06	0.03
Comselogoside	0.10	0.08	0.08	0.07	0.07	0.10	0.07	0.06	0.10	0.05	0.10	0.10	0.07	0.05	0.02	0.07	0.07	0.07	0.08	0.07
Oleuropein	2.45	0.40	0.32	1.78	5.80	13.75	22.20	9.81	2.81	3.84	17.46	22.74	5.34	7.24	29.69	23.57	6.28	9.77	23.16	9.06
EA	0.07	0.02	0.05	0.28	0.21	0.17	0.11	0.42	0.09	0.06	0.08	0.11	0.04	0.01	0.04	0.10	0.08	0.08	0.05	0.38
Lucidumoside C	0.04	0.01	<0.01	0.02	1.06	0.38	0.13	0.04	0.73	1.74	0.21	0.05	0.63	0.97	0.05	0.30	0.85	0.46	0.21	0.04
Ligustroside	0.11	0.01	0.01	0.13	0.20	0.44	1.00	0.40	0.18	0.38	0.80	0.99	0.10	0.26	0.74	0.45	0.10	0.19	0.24	0.19
Oleacein	0.20	0.07	0.18	0.42	0.32	0.63	0.67	0.62	0.07	0.05	0.34	0.40	0.05	0.02	0.04	0.17	0.23	0.30	0.41	0.39
6-O-[...] secologanoside	0.03	0.03	0.04	0.04	0.03	0.04	0.04	0.03	0.02	0.03	0.04	0.04	0.03	0.03	0.02	0.03	0.02	0.03	0.03	0.03
OleAgly (isomers I+II)	0.09	0.01	0.02	0.20	0.30	0.34	0.39	0.70	0.24	0.10	0.43	0.46	0.12	0.03	0.15	0.31	0.24	0.14	0.23	0.49
LigAgly (isomer II)	0.02	<0.01	<0.01	0.03	0.04	0.04	0.05	0.07	0.04	0.04	0.05	0.06	0.01	<0.01	0.02	0.02	0.02	0.02	0.02	0.03
Total secoiridoids	*5.66*	*2.56*	*2.83*	*4.62*	*9.85*	*18.71*	*27.85*	*13.77*	*6.18*	*9.25*	*22.29*	*27.45*	*8.68*	*11.85*	*33.12*	*27.91*	*9.95*	*13.85*	*27.54*	*12.27*
Rutin	0.12	0.08	0.04	0.06	0.31	0.36	0.28	0.26	0.23	0.26	0.31	0.31	0.15	0.15	0.16	0.15	0.25	0.27	0.35	0.24
Luteolin 7-O-glucoside	0.18	0.16	0.14	0.17	0.18	0.26	0.20	0.22	0.14	0.15	0.15	0.15	0.17	0.12	0.24	0.22	0.35	0.53	0.51	0.32
Luteolin glucoside (isomer I)	0.02	0.01	0.02	0.02	0.05	0.06	0.03	0.04	0.03	0.05	0.04	0.03	0.02	0.02	0.03	0.01	0.09	0.16	0.12	0.07
Luteolin glucoside (isomer II)	0.01	<0.01	0.01	0.01	0.01	0.02	0.01	0.01	<0.01	0.01	<0.01	<0.01	0.01	0.01	0.01	<0.01	0.01	0.03	0.02	0.01
Luteolin	0.11	0.28	0.22	0.09	0.02	0.02	0.01	0.02	0.04	0.06	0.02	0.02	0.02	0.03	0.03	0.02	0.08	0.03	0.02	0.05
Total flavonoids	*0.44*	*0.54*	*0.42*	*0.35*	*0.57*	*0.71*	*0.53*	*0.54*	*0.45*	*0.52*	*0.52*	*0.52*	*0.35*	*0.33*	*0.46*	*0.40*	*0.78*	*1.02*	*1.01*	*0.69*
Hydroxy maslinic acid	0.03	0.04	0.04	0.04	0.01	0.03	0.03	0.03	0.08	0.01	0.02	0.01	0.03	0.05	0.06	0.04	0.03	0.02	0.02	0.02
Maslinic acid	12.53	12.68	12.60	14.93	9.18	11.43	12.21	14.96	8.73	8.92	9.53	9.76	7.66	9.25	9.98	9.60	10.73	13.17	11.81	14.58
Betulinic acid	0.04	0.04	0.04	0.04	0.03	0.03	0.04	0.04	0.02	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.03	0.04	0.04	0.04
Oleanolic acid	4.06	4.05	4.22	4.96	3.58	4.67	4.63	5.34	2.71	3.10	3.25	3.51	2.96	4.05	4.56	4.45	4.31	5.06	4.69	5.12
Total triterpenic acids	*16.67*	*16.80*	*16.90*	*19.98*	*12.81*	*16.15*	*16.91*	*20.38*	*11.54*	*12.06*	*12.82*	*13.32*	*10.68*	*13.39*	*14.64*	*14.14*	*15.09*	*18.29*	*16.55*	*19.77*
β+γ-tocopherols	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	<0.01	0.02	0.01	0.01	<0.01
α-tocopherol	0.32	0.17	0.19	0.14	0.03	0.04	0.06	0.06	0.02	0.02	0.05	0.07	0.06	0.10	0.03	0.09	0.20	0.16	0.24	0.16
Total tocopherols	*0.32*	*0.17*	*0.19*	*0.14*	*0.03*	*0.05*	*0.06*	*0.07*	*0.02*	*0.02*	*0.05*	*0.07*	*0.06*	*0.11*	*0.03*	*0.09*	*0.22*	*0.17*	*0.25*	*0.17*
Unknown 1	0.32	0.31	0.29	0.41	0.44	0.63	0.66	0.61	0.44	0.49	0.59	0.61	0.76	1.01	1.29	1.15	0.70	0.81	1.06	0.93
Unknown 2	0.35	0.19	0.06	0.02	1.40	0.29	0.04	0.02	1.18	0.77	0.19	0.04	0.29	0.13	0.04	0.21	0.28	0.12	0.04	<0.01
Unknown 3	0.02	0.01	<0.01	0.02	0.99	0.41	0.09	0.05	0.65	0.64	0.19	0.09	0.17	0.14	0.02	0.15	0.44	0.21	0.07	0.02
Total unknown	*0.69*	*0.52*	*0.35*	*0.45*	*2.84*	*1.33*	*0.79*	*0.68*	*2.28*	*1.91*	*0.97*	*0.74*	*1.22*	*1.27*	*1.36*	*1.51*	*1.43*	*1.13*	*1.17*	*0.95*
TOTAL	**29.82**	**23.93**	**24.93**	**32.35**	**37.12**	**49.38**	**57.37**	**49.64**	**35.74**	**43.51**	**57.84**	**63.40**	**25.39**	**32.37**	**54.95**	**52.34**	**48.71**	**56.31**	**67.57**	**54.64**

	Lechin				Loaime Alhama				Loaime Tiena				Lucio				Manzanilla			
Dehydration T (°C)	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**
Quinic acid	10.23	8.53	8.42	10.92	10.14	8.57	13.19	10.59	11.02	11.68	13.97	13.97	13.34	13.95	15.07	14.76	8.72	9.75	11.01	14.99
Total acids and derivatives	*10.23*	*8.53*	*8.42*	*10.92*	*10.14*	*8.57*	*13.19*	*10.59*	*11.02*	*11.68*	*13.97*	*13.97*	*13.34*	*13.95*	*15.07*	*14.76*	*8.72*	*9.75*	*11.01*	*14.99*
HTY glucoside	1.50	1.75	1.52	1.46	1.11	0.89	1.49	1.80	0.13	0.16	0.14	0.15	1.63	2.04	3.06	3.19	0.38	0.63	0.63	0.68
Total simple phenols	*1.50*	*1.75*	*1.52*	*1.46*	*1.11*	*0.89*	*1.49*	*1.80*	*0.13*	*0.16*	*0.14*	*0.15*	*1.63*	*2.04*	*3.06*	*3.19*	*0.38*	*0.63*	*0.63*	*0.68*
Acyclodihydro EA hexoside	0.50	0.45	0.43	0.38	0.36	0.70	0.34	0.31	0.47	0.62	0.58	0.47	0.35	0.36	0.34	0.33	0.40	0.45	0.43	0.38
Oleoside/secologanoside	1.73	1.65	1.45	1.43	1.18	0.92	1.33	1.12	0.07	0.10	0.07	0.05	0.41	0.44	0.52	0.51	0.25	0.34	0.28	0.32
EA glucoside	1.71	1.29	1.16	0.66	1.74	1.29	1.19	1.21	0.14	0.55	0.15	0.09	0.84	0.73	1.11	0.75	0.58	0.90	0.59	0.17
Neo-nuzhenide	0.03	0.03	0.03	0.02	0.04	0.03	0.04	0.03	<0.01	0.01	<0.01	<0.01	0.01	0.02	0.02	0.02	0.04	0.04	0.04	0.04
Hydroxyoleuropein	0.06	0.17	0.08	0.05	0.42	0.37	0.16	0.12	0.04	0.13	0.08	0.05	0.19	0.21	0.15	0.07	0.17	0.54	0.28	0.07
Verbascoside	0.24	0.28	0.41	0.41	<0.01	<0.01	0.01	0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	0.05	0.05	<0.01	<0.01	<0.01	<0.01
Oleuropein glucoside	0.04	0.10	0.04	0.04	0.22	0.04	0.17	0.17	0.01	0.03	0.01	0.01	0.08	0.12	0.13	0.11	0.04	0.07	0.05	0.04
Caffeoyl 6-oleoside	0.09	0.11	0.06	0.05	0.48	0.02	0.45	0.42	0.20	0.35	0.34	0.21	0.27	0.43	0.47	0.39	0.15	0.29	0.28	0.19
Caffeoyl 6-secologanoside	0.01	0.02	0.02	0.02	0.07	0.01	0.16	0.08	0.05	0.05	0.05	0.04	0.05	0.09	0.10	0.06	0.03	0.06	0.04	0.04
Comselogoside	0.07	0.05	0.04	0.05	0.43	0.01	0.45	0.44	0.40	0.43	0.45	0.50	0.40	0.46	0.45	0.46	0.22	0.29	0.32	0.30
Oleuropein	14.04	19.98	21.54	19.38	8.95	3.73	12.99	13.37	0.78	2.27	1.05	0.76	2.84	7.67	10.24	11.04	0.70	3.83	4.66	5.98
EA	0.03	0.02	0.04	0.09	0.08	0.12	0.41	0.36	0.06	0.17	0.20	0.35	0.09	0.05	0.24	0.35	0.09	0.15	0.25	0.55
Lucidumoside C	0.61	0.71	0.21	0.17	1.94	0.87	0.07	0.05	0.24	0.16	0.04	0.02	0.53	0.43	0.21	0.06	0.95	0.91	0.24	0.05
Ligustroside	0.30	0.60	0.48	0.40	0.18	0.14	0.20	0.19	0.04	0.28	0.07	0.04	0.14	0.32	0.35	0.29	0.10	0.44	0.33	0.32
Oleacein	0.28	0.13	0.21	0.22	0.08	0.03	0.68	0.55	0.04	0.11	0.11	0.12	0.04	0.06	0.19	0.24	0.03	0.13	0.24	0.38
6-O-[...] secologanoside	0.11	0.09	0.09	0.10	0.18	0.02	0.21	0.17	0.03	0.03	0.04	0.04	0.10	0.11	0.13	0.12	0.06	0.08	0.09	0.11
OleAgly (isomers I+II)	0.15	0.05	<0.01	0.12	0.26	0.03	0.76	0.66	0.04	0.19	0.12	0.23	0.18	0.21	0.67	0.48	0.05	0.16	0.17	0.58
LigAgly (isomer II)	0.01	0.01	0.01	0.01	0.02	<0.01	0.04	0.05	0.05	0.14	0.06	0.08	0.03	0.03	0.05	0.04	0.02	0.04	0.04	0.08
Total secoiridoids	*20.01*	*25.75*	*26.30*	*23.60*	*16.61*	*8.34*	*19.66*	*19.30*	*2.66*	*5.64*	*3.43*	*3.10*	*6.55*	*11.75*	*15.42*	*15.39*	*3.90*	*8.72*	*8.33*	*9.60*
Rutin	0.54	0.52	0.40	0.41	0.46	0.08	0.42	0.37	0.12	0.12	0.09	0.06	0.22	0.24	0.26	0.25	0.06	0.04	0.04	0.04
Luteolin 7-O-glucoside	0.20	0.21	0.14	0.19	0.17	0.14	0.18	0.27	0.22	0.26	0.19	0.19	0.14	0.17	0.15	0.18	0.04	0.04	0.05	0.06
Luteolin glucoside (isomer I)	0.08	0.07	0.06	0.06	0.05	0.02	0.04	0.03	0.03	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01
Luteolin glucoside (isomer II)	0.01	0.01	0.01	0.01	0.02	<0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01
Luteolin	0.09	0.03	0.08	0.11	0.04	0.03	0.02	0.02	0.15	0.10	0.15	0.21	0.04	0.03	0.01	0.01	0.02	0.01	0.02	0.02
Total flavonoids	*0.93*	*0.83*	*0.69*	*0.78*	*0.74*	*0.28*	*0.67*	*0.70*	*0.55*	*0.53*	*0.47*	*0.48*	*0.42*	*0.47*	*0.44*	*0.47*	*0.13*	*0.10*	*0.12*	*0.12*
Hydroxy maslinic acid	0.04	0.03	0.03	0.04	0.02	0.03	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01
Maslinic acid	9.35	10.70	9.84	12.46	12.07	10.34	14.97	13.48	9.94	10.02	8.54	9.59	7.79	10.86	10.11	9.17	8.92	7.80	7.38	10.53
Betulinic acid	0.05	0.04	0.04	0.04	0.05	0.03	0.05	0.04	0.04	0.04	0.03	0.04	0.03	0.04	0.04	0.04	0.02	0.03	0.03	0.04
Oleanolic acid	5.34	4.57	3.48	5.42	5.07	3.77	5.47	4.94	4.42	4.84	3.86	3.88	1.97	3.64	3.40	2.79	2.61	1.99	1.92	3.44
Total triterpenic acids	*14.78*	*15.34*	*13.39*	*17.97*	*17.21*	*14.17*	*20.51*	*18.48*	*14.40*	*14.91*	*12.44*	*13.51*	*9.81*	*14.56*	*13.57*	*12.02*	*11.57*	*9.82*	*9.34*	*14.02*
β+γ-tocopherols	0.01	0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	0.01	0.01	0.01	<0.01	<0.01	<0.01	<0.01	<0.01
α-tocopherol	0.22	0.30	0.26	0.21	0.21	0.08	0.18	0.29	0.09	0.22	0.12	0.08	0.30	0.13	0.28	0.22	0.05	0.06	0.04	0.08
Total tocopherols	*0.23*	*0.31*	*0.27*	*0.22*	*0.21*	*0.08*	*0.18*	*0.29*	*0.09*	*0.22*	*0.12*	*0.08*	*0.31*	*0.14*	*0.29*	*0.23*	*0.05*	*0.06*	*0.05*	*0.08*
Unknown 1	0.48	0.40	0.36	0.47	0.81	0.82	1.05	0.82	0.29	0.28	0.30	0.35	0.47	0.48	0.69	0.76	0.50	0.64	0.65	0.90
Unknown 2	1.22	0.18	0.17	0.23	0.77	0.17	0.10	0.05	0.40	0.15	0.04	0.01	0.35	0.23	0.04	0.02	0.71	0.13	0.14	0.03
Unknown 3	0.48	0.12	0.07	0.12	1.19	0.08	0.29	0.17	0.27	0.10	0.05	0.02	0.35	0.32	0.17	0.07	0.47	0.20	0.14	0.05
Total unknown	*2.19*	*0.69*	*0.61*	*0.82*	*2.77*	*1.07*	*1.44*	*1.05*	*0.95*	*0.53*	*0.39*	*0.38*	*1.17*	*1.03*	*0.90*	*0.85*	*1.68*	*0.96*	*0.93*	*0.98*
TOTAL	**49.87**	**53.21**	**51.19**	**55.75**	**48.79**	**33.40**	**57.13**	**52.22**	**29.81**	**33.67**	**30.96**	**32.66**	**33.22**	**43.94**	**48.75**	**46.91**	**26.43**	**30.04**	**30.40**	**40.48**

	Nevadillo				Ombliguillo				Picual				Picual Huétor				Picudo			
Dehydration T (°C)	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**	**35**	**55**	**75**	**100**
Quinic acid	20.53	18.56	18.94	19.89	12.95	13.56	15.25	13.95	11.21	11.27	10.27	13.26	23.88	23.01	22.98	26.19	18.62	18.80	22.01	23.83
Total acids and derivatives	*20.53*	*18.56*	*18.94*	*19.89*	*12.95*	*13.56*	*15.25*	*13.95*	*11.21*	*11.27*	*10.27*	*13.26*	*23.88*	*23.01*	*22.98*	*26.19*	*18.62*	*18.80*	*22.01*	*23.83*
HTY glucoside	0.23	0.13	0.33	0.44	0.10	0.07	0.10	0.12	0.50	0.67	1.41	1.39	1.54	1.60	1.42	2.01	0.48	1.33	0.77	0.73
Total simple phenols	*0.23*	*0.13*	*0.33*	*0.44*	*0.10*	*0.07*	*0.10*	*0.12*	*0.50*	*0.67*	*1.41*	*1.39*	*1.54*	*1.60*	*1.42*	*2.01*	*0.48*	*1.33*	*0.77*	*0.73*
Acyclodihydro EA hexoside	0.34	0.32	0.29	0.30	0.35	0.35	0.33	0.33	0.41	0.45	0.47	0.43	0.42	0.44	0.42	0.37	0.32	0.35	0.33	0.24
Oleoside/secologanoside	0.63	0.49	0.53	0.56	0.41	0.42	0.43	0.47	0.26	0.40	0.80	0.43	0.96	0.81	0.45	0.64	0.25	0.36	0.20	0.16
EA glucoside	0.44	0.34	0.30	0.31	0.69	0.53	0.70	0.76	0.70	1.09	1.08	0.23	0.72	0.54	0.24	0.17	0.22	0.77	0.29	0.08
Neo-nuzhenide	0.01	0.01	0.01	0.03	<0.01	<0.01	<0.01	0.01	0.01	0.01	0.04	0.02	0.01	0.01	<0.01	0.01	0.01	0.01	<0.01	<0.01
Hydroxyoleuropein	0.23	0.31	0.12	0.05	0.36	0.38	0.20	0.06	0.08	0.14	0.04	0.02	0.07	0.05	0.01	0.01	0.07	0.34	0.23	0.08
Verbascoside	<0.01	<0.01	0.01	0.02	<0.01	<0.01	0.01	0.02	0.01	0.03	0.07	0.11	0.26	0.30	0.37	0.84	<0.01	<0.01	<0.01	0.01
Oleuropein glucoside	0.03	0.03	0.03	0.05	0.06	0.04	0.05	0.09	0.02	0.02	0.04	0.03	0.04	0.04	0.04	0.05	0.02	0.03	0.02	0.01
Caffeoyl 6-oleoside	0.03	0.02	0.03	0.05	0.13	0.16	0.20	0.24	0.19	0.20	0.30	0.24	0.14	0.14	0.09	0.06	0.30	0.40	0.28	0.18
Caffeoyl 6-secologanoside	0.01	0.01	0.01	0.01	0.03	0.03	0.04	0.03	0.03	0.04	0.09	0.06	0.03	0.03	0.02	0.03	0.04	0.11	0.06	0.04
Comselogoside	0.03	0.01	0.04	0.04	0.21	0.24	0.26	0.30	0.20	0.22	0.17	0.18	0.10	0.10	0.04	0.04	0.39	0.46	0.37	0.36
Oleuropein	4.27	2.41	7.64	8.22	1.87	1.46	5.18	11.80	1.45	2.25	19.55	7.82	14.30	10.35	6.85	10.96	1.46	3.76	1.68	1.23
EA	0.09	0.09	0.09	0.38	0.19	0.34	0.41	0.49	0.04	0.03	<0.01	0.31	0.04	0.06	0.12	0.13	0.13	<0.01	0.13	0.27
Lucidumoside C	0.56	0.40	0.03	0.01	1.30	1.03	0.17	0.03	0.32	0.46	0.02	0.01	0.20	0.11	0.02	0.03	0.46	0.55	0.20	0.04
Ligustroside	0.34	0.37	0.29	0.36	0.22	0.20	0.23	0.42	0.09	0.24	1.01	0.28	0.31	0.27	0.12	0.20	0.03	0.17	0.07	0.20
Oleacein	0.14	0.19	1.05	1.59	0.06	0.04	0.44	0.60	0.03	0.02	<0.01	0.07	0.05	0.06	0.12	0.06	0.05	0.22	0.15	0.05
6-O-[...] secologanoside	0.03	0.03	0.03	0.03	0.03	0.06	0.05	0.05	0.03	0.03	0.04	0.04	0.08	0.07	0.05	0.07	0.05	0.07	0.07	0.07
OleAgly (isomers I+II)	0.08	0.06	0.20	2.23	0.10	0.12	0.88	2.41	0.16	0.16	0.12	1.19	0.81	0.48	0.66	1.04	0.05	0.17	0.04	0.06
LigAgly (isomer II)	0.04	0.04	0.04	0.38	0.07	0.06	0.14	0.27	0.04	0.03	0.01	0.12	0.05	0.04	0.04	0.08	0.02	0.04	0.02	0.02
Total secoiridoids	*7.31*	*5.12*	*10.74*	*14.62*	*6.05*	*5.46*	*9.72*	*18.39*	*4.06*	*5.81*	*23.87*	*11.58*	*18.59*	*13.88*	*9.67*	*14.79*	*3.88*	*7.81*	*4.14*	*3.08*
Rutin	0.24	0.16	0.26	0.25	0.17	0.12	0.15	0.19	0.13	0.15	0.20	0.15	0.30	0.27	0.21	0.23	0.31	0.37	0.21	0.14
Luteolin 7-O-glucoside	0.25	0.20	0.24	0.32	0.20	0.17	0.25	0.30	0.10	0.11	0.09	0.06	0.09	0.08	0.05	0.05	0.20	0.15	0.22	0.23
Luteolin glucoside (isomer I)	0.05	0.03	0.02	0.03	0.03	0.03	0.03	0.03	0.01	0.01	0.01	<0.01	0.02	0.02	0.01	0.01	<0.01	0.01	0.03	0.02
Luteolin glucoside (isomer II)	0.02	0.01	0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	<0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.01
Luteolin	0.08	0.14	0.12	0.06	0.05	0.04	0.03	0.02	0.05	0.04	0.01	0.02	0.01	0.02	0.05	0.02	0.18	0.08	0.14	0.16
Total flavonoids	*0.64*	*0.54*	*0.65*	*0.68*	*0.47*	*0.38*	*0.49*	*0.55*	*0.30*	*0.31*	*0.31*	*0.23*	*0.44*	*0.40*	*0.33*	*0.32*	*0.71*	*0.63*	*0.62*	*0.57*
Hydroxy maslinic acid	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.02	<0.01	<0.01	<0.01	<0.01	0.01	<0.01	0.01	0.01	0.01	0.01	0.01	0.01
Maslinic acid	11.22	10.99	9.21	9.02	14.44	13.30	13.25	13.76	6.42	5.40	5.62	3.99	7.49	2.20	2.33	4.67	10.06	9.74	9.90	9.87
Betulinic acid	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.03	0.02	0.03	0.03	0.04	0.03	0.03	0.03	0.04	0.04	0.03	0.04
Oleanolic acid	4.42	3.57	2.80	2.33	5.90	5.62	5.22	5.27	1.97	1.62	1.44	1.43	3.24	1.22	1.58	1.52	4.87	4.30	4.64	4.30
Total triterpenic acids	*15.70*	*14.61*	*12.07*	*11.40*	*20.41*	*18.99*	*18.54*	*19.10*	*8.42*	*7.05*	*7.09*	*5.45*	*10.78*	*3.45*	*3.95*	*6.22*	*14.97*	*14.08*	*14.58*	*14.22*
β+γ-tocopherols	0.01	0.01	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.05	0.03	0.03	0.01	0.02	0.12	0.01	0.01	<0.01	<0.01	<0.01	<0.01
α-tocopherol	0.12	0.12	0.09	0.11	0.10	0.06	0.03	0.06	0.53	0.56	0.72	0.34	0.53	0.46	0.71	0.40	0.05	0.04	0.01	0.05
Total tocopherols	*0.12*	*0.13*	*0.09*	*0.11*	*0.10*	*0.06*	*0.04*	*0.06*	*0.57*	*0.59*	*0.75*	*0.35*	*0.54*	*0.58*	*0.73*	*0.41*	*0.05*	*0.04*	*0.01*	*0.05*
Unknown 1	0.42	0.39	0.45	0.49	0.47	0.51	0.63	0.66	0.28	0.36	0.48	0.43	0.43	0.40	0.38	0.41	0.48	0.63	0.50	0.59
Unknown 2	1.21	0.59	0.16	0.04	1.16	0.66	0.27	0.03	0.15	0.05	<0.01	<0.01	0.04	0.03	0.02	0.01	1.11	0.48	0.28	0.04
Unknown 3	0.43	0.32	0.10	0.04	0.75	0.77	0.32	0.07	0.29	0.13	0.01	0.01	0.15	0.14	0.09	0.01	0.68	0.27	0.16	0.04
Total unknown	*2.07*	*1.30*	*0.72*	*0.57*	*2.38*	*1.95*	*1.22*	*0.76*	*0.73*	*0.53*	*0.48*	*0.43*	*0.62*	*0.57*	*0.49*	*0.43*	*2.27*	*1.37*	*0.94*	*0.67*
TOTAL	**46.59**	**40.39**	**43.53**	**47.69**	**42.46**	**40.46**	**45.34**	**52.93**	**25.79**	**26.24**	**44.18**	**32.70**	**56.38**	**43.49**	**39.56**	**50.36**	**40.99**	**44.06**	**43.08**	**43.15**

Abbreviations. HTY: hydroxytyrosol; EA: elenolic acid; OleAgly: oleuropein aglycone; LigAgly: ligstroside aglycone; 6-O-[...] secologanoside: 6-O-[(2E)-2,6-Dimethyl-8-hydroxy-2-octenoyloxy] secologanoside

Chapter 10

Exploring the capability of LC-MS and GC-MS multi-class methods to discriminate olive oils from different geographical indications and to identify potential origin markers

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Abstract: Looking for a strategy to authenticate the declared origin of commercial extra virgin olive oils (EVOOs), 126 samples from six different Mediterranean geographical indications (GIs) were analyzed by means of two different platforms (LC-ESI-QTOF MS (in positive and negative polarity) and GC-APCI-QTOF MS (in positive mode)) combined to chemometrics. The sample treatment and chromatographic/detection conditions (in both platforms) were chosen to enable the comprehensive characterization of the complete minor fraction of the oils within a single run. Noticeable discrimination among the six evaluated GIs (Priego de Córdoba and Baena (Spain), Kalamata (Greece), Toscano (Italy), and Ouazzane and Meknès (Morocco)) was achieved building two-class PLS-DA models which considered the data coming from both platforms. The contribution of a few thousand molecular features to the statistical models was evaluated in depth and several compounds were pointed out as possible GIs distinctive markers. The complementarity of the different approaches was discussed and diverse strategies were used to annotate the classifiers. Characteristic composition patterns were defined for each GI.

Practical applications: Protected Designation of Origin (PDO) and Protected Geographical Indication (PGI) labels are important tools to promote high quality EVOOs, assuring the connection to a particular territory and the unique combination of natural and human factors which make possible to obtain matchless oils. In this context, it is imperative to furnish the control labs with innovative tools and methods which are able to provide extensive information about the EVOOs' minor fraction (of unquestionable importance regarding its overall quality) in just one run and to give the chance to find and identify (and validate) origin markers. The utility of validated classifiers to authenticate the belonging (or not) of an EVOO to a particular GI is not open to debate. The consumers' confidence will be perceptibly undermined if the geographical name is used on products not having the expected qualities or if the production specifications are sometimes not followed by producers.

Keywords: virgin olive oil; protected geographical indications; liquid chromatography; gas chromatography; mass spectrometry; chemometrics.

1. INTRODUCTION

According to the International Olive Council statistics from the 2016-17 crop year, around 97% of the worldwide olive oil was produced by countries from the Mediterranean basin [1], but more than 26% was consumed by countries from the rest of the world, such as the United States, Brazil or Japan [2]. The globalization of the olive sector has resulted in a very competitive worldwide marketplace, where fraudulent practices (category mislabeling or even adulteration with cheaper oils) undermine public confidence. Nevertheless, consumers' awareness is rapidly growing and they are willing to pay higher prices for high-quality traceable products with distinctive premium characteristics [3,4].

The expansion of olive growing in emerging areas as well as the increase in crop productivity over the last years [5,6], has pushed olive oil producers to seek differentiation strategies to improve profitability and market competitiveness. One of them is the implementation of geographical indications (GIs) which intend to promote olive oils' specific attributes linked to the particular environment of a region (including natural and human factors) [7,8]. Since 1992, the European Union recognizes authenticity of agricultural products or foodstuffs produced and/or processed in a specific geographical area with two labels: Protected Designations of Origin (PDOs) if the entire production cycle is carried out in a specific territory, and Protected Geographical Indications (PGIs) if a single phase of production takes place in that region. In that way, European regulations want to benefit the rural economy by encouraging the traditional agricultural production linked to a local know-how and a cultural identity [9]. Apart from allowing producers to take advantage of a time-honored reputation, PDO/PGI labeling provides product-specific information and reduces the uncertainty faced by consumers in their food choice decision process.

The European Regulation (EU) 1151/2012 protects registered names from misuse and imitation and establishes quality schemes to ensure traceability and genuineness of the products [10]. However, the verification of the declared origin entails a great challenge for the analytical community since standardized parameters are not useful to discriminate origin-labeled oils from other extra virgin olive oils (EVOOs). In addition, the considerable variability in olive oil composition, depending not only on its geographical origin but also on other agro-technological parameters (such as seasonal climate and processing/storage means), makes the establishment of specific physico-chemical patterns for each PDO/PGI very challenging. It is possible to classify the strategies that have been proposed for geographical origin authentication so far into two main categories [3,11]: those based on trace elemental composition or the determination of stable isotope ratios; and those based on the determination of organic compounds (including the establishment of global indices, the application of separative techniques and direct spectroscopic measurements). Regardless of the selected strategy, the use of chemometrics to process the huge amount of data provided by advanced analytical instruments has become commonplace to extract relevant information from the results and to build classification models [12]. For instance, evidences of the usefulness of multi-element analysis combined with principal component analysis (PCA) or linear discriminant analysis (LDA) in the study of olive oil traceability have been widely reported [13,14]. Likewise, spectroscopic techniques such as near or mid-infrared spectroscopy fingerprinting along with modeling and classification methods (partial least squares-discriminant analysis (PLS-DA) and soft independent modeling of class analogies (SIMCA)) have shown their capability to trace VOO origin [15].

Having in mind the complexity of VOO matrix, the use of different metabolomic approaches giving comprehensive information about the sample under study (mainly, but not exclusively, based on MS or NMR and chemometrics) has been proposed as a way to address the problem of origin mislabeling in the olive oil sector [16]. In this regard, pattern recognition techniques applied

to the ^{1}H NMR data of the olive oil unsaponifiable fraction have revealed valuable information for the geographical characterization of VOOs [17,18]. Phenolic compounds profiling combined with PCA and LDA has been pointed out as a very promising approach in origin authentication studies too [19,20]. In another recently published application, selected ion flow tube MS (SIFT-MS) has been used to collect VOO volatile fingerprints, which followed by PLS-DA, provided a strong discrimination among Mediterranean PDO/PGIs [21]. It is worthy to mention some other interesting examples illustrating the differentiation of VOO samples according to their geographical provenance based on the determination of specific chemical classes (triacylglycerols [22], fatty acids [23], esterified sterols [24] or volatile compounds [25,26]).

In the current work, non-targeted metabolomic approaches have been tested for achieving the discrimination of EVOOs from different GIs and the identification of potential origin markers. Two multi-class methodologies (LC and GC coupled to high resolution MS) allowing the simultaneous evaluation within a single run of VOO minor compounds belonging to different chemical classes (phenolic and triterpenic compounds, tocopherols, sterols and free fatty acids, among others) have been applied to the analysis of a sample-set composed by 126 oil samples from six different Mediterranean GIs (from 4 different countries). Robust two-class models were built with the use of supervised chemometrics, pointing at the molecular features with a most remarkable influence for each GI.

2. MATERIALS AND METHODS

2.1. Reagents and standards

Gradient grade ethanol (EtOH) LiChrosolv® supplied by Merck (Madrid, Spain) and deionized water produced by a Millipore Milli-Q system (Bedford, MA, USA) were used for sample preparation. Acetonitrile (ACN) and acetic acid (AcH) from Sigma-Aldrich (St. Louis, MO, USA) were used for LC mobile phases preparation. 1 mL ampules of derivatization reagent for GC (*N,O*-Bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane, (BSTFA + 1% TMCS)) were supplied by Sigma-Aldrich too. A standard mixture containing 29 compounds from the VOO minor fraction (dissolved in EtOH/H_2O (80:20, v/v)) was used for identity confirmation. All of them were acquired from Sigma-Aldrich. The mix included: α-, β-, γ- and δ- tocopherols, erythrodiol, uvaol, betulinic acid, oleanolic acid, maslinic acid, ursolic acid, β-sitosterol, campesterol, stigmasterol, luteolin, apigenin, rutin, pinoresinol, oleuropein, hydroxytyrosol, tyrosol, quinic acid, ferulic acid, vanillic acid, vanillin, *p*-coumaric acid, palmitoleic acid, oleic acid, linoleic acid and linolenic acid. In addition, isolated fractions of oleuropein and ligstroside aglycones, the decarboxymethylated form of both oleuropein and ligstroside aglycones, elenolic acid, acetylated hydroxytyrosol and acetoxypinoresinol were also analyzed to generate a list of known target analytes ("Analyte List") which could help to identify potential GI markers. All the samples and stock solutions were stored at -20 ºC.

2.2. Samples

126 EVOO samples from six different Mediterranean regions belonging to four different countries were analyzed in this work. The sample set included commercial EVOOs purchased from local stores from two Spanish PDOs (Priego de Córdoba, 25 samples and Baena, 19 samples), one Greek PDO (Kalamata, 15 samples) and one Italian PGI (Toscano, 20 samples). In addition, 25 samples from Ouazzane PGI, purchased directly from the producers "Groupement d'Intérêt Economique Femmes du Rif" and 22 samples from Meknès (GI recognition under evaluation),[7] kindly supplied by Agro-Pôle Olivier National School of Agriculture of Meknès (Morocco) were included in this study.

Equivalent volumes of all bottles from the same region were mixed into six representative blends of each GI and were used for experimental conditions optimization. A global quality control (QC) sample was prepared by mixing equal amounts of the individual blends and interspersed in the analytical sequence at regular intervals (every 20 and 10 injections in LC-MS and GC-MS, respectively) to evaluate the performance of the analytical systems.

2.3. Equipment and software

LC-MS analyses were carried out in an Elute UHPLC (Bruker Daltonik GmbH, Bremen, Germany) equipped with a binary pump which was coupled to a Compact™ QqTOF mass spectrometer (Bruker Daltonik) by an electrospray ionization (ESI) source. GC-MS analyses were conducted in a Bruker 450-GC with the same detection system as in LC. In this case, the coupling was made through an atmospheric pressure chemical ionization (APCI) source, which presents a great advantage over the "classical" electron impact (EI) GC-MS systems as it can preserve the pseudo-molecular ion information.

Instrument control and data acquisition were performed using Compass HyStar (Bruker Daltonik). Compass DataAnalysis 4.4 (Bruker Daltonik) was used for data visualization and MetaboScape® 3.0 (Bruker Daltonik) was used for molecular features selection, bucketing, filtering, scaling, normalization and statistical treatment of the LC-MS and GC-MS data. The proper performance of the analytical platforms to provide accurate information was evaluated with Microsoft Excel 2013 (Microsoft Corporation, Redmond, WA, USA).

2.4. Sample treatment

The isolation of the VOO minor fraction from the samples was performed according to a previously proposed LLE methodology [27] with slight modifications. Briefly, 1 (± 0.01 g) of VOO was extracted three successive times (vortex shaking, centrifugation and supernatant collection) with 10 mL of EtOH/H_2O (60:40, v/v) and two 10 mL portions of EtOH/H_2O (80:20, v/v). After solvent evaporation, the residue was redissolved with 1 mL of EtOH/H_2O (80:20, v/v) and filtered through a Clarinert™ 0.22 µm nylon syringe filter from Agela Technologies (Wilmington, DE, USA). A further derivatization step of VOO extracts (as well as standard solutions) was required for GC-MS

analyses. To that end, glass vials containing 50 µL of extract were dried in a vacuum centrifuge concentrator (Eppendorf, Hamburg, Germany). Subsequently, the residue was reconstituted in 75 µL BSTFA + 1% TMCS and kept at room temperature for 1 h before injection into the chromatograph.

2.5. LC-MS conditions

The extracts were eluted in LC using an Intensity Solo C18 column (2.1 × 100 mm, 1.8 µm) (Bruker Daltonik), protected with an AQUITY UPLC BEH C18 VanGuard pre-column (2.1 × 5 mm, 1.7 µm) (Waters, Manchester, UK), with water (phase A) and ACN (phase B), both of them acidified with 0.5% of AcH. The mobile phase gradient was designed as follows: 0 to 2 min, 5%–30% B; 2 to 7 min, 30%–50% B; 7 to 8 min, 50%–90% B; 8 to 8.2 min, 90%–95% B, 8.2 to 10 min, 95%–99.9% B (kept for 3.9 min), and 13.9 to 14 min, 99.9%–5% B. Thus, the analysis time was 14 min plus 2 post-run min for column stabilization. The flow rate was set at 0.4 mL/min; it was just increased at 0.6 mL/min (and kept at this value) from 10 to 14 min to speed up the elution of the most apolar components. After that, it was set again to the initial value. The injection volume was 2 µL.

MS detection conditions were optimized for the analytes under study in both positive and negative ionization modes. Regarding the ESI source, the main parameters were capillary voltage (set at 3500 V in negative polarity and 4500 V in positive ion mode), nebulizer pressure (2 bar), drying gas flow (8 L/min) and drying temperature (200 ºC). When the QTOF analyzer was coupled to the LC system, its tune parameters were set as follows: 100 Vpp for both 1 and 2 RF funnels, 50 Vpp for hexapole RF, 5 eV for quadrupole ion energy, 6 eV for collision energy, 1000 Vpp for collision RF, transfer time of 70 µs and pre pulse storage of 8 µs. Full scan spectra was recorded (from 30 to 1000 *m/z*) at a scan rate of 1 Hz.

Some auto MS/MS analyses were performed in order to obtain information about the fragmentation pattern of the proposed markers that could help to suggest their tentative identity. Precursor ions were collected in a cycle time of 1 s, with an absolute threshold of 1500 counts and collision energy stepping factors fluctuating between 0.2 and 0.8%. In those analyses, the spectra rate was set at 8 Hz.

2.6. GC-MS methodology

Separation in GC was carried out in a BR-5 column (30 m × 0.25 mm i.d., 0.25 µm) (Bruker Daltonik) with a constant He flow of 1 mL/min. 1 µL of the silylated extract was injected at a split ratio of 1:20 with an injector temperature of 250 ºC. Analytes were separated with the oven temperature initially kept at 150 °C for 5 min and ramped afterwards to 320 ºC at a rate of 4° C/min (keeping that value for 5 extra min), according to the previously reported chromatographic conditions [27]. The transfer line was kept at 290 ºC. The optimized source parameters for the APCI interface were: 2000 V for capillary voltage, 2000 nA for corona discharge, 3.5 bar for nebulizer pressure, 2.5 L/min for dry gas flow and dry temperature of 280 ºC. Tune parameters of the QqTOF analyzer in GC-MS analyses were set as follows: 200 Vpp for funnel 1 RF, 100 Vpp for funnel 2 RF,

50 Vpp for hexapole RF, 4 eV for the quadrupole ion energy, 8 eV for the collision energy, 300 Vpp for the collision RF, transfer time of 100 μs and pre pulse storage of 2 μs. Full scan spectra was recorded (from 30 to 1000 *m/z*) at a scan rate of 3 Hz.

2.7. Data treatment

Raw data was imported into MetaboScape® 3.0 (Bruker Daltonik) which was able to accomplish the entire data treatment, from pre-processing to multivariate analysis. The first stage of the data treatment included three different steps. The automatic internal mass calibration (step a) was performed by comparison with appropriate calibration lists (containing clusters of sodium formate/acetate for LC-MS files and common cyclic-siloxanes from the background for GC-MS files). Non-linear retention time (Rt) alignment (step b) was conducted by means of the T-ReX 3D (Time aligned Region Complete eXtraction) algorithm, which automatically extracts and combines isotopes, adducts and fragments belonging to the same compound into one feature. All compounds detected and clustered in the analyses were presented in a data matrix, together with their Rt, measured *m/z*, molecular weight, detected ions, intensity found in each sample and some additional info regarding their identity (step c). These annotated data matrices of putative compounds are hereinafter referred to as bucket tables.

The main parameters selected to process raw data are presented in Table S1 (Supporting information). It includes the intensity threshold and minimum peak length for peak detection as well as different bucketing parameters, such as Rt and mass ranges, extracted ion chromatograms (EIC) correlation (only if the correlation of two monoisotopes' EICs was higher than the threshold, two ions were considered as potential adducts or fragments of the same compound) and expected adducts and/or neutral losses (primary, seed and common ions). While common ions were useful for reliable interpretations, the presence of at least one of the primary or seed ions in the spectra was required before completing the extraction of any feature. Table S1 also shows the total number of compounds included in the bucket tables (from GC-MS in positive polarity and LC-MS in positive and negative polarity, respectively); moreover, as can be seen, the software artificially created another combined bucket list for LC-MS measurements, joining together features acquired by using both polarities, based on Rts and putative neutral masses. Additional filters were applied to discard features that were not present in at least 25% of the samples of one GI and features from blanks were subtracted in order to avoid the presence of "false compounds" (compounds not appertaining to the EVOO samples) in the bucket table. Moreover, QC replicates, standard solutions and blanks were logically excluded from the sample table before performing the statistical treatment of the data.

Constant sum normalization of peak intensity (also called normalization to the sum of buckets), which is one of the most commonly used normalization methods in Metabolomics [28], was applied as pre-processing step to reduce systematic variations. Normalization to an internal standard (IS) (3,4-dihydroxyphenylacetic) was also considered, but finally discarded because it did

not lead to better results. Unsupervised statistical analysis was carried out by PCA, which simplifies the complexity in high-dimensional data while retaining trends and patterns. Four PCA models were built with the data matrices from the four bucket tables (GC-MS in positive polarity and LC-MS in negative, positive and combined polarities). Afterwards, supervised chemometric methods were applied to the four just mentioned data matrices. PLS-DA was used to create two-class models by facing each GI to the rest, in order to find the most relevant features contributing to class separation. PLS-DA optimizes the separation between different groups of samples, which is accomplished by linking two data matrices X (*i.e.* raw data) and Y (*i.e.* groups). Full cross validation was used when establishing all the unsupervised and supervised statistical models to test their significance. MetaboScape automatically keeps a portion of analyses out of model calculation, calculates a number of parallel models from the reduced data, predicts the omitted data by the different models and, finally, compares the predicted values with the actual ones.

Compounds that could be considered as potential origin markers were later subjected to a semi-quantitative evaluation in all the samples and their identity was additionally investigated. The software package included different tools to achieve a confident annotation of the compounds pointed out as possible markers. First, SmartFormula 3D algorithm was used to predict their molecular formulas. This tool provides two parameters to evaluate the quality of the prediction: the difference between measured and calculated *m/z* and the mSigma value, which denotes the goodness of fit between the measured and theoretical isotopic pattern. Compound Crawler tool was used afterwards to look for possible structures in online databases (ChEBI, ChemSpider and PubChem) for compound annotation. Additionally, MetFrag algorithm [29,30] was used to perform *in-silico* fragmentation of the compound structures with subsequent MS/MS fragment peak matching. Another alternative for the annotation of buckets was to compare assigned MS/MS spectra with in-house and commercial spectral libraries, containing reference spectra and additional information of well-known compounds.

3. RESULTS AND DISCUSSION

3.1. Extensive coverage of VOO minor fraction: extraction protocol and LC-MS and GC-MS conditions

The sample preparation protocol as well as the LC-MS and GC-MS multi-analyte methodologies applied in this study were adapted from those presented in a previous work [27]. As mentioned in section 2.4, the LLE protocol used for preparing the EVOO extracts was the result of a modification carried out to simplify the procedure and to facilitate the supernatant collection after the centrifugation step. Firstly, the order of the extractant solvents was inverted. Thus, the EtOH/H_2O (60:40, v/v) mixture, which presents higher density than the oily phase, was used in the first place, followed by two consecutive extraction steps with the less dense EtOH/H_2O (80:20, v/v) mixture. In that way, four 6 mL portions of extractant solvent were replaced by three 10 mL portions

of the EtOH/H_2O mixtures, which led to equivalent extraction rates. The repeatability of the modified sample preparation procedure was carefully checked for 6 replicates of the QC, obtaining relative standard deviation (%RSD) lower than 3.5% for the total area of the chromatogram (value achieved considering the LC-MS measurements in negative polarity).

The optimal chromatographic conditions were the result of adapting very recently published methodologies [27] to the columns and platforms used in this study. Slight changes were made regarding GC settings, whereas in LC, the re-optimization was more extensive. The use of a narrower column with a smaller particle size, allowed the reduction of the solvent flow and the run time in more than 50% with comparable resolution to the primary method. Any modification of the separation conditions searched out to get information about as many compounds as possible within the same run with the best attainable analytical performance (and shortest analysis time).

Coupling and MS conditions (source and transfer parameters) had to be optimized for the Compact™ QqTOF MS detector. They were thoroughly designed to ensure the proper ionization of the analytes, paying special attention to the drying temperature and gas flow in both the ESI and APCI sources (for LC-MS and GC-MS analyses, respectively). Regarding the detection conditions of the QqTOF analyzer, the creation of adapted or specific segments for analytes with different chemical properties was not recommended since a speedy and frequent switching would need extra calibration and time. Therefore, a compromise solution to enable the detection of chemical substances in a wide range of polarities/volatilities covered by these two methodologies was reached after a deep characterization of the standard mixture containing 29 compounds, the isolated fractions of VOO phenolic compounds as well as the representative blends of the six GIs. The optimized parameters were presented in section 2.5 and 2.6. Fig. S1 shows the EICs obtained for the QC sample with the adjusted methodologies in each platform and polarity. With the finally selected conditions, the goal of monitoring a very large number of compounds from the VOO minor fraction was accomplished. The total number of compounds extracted in the bucket tables is given in Table S1. The GC-MS method was suited to give information about 5070 analytes, whereas LC-MS was appropriate for determining 2198 (in negative polarity) and 2647 compounds (in positive polarity). As previously stated, the software artificially created a combined-polarity bucket table for LC-MS measurements. The sum of total buckets found for LC-MS data in negative and positive polarities (4845) and the number of buckets in the combined table (4737 buckets) differed in 108 features, what indicates that MetaboScape automatically identified 108 compounds in both ionization modes.

The identification of the monitored features was definitely not our aim at this stage of the study; however, our prior knowledge indicated that, at least, compounds belonging to six chemical classes from VOO minor fraction could be determined (phenolic and triterpenic compounds, tocopherols, sterols, free fatty acids and some hydrocarbons).

3.2. Statistical models and achieved classifications

Raw data provided by the GC-MS and LC-MS platforms were processed using the parameters presented in Table S1. Those values were chosen in an attempt to avoid some of the most common issues encountered when building classification models and trying to have a reasonable number of variables in the bucket table without over-fitting the models [31,32]. The total number of obtained compounds was established (as commented in section 2.7) after applying the filters and excluding the blank's buckets. The software being used considered some quality indicators to check the performance of the processing stage. SD of the mass calibration for each analysis presented values lower than 0.0002 ppm, 0.0007 and 0.0005 ppm in GC-MS, LC-MS (negative polarity) and LC-MS (positive polarity), respectively. Moreover, chromatogram alignment SD values did not exceed 2.56 s, 1.17 s and 0.99 s in GC-MS, LC-MS (negative polarity) and LC-MS (positive polarity), respectively.

Before continuing with the statistical analysis, system stability was checked along the sequences to ensure the validity of the obtained data sets [33]. For that purpose, 14 QC injections were intercalated in each sequence, every 10 samples in GC-MS and every 20 samples in LC-MS. In spite of the different intervals, the total number of QC injected when using the three methodologies (GC-MS in positive and LC-MS in both polarities) was the same since each sample was analyzed once in GC-MS (52 min run time) and twice in LC-MS (15 min run time). Firstly, the visual inspection of the overlapped chromatograms was undertaken, and secondly, different control charts were built following the strategy reported by Gika *et al.* [34]. On the one hand, the total intensity of the chromatograms (sum of every feature's intensity) was plotted against the run order of each QC. As shown in Fig. S2 (A), all the obtained values for every QC in each sequence of analysis were between the ±2SD limits. On the other hand, several features were selected to build individual control charts, trying to cover examples of compounds with different *m/z* signals and diverse retention times (Rt). As observed in Fig. S2 (B), every tested feature met the quality requirements described in the just mentioned publication [34]: *"one QC out of 3SD limit, two or more subsequent QC samples out of 2SD limit, five to six subsequent QC samples out of the SD limit on the same side of the mean, 8 subsequent QC samples with a trend towards increasing or decreasing with time"*. Therefore, the overall repeatability of the different methods used within the study could be considered satisfactory and the datasets suitable for the statistical analysis.

The next step was to obtain a general overview of the datasets using unsupervised multivariate analysis. PCA was used to describe the variance in each data matrix in a lower dimensional space by reducing the number of considered variables with minor loss of information. Thus, four PCA models were established with the four data matrices (GC-MS$^+$, LC-MS$^-$, LC-MS$^+$ and LC-MS- combined). The clustering of the QC replicates in the center of the projection indicating proper normalization and good data precision was initially verified. The 3D scores plots showing the three first principal components of the built models are presented in Fig. 1. The clustering of the samples coming from the same GI is easily noticeable. It is worth mentioning that samples from the same country are grouped in adjacent clusters (Priego de Córdoba and Baena (Spanish) and Meknès

and Ouazzane (Moroccan)). Table 1 provides relevant descriptive information to compare the four PCA models. The model from LC-MS in negative polarity explained 90 percent of the variance with the lowest number of components, 13. In addition, its first four components explained the highest percentage of variance, 69.2%. On the other hand, the GC-MS model presented the best R^2 (parameter that estimates the goodness of the fit) and the model of LC-MS in positive polarity presented the best Q^2 (which estimates the goodness of the prediction).

Table 1. Descriptive information of the unsupervised PCA models

	LC-MS			GC-MS
Polarity	Negative	Positive	Combined	Positive
n $PC_{90\%}$	13	17	19	19
R^2	0.8702	0.9099	0.9189	0.9811
Q^2	0.6959	0.8387	0.8057	0.5315
Explained variance	PC1: 30.2% PC2: 20.7% PC3: 13.9% PC4: 4.4%	PC1: 28.1% PC2: 19.0% PC3: 11.0% PC4: 5.3%	PC1: 24.1% PC2: 20.6% PC3: 11.6% PC4: 5.4%	PC1: 30.3% PC2: 17.2% PC3: 10.4% PC4: 6.4%

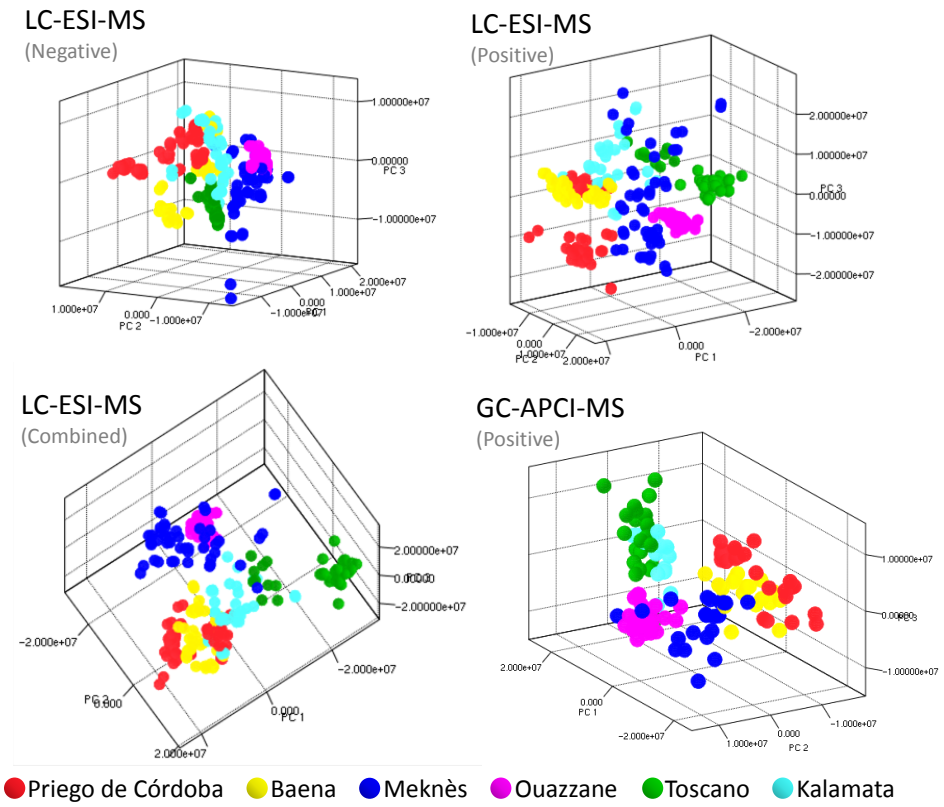

Figure 1. 3D scores plots showing the three first principal components of the PCA models established with data coming from each platform and polarity.

In a subsequent stage of the data treatment, PLS-DA was applied to the four data matrices to build two-class models to discriminate each GI from the rest. Table S2 includes R^2 and Q^2 quality parameters for the 24 established models (6 models with each data matrix) and Fig. S3 shows their scores plots. PLS-DA is a dimension reduction tool which filters out variables with little relevance to explain the preselected groups' discrimination. However, unlike in PCA plots where the PCs point along the directions of maximum variance in the X space, the PCs now highlight that part of the variance which is most relevant for modelling the data. As seen in Fig. S3, the best class separation was achieved for Ouazzane, Kalamata and Toscano GIs, while Priego de Córdoba and Baena, the two Spanish (specifically Andalusian) PDOs were the most hardly separable. In all the proposed models except for the ones using GC-MS data, the obtained quality parameters for fitness and prediction were higher than 0.9. The most favorable R^2 and Q^2 values were found for the models using combined information (from both polarities) achieved by LC-MS. In spite of the good quality parameters of the models achieved with combined information, there was one case in which no total class separation was attained: the model built to discriminate Baena samples from the rest, where one sample from Meknès fell in the middle of Baena group (R^2: 0.998995, Q^2: 0.981470).

The variable importance selection method in PLS-DA is based on Variable Importance in the Projection (VIP) scores, which estimate the importance of each variable to explain class separation. Thus, compounds with the highest VIP values had the greatest contribution to GI discrimination in the two-class models and could be pointed out as potential markers for a given GI. Table 2 shows the 5 compounds with the highest VIP value for each PLS-DA two-class model, together with characteristic information that helped to annotate the compound (when possible). More analytes were logically included in the VIP lists generated by the software for each model/platform/polarity, but we only considered those with the highest VIP values to simplify the overview. The next sections of the paper will describe the followed strategies to reach the identity of the markers, the complementarity of the different platforms/polarities and their possible use in semi-quantitative approaches.

3.3. Markers identification: complementarity of different platforms and polarities

Once the potential origin markers were selected according to their VIP value for each established PLS-DA model, they were further investigated to give them a plausible identity by using the annotation tools provided by MetaboScape.

The identity of most of the markers chosen when using the data from the LC-MS platform operating in negative polarity was found without excessive difficulties. 11 out of the 15 found markers were available as pure standards or as isolated fractions from VOO and they were automatically annotated with the Analyte List previously created with experimental data (*m/z* signals, isotopic patterns and Rts). As can be seen in Table 2 (a), all the established markers in LC-MS in negative polarity were substances which belong to phenolic compounds chemical class. The

identification of hydroxy decarboxymethyl oleuropein aglycone and three elenolic acid-related compounds (hydroxy decarboxymethyl elenolic acid, desoxy elenolic acid and hydroxy elenolic acid) had been previously accomplished by our research team [27] and the reported *m/z* signals and relative Rts allowed the unambiguous annotation of these four markers. Besides, elenolic acid (isomer a), decarboxymethyl oleuropein aglycone, hydroxytyrosol, luteolin, acetoxypinoresinol and different isomers of oleuropein and ligstroside aglycones could be mentioned as examples of origin markers. The fact that the same substance appeared as classifier for different GIs is perfectly explainable, since its significance can be related to its high or low concentrations and/or its relative concentration ratio with some other markers.

Table 2. GI markers pointed out by the PLS-DA models

(a)	LC-MS negative						
	Rt	**Measured *m/z***	**Δ*m/z* [mDa]**	**mSigma**	**Molecular Formula**	**Name**	**VIP value**
Priego de Córdoba	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	15.85
	3.11	257.0670	-0.631	12.3	$C_{11}H_{14}O_7$	hydroxy elenolic acid	11.29
	4.37	319.1187	-0.056	6.2	$C_{17}H_{20}O_6$	decarboxymethyl oleuropein aglycone	9.99
	1.93	153.0551	-0.594	0.2	$C_8H_{10}O_3$	hydroxytyrosol	7.84
	3.59	335.1136	-0.318	7.3	$C_{17}H_{20}O_7$	hydroxy decarboxymethyl oleuropein aglycone	7.83
Baena	4.25	285.0406	0.115	4.1	$C_{15}H_{10}O_6$	luteolin	13.86
	1.93	153.0551	-0.594	0.2	$C_8H_{10}O_3$	hydroxytyrosol	13.68
	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	12.00
	2.10	199.0607	-0.473	8.5	$C_9H_{12}O_5$	hydroxy decarboxymethyl elenolic acid	8.50
	4.37	319.1187	-0.056	6.2	$C_{17}H_{20}O_6$	decarboxymethyl oleuropein aglycone	8.00
Meknès	4.37	319.1187	-0.056	6.2	$C_{17}H_{20}O_6$	decarboxymethyl oleuropein aglycone	12.51
	3.59	335.1136	-0.318	7.3	$C_{17}H_{20}O_7$	hydroxy decarboxymethyl oleuropein aglycone	11.74
	4.65	377.1241	-0.056	10.0	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer a)	10.50
	4.25	285.0406	0.115	4.1	$C_{15}H_{10}O_6$	luteolin	9.22
	1.93	153.0551	-0.594	0.2	$C_8H_{10}O_3$	hydroxytyrosol	8.94
Ouazzane	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	11.91
	3.15	225.0770	-0.037	10.0	$C_{11}H_{14}O_5$	desoxy elenolic acid	10.63
	4.65	377.1241	-0.056	10.0	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer a)	8.06
	5.86	377.1240	-0.144	10.9	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer c)	8.03
	7.06	361.1292	-0.058	9.5	$C_{19}H_{22}O_7$	ligstroside aglycone (isomer b)	7.70
Toscano	5.86	377.1240	-0.144	10.9	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer c)	12.14
	7.06	361.1292	-0.058	9.5	$C_{19}H_{22}O_7$	ligstroside aglycone (isomer b)	8.24
	5.69	377.1241	-0.114	6.9	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer b)	8.03
	6.22	377.1241	-0.060	8.5	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer d)	7.33
	4.90	415.1397	-0.110	1.2	$C_{22}H_{24}O_8$	acetoxypinoresinol	6.95
Kalamata	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	11.21
	7.06	361.1292	-0.058	9.5	$C_{19}H_{22}O_7$	ligstroside aglycone (isomer b)	8.96
	5.71	361.1292	-0.074	10.1	$C_{19}H_{22}O_7$	ligstroside aglycone (isomer a)	7.59
	3.11	257.0670	-0.631	12.3	$C_{11}H_{14}O_7$	hydroxy elenolic acid	7.23
	5.69	377.1241	-0.114	6.9	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer b)	7.05

m/z corresponding to [M-H]- MS signal

(b)	LC-MS positive						
	Rt	*m/z*	Δ*m/z* [mDa]	mSigma	Molecular Formula	Name	VIP value
Priego de Córdoba	8.91	217.1592	-0.462	11.0	$C_{15}H_{20}O$	hexyl cinnamaldehyde	10.97
	5.22	267.1231	0.394	2.1	$C_{14}H_{18}O_5$	unknown 1	10.92
	7.86	231.1385	0.500	3.3	$C_{15}H_{18}O_2$	unknown 2	9.89
	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	9.40
	4.04	243.0868	0.517	10.4	$C_{11}H_{14}O_6$	elenolic acid (isomer b)	8.12
Baena	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	13.87
	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	8.05
	5.22	267.1231	0.394	2.1	$C_{14}H_{18}O_5$	unknown 1	7.83
	8.91	217.1592	-0.462	11.0	$C_{15}H_{20}O$	hexyl cinnamaldehyde	7.04
	7.86	231.1385	0.500	3.3	$C_{15}H_{18}O_2$	unknown 2	6.30
Meknès	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	13.03
	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	12.17
	7.86	231.1385	0.500	10.7	$C_{15}H_{18}O_2$	unknown 2	10.30
	8.91	217.1592	-0.462	11.0	$C_{15}H_{20}O$	hexyl cinnamaldehyde	7.19
	8.75	215.1436	0.543	8.9	$C_{15}H_{18}O$	unknown 3	6.93
Ouazzane	7.86	231.1385	0.500	10.7	$C_{15}H_{18}O_2$	unknown 2	14.49
	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	7.96
	5.71	363.1444	0.492	9.1	$C_{19}H_{22}O_7$	ligstroside aglycone (isomer a)	7.22
	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	6.60
	2.64	171.1015	-0.038	6.4	C9H14O3	unknown 4	6.20
Toscano	7.86	231.1385	0.500	10.7	$C_{15}H_{18}O_2$	unknown 2	14.98
	8.91	217.1592	-0.462	11.0	$C_{15}H_{20}O$	hexyl cinnamaldehyde	10.68
	7.40	357.1341	0.871	21.8	$C_{20}H_{20}O_6$	unknown 5	10.08
	8.55	235.1333	0.465	11.1	$C_{14}H_{18}O_3$	unknown 6	5.62
	8.75	215.1436	0.543	8.9	$C_{15}H_{18}O$	unknown 3	5.40
Kalamata	7.86	231.1385	0.500	3.3	$C_{15}H_{18}O_2$	unknown 2	12.87
	6.44	235.1697	0.500	11.2	$C_{15}H_{22}O_2$	4-octylbenzoic acid	7.89
	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	7.26
	3.60	234.0868	0.496	1.4	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	6.46
	6.16	301.0714	0.687	5.3	$C_{16}H_{12}O_6$	diosmetin	6.16

m/z corresponding to $[M+H]^+$ MS signal

(c)	LC-MS combined						
	Rt	*m/z*	Δ*m/z* [mDa]	mSigma	Molecular Formula	Name	VIP value
Priego de Córdoba	8.91	217.1592	-0.462	11.0	$C_{15}H_{20}O$	hexyl cinnamaldehyde	11.62
	5.22	267.1231	0.394	2.1	$C_{14}H_{18}O_5$	unknown 1	10.77
	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	10.54
	7.86	231.1385	0.500	3.3	$C_{15}H_{18}O_2$	unknown 2	10.53
	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	9.63
Baena	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	15.05
	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	8.97
	1.93	153.0551	-0.594	0.2	$C_8H_{10}O_3$	hydroxytyrosol	8.96
	5.22	267.1231	0.394	2.1	$C_{14}H_{18}O_5$	unknown 1	7.84
	2.10	199.0607	-0.473	8.5	$C_9H_{12}O_5$	hydroxy decarboxymethyl elenolic acid	7.07
Meknès	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	13.20
	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	12.29
	7.86	231.1385	0.500	10.7	$C_{15}H_{18}O_2$	unknown 2	10.87
	3.59	335.1136	-0.318	7.3	$C_{17}H_{20}O_7$	hydroxy decarboxymethyl oleuropein aglycone	10.07
	4.37	319.1187	-0.056	6.2	$C_{17}H_{20}O_6$	decarboxymethyl oleuropein aglycone	9.98

	7.86	231.1385	0.500	10.7	$C_{15}H_{18}O_2$	unknown 2	15.75
	5.16	271.0605	-0.892	9.8	$C_{15}H_{10}O_5$	apigenin	8.56
Ouazzane	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	7.93
	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	7.79
	3.15	225.0770	-0.037	10.0	$C_{11}H_{14}O_5$	desoxy elenolic acid	7.07
	7.86	231.1385	0.500	10.7	$C_{15}H_{18}O_2$	unknown 2	17.02
	8.91	217.1592	-0.462	11.0	$C_{15}H_{20}O$	hexyl cinnamaldehyde	12.16
Toscano	7.40	357.1341	0.871	21.8	$C_{20}H_{20}O_6$	unknown 5	11.23
	5.86	377.1240	-0.144	10.9	$C_{19}H_{22}O_8$	oleuropein aglycone (isomer c)	6.98
	8.75	215.1436	0.543	8.9	$C_{15}H_{18}O$	unknown 3	6.36
	7.86	231.1385	0.500	3.3	$C_{15}H_{18}O_2$	unknown 2	12.02
	3.60	241.0720	0.225	6.0	$C_{11}H_{14}O_6$	elenolic acid (isomer a)	10.5
Kalamata	7.06	361.1292	-0.058	9.5	$C_{19}H_{22}O_7$	ligstroside aglycone (isomer b)	8.52
	4.25	287.0556	0.615	5.4	$C_{15}H_{10}O_6$	luteolin	8.38
	6.44	235.1697	0.500	11.2	$C_{15}H_{22}O_2$	4-octylbenzoic acid	7.34

m/z corresponding to $[M-H]^-$ or $[M-H]^+$ depending on the predominant signal found by Metaboscape 3.0

(d)	GC-MS						
	Rt	***m/z***	**Δ*m/z* [mDa]**	**mSigma**	**Molecular Formula ***	**Name**	**VIP value**
	15.39	315.1257	-0.203	2.4	$C_{14}H_{22}O_6Si$	elenolic acid (isomer I)	26.96
	34.54	427.3236	2.830	18.9	$C_{24}H_{46}O_4Si$	glyceryl linoleate	19.99
Priego de Córdoba	38.23	281.1385	0.298	0.3	$C_{14}H_{24}O_2Si_2$	oleuropein aglycone (isomer I)	18.94
	30.44	193.1036	0.565	29.8	$C_{11}H_{16}OSi$	decarboxymethyl ligstroside aglycone	16.40
	43.84	397.3825[b]	-0.32	18.1	$C_{29}H_{48}$	β-sitosterol	11.69
	15.39	315.1257	-0.203	2.4	$C_{14}H_{22}O_6Si$	elenolic acid (isomer I)	26.48
	30.44	193.1036	0.565	29.8	$C_{11}H_{16}OSi$	decarboxymethyl ligstroside aglycone	17.13
Baena	34.54	427.3236	2.830	18.9	$C_{24}H_{46}O_4Si$	glyceryl linoleate	16.39
	19.64	327.2710	-0.372	9.4	$C_{19}H_{38}O_2Si$	palmitoleic acid	15.00
	8.75	193.1038	-0.166	8.9	$C_{11}H_{16}OSi$	tyrosol	11.36
	38.23	281.1385	0.298	0.3	$C_{14}H_{24}O_2Si_2$	oleuropein aglycone (isomer I)	26.09
	35.41	193.1035	-0.499	5.8	$C_{11}H_{16}OSi$	ligstroside aglycone (isomer I)	18.39
Meknès	13.69	281.1384	-0.343	11.1	$C_{14}H_{24}O_2Si_2$	hydroxytyrosol	15.86
	19.64	327.2710	-0.372	9.4	$C_{19}H_{38}O_2Si$	palmitoleic acid	14.33
	43.84	397.3825[#]	-0.32	18.1	$C_{29}H_{48}$	β-sitosterol	12.46
	13.69	281.1384	-0.343	11.1	$C_{14}H_{24}O_2Si_2$	hydroxytyrosol	23.42
	43.84	397.3825[#]	-0.32	18.1	$C_{29}H_{48}$	β-sitosterol	20.48
Ouazzane	37.60	193.1037	0.535	9.8	$C_{11}H_{16}OSi$	ligstroside aglycone (isomer III)	20.18
	36.76	193.1038	0.549	8.2	$C_{11}H_{16}OSi$	ligstroside aglycone (isomer II)	19.69
	38.23	281.1385	0.298	0.3	$C_{14}H_{24}O_2Si_2$	oleuropein aglycone (isomer I)	16.74
	19.64	327.2710	-0.372	9.4	$C_{19}H_{38}O_2Si$	palmitoleic acid	25.77
	44.66	499.4318	-1.300	6.0	$C_{33}H_{58}OSi$	cycloartenol	24.09
Toscano	30.44	193.1036	0.565	29.8	$C_{11}H_{16}OSi$	decarboxymethyl ligstroside aglycone	21.84
	34.54	427.3236	2.830	18.9	$C_{24}H_{46}O_4Si$	glyceryl linoleate	19.91
	8.75	193.1038	-0.166	8.9	$C_{11}H_{16}OSi$	tyrosol	15.12
	44.00	395.3666[#]	-0.606	10.9	$C_{29}H_{46}$	Δ^5-avenasterol	26.07
	34.54	427.3236	2.830	18.9	$C_{24}H_{46}O_4Si$	glyceryl linoleate	20.31
Kalamata	44.66	499.4318	-1.300	6.0	$C_{33}H_{58}OSi$	cycloartenol	18.77
	45.50	513.4473	-1.269	6.3	$C_{34}H_{60}OSi$	methylencycloartanol	18.73
	47.03	511.3958	-0.781	8.1	$C_{33}H_{54}O_2Si$	oleanolic acid	18.69

*of the detected *m/z*

m/z corresponding to $[M-nH+nTMS]^+$

[#] *m/z* corresponding to $[M-H+TMS-OTMS]^+$

For the markers obtained from models using LC-MS data in positive polarity, the assignment of a tentative identity was much harder. In this case, just 5 out of the 14 selected markers were available in our experimentally created Analyte List (apigenin, luteolin, elenolic acid (isomers a and b) and ligstroside aglycon (isomer a)). The MS/MS library search allowed the annotation of diosmetin (a luteolin derivative commonly found in VOO) [27] and MetFrag supported two possible candidate structures provided by Compound Crawler for *m/z* 217.1592 (Rt 8.91 min) and *m/z* 235.1333 (Rt 6.44 min), which were tentatively annotated as hexyl cinnamaldehyde and 4-octylbenzoic acid, respectively (Fig. S4). The identity of 6 markers could not be figured out so far, as seen in Table 2 (b). The list of possible structures matching the calculated molecular formula was very extensive and the interpretation of the very fragmented MS/MS spectra did not conduct to an unequivocal identification. Even though the identity of some markers could not be revealed, the achieved results in LC-MS in positive polarity were crucial to confirm the significance of markers previously identified in negative polarity and to add some others to the potential classifiers list. Moreover, in the strict sense, it is not absolutely necessary to fully identify an efficient GI marker before using it for classification purposes.

The LC-MS combined model led us to pay attention to 16 substances. In general, they were compounds which had been marked as significant ones in negative or positive polarity. It was very interesting to evaluate their impact in the classification models which had been built considering both polarities in the data treatment (Table 2 (c)).

The identification of compounds from the VOO minor fraction when using GC-MS was not very straightforward, due to the derivatization reaction (trimethylsilylation) used to increase the volatility of the analytes. Despite the fact that the fragmentation occurring in the APCI interface (which is categorized as a soft ionization source) generates complex spectra, they can be decoded taking into account that $[M\text{-}nH+nTMS+H]^+$, $[M\text{-}nH+nTMS\text{-}mOTMS+H]^+$, $[M\text{-}nH+nTMS\text{-}mCOOTMS+H]^+$, etc. are frequent MS signals *(notice that TMS is used as the abbreviation of the trimethylsilyl group (C_3H_9Si)).* 15 compounds were designated as possible classifiers for the 6 two-class models. Some of them were easily identified since they were included in our Analyte List: eight phenolic compounds (elenolic acid (isomer I), hydroxytyrosol, tyrosol, one isomer of oleuropein aglycone, three of ligstroside aglycone and decarboxymethyl ligstroside aglycone); one fatty acid (palmitoleic acid); one sterol (β-sitosterol); and one triterpenic compound (oleanolic acid). On the contrary, the identity of 4 out of the 15 markers pointed out by the models using GC-MS data which were not available as pure standards or isolated fractions had to be investigated bearing in mind the adducts usually observed in APCI (mentioned above). $[M\text{-}H+TMS]^+$ and $[M\text{-}H+TMS\text{-}OTMS]^+$ signals were found for compounds at min 44.0, 44.6 and 45.50, and the assigned molecular formulas ($C_{29}H_{48}O$, $C_{30}H_{50}O$ and $C_{31}H_{52}O$) corresponded to three sterols commonly found in VOO[27] (Δ^5-avenasterol, cycloartenol and methylencycloartanol, respectively). Moreover, the compound eluting at min 34.54, with *m/z* 427.3236 ($C_{24}H_{47}O_4Si$) showed the signals $[M\text{-}H+TMS+H]^+$, $[M\text{-}2H+2TMS+H]^+$ and $[M\text{-}2H+2TMS\text{-}OTMS+H]^+$ in its MS spectra and was

tentatively annotated as glyceryl linoleate (a triacylglycerol derivative which may arise as a result of incomplete biosynthesis or hydrolytic reactions) [35] with neutral molecular formula $C_{21}H_{38}O_4$. In general, the GC-MS models guided us to discover different classifiers (belonging to diverse chemical families) of those achieved by LC-MS; this fact clearly demonstrated the complementarity of both platforms. The isomers of elenolic acid, oleuropein and ligstroside aglycones are denoted in Roman numerals in Table 2 (d) to distinguish them of those detected in LC-MS, since an exact parallel between them cannot be drawn.

3.4. Establishing GI characteristic compositional patterns

Fig. 2 summarizes the most influential markers found for each GI combining the information obtained by all the models built with the data from all the used platforms. The most remarkable features to distinguish the EVOOs coming from Priego de Córdoba were the following ones: very high relative levels of isomers of elenolic acid and its derivative hydroxy elenolic acid; very high abundance of apigenin and unknowns 1 ($C_{14}H_{18}O_5$) and 2 ($C_{15}H_{18}O_2$); and high levels of the decarboxymethylated forms of oleuropein and ligstroside aglycones and hydroxy decarboxymethyl oleuropein aglycone. In contrast, these oils exhibited low relative concentrations of glyceryl linoleate, oleuropein aglycone I, β-sitosterol, the marker tentatively annotated as hexyl cinnamaldehyde and hydroxytyrosol. All these results can be seen in Fig. S5, where different box plots - for graphically depicting the groups of samples - have been included. Baena high quality oils were distinguished from the others by the extremely high levels of flavonoids (luteolin and apigenin) and hydroxytyrosol and high relative abundance of hydroxy decarboxymethyl elenolic acid. Besides, they displayed low levels of decarboxymethyl oleuropein and ligstroside aglycones, tyrosol and unknown 1. Baena oils were also very poor regarding glyceryl linoleate and palmitoleic acid. Moroccan EVOOs coming from Meknès were defined by remarkably low levels of flavonoids, decarboxymethyl oleuropein aglycone and its hydroxylated derivative, but unusually high abundance of oleuropein and ligstroside aglycones. Other important but less influential features were their low levels of simple phenols (specifically of hydroxytyrosol) and palmitoleic acid, and the high relative concentration of β-sitosterol and unknown 3 ($C_{15}H_{18}O$). As far as EVOOs coming from Ouazzane are concerned, it is possible to describe their characteristic pattern as follows: extremely high concentration of desoxy elenolic acid, elevated relative levels of hydroxytyrosol and β-sitosterol, and quite low relative concentration of unknown 2, elenolic acid (isomer a) and flavonoids. This description can be completed adding notably raised abundance of unknown 4 ($C_9H_{14}O_3$) and relatively high concentration of different isomers of ligstroside and oleuropein aglycones. Italian olive oils from Toscano PGI showed characteristic upside levels of palmitoleic acid, cycloartenol and hexyl cinnamaldehyde, high relative concentrations of decarboxymethyl ligstroside aglycone and acetoxypinoresinol and low levels of some ligstroside and oleuropein aglycone isomers and unknown 3. Apart from that, they displayed high abundance of unknowns 2, 5 ($C_{20}H_{20}O_6$) and 6 ($C_{14}H_{18}O_3$). Greek EVOOs from Kalamata presented considerably high relative concentration of Δ^5-avenasterol, glyceryl linoleate, oleanolic acid, 4-octylbenzoic acid and

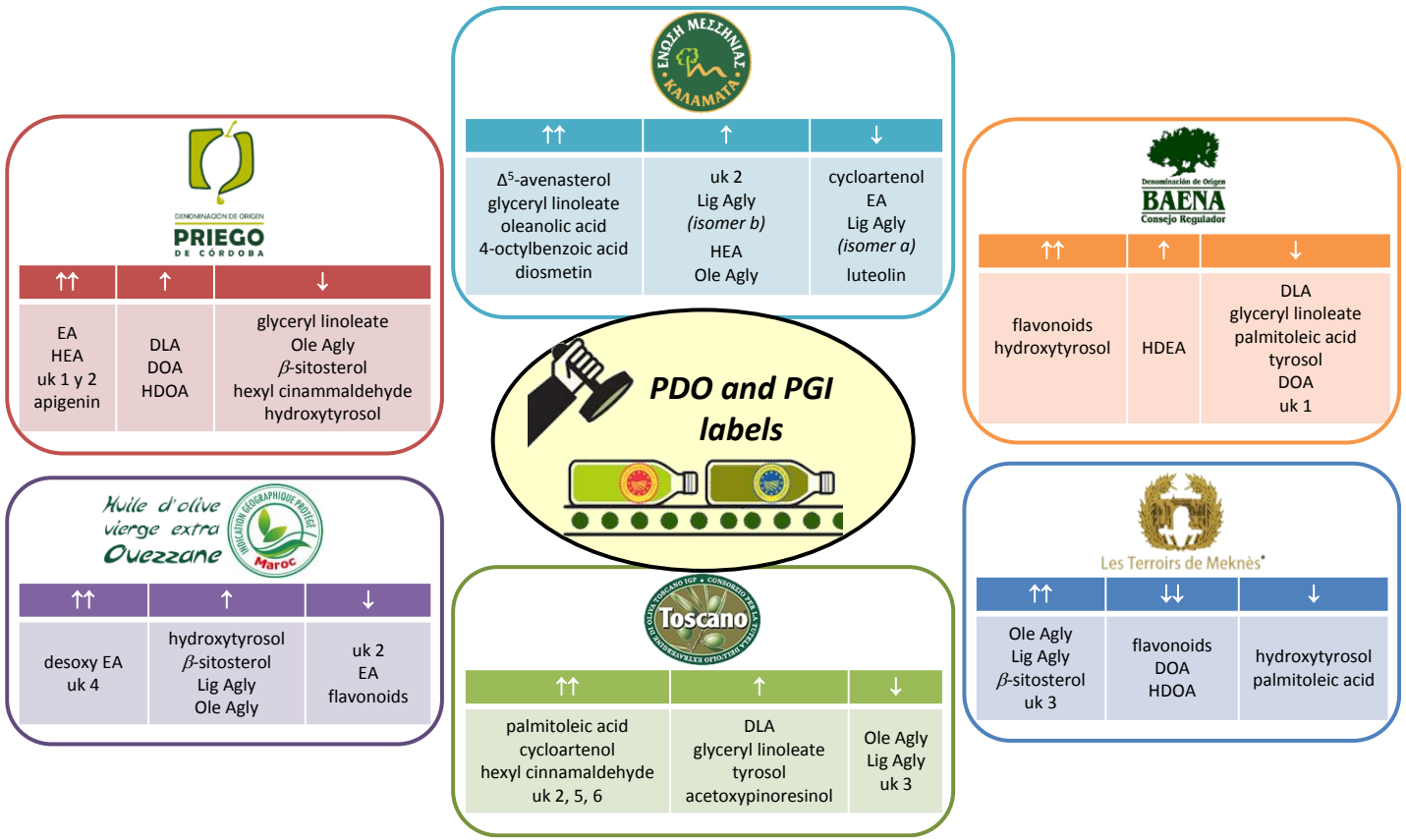

Figure 2. Summary of the most influential markers found for each GI combining the information obtained by all the models built with the data from all the used platforms. Ole Agly: oleuropein aglycone; DOA: decarboxymethyl oleuropein aglycone; HDOA: hydroxy decarboxymethyl oleuropein aglycone; Lig Agly: ligstroside aglycone; DLA: decarboxymethyl ligstroside aglycone; EA: elenolic acid; HEA: hydroxy elenolic acid; HDEA: hydroxy decarboxymethyl elenolic acid; uk: unknown; ↑↑ very high, ↑ high and ↓ low content.

diosmetin. Besides, they showed high levels of unknown 2, ligstroside aglycone (isomer b) (contrasting with low levels of the isomer a), hydroxy elenolic acid and oleuropein aglycone, and low abundance of cycloartenol, methylencycloartanol, elenolic acid and luteolin.

As stated, the main aim of this contribution was to attain the discrimination of EVOOs from different GIs by using multi-class metabolomic approaches and to identify potential origin markers. Establishing specific quantitative ranges of the markers is beyond the scope of this work and, from our point of view, would not represent an appropriate strategy, since there are plentiful factors affecting their concentration. It is more advisable to estimate the relative levels of several classifiers (which ones are the most abundant and which can be considered as the less profuse). In addition, it seems imperative to say that the need of validating the markers using samples from other seasons (and geographical locations) is evident. However, this is not a detriment to the compelling achievements of this project.

4. CONCLUSIONS

High quality olive oil labeling provides product-specific information and reduces quality uncertainty faced by consumers in their EVOO-choice decision process. Therefore, it is very pertinent to develop powerful and reliable methodologies assuring the geographical origin and overall quality of the GI oils. In this case, we focused on the massive coverage of VOO minor fraction and the use of non-targeted LC-ESI-QTOF MS (in positive and negative polarity) and GC-APCI-QTOF MS (in positive mode) methodologies. The capabilities of MetaboScape gave us the chance to build statistical models (two-class PLS-DA models) to discriminate among the selected samples pointing at potential classifiers (which were identified to a great extent, thanks to the annotation tools included within the software package and the use of specific standard mixtures, isolated VOO fractions and representative oil blends). The different polarities and platforms logically drove to diverse makers, taking advantage of their complementarity and, consequently, enriching the outcomes of the project. The definition of distinct compositional patterns of each GI was a very valuable accomplishment.

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Table S1. Selected processing parameters in Metaboscape 3.0.

	GC-APCI MS (positive)	LC-ESI MS (negative)	LC-ESI MS (positive)
Intensity threshold [counts]	5000	20000	35000
Minimum peak length [spectra]	9	4	
Rt range [min]	7.5 - 50	0.5 - 14	
Mass range [*m/z*]	50 - 1000	50 - 700	
EIC correlation	0.8	0.7	
Primary Ion	$[M+H]^+$	$[M-H]^-$	$[M+H]^+$
Seed Ions	$[M]^+$	$[M+Cl]^-$	$[M+Na]^+$, $[M+K]^+$
Common Ions	$[M+H-C_3H_8Si]^+$, $[M+H-C_6H_{16}Si_2]^+$, $[M+H-C_9H_{24}Si_3]^+$, $[M+H-C_{12}H_{32}Si_4]^+$, $[M+H-C_3H_9OSi]^+$	$[M-H-H_2O]^-$	$[M-H_2O+H]^+$
# of Buckets	5070	2198	2647
# of Buckets Combined Polarity	-	4737	

Table S2. Quality parameters of the two-class PLS-DA models.

	LC-MS (negative)	LC-MS (positive)	LC-MS (combined)	GC-MS (positive)
Priego de Córdoba	R^2: 0.986705	R^2: 0.997092	R^2: 0.997285	R^2: 0.803703
	Q^2: 0.963632	Q^2: 0.983695	Q^2: 0.982388	Q^2: 0.666292
Baena	R^2: 0.951771	R^2: 0.985733	R^2: 0.998995	R^2: 0.758957
	Q^2: 0.919694	Q^2: 0964519	Q^2: 0.981470	Q^2: 0.553767
Meknès	R^2: 0.944714	R^2: 0.976262	R^2: 0.989293	R^2: 0.973982
	Q^2: 0.913383	Q^2: 0.949756	Q^2: 0.971819	Q^2: 0.830445
Ouezzane	R^2: 0.994020	R^2: 0.981932	R^2: 0.999037	R^2: 0.964705
	Q^2: 0.985885	Q^2: 0.967208	Q^2: 0.993752	Q^2: 0.902717
Toscano	R^2: 0.996381	R^2: 0.995584	R^2: 0.998799	R^2: 0.973336
	Q^2: 0.977768	Q^2: 0.987154	Q^2: 0.990977	Q^2: 0.894252
Kalamata	R^2: 0.995528	R^2: 0.998477	R^2: 0.997330	R^2: 0.996639
	Q^2: 0.974758	Q^2: 0.986167	Q^2: 0.981570	Q^2: 0.964195

R^2: Goodness of the fit
Q^2: Goodness of the prediction

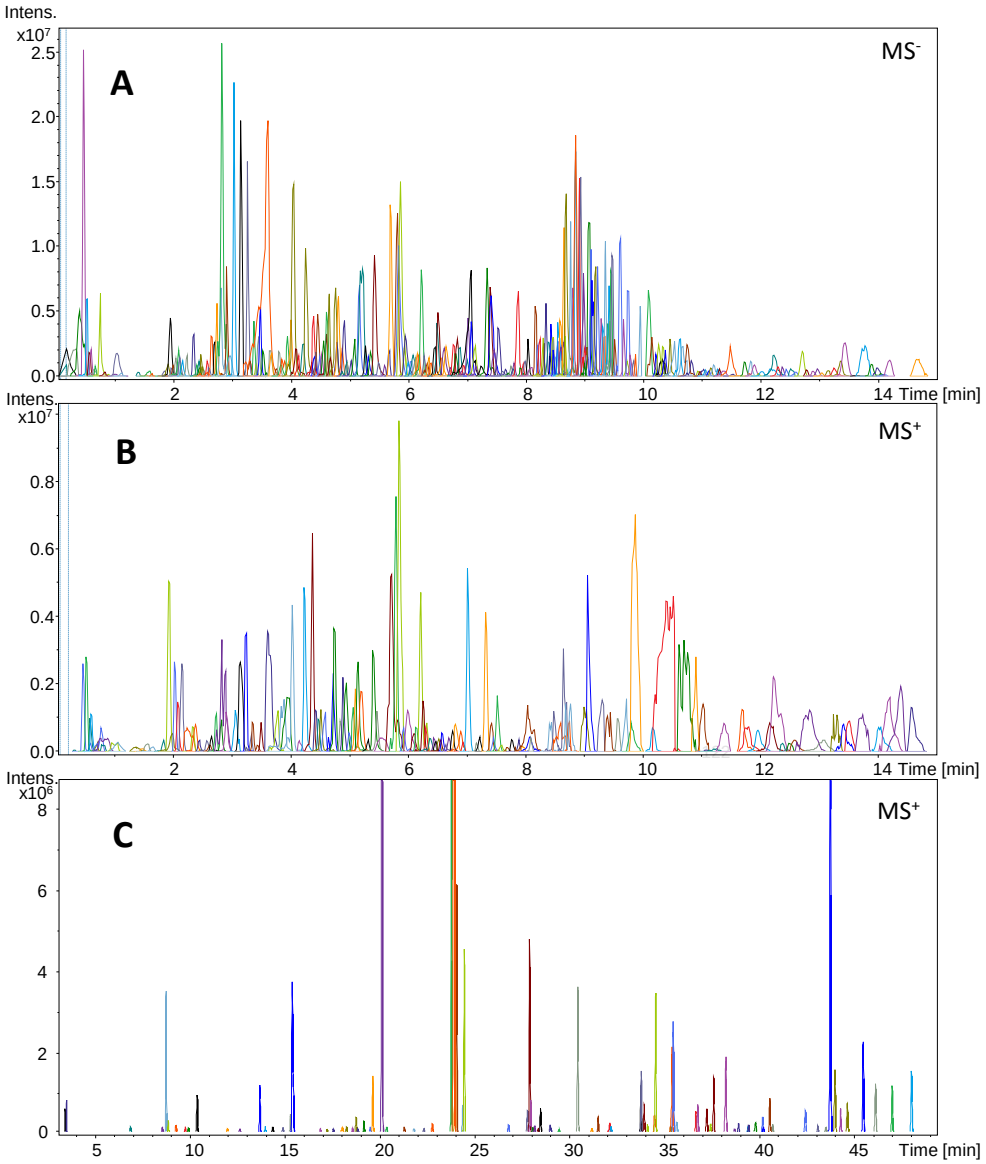
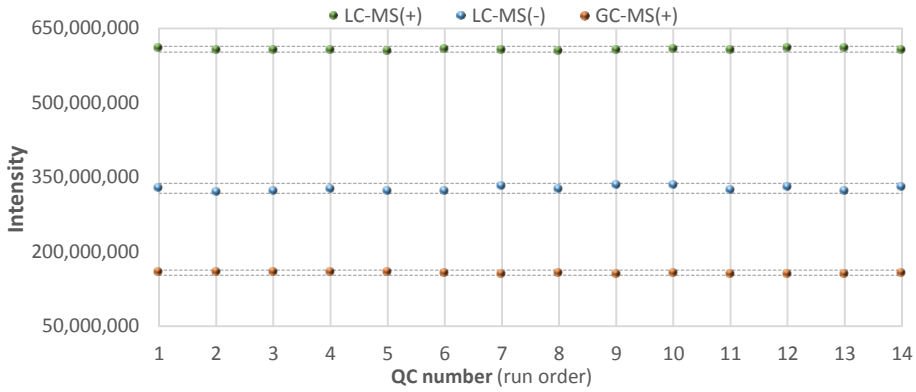


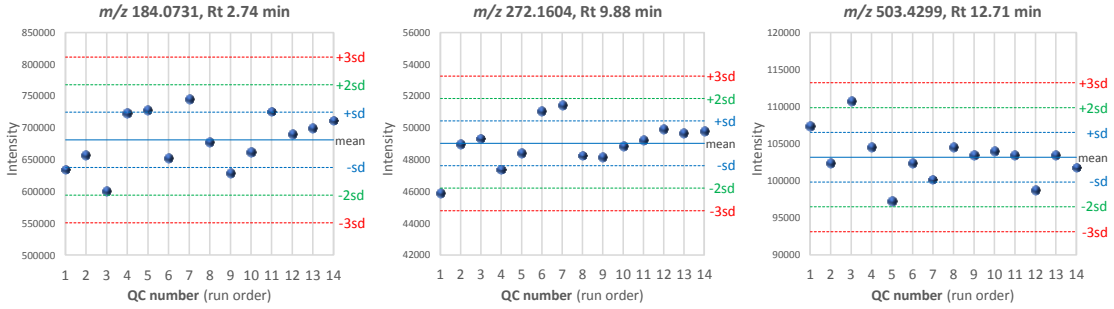
Figure S1. Extracted Ion Chromatograms (EIC) of the QC sample analyzed by LC-ESI-MS in negative (A) and positive (B) polarity, and by GC-APCI-MS (C).

A) Control charts for the total intensity of the chromatograms obtained by applying the three described methods

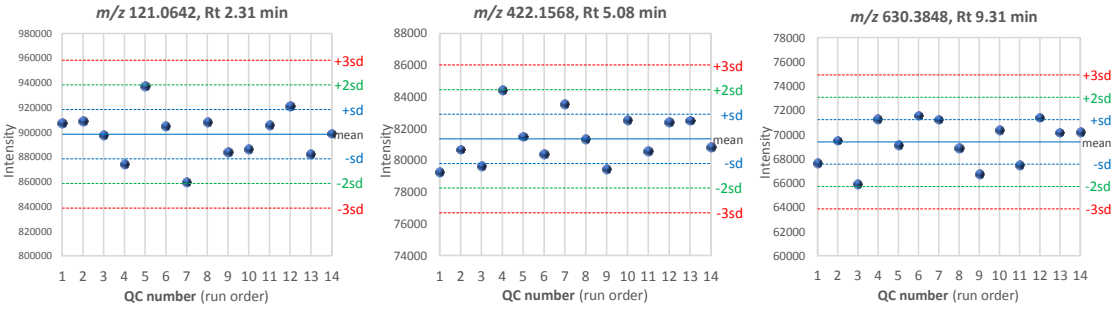


B) Control charts for three selected compounds when using the three described methodologies

I. LC-MS (negative polarity)



II. LC-MS (positive polarity)



III. GC-MS (positive polarity)

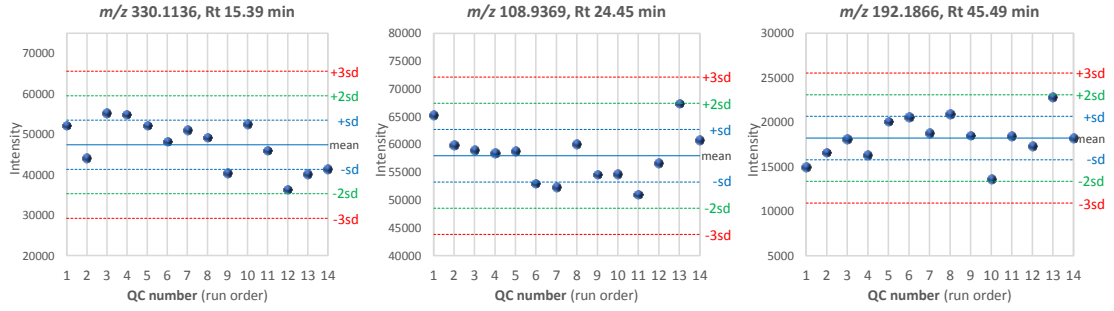


Figure S2. Control charts showing system stability along the sequences of analysis.

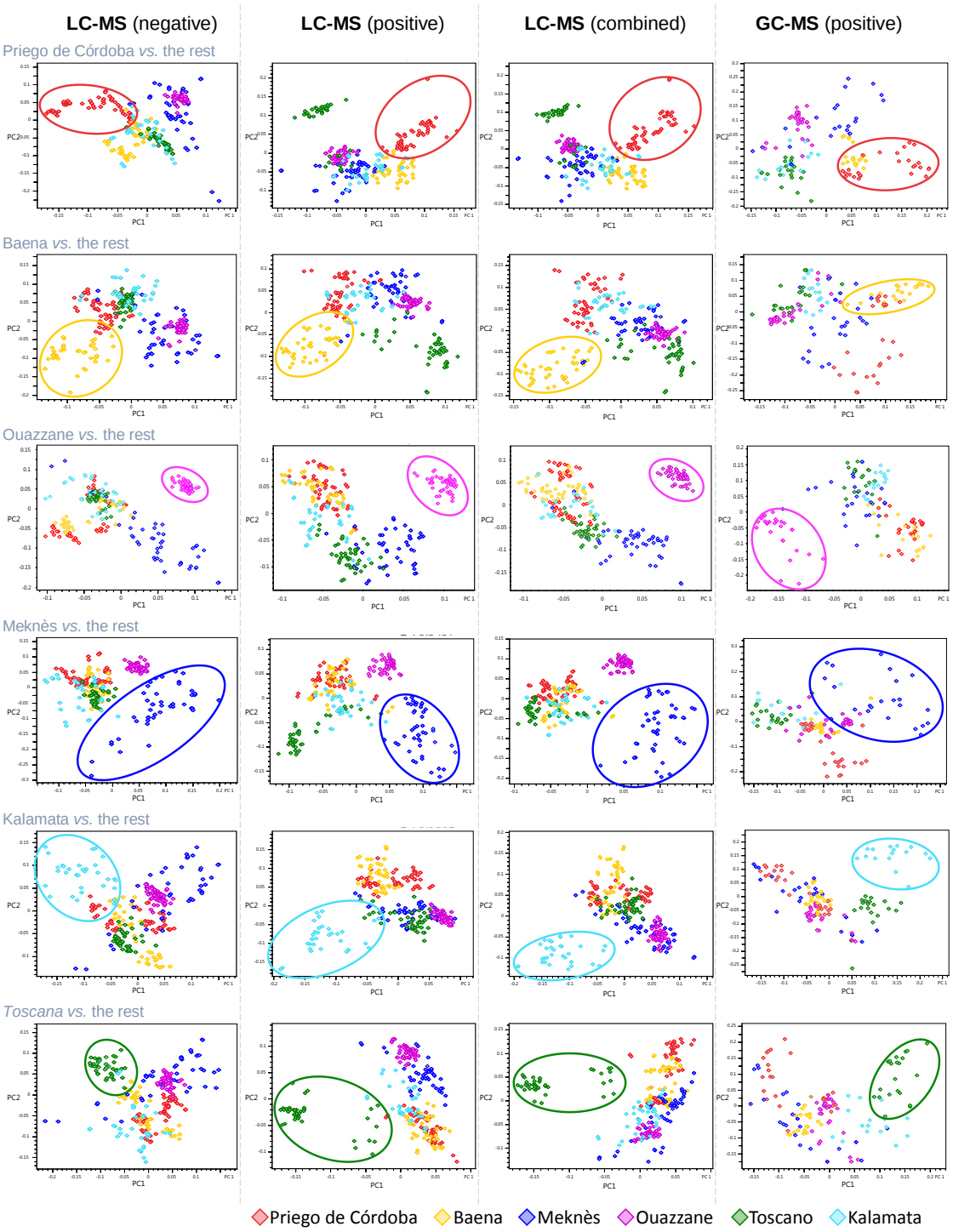
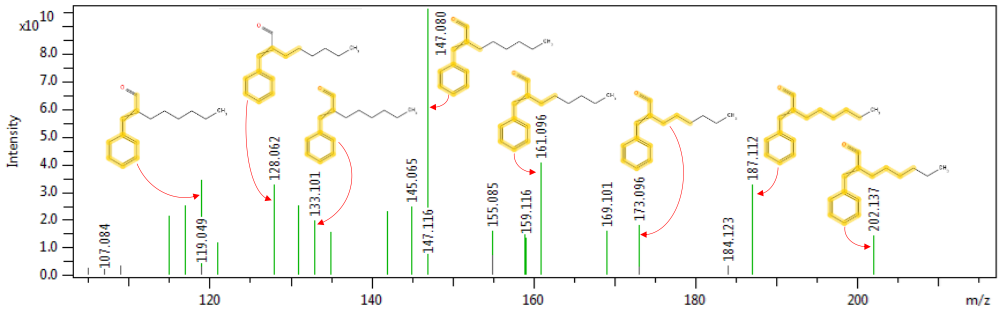


Figure S3. Scores plots of the PLS-DA two-class models.

I) **Hexyl cinnamaldehyde** (2-bezylideneoctanal) 217.1592 *m/z* Rt: 8.91 min



II) **4-octylbenzoic acid** 235.1697 *m/z* Rt: 6.44 min

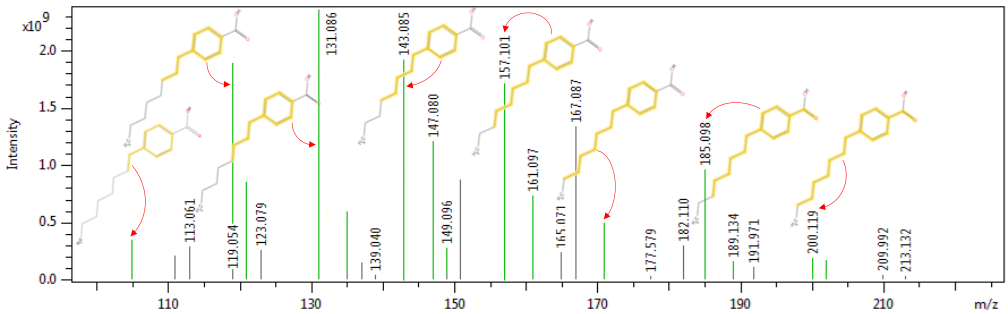
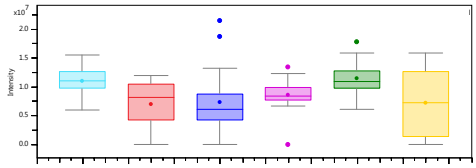


Figure S4. MetFrag *in-silico* fragmentation for two tentatively annotated markers in LC-MS$^+$.

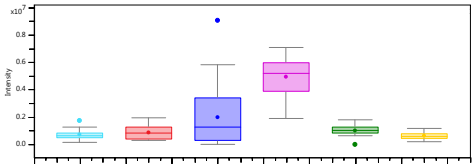
Figure S5. Boxplots for the markers pointed out by the PLS-DA models, summarizing all the intensities of the samples (grouped by the GI to which they belong) in a box-whiskerplot

I. Markers from GC-APCI-MS models

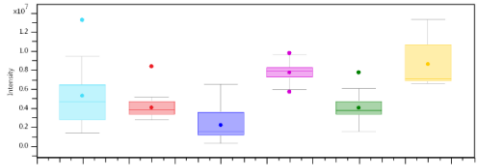
Tyrosol



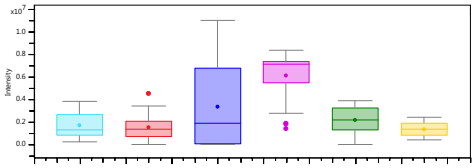
Ligstroside aglycone (isomer II)



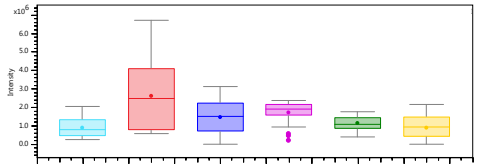
Hydroxytyrosol



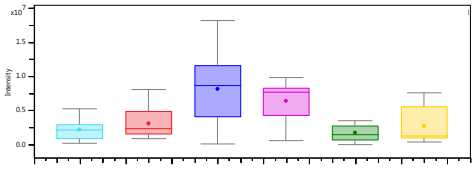
Ligstroside aglycone (isomer III)



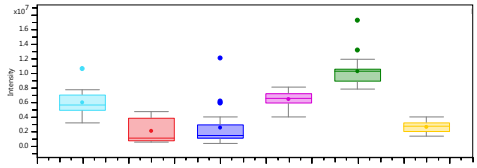
Elenolic acid (isomer I)



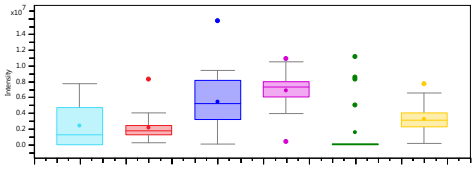
Oleuropein aglycone I



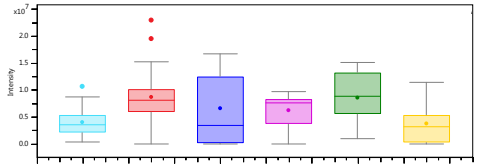
Palmitoleic acid



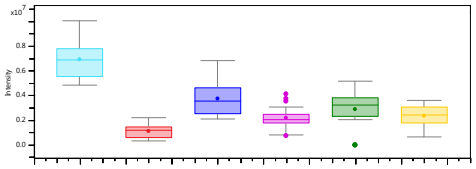
β-sitosterol



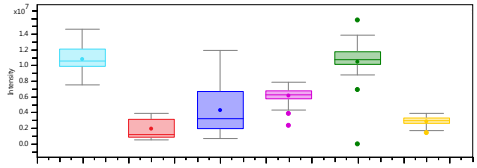
Decarboxymethyl ligstroside aglycone



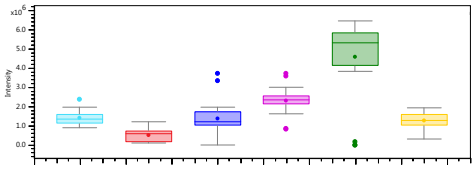
Δ^5-avenasterol



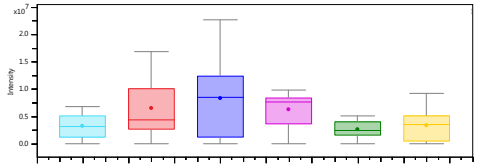
Glyceryl linoleate



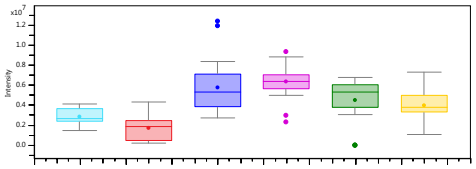
Cycloartenol



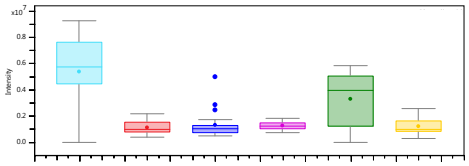
Ligstroside aglycone (isomer I)



Methylencycloartanol

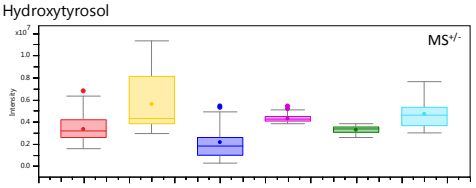


Oleanolic acid

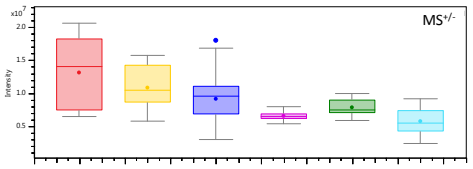


II. Markers from LC-ESI-MS models

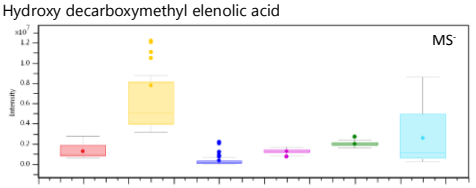
Hydroxytyrosol



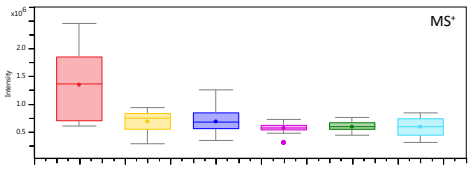
Elenolic acid (isomer a)



Hydroxy decarboxymethyl elenolic acid



Elenolic acid (isomer b)



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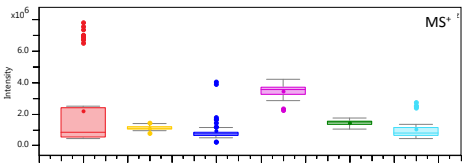
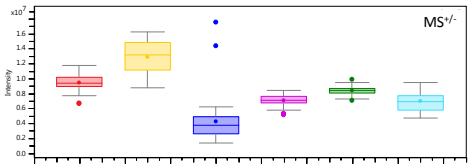


Luteolin



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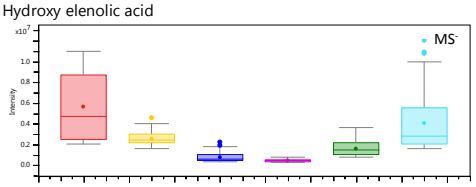
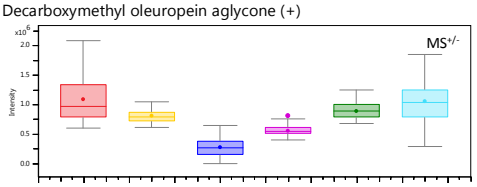


Decarboxymethyl oleuropein aglycone (+)



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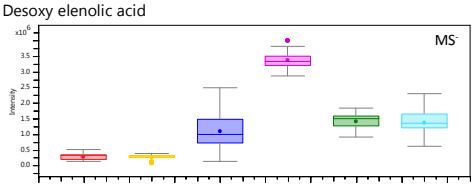
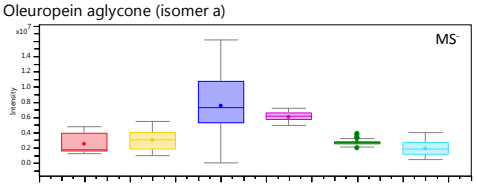
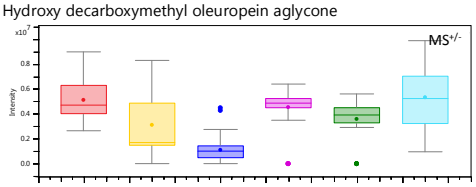


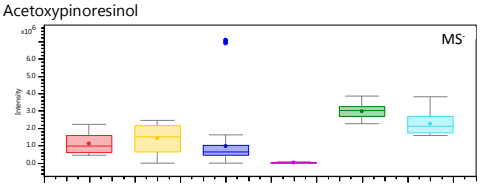
Oleuropein aglycone (isomer a)



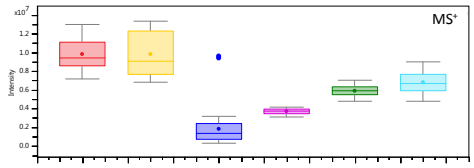
Hydroxy decarboxymethyl oleuropein aglycone



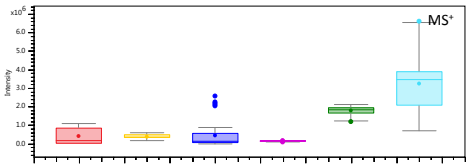
Acetoxypinoresinol



Apigenin



4-octylbenzoic acid



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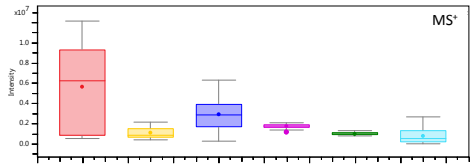
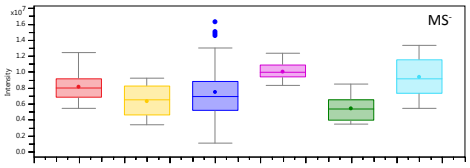
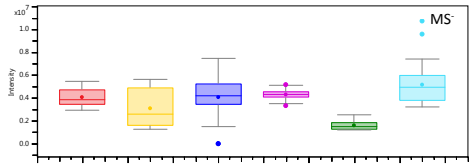


Ligstroside aglycone (isomer b)



Oleuropein aglycone (isomer b)



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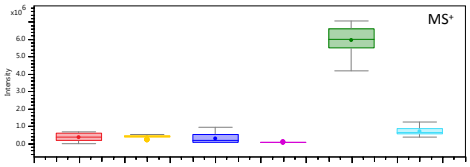
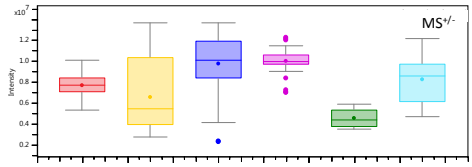


Oleuropein aglycone (isomer c)



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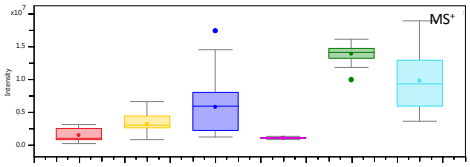
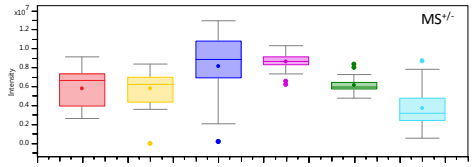


Ligstroside aglycone (isomer a)



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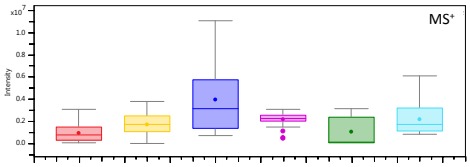
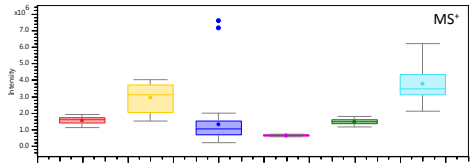
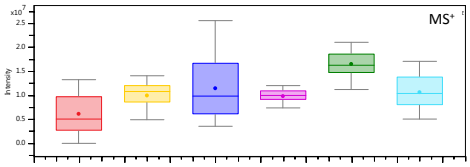


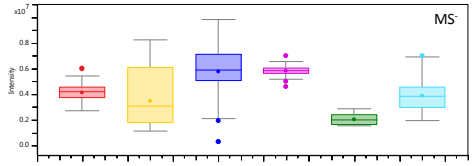
Diosmetin



Hexyl cinnamaldehyde



Oleuropein aglycone (isomer d)



Chapter 11

Unravelling the distribution of secondary metabolites in *Olea Europaea* L.: Exhaustive characterization of eight olive-tree derived matrices by complementary platforms (LC-ESI/APCI-MS and GC-APCI-MS)

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Molecules **(under review)**

Abstract: In order to understand the distribution of the main secondary metabolites found in *Olea europaea* L., 8 different samples (olive leaf, stem, seed, fruit skin and pulp, as well as virgin olive oil, olive oil obtained from stoned and dehydrated fruits and olive seed oil) coming from a Picudo *cv.* olive tree were analyzed. All the experimental conditions were selected so as to assure the maximum coverage of the metabolome of the samples under study within a single run. The use of LC and GC with high resolution MS (through different ionization sources, ESI and APCI) and the annotation strategies within MetaboScape 3.0 software allowed the identification of around 150 compounds in the profiles, showing great complementarity between the evaluated methodologies. The identified metabolites belonged to different chemical classes: triterpenic acids and dialcohols, tocopherols, sterols, free fatty acids and several sub-types of phenolic compounds. The suitability of each platform and polarity (negative and positive) to determine each family of metabolites was evaluated in-depth, finding, for instance, that LC-ESI-$MS^{(+)}$ was the most efficient choice to ionize phenolic acids, secoiridoids, flavonoids and lignans and LC-APCI-MS was very appropriate for pentacyclic triterpenic acids ($MS^{(-)}$) and sterols and tocopherols ($MS^{(+)}$). Afterwards, a semi-quantitative comparison of the selected matrices was carried out, establishing their typical features (*e.g.* fruit skin was pointed out as the matrix with the highest relative amounts of phenolic acids, triterpenic compounds and hydroxylated fatty acids, and seed oil was distinctive for its high relative levels of acetoxypinoresinol and tocopherols).

Keywords: *Olea europaea* L.; liquid chromatography; gas chromatography; mass spectrometry; secondary metabolites.

1. INTRODUCTION

Olive tree (*Olea europaea* L.), which has accompanied mankind since prehistoric times, has played a fundamental role in the economic, social and cultural spheres of Mediterranean civilizations [1,2]. Nowadays, along with the consumption of olives and olive oil in the diet, the use of different olive fractions with therapeutic purposes is still deeply rooted in traditional medicine from many parts of the world. It is now known that some of these traditional usages are supported by scientific evidences. In fact, different *in vitro* and *in vivo* studies carried out on plant materials or isolated components from olive tree and virgin olive oil (VOO) have demonstrated their health-promoting effects against inflammatory and age-dependent ailments such as cardiovascular and neurodegenerative diseases, diabetes or cancer, among others [3–7]. The phytochemical characterization of these matrices has revealed the presence of a plethora of bioactive secondary metabolites belonging to different chemical classes, mainly phenolic and triterpenic compounds, tocopherols, sterols and pigments [3,4]. Some of these phytonutrients found in olive fruits are transferred into the VOO [8–10] and are considered the main responsible of the healthy benefits derived from its consumption [11,12]. Logically, the rest of them remain in the VOO processing by-products, which have been pointed out as very valuable sources of bioactive compounds [13–15].

Both effluents (olive mill wastewater) and solid wastes (olive pomace) containing phenolic compounds, organic acids and lipids, are harmful to the environment. Consequently, some of the current VOO by-products management practices involve bioconversion to reduce their environmental impact or the recovery of those phytochemicals with potential applications in food, pharmaceutical and cosmetic industries [16–18]. Olive leaves (either coming from pruning or harvested olive fruits washing in table olive or VOO industries) represent the other major olive tree derived by-product and are also very rich in valuable metabolites; different reviews addressing olive leaf characterization, extraction techniques and applications can be found in literature [19,20]. Olive stones from pitted olive table industry have also been identified as a source of proteins and phenols with industrial applications [21].

The transformation of olive fruit and the valorization of by-products are currently considered as parts of the same integral cycle of olive grove exploitation. New environmentally friendly extraction techniques of high value-added compounds from olive derived residues are emerging as a way to increase the profitability of olive sector [22]. Moreover, formerly unexplored products such as olive seed oil and novel processing methods are being investigated in an attempt to take advantage of all olive tree derived matrices with zero waste generation [23–25]. It is clear that the exhaustive characterization of every olive tree fraction (olive fruit organs and resulting oils, as well as leaves and stems) is crucial when looking for new applications or new sources of bioactive compounds [26].

Different metabolomic approaches, mainly based on nuclear magnetic resonance (NMR) [27,28] and mass spectrometry (MS) [29], have been applied to the study of small metabolites in olive tree matrices. The use of separative techniques such as liquid (LC) or gas (GC) chromatography prior to MS detection is commonplace when analyzing complex plant derived samples [30]. Both LC/GC-MS based metabolic profiling approaches (primarily focused on the polar phenols fraction) have been used to study olive plant organs (leaves, stems, wood, roots) [31–33], olive fruits [34,35], and VOO [36,37]. Different coupling interfaces can be used depending on the physicochemical properties of the analytes under study [38]. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources are the most frequently chosen for LC-MS analyses. Both of them can offer complementary information; for instance, whereas ESI has been the most commonly used interface for phenols profiling [33,34,36], APCI has demonstrated some advantages for the detection of specific families of compounds such as tocopherols or sterols and has also proved to be suitable for phenolic compounds determination [39–41]. In GC-MS, electron impact (EI) is the most used ionization source because, at 70 eV, it produces a characteristic fragmentation pattern that enables identification of compounds by means of mass spectral library search. However, the use of softer ionization techniques such as CI or APCI, which can preserve the pseudo-molecular ion information, is becoming increasingly popular since they allow the identification of unknown compounds missing in commercial libraries [42].

In this study, multi-analyte methods were applied to the metabolic profiling of 8 matrices coming from a Picudo *cv.* olive tree, including plant materials (leaves, stems and fruit epicarp, mesocarp and seed) and oils (VOO, olive oil obtained from stoned and dehydrated fruits and olive seed oil). Sample preparation consisted in the application of a very unselective protocol aiming the extraction of as many compounds as possible. The resulting extracts were analyzed by LC-QTOF MS (coupled through two kinds of interfaces, ESI and APCI) and GC-APCI-QTOF MS (after derivatization of the prepared extracts) in order to compare the analytical performance of each platform and maximize the achieved information. Our final goal was to understand the distribution of the detected compounds on the studied matrices.

2. RESULTS AND DISCUSSION

2.1. Comprehensive qualitative determination of the matrices under study

In a first stage of the study, 50 standards or isolated fractions of compounds already detected in *Olea europaea* L. matrices were analyzed by GC-APCI MS, LC-ESI MS and LC-APCI MS, in order to create an analyte list with the *m/z* and retention time (Rt) of known molecules which could help to achieve the identification of as many compounds as possible in the selected samples. Afterwards, all the prepared extracts were analyzed by using the three described methodologies. LC-MS analyses were conducted at least 4 times with each interface (ESI and APCI), in positive and negative polarities, both in normal MS and auto MS/MS modes. Fig. S1 (supplementary materials) shows typical chromatograms obtained with each platform and polarity when the olive oil obtained from stoned and dehydrated fruits is analyzed.

All the acquired files were imported into MetaboScape. Apart from selecting the optimal threshold for features selection depending on the intensity of the obtained chromatograms, the choice of the target ions was carefully optimized in order to correctly detect potential adducts or fragments belonging to each compound. When using negative polarity in LC-MS analyses, the pseudo-molecular ion $[M-H]^-$ was the major signal found in the spectra regardless of the interface. On the contrary, in positive ion mode, $[M+H]^+$ was not the prevalent MS signal in many cases; water losses were very common ($[M-H_2O+H]^+$) and alkali adducts (mainly $[M+Na]^+$ and $[M+K]^+$) were also frequently found, especially with the ESI source. Regarding GC-APCI MS signals, most of the compounds presented the *m/z* of the totally silylated molecule in their spectra, but MS signals corresponding to the loss of trimethylsilyl groups ($-C_3H_8Si$) as well as $-OC_3H_9Si$ losses were also commonly found. Accordingly, considering X as the completely silylated molecule, $[X+H]^+$, $[X-C_3H_8Si+H]^+$, $[X-C_6H_{16}Si_2+H]^+$, $[X-C_9H_{24}Si_3+H]^+$, $[X-C_{12}H_{32}Si_4+H]^+$ and $[X-OC_3H_9Si+H]^+$ were defined as additionally possible ions (referred to as 'common ions') for features selection in MetaboScape. The rest of the extraction parameters, among which peak length and peak correlation stand out, were selected so as to have a reasonable number of putative compounds (approximately 2000) in each data matrix of the 5 resulting ones (one for each experiment: 2

interfaces and 2 polarities in LC-MS and one in GC-MS; in other words, 1 for LC-ESI$^{(+)}$, 1 for LC-ESI$^{(-)}$, 2 for LC-APCI in positive and negative polarity, respectively, and 1 for GC-APCI in positive polarity).

Afterwards, all the available annotation strategies were applied in an attempt to give plausible identities to as many compounds as possible in the analyzed extracts. Fig. 1a shows a comparison between the total number of annotated compounds accomplished by using the different platforms employed in this study (having into account both polarities to calculate the number corresponding to LC couplings). The GC-APCI-MS hyphenation gave the fewest number of putative compound identifications in the olive derived samples (58), although 11 of them could only be detected with this platform. With LC-MS methodologies, 137 and 130 compounds were identified using ESI and APCI sources, respectively; 126 being detected with both of them. As can be seen from Fig. 1b, 129 was the highest number of compounds that could be identified in one run, specifically, operating the LC-ESI-MS platform in positive ionization mode. When using the negative mode for LC-ESI-MS analyses, only one compound less could be identified, 120 substances being correctly annotated by using both polarities. In the case of LC-APCI-MS analyses, negative ionization mode allowed the identification of 111 compounds whilst 83 were identified in positive polarity; 64 of them being detected in both polarities.

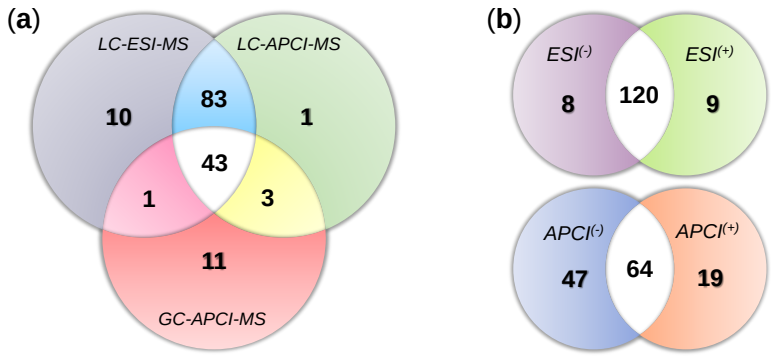

Figure 1. Venn diagrams showing total and overlapping numbers of identified compounds achieved with each platform and MS polarity. (a) LC-ESI-MS vs. LC-APCI-MS vs. GC-APCI-MS (combining together both ionization modes in LC-MS experiments); (b) Positive (+) vs. negative (-) polarity in LC-ESI-MS and LC-APCI-MS platforms.

Table S1 shows the detected compounds in LC-MS using both ESI and APCI sources. It includes the assigned names, the calculated neutral molecular formulas (M), Rts and MS signals detected when using each interface in both positive and negative polarities. The presented *m/z*, error (difference between the observed mass and the theoretical one) and mSigma (goodness of fit between the measured and the calculated isotopic pattern) correspond to the major ion detected (appearing first in the row) in the 'ESI MS signal' column (when available). The last column indicates if the compound identity was confirmed with the corresponding analytical standard or isolated fraction, if the identification was tentatively achieved with MetaboScape annotation tools (MetFrag or MS/MS library search), or if the peak assignment was done by contrasting previously published

information about compounds already detected in olive-related matrices. A total of 141 annotated compounds, belonging to 7 different chemical families, are presented in Table S1.

Organic acids. The presence of quinic acid in the extracts was confirmed by analyzing the corresponding pure standard. In addition, the compound eluting at 0.7 min (calculated molecular formula $C_6H_8O_7$), was tentatively annotated as citric acid (as previously reported in literature [33]).

Phenolic acids and aldehydes. 5 cinnamic acids (caffeic, *p*-coumaric, ferulic, sinapic and t-cinnamic acids), 8 benzoic acids (gallic, protocatechuic, gentisic, 4-hydroxybenzoic, 4-hydroxyphenylacetic, vanillic, syringic and homovanillic acids) and vanillin (a benzoic aldehyde) were annotated by matching with our in-house created analyte list. Moreover, 3,4,5-trimethoxybenzoic (known as eudesmic acid) and verbascoside (also known as acteoside), which is a hydroxycinnamic acid derivative, were tentatively identified in accordance with previous reports [34].

Coumarins. 2 coumarins, aesculetin and aesculin (aesculetin 6-O-glucoside) were found in the analyzed extracts. Suggested peaks agreed with relative Rts found by other authors [32,33].

Simple phenols and derivatives. The most popular substances of those found in olive matrices belonging to this family are hydroxytyrosol and tyrosol, which were unequivocally annotated by comparison with their analytical standards. Different derivatives of both of them (oxidized and acetylated hydroxytyrosol as well as hydroxytyrosol and tyrosol glucosides) were also found in some of the studied samples. Their identities were assigned having into account the changes of polarity caused by their distinctive functional groups and the way in which they theoretically should influence the eluting order. Another phenolic alcohol (3,4-dihydroxyphenylglycol) widely described in *Olea europaea* L. related matrices [34] and 2-phenethyl *β*-primeveroside, which has been previously isolated from olive cells [43], were also detected in the evaluated extracts. Besides, the peak with Rt 8.0 min and calculated molecular formula $C_{17}H_{26}O_4$, was tentatively annotated as gingerol, a natural methoxyphenol which, as far as we know, has not been detected in olive tissues before. The outcome of MetFrag (conducted on LC-ESI-MS/MS$^{(-)}$ data) that helped to gingerol's fragments assignment is represented in Fig. S2a.

Secoiridoids and derivatives. This chemical class was represented by 49 compounds in total; the identity of 15 of them was confirmed with the corresponding pure standard or isolated fraction. Secoiridoids are complex phenols characterized by the presence of elenolic acid in their structure and they can occur in glycosidic or aglycone forms (as a result of enzymatic hydrolysis). Lots of intermediates and derived products can be found in olive tree derived matrices, resulting from their biosynthetic and degradation pathways [44]. One sub-group of secoiridoids included 20 compounds belonging to the oleuropein family (which presents hydroxytyrosol in their structure): oleuropein, hydroxyoleuropein, dihydrooleuropein (two isomers), oleuropein glucoside, oleuropein aglycone (six isomers), methyloleuropein aglycone, dimethyloleuropein aglycone, 10-hydroxyoleuropein aglycone (two isomers), dehydrooleuropein aglycone,

decarboxymethyloleuropein aglycone (oleacein), hydroxydecarboxymethyloleuropein aglycone, methyldecarboxymethyloleuropein aglycone and hydroxytyrosol acyclodihydroelenolate. Another sub-group corresponded to the 9 homologous tyrosol derivatives (ligstroside family): ligstroside, ligstroside aglycone (six isomers), decarboxymethylligstroside aglycone (oleocanthal) and hydroxydecarboxymethylligstroside aglycone. The third sub-group was comprised of elenolic acid and 19 related compounds, including hydroxyelenolic acid (three isomers), desoxyelenolic acid (two isomers), decarboxymethylelenolic acid, hydroxydecarboxymethyl elenolic acid (two isomers), the decarboxylated form of hydroxyelenolic acid (two isomers), elenolic acid methylester, acyclodihydroelenolic acid hexoside, elenolic acid glucoside (also known as oleoside 11-methylester), oleoside or secologanoside (which are double-bond positional isomers), nuzhenide, comselogoside (two isomers), cafselogoside and lucidumoside C.

Flavonoids. Flavonoids, which are widespread in plants and fruits, can have lots of structural variations that generate different sub-classes. In the analyzed extracts, 8 flavonoids were found in aglycone form: a flavanone (naringenin), a flavanol (gallocatechin), a flavonol (quercetin), 2 flavanonols (dihydrokaempferol and taxifolin) and 3 flavones (luteolin, diosmetin and apigenin). MetFrag and MS/MS library search allowed the tentative annotation of $C_{15}H_{12}O_6$ (min 3.6) as a kaempferol derivative, not previously detected in *Olea europaea* L. matrices (See Fig. S2b). Eleven flavonoid glycosides were also identified in the evaluated samples. 3 luteolin glucosides were detected at Rt 2.8, 3.1 and 3.2 min; the first one was identified as luteolin 7-O-glucoside (confirmed with the pure standard), the second one was annotated as luteolin 4'-O-glucoside (according to the Bruker Sumner MetaboBASE Plant Library) and the third one could be a different positional isomer or another kind of glycoside. The presence of rutin, quercetin 4'-O-glucoside and apigenin 7-O-glucoside was confirmed with their pure standards too. A luteolin diglucoside isomer, cyanidin 3-O-glucoside, luteolin 7-O-rutinoside, apigenin 7-O-rutinoside and chrysoeriol 7-O-glucoside were also found in the evaluated olive tree derived samples. Tentative identification of positional isomers for those compounds which are not present in spectral libraries, was carried out on the basis of previously published reports [3,34].

Lignans. Pinoresinol, hydroxypinoresinol, acetoxypinoresinol and syringaresinol, which have been widely described in olive oil and tissues, were also identified by LC-MS.

Pentacyclic triterpenes. 3 triterpenic acids (maslinic, betulinic and oleanolic acids) and 2 triterpenic alcohols (erythrodiol and uvaol) were found in the extracts and unequivocally annotated thanks to our analyte list. Additionally, the peak eluting at 8.0 min (calculated molecular formula $C_{30}H_{48}O_5$) was tentatively assigned to a maslinic acid monohydroxylated derivative, which has been described as a product of maslinic acid metabolism [45]. Its fragmentation pattern was characterized by a major signal corresponding to the dehydroxylated molecule and the decarboxylated maslinic acid moiety, which was also predominant in maslinic acid MS/MS spectrum.

Tocopherols. The 4 forms of tocopherols (*α*-, *β*-, *γ*- and *δ*-) where found in some of the analyzed samples and annotated by comparison with their pure standard. Nevertheless, *β*- and *γ*-structural isomers could not be resolved in reverse-phase LC and coeluted in min 12.7.

Sterols. Stigmasterol, campesterol and *β*-sitosterol were annotated by matching with the in-house created analyte list. 2 lupeol isomers, cycloartenol, stigmastadienol, Δ^5-avenasterol, citrostadienol and methylencycloartanol were also found in some of the prepared extracts; peak assignment was performed based on the occurrence and relative Rts described in previous reports [46–48].

Fatty acids and derivatives. Some of the most common fatty acids occurring in olive fruits and oils (stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), palmitic (C16:0) and palmitoleic (C18:1) acids) were detected with the proposed LC-MS methodologies. Azelaic acid, which is a derived product from oleic acid oxidation, as well as different hydroxylated fatty acid derivatives (hydroxydecanoic, hydroxyoctadecatrienoic, hydroxyoctadecadienoic, hydroxyoctadecenoic, hydroxyoctadecanoic, hydroxyeicosanoic, dihydroxyhexadecanoic, dihydroxyoctadecanoic, dihydroxyoctadecadienoic, trihydroxyoctadecadienoic, trihydroxyoctadecenoic and trihydroxyoctadecanoic acids), were also tentatively identified some of the evaluated samples. Those compounds have been reported as auto-oxidation products in heated edible fats [49], although some of them have been also found in olive leaves [50]. To the best of our knowledge, this is the first time that so many members of this family are found in olive derived matrices.

Table S2 lists the 58 compounds detected with GC-APCI-MS. It includes names, M and Rts of the assigned peaks, as well as the qualitative information used for identification purposes: *m/z*, error, mSigma, calculated molecular formula and chemical arrangement corresponding to that formula, together with some other MS signals which helped to confirm the proposed identity (with their molecular formula between brackets). The most abundant *m/z* of each compound is presented in bold letters.

As already mentioned, the number of compounds annotated using this platform was much lower than with the LC-MS couplings. On the one hand, all the glycosylated forms were undetectable by this methodology (under the selected conditions) and on the other hand, most secoiridoid derivatives presented a very similar in-source fragmentation that prevented the straightforward identification of all the individual molecules detected by LC-MS. Compound identification when using this GC-MS methodology was partially discussed in a previous report [37]; nevertheless, the use of a high resolution analyzer together with the APCI interface (which produced lower in-source fragmentation than the EI source used in the just mentioned publication) allowed the confirmation of some tentatively assigned identities.

Between those compounds exclusively detected with GC, we found squalene, a well-known hydrocarbon from VOO [37]; arachidic or eicosanoic acid (C20:0), whose hydroxylated derivative

was tentatively identified with the LC-MS platforms; and glyceryl linoleate, which could come from triacyglycerols degradation. Besides, additional isomers of apigenin, luteolin, maslinic acid and elenolic acid (two isomers in this case) were detected in the analyzed extracts. In the case of both flavonoids, the detected isomers eluted earlier than the peak of the corresponding pure standard.

2.2. Comparison of the potential of the evaluated analytical platforms

One of the main objectives of the present work was to evaluate the adequacy of each tested methodology to determine different chemical classes of metabolites found in olive tree derived samples. Apart from the number of analytes which could be detected and tentatively annotated by using each platform and polarity (already discussed in Section 2.1. and clearly depicted in Fig. 1), the efficiency of the ionization in each case was deeply evaluated. To illustrate this comparison, Fig. 2 shows the efficiency of all the tested couplings when detecting different classes of compounds found in the oil produced from stoned and dehydrated olives. Two reasons made us selecting this sample to perform the comparison shown in the figure: (i) it was the matrix containing the second major number of total compounds (as it will be further described in Section 2.3.), and (ii) it was the richest sample in terms of number of substances identified with the GC-MS platform. Bearing these two factors in mind, it could be considered as a very appropriate instance to illustrate the platforms comparison. In any case, similar charts and numerical comparisons were carried out for the rest of the matrices, corroborating the displayed observations regarding the efficiency of each platform to ionize every chemical family.

Fig. 2a displays the normalized peak areas achieved for each chemical class with the five evaluated approaches (LC-ESI-MS$^{(-/+)}$, LC-APCI-MS$^{(-/+)}$ and GC-APCI-MS$^{(+)}$). To facilitate the comparison, the highest area value (sum of all the compounds belonging to each group described in Section 2.1.) was considered as 100, and the obtained areas with the rest of the tested platforms were expressed as a percentage of that value. It can be seen that the LC-ESI-MS platform, when working in positive ionization mode, produced the highest ionization rate for phenolic acids and aldehydes, secoiridoids and derivatives, flavonoids and lignans. The LC-ESI-MS methodology in negative polarity was the most appropriate one to detect the group of organic acids and coumarins, although it also showed relatively good efficiency when detecting secoiridoids and related substances. LC-ESI-MS$^{(-)}$ was also the second option to ionize phenolic acids and aldehydes with a suitable degree of effectiveness, and the third one (with very similar efficacy if compared with LC-ESI-MS$^{(+)}$) for simple phenols and derivatives. The ESI interface (in any of both, positive or negative, polarities) was not useful for the determination of sterols. The LC-APCI-MS coupling used in negative ionization mode gave the best ionization rate for pentacyclic triterpenes (even though the alcohols were not ionizable in MS$^{(-)}$), while, in positive polarity, it was the best option for tocopherols and sterols detection. As expected, LC-APCI conjunction resulted to be inadvisable for the detection of the most polar compounds. The GC-APCI-MS method was the best option for simple phenols and fatty acids-related analytes. It also gave good results for the rest of the considered chemical classes (in particular for lignans, tocopherols and sterols (if compared with

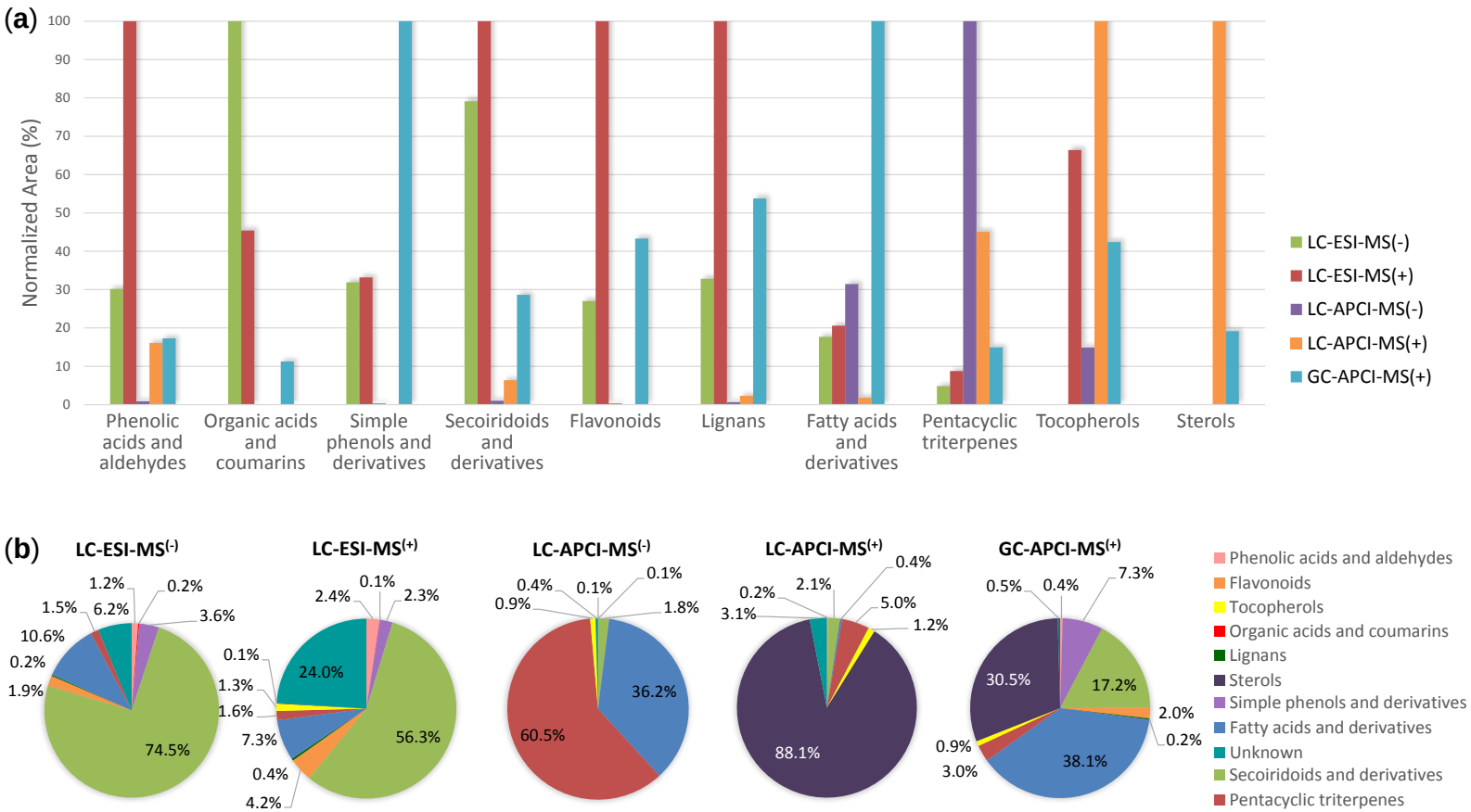

Figure 2. (a) Bars graph representing the sum of areas (in a normalized axis) of the compounds found in the oil obtained from stoned and dehydrated olives (grouped by chemical class), by means of each tested platform and polarity; (b) Pie charts showing the share of every chemical class (in terms of area (% of the total area)) in the chromatograms obtained with each employed methodology for the same sample than in part (a).

the other approaches)), except for the previously mentioned fact that it was not possible to determine glycosylated compounds by means of this coupling (hence the lower number of annotated metabolites in this platform). That means that the respective values shown in Fig. 2 regarding the GC-APCI-MS platform do just consider aglycone forms.

Pie charts presented in Fig.2b show the percentage (in terms of area) corresponding to each determined chemical class over the total area of the chromatograms obtained by means of the 5 methodologies used in this study. In view of the fact that some compounds remained as "unknown" (although we were able to assign them a molecular formula), we decided to include these substances in the systematic analytical comparison; doing it so we could have an idea about the percentage of the total area corresponding to non-identified substances in each platform (please, note that the analytes comprised in the unknown fraction are different in LC-MS than in GC-MS). Secoiridoids and derivatives group represented the highest area fraction of the chromatograms acquired with the ESI source in LC-MS, followed by fatty acids and derivatives, and the rest of phenolic compounds (simple phenols, flavonoids, phenolic acids and aldehydes and lignans) in different proportions depending on the selected MS polarity. The area corresponding to unknown peaks was also appreciable, accounting for 6% of the total area in negative polarity and for almost a quarter of the entire chromatogram in positive polarity. Pentacyclic triterpenes constituted around 1.5% of both (negative and positive) LC-ESI-MS chromatograms. As revealed in Fig. 2a, the APCI interface in LC-MS produced better ionization for the less polar compounds. Therefore, in negative polarity, one third of the whole chromatogram area corresponded to fatty acids and derivatives, and almost the other two thirds were taken up by triterpenic acids. When using LC-APCI-MS$^{(+)}$, nearly 90% of the total area corresponded to sterols, 5% to pentacyclic triterpenes and around 3% to the unknown fraction. The chromatogram obtained by means of the GC-APCI-MS platform was more proportionally distributed. In this case, fatty acids and derivatives accounted for 38.1%, sterols for 30.5%, secoiridoids for 17.2%, simple phenols and derivatives for 7.3%, pentacyclic triterpenes for 3.0% and flavonoids for 2% of the chromatogram area. Logically, minor chemical classes such as organic acids, coumarins and lignans represented less than 1% regardless of the platform. It is also worth mentioning that tocopherols constituted around 1% of the total area obtained by all the evaluated methodologies, excluding LC-ESI-MS$^{(-)}$ (with which they were hardly detectable).

2.3. Establishing the relative prevalence of each determined chemical class in the samples under evaluation

Fig. 3 presents the relative distribution of each determined chemical class in the evaluated samples. A similar strategy to the one described before was applied to facilitate the comparison. Thus, the integrated areas were normalized to the major value found for each family of compounds; in a subsequent step, the areas found in the rest of the matrices were expressed as a percentage of the richest one. As the peak intensity depends on the ionization rate of each individual substance, the followed strategy cannot be used to establish a comparison among

different compound classes. The comparative purpose in this case, was consequently semi-quantitative and, as stated, just pertinent to collate the different samples considering each chemical class separately. Establishing absolute quantitative values of each analyte in every substance class was beyond the goal of this study.

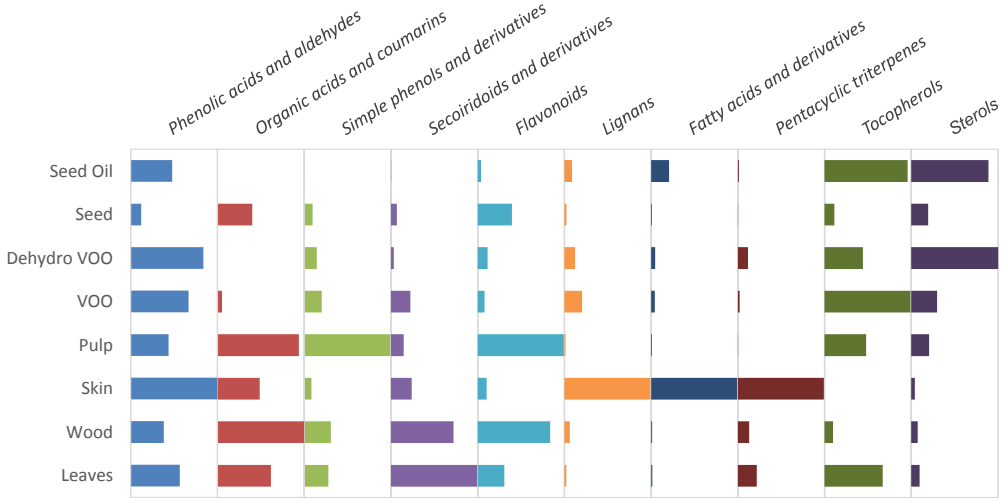

Figure 3. Bars graph representing relative distributions of each evaluated chemical class in the eight studied olive tree derived samples from Picudo *cv.*

The distribution of the determined metabolites in the eight analyzed samples can be checked in Table S3 (all the given values are % referred to the richest sample regarding each analyte). In order to obtain comparable results among matrices, all the reported relative areas were integrated in chromatograms obtained by means of the same platform. Nevertheless, each chemical class was determined in the most favorable coupling (the one giving the maximum number of identified compound and good ionization rate avoiding saturation in any matrix): organic acids, coumarins and phenolic compounds (phenolic acids and aldehydes, simple phenols, secoiridoids, flavonoids and lignans) in LC-ESI-MS$^{(-)}$; fatty acids and derivatives as well as triterpenic acids in LC-APCI-MS$^{(-)}$; and triterpenic alcohols, tocopherols and sterols in LC-APCI-MS$^{(+)}$.

Phenolic acids and derivatives were quite distributed over all the evaluated samples, fruit skin and olive oils (obtained by any of the two procedures described in Section 3.2) being the richest matrices. The content of the oils in terms of organic acids was very low, probably because they are the most hydrophilic compounds among all the determined metabolites. Coumarins were almost exclusively found in stems; finding these substances in wood tissues is in good agreement with what was previously described by other authors for some olive tree varieties [31].

With regard to simple phenols, the glycosidic forms were mostly found in olive tissues, since they are generally hydrolyzed during oil extraction (for example, by the β-glucosidase action [8]). VOO was the richest matrix in terms of 3,4-dihydroxyphenylglycol. On the contrary, if compared with the oil obtained from stoned and dehydrated olives, the oil produced by the two-phase

extraction method presented a reduced amount of the other two phenyl alcohols (tyrosol and hydroxytyrosol) and the acetylated derivative of hydroxytyrosol, but a higher content of the oxidized one. In addition, VOO was richer in terms of aglycones of oleuropein and ligstroside derivatives and had lower concentration of elenolic acid derivatives than the oil obtained from stoned and dehydrated olives; this can be seen in detail in Fig. S3. It could suggest that either the thermal process involved in the dehydration of the stoned fruits is breaking down the secoiridoids into their degradation products (phenolic alcohols, elenolic acid and derivatives) [8], or that the absence of water during the oil extraction is detrimental to the transfer of secoiridoids from the pulp to the oily phase. Fig. S3 also shows how the glycosylated secoiridoids were more abundant in tissues than in the oils for the same reason as for the glycosylated simple phenols. Skin and seeds were the poorest olive tissues in terms of secoiridoids, being nuzhenide the most prevalent secoiridoid found in the latter one, as previously reported by different authors [51,52]. Glycosylated flavonoids were predominantly distributed between leaves and stems. The aglycones were also present in olive oils, more abundantly in VOO. Seeds and seed oil were the poorest matrices in terms of this chemical class, but they contained noticeable amounts of lignans, seed oil being the richest matrix regarding acetoxypinoresinol. Olive fruit skin was the matrix with the highest content of the other three evaluated lignans (syringaresinol, pinoresinol and hydroxypinoresinol).

Although fatty acids are usually found as part of triacylglycerols, they were detected free in the three kind of oils evaluated in this study. Moreover, the compounds tentatively annotated as fatty acid hydroxylated derivatives, were found in high relative amounts in olive skin. As far as triterpenic compounds are concerned, olive skin was the richest matrix, followed by leaves, except for betulinic acid that was found at a higher relative concentration level in the stems. The oil obtained from stoned and dehydrated olives presented higher relative triterpenoids content than the VOO obtained from the conventional procedure. Besides, those compounds were found at very low relative levels in olive seed and pulp (what is in agreement with previous findings [53]). Regarding tocopherols, VOO was the matrix containing the highest relative amount of α-tocopherol, while δ-, β- and γ- tocopherols were higher, in relative terms, in the seed oil and the oil obtained from stoned and dehydrated olives. The latter was the richest matrix in terms of sterols, followed by VOO (considering the overall distribution of all the determined sterols). Just campesterol, citrostadienol and β-sitosterol were found at higher relative levels in seed oil. Some sterols were found at low relative concentrations in pulp and seeds; they were almost missing from the rest of olive tree derived tissues (leaves, stems and olive skin).

3. MATERIALS AND METHODS

3.1. Chemicals and standards

Deionized water produced by a Millipore Milli-Q system (Bedford, MA, USA) and acetonitrile of LC-MS grade supplied from Sigma-Aldrich (St. Louis, MO, USA), were acidified with 0.5% acetic

acid (provided by Sigma-Aldrich too), and used as mobile phases in LC. Gradient grade ethanol for sample preparation was purchased from Merck (Madrid, Spain). *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane, (BSTFA + 1% TMCS) used as derivatization reagent and 43 pure standards of metabolites found in *Olea europaea* matices were acquired from Sigma-Aldrich. The list of standard compounds included: phenolic compounds (hydroxytyrosol, tyrosol, oleuropein, luteolin, luteolin 7-O-glucoside, apigenin, apigenin 7-O-glucoside, quercetin, quercetin 4-O-glucoside, rutin, pinoresinol, vanillin and quinic, gallic, protocatechuic, gentisic, 4-hydroxybenzoic, 4-hydroxyphenylacetic, vanillic, caffeic, syringic, homovanillic, *p*-coumaric, sinapic, ferulic, and *t*-cinnamic acids); triterpenic compounds (maslinic, betulinic, oleanolic and ursolic acids, erythrodiol and uvaol); fatty acids (palmitoleic, oleic, linoleic and linolenic acids); tocopherols (*α*-, *β*-, *γ*- and *δ*- tocopherols), and sterols (stigmasterol, campesterol and *β*-sitosterol). Additionally, isolated fractions of secoiridoids (oleuropein and ligstroside aglycones, oleacein and oleocanthal), elenolic acid, acetylated hydroxytyrosol and acetoxypinoresinol, which were not commercially available, were also used for identification purposes.

3.2. Samples and sample treatment

Sampling of olive fruits, leaves and stems, was performed on the same Picudo *cv.* olive tree mucho grown in Castillo de Locubín (Jaén, Spain (at approximately 750 m above sea level)) in January 2017. The olive tree cultivar was declared by the producer and had been previously certified. In total, 8 different samples (tissues and oils) were analyzed in this study. Leaves and stems were dried at ambient temperature and stored in a fresh dark place. A portion of 5 kg of fresh fruits was processed by means of an Abencor® laboratory oil mill (MC2 Ingeniería y Sistemas, Seville, Spain) to obtain VOO at laboratory scale by the conventional two-phase process, which involves three steps: (i) crushing of entire fruits, (ii) malaxation of the paste and (iii) centrifugation for oil separation. Official IOC determinations were carried out to confirm the belonging of the oil to VOO category. The rest of the fruits were manually deconstructed to obtain different tissues and oils. Firstly, fruits were stoned and the obtained pits were broken with a hammer to extract the olive seed contained inside. Next, half of the stoned fruits were peeled to obtain olive skin and pulp separately, which were straightaway frozen and freeze-dried. The other half of the stoned fruits were dried at 50 °C in an oven until constant weight. Olive seeds and dehydrated stoned fruits were further processed by mechanical pressing to obtain two new kinds of oils. Fig. 4 shows a diagram of the procedure followed to prepare the samples.

Both kinds of samples (tissues and oils) were subjected to a very unselective sample treatment, trying to extract compounds belonging to different chemical classes in a wide range of polarity. Thus, two ethanol/water mixtures were applied in a liquid-liquid or solid-liquid extraction protocol adapted from that previously suggested by our research team [37,54]. For liquid samples, 1 g of oil was extracted three times with 6 mL ethanol/water mixtures, whereas for solid samples, 0.5 g of the grinded and sieved tissue were extracted three times with 10 mL of the extractant agent. In

both cases, the first extraction step was done with ethanol/water (60:40, v/v), while for the last two steps, ethanol/water (80:20, v/v) was used instead. Olive tissues extraction was carried out in an ultrasonic bath from J.P. Selecta (Barcelona, Spain) for 30 min whilst 4 min of vortex shaking where enough to mix the phases in oily samples and to assure an efficient extraction. After collecting together the supernatants from the three extraction steps, the solvent was evaporated in a rotavap and the residue was reconstituted in the appropriate volume of ethanol/water (80:20, v/v) (1 mL for the oils and 5 mL for the olive tissues). For GC analyses, a 50 µL aliquot of the prepared extracts was dried and then derivatized with 75 µL of BSTFA + 1% TMCS (keeping it at ambient temperature for 1 h) before injection into the chromatograph, following a previously reported strategy [37,54].

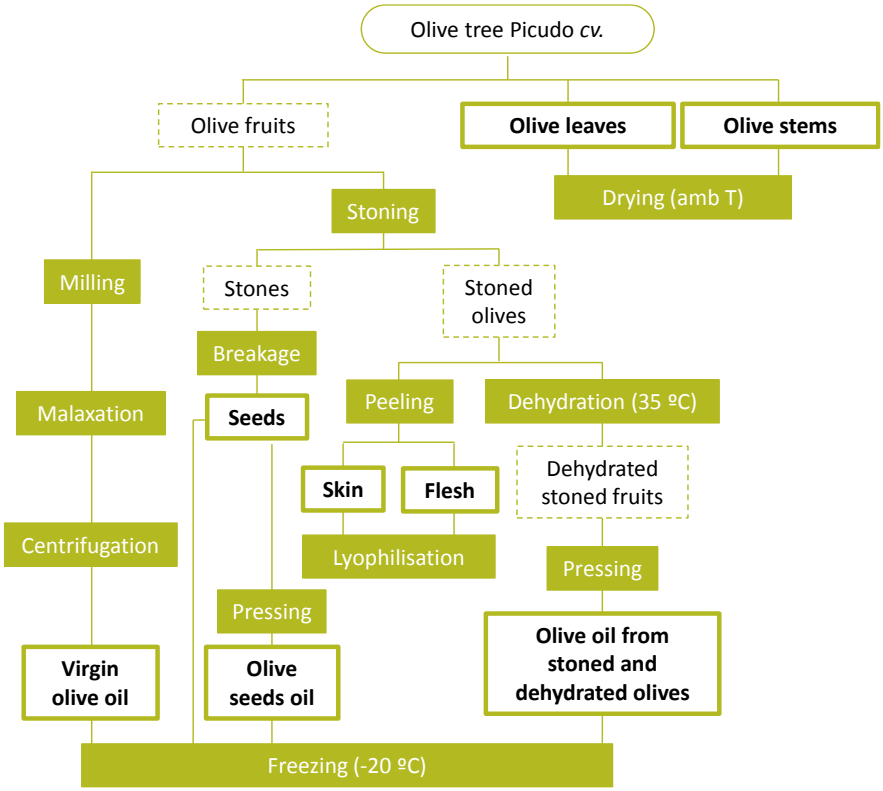

Figure 4. Diagram of the procedure followed to obtain the 8 samples studied in this work, including intermediate products (dotted lines) and employed processes (shaded boxes).

3.3. GC-MS and LC-MS methodologies

GC-MS analyses were carried out in a Bruker 450-GC (Bruker Daltonik GmbH, Bremen, Germany) coupled to a Compact™ QqTOF mass spectrometer (Bruker Daltonik) through an APCI source. 1 µL of the silylated extract was injected at a split ratio of 1:20 with an injector temperature of 250 ºC. Analytes were separated in a BR-5 column (30 m × 0.25 mm i.d., 0.25 µm) (Bruker Daltonik) with 1 mL/min of He as carrier gas and a linear temperature gradient from 150 to 320 °C

at a rate of 4° C/min. The experimental conditions of the GC-MS method were described elsewhere to determine minor components of VOO [54].

LC-MS analyses were performed in an Elute UHPLC (Bruker Daltonik) coupled to the same MS detector as in GC-MS. Two different interfaces were used in this case, APCI and ESI. Analytes were eluted slightly modifying the previously published conditions [54], in an Intensity Solo C18 column (2.1 × 100 mm, 1.8 µm) (Bruker Daltonik), using acidified water (phase A) and ACN (phase B) with the following gradient: 0 to 2 min, 5%–30% B; 2 to 7 min, 30%–50% B; 7 to 8 min, 50%–90% B; 8 to 8.2 min, 90%–95% B, 8.2 to 10 min, 95%–99.9% B (kept for 5.9 min), and 15.9 to 16 min, 99.9%–5% B (kept for 2 post-run min). The flow rate was 0.4 mL/min from 0 to 10 min, and 0.6 mL/min from min 10 to the end of the run. The already reported detection conditions for ESI-QqTOF MS [54] were also used in this study. When the LC-MS coupling was done through the APCI interface, nebulizer pressure was set at 2 bars; drying gas flow and temperature were set at 2 L/min and 300 °C, apiece; capillary voltages were set at 2,500 V in negative polarity and 2,000 V in positive one; and 6,000 and 10,000 nA were chosen for corona current in negative and positive ionization modes, respectively. Auto MS/MS fragmentation was also carried out in LC-MS analyses in order to facilitate compound identification. An absolute threshold of 1500 counts and a cycle time of 1 s were selected for precursor ions collection; collision energy stepping factors fluctuated between 0.2 and 0.8%.

GC-MS analyses were internally calibrated by comparison with known *m/z* from common cyclic-siloxanes found in the background. In LC-MS, an external calibrant was directly pumped into the interface at the beginning of each run using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA), equipped with a Hamilton syringe (Reno, Nevada, USA). The calibrant for LC-ESI MS analyses consisted in a mix of clusters of sodium formate and acetate, while the one used in LC-APCI MS was a mixture of analytical standards (available in our lab) including an APCI tuning mix and six pesticides of known *m/z* in the range from 121 to 955.

Data acquisition was done with the software Compass HyStar and data treatment with DataAnalysis 4.4 and MetaboScape® 3.0 (the three of them from Bruker Daltonik). The latter one automatically recalibrated the acquired MS data and performed the molecular features selection, bucketing, filtering and scaling. MetaboScape incorporates different tools that helped to identify the compounds found in the chromatograms: SmartFormula, which determines the molecular formula of each detected compound from its exact mass and isotopic pattern (having into account all found adducts); Compound Crawler, which searches molecular structures for given molecular formulas in local (AnalyteDB) and public online databases (ChEBI, ChemSpider and PubChem); and MetFrag, which performs *in-silico* fragmentation of the potential structures and compares them with the acquired MS/MS spectra [55,56]. This software also allows annotation by comparison with previously created analyte lists and MS/MS spectral libraries (Bruker Sumner MetaboBASE Plant Libraries 1.0 and Bruker HMDB Metabolite Library).

3. CONCLUSIONS

Eight interesting matrices coming from a Picudo *cv.* olive tree have been analyzed by powerful LC-ESI/APCI-QTOF MS and GC-APCI-QTOF MS methodologies, providing a comprehensive coverage of their secondary metabolites (141 substances identified in LC-MS and 58 in GC-MS) and giving reliable information about their phytochemical distribution. The suitability of each platform and polarity to determine each family of metabolites was systematically evaluated. When the selected matrices were compared by using a semi-quantitative approach, fruit skin resulted to be the matrix with the highest relative amounts of phenolic acids, triterpenic and fatty acid hydroxylated substances, exhibiting remarkable relative content of lignans too. Coumarins were almost exclusively found in stems. The glycosidic simple phenols and glycosylated secoiridoids were more abundant in tissues, as well as the glycosilated flavonoids (predominantly distributed between leaves and stems). VOO was the matrix showing highest relative content of 3,4-dihydroxyphenylglycol, aglycones of oleuropein and ligstroside derivatives, flavonoids (aglycones) and α-tocopherol. The oil obtained from stoned and dehydrated olives (in comparison with VOO) had relatively raised levels of tyrosol, hydroxytyrosol, the acetylated derivative of hydroxytyrosol, sterols and elenolic acid derivatives. Seed oil stood out for its notable levels of acetoxypinoresinol and tocopherols.

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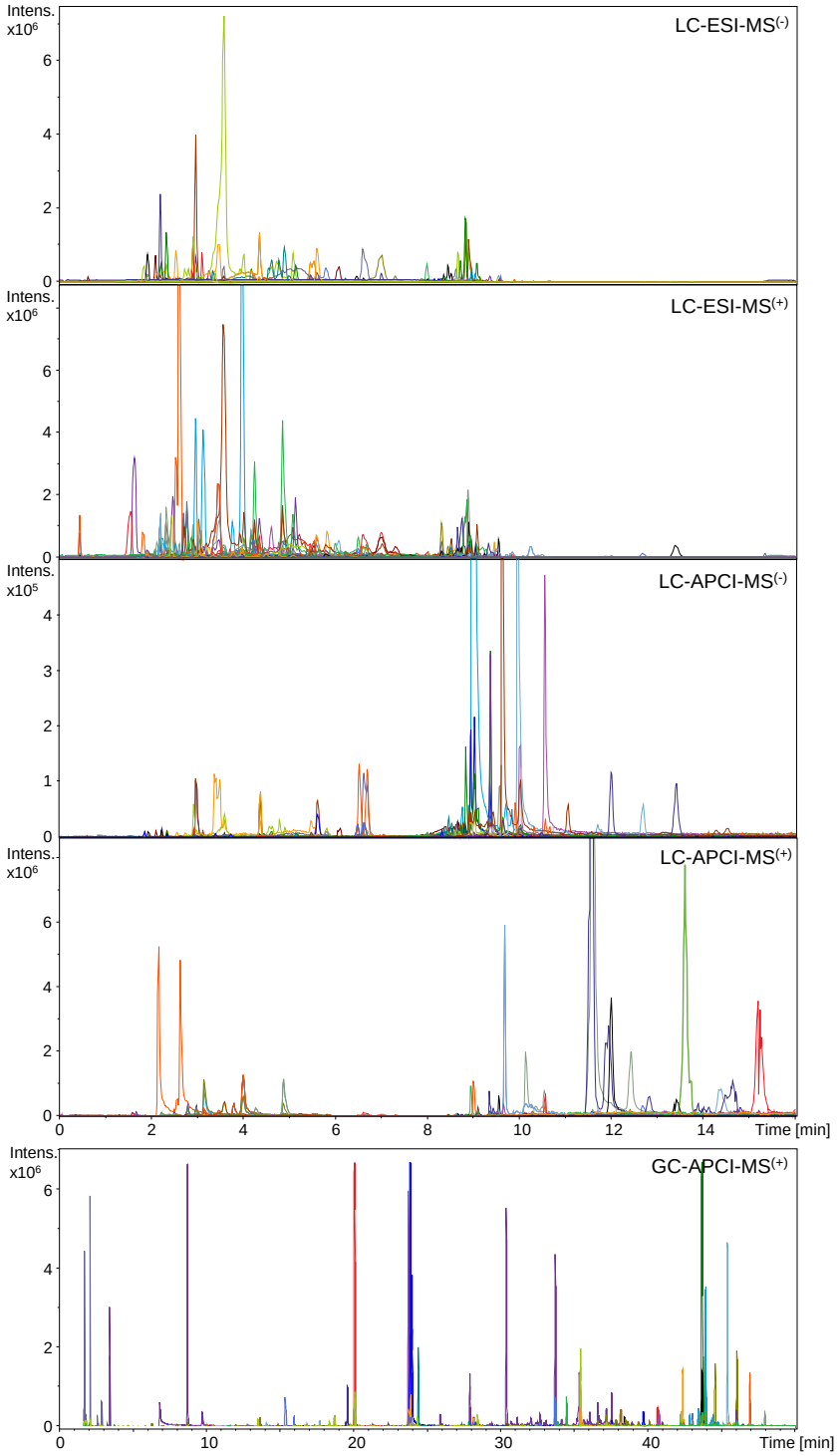
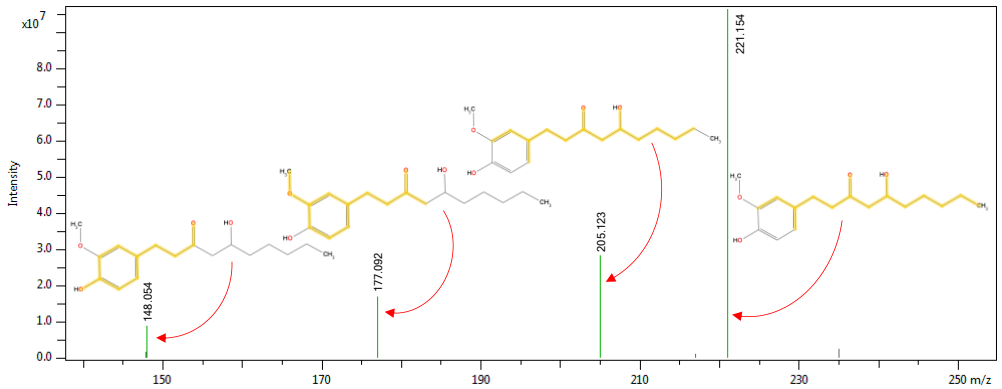


Figure S1. Extracted Ion Chromatograms (EICs) of all the identified compounds in olive oil obtained from stoned and dehydrated fruits, when it is analyzed by means of each evaluated platform and polarity.

A) Gingerol



B) Dihydrokaempferol

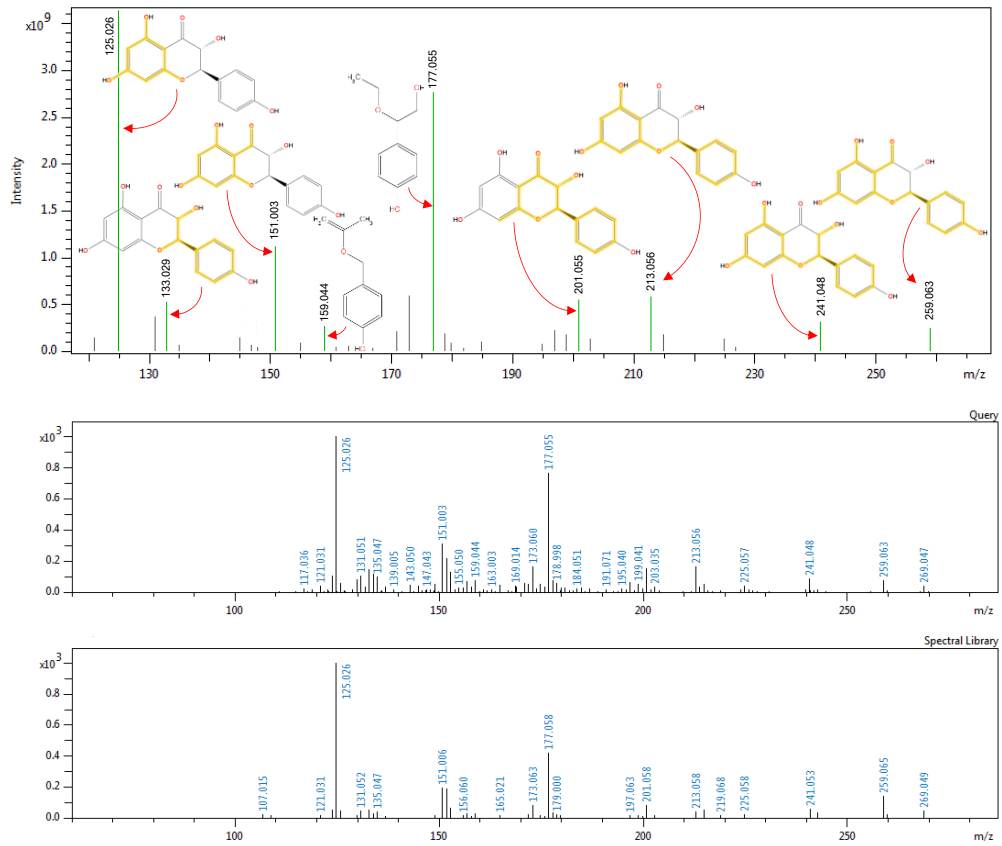


Figure S2. MetFrag in-silico fragmentation for two tentatively annotated metabolites (A and B), and spectral library match for compound B.

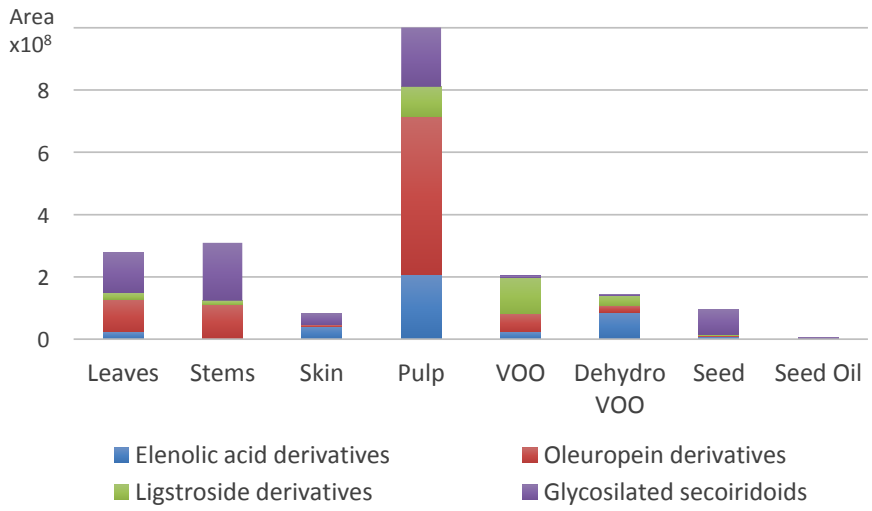


Figure S3. Distribution of secoiridioids in the eight matrices under study (representation of the sum of absolute peak areas).

Table S1. List of compounds detected with LC-MS methodologies.

Compound	Neutral Molecular formula	Rt (min)	Negative					Positive					ID
			m/z	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	*m/z*	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	
quinic acid	$C_7H_{12}O_6$	0.6	191.0555	-0.621	8.8	$[M-H]^-$	-	193.0710	0.318	8.7	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	S
citric acid	$C_6H_8O_7$	0.7	191.0191	-0.651	3	$[M-H]^-$	-	175.0238	0.052	8.4	$[M-H_2O+H]^+$, $[M+H]^+$	-	L
3,4-dihydroxyphenylglycol	$C_8H_{10}O_4$	0.8	151.0399	-0.342	8.9	$[M-H_2O-H]^-$, $[M-H]^-$	-	153.0544	-0.235	20.1	$[M-H_2O+H]^+$, $[M+H]^+$	$[M+H]^+$	L
oxydized hydroxytyrosol	$C_8H_8O_3$	1.0	151.0398	-0.808	15.6	$[M-H]^-$	$[M-H]^-$	153.0536	0.092	8.9	$[M+H]^+$, $[M-H_2O+H]^+$	-	L
hydroxytyrosol glucoside	$C_{14}H_{20}O_8$	1.7	315.1085	-0.065	3.8	$[M-H]^-$	$[M-H]^-$	317.1235	0.357	5.7	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	L
hydroxy decarboxymethyl elenolic acid isomers I and II	$C_9H_{12}O_5$	1.7, 2.1	199.0607	-0.473	8.5	$[M-H]^-$	$[M-H]^-$	183.0654	0.230	9	$[M-H_2O+H]^+$, $[M+H]^+$, $[M+Na]^+$	-	L
gallic acid	$C_7H_6O_5$	1.8	169.0140	-0.793	26.6	$[M-H]^-$	-	-	-	-	-	-	S
acyclodihydroelenolic acid hexoside	$C_{17}H_{28}O_{11}$	1.8	407.1560	0.105	11.1	$[M-H]^-$	$[M-H]^-$	409.1701	-0.476	6.3	[M+H]+, $[M-H_2O+H]$+, [M+Na]+	$[M+H]^+$	L
decarboxylated form of hydroxy elenolic acid isomers I and II	$C_{10}H_{14}O_5$	1.9, 2.2	213.0768	-0.044	9.2	$[M-H]^-$, $[M-H_2O-H]^-$	[M-H]-, $[M-H-H_2O]^-$	197.0813	0.614	15.2	$[M-H_2O+H]^+$, $[M+H]^+$, $[M+Na]^+$	$[M-H_2O+H]^+$, $[M+H]^+$	L
hydroxytyrosol	$C_8H_{10}O_3$	1.9	153.0551	-0.594	0.2	$[M-H]^-$	$[M-H]^-$	155.0700	-0.276	7.7	$[M+H]^+$	$[M+H]^+$	S
protocatechuic acid	$C_7H_6O_4$	1.9	153.0190	-0.309	8.2	$[M-H]^-$	$[M-H]^-$	155.0338	-0.644	12.5	$[M+H]^+$, $[M-H_2O+H]^+$	-	S
tyrosol glucoside	$C_{14}H_{20}O_7$	2.0	299.1139	0.191	1.6	$[M-H]^-$	$[M-H]^-$	301.1292	0.870	19.2	$[M+H]^+$	-	L
aesculin	$C_{15}H_{16}O_9$	2.0	339.0720	0.026	17.2	$[M-H]^-$	-	341.0877	1.033	0.4	$[M+H]^+$	$[M+H]^+$	L
dihydro oleuropein	$C_{25}H_{36}O_{13}$	2, 2.9	543.2082	-1.109	28.6	$[M-H]^-$, $[M-H_2O-H]^-$	[M-H]-, $[M-H-H_2O]^-$	-	-	-	-	-	L
oleoside/secologanoside	$C_{16}H_{22}O_{11}$	2.1	389.1091	0.014	4.7	$[M-H]^-$	$[M-H]^-$	391.1165	0.163	7.6	$[M+H]^+$	-	L
eudesmic acid	$C_{10}H_{12}O_5$	2.2	211.0607	0.236	28.9	$[M-H]^-$	$[M-H]^-$	231.0766	0.092	3.5	$[M+H]^+$	-	L
tyrosol	$C_8H_{10}O_2$	2.3	137.0608	-0.006	6.2	$[M-H]^-$	$[M-H]^-$	121.0642	-0.628	4.6	$[M-H_2O+H]^+$, $[M+H]^+$, $[M+Na]^+$	-	S
gentisic acid	$C_7H_6O_4$	2.3	153.0119	-0.309	8.2	$[M-H]^-$	-	155.0338	-0.644	20.1	$[M+H]^+$	-	S
luteolin diglucoside	$C_{27}H_{30}O_{16}$	2.3	609.1461	1.746	30.1	$[M-H]^-$	-	611.1608	-0.409	20.1	$[M+H]^+$	-	L
cyanidin 3-O-glucoside	$C_{21}H_{22}O_{11}$	2.3	449.1095	0.530	3.4	$[M-H]^-$	$[M-H]^-$	451.1217	-0.755	17.3	$[M+H]^+$	-	L
4-hydroxybenzoic acid	$C_7H_6O_3$	2.4	137.0246	0.339	8.2	$[M-H]^-$	-	139.0384	-0.512	5.5	$[M+H]^+$	$[M+H]^+$	S
elenolic acid glucoside	$C_{17}H_{24}O_{11}$	2.4	403.1246	0.093	9.3	$[M-H]^-$	$[M-H]^-$	405.1396	-0.383	21.2	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	L
4-hydroxyphenylacetic acid	$C_8H_8O_3$	2.4	151.0397	-0.279	5.6	$[M-H]^-$	$[M-H]^-$	153.0542	-0.133	7.2	$[M+H]^+$	-	S
unknown 1	$C_{16}H_{26}O_{10}$	2.4	377.1453	0.000	5.7	$[M-H]^-$	$[M-H]^-$	379.1604	0.500	23.3	$[M+H]^+$	-	-
aesculetin	$C_9H_6O_4$	2.5	177.0188	-0.923	3.3	$[M-H]^-$	$[M-H]^-$	179.0340	-0.163	27.6	$[M+H]^+$	$[M+H]^+$	L
vanillic acid	$C_8H_8O_4$	2.5	167.0345	-0.459	7.1	$[M-H]^-$	$[M-H]^-$	169.0497	-0.868	30.6	$[M+H]^+$	-	S
caffeic acid	$C_9H_8O_4$	2.5	179.0347	-0.310	8.3	$[M-H]^-$	-	181.0495	-0.036	29.1	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	S
gallocatechin	$C_{15}H_{14}O_7$	2.6	305.0702	3.507	27.6	$[M-H]^-$	-	-	-	-	-	-	L
unknown 2	$C_9H_{14}O_3$	2.6	169.0869	0.135	17.1	$[M-H]^-$	-	171.1016	0.012	6.2	$[M+H]^+$, $[M-H_2O+H]^+$, $[M+Na]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	-
decarboxymethyl elenolic acid	$C_9H_{12}O_4$	2.6	183.0658	-0.526	8	$[M-H]^-$	[M-H]-, $[M-H-H_2O]^-$	185.0812	0.348	7.8	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	L
phenylethyl primeveroside	$C_{19}H_{28}O_{10}$	2.6	415.1612	0.102	3.8	$[M-H]^-$	$[M-H]^-$	417.1749	-2.830	41.3	$[M+H]^+$	$[M+H]^+$	L
unknown 3	$C_{18}H_{34}O_{14}$	2.6	473.1863	1.278	40.3	$[M-H]^-$	$[M-H]^-$	475.2054	0.976	30.2	$[M+H]^+$	-	-
syringic acid	$C_9H_{10}O_5$	2.6	197.0452	-0.004	12.4	$[M-H]^-$	$[M-H]^-$	199.0601	-0.402	8.9	$[M+H]^+$	-	S
homovanillic acid	$C_9H_{10}O_4$	2.7	181.0502	-0.778	13.6	$[M-H]^-$	$[M-H]^-$	183.0655	0.051	36.9	$[M+H]^+$	$[M+H]^+$	S

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			m/z	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	*m/z*	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	
rutin	$C_{27}H_{30}O_{16}$	2.7	609.1464	0.278	5	$[M-H]^-$	$[M-H]^-$	611.1615	0.290	25.9	$[M+H]^+$	-	S
luteolin rutinoside	$C_{27}H_{30}O_{15}$	2.7	593.1516	0.047	27.3	$[M-H]^-$	$[M-H]^-$	595.1671	0.805	26.2	$[M+H]^+$	-	L
hydroxy oleuropein	$C_{25}H_{32}O_{14}$	2.7	555.1717	-0.391	4.7	$[M-H]^-$	$[M-H]$-, $[M-H-H_2O]^-$	-	-	-	-	-	L
p-coumaric acid	$C_9H_8O_3$	2.8	163.0397	-0.107	11.8	$[M-H]^-$	$[M-H]^-$	165.0545	-0.084	7.3	$[M+H]^+$, $[M-H_2O+H]^+$, $[M+Na]^+$	$[M+H]^+$	S
quercetin 4-O-glucoside	$C_{21}H_{20}O_{12}$	2.8	463.0882	-0.010	29.3	$[M-H]^-$	$[M-H]^-$	465.1007	-2.035	30.8	$[M+H]^+$	$[M+H]^+$	S
luteolin 7-O-glucoside	$C_{21}H_{20}O_{11}$	2.8	447.0933	0.009	11	$[M-H]^-$	$[M-H]^-$	449.1078	-0.586	17.2	$[M+H]^+$	$[M+H]^+$	S
verbascoside	$C_{29}H_{36}O_{15}$	2.8	623.1981	-0.003	14.6	$[M-H]^-$	-	625.2139	-0.840	31.7	$[M+H]^+$	-	L
oleuropein glucoside	$C_{31}H_{42}O_{18}$	2.9	701.2303	0.503	7.7	$[M-H]^-$	$[M-H]^-$	-	-	-	-	-	L
apigenin 7-O-rutinoside	$C_{27}H_{30}O_{14}$	2.9	577.1568	1.094	29.5	$[M-H]^-$	-	579.1701	-0.732	18.6	$[M+H]^+$	$[M+H]^+$	SL
hydroxy elenolic acid isomers I, II, III	$C_{11}H_{14}O_7$	2.9, 3.1, 3.3	257.0669	0.187	12.4	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H-H_2O]$-, $[M-H]^-$	259.0816	1.412	12.9	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	L
nuzhenide	$C_{31}H_{42}O_{17}$	2.9	685.2346	-0.005	5.8	$[M-H]^-$	$[M-H]^-$	507.1867	0.600	12.9	$[M-C_6H_{12}O_6+H]^+$	-	L
sinapic acid	$C_{11}H_{12}O_5$	2.9	223.0615	-0.045	9.9	$[M-H]^-$	$[M-H]^-$	225.0765	0.482	11.8	$[M+H]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	S
cafselogoside	$C_{25}H_{28}O_{14}$	2.9	551.1409	0.307	8	$[M-H]^-$, $[M-H_2O-H]^-$	-	-	-	-	-	-	L
unknown 4	$C_{11}H_{16}O_6$	3.0	243.0876	0.141	11.1	$[M-H]^-$	$[M-H]^-$	245.1025	0.486	13.7	$[M+H]^+$, $[M+Na]^+$, $[M+K]^+$	$[M-H_2O+H]^+$, $[M+H]^+$	-
vanillin	$C_8H_8O_3$	3.0	151.0396	-1.103	9.8	$[M-H]^-$	$[M-H]^-$	153.0543	0.567	14.1	$[M+H]^+$	$[M+H]^+$	S
ferulic acid	$C_{10}H_{10}O_4$	3.1	193.0503	-0.570	7.6	$[M-H]^-$	$[M-H]^-$	195.0658	0.597	9.6	$[M+H]^+$, $[M-H_2O+H]^+$	$[M-H_2O+H]^+$, $[M+H]^+$	S
luteolin 4'-O-glucoside	$C_{21}H_{20}O_{11}$	3.1	447.0934	0.075	13.4	$[M-H]^-$	$[M-H]^-$	449.1057	-0.638	16.2	$[M+H]^+$	$[M+H]^+$	SL
taxifolin	$C_{15}H_{12}O_7$	3.1	303.0509	0.079	18.3	$[M-H]^-$	$[M-H]$-, $[M-H-H_2O]^-$	305.0661	0.206	27	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	L
apigenin 7-O-glucoside	$C_{21}H_{20}O_{10}$	3.1	431.0990	0.667	26.9	$[M-H]^-$	$[M-H]^-$	433.1124	-0.506	5.5	$[M+H]^+$	$[M+H]^+$	S
quercetin glucoside isomer	$C_{21}H_{20}O_{12}$	3.1	463.0884	-0.068	6.1	$[M-H]^-$	-	465.1001	-2.035	30.8	$[M+H]^+$	-	L
desoxy elenolic acid	$C_{11}H_{14}O_5$	3.1, 3.6	225.0771	-0.292	7	$[M-H]^-$	$[M-H]^-$	227.0913	-0.415	21.3	$[M+H]^+$	$[M+H]$+, $[M-H_2O+H]^+$	L
luteolin glucoside isomer	$C_{21}H_{20}O_{11}$	3.2	447.0914	-0.259	11	$[M-H]^-$	$[M-H]^-$	449.1056	-0.749	9.4	$[M+H]$+	$[M+H]^+$	L
chrysoeriol 7-O-glucoside	$C_{22}H_{22}O_{11}$	3.2	461.1078	-0.885	31.8	$[M-H]^-$	$[M-H]^-$	463.1235	-0.537	20.3	$[M+H]^+$	$[M+H]^+$	SL
comselogoside isomers I and II	$C_{25}H_{28}O_{13}$	3.2, 3.6	535.1457	0.014	37.5	$[M-H]^-$	$[M-H]$-, $[M-H-H_2O]^-$	537.1527	-3.132	39.1	$[M+H]^+$	$[M+H]^+$	L
10-hydroxy oleuropein aglycone isomers I and II	$C_{19}H_{22}O_9$	3.3, 4.1	393.1197	-0.871	35.9	$[M-H]^-$	$[M-H]^-$	395.1331	1.180	20.6	$[M+H]^+$	-	L
hydroxytyrosol acyclodihydroelenolate	$C_{19}H_{26}O_8$	3.3	381.1560	0.482	22.1	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H]$-, $[M-H-H_2O]$-	365.1599	-1.575	47.2	$[M-H_2O+H]^+$, $[M+H]^+$	-	L
oleuropein	$C_{25}H_{32}O_{13}$	3.4	539.1769	-0.116	11.1	$[M-H]^-$	$[M-H]$-, $[M+Cl]$-	523.1764	-4.748	53.6	$[M-H_2O+H]^+$, $[M+H]^+$	$[M-H_2O+H]^+$, $[M+H]^+$	S
azelaic acid	$C_9H_{16}O_4$	3.4	187.0971	-0.535	2.6	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H]^-$	189.1125	-1.125	7.7	$[M+H]^+$, $[M+Na]^+$	-	T
hydroxytyrosol acetate	$C_{10}H_{12}O_4$	3.4	195.0658	-0.522	8.2	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H]^-$	197.0814	0.588	14.2	$[M+H]^+$, $[M-H_2O+H]^+$, $[M+K]^+$, $[M+Na]^+$	-	I
hydroxypinoresinol	$C_{20}H_{22}O_7$	3.6	373.1294	0.079	7.3	$[M-H]^-$	$[M-H]^-$	375.1451	0.479	17.8	$[M+H]^+$	-	L
hydroxy decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_7$	3.6	335.1135	-0.183	18.3	$[M-H]^-$	$[M-H]^-$	337.1275	-0.700	19.1	$[M+H]^+$	-	L
elenolic acid	$C_{11}H_{14}O_6$	3.6	241.0720	0.271	1.7	$[M-H]^-$	$[M-H]^-$	225.0765	0.716	10	$[M-H_2O+H]^+$, $[M+H]^+$, $[M+K]^+$, $[M+Na]^+$	$[M-H_2O+H]^+$, $[M+H]^+$	I
dihydrokaempferol	$C_{15}H_{12}O_6$	3.6	287.0565	0.337	11.3	$[M-H]^-$	$[M-H]$-, $[M-H-H_2O]$-	289.0708	-0.230	23.6	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	Met Frag/SL

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			m/z	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	*m/z*	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	
lucidumoside C	$C_{27}H_{36}O_{14}$	3.7	583.2031	-0.235	2.8	$[M-H]^-$	$[M-H]^-$	-	-	-	-	-	L
ligstroside	$C_{25}H_{32}O_{12}$	3.9	523.1820	-0.179	3.5	$[M-H]^-$	$[M-H]^-$	507.1852	-0.900	15.9	$[M-H_2O+H]^+$	-	L
luteolin	$C_{15}H_{10}O_6$	4.2	285.0405	0.136	2.4	$[M-H]^-$	$[M-H]^-$	287.0557	0.723	2.9	$[M+H]^+$	$[M+H]^+$	S
quercetin	$C_{15}H_{10}O_7$	4.3	301.0351	-0.194	17	$[M-H]^-$	$[M-H]^-$	303.0506	-0.923	34.5	$[M+H]^+$	$[M+H]^+$	S
hydroxy decanoic acid	$C_{10}H_{20}O_3$	4.3	187.1338	0.187	0.4	$[M-H]^-$	$[M-H]^-$	171.1379	-0.312	28.3	$[M-H_2O+H]^+$, $[M+H]^+$, $[M+Na]^+$	-	T
decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_6$	4.4	319.1187	-0.018	9.9	$[M-H]^-$	$[M-H]^-$	321.1338	0.718	27.2	$[M+H]^+$	-	I
oleuropein aglycone (six isomers)	$C_{19}H_{22}O_8$	4.4, 4.6, 5.7, 5.8, 6.2, 7.5	377.1242	0.069	3.1	$[M-H]^-$	[M-H]-, $[M-H-H_2O]$-	379.1387	-0.095	4.3	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	I
syringaresinol	$C_{22}H_{26}O_8$	4.4	417.1551	-2.794	29.3	$[M-H]^-$	-	419.1691	-0.944	29.6	$[M-H_2O+H]^+$	-	L
elenolic acid methylester	$C_{12}H_{16}O_6$	4.5	255.0876	-0.273	12.4	$[M-H]^-$	-	257.1022	0.407	38.1	$[M-H_2O+H]^+$, $[M+H]^+$	-	L
pinoresinol	$C_{20}H_{22}O_6$	4.7	357.1337	-0.625	20.7	$[M-H]^-$	$[M-H]^-$	359.1489	-4.095	17.6	$[M+H]^+$	$[M+H]^+$	S
t-cinnamic acid	$C_9H_8O_2$	4.8	147.0447	-0.772	15.6	$[M-H]^-$	$[M-H]^-$	131.0485	0.060	12.5	$[M-H_2O+H]^+$, $[M+H]^+$	-	S
acetoxypinoresinol	$C_{22}H_{24}O_8$	4.9	415.1404	-1.632	8.2	$[M-H]^-$	-	417.1539	-0.515	15.2	$[M+H]^+$	-	I
trihydroxy octadecadienoic acid	$C_{18}H_{32}O_5$	5.0	327.2180	0.034	6.9	$[M-H]^-$	$[M-H]^-$	329.2330	-0.216	15.9	[M+H]+	-	T
trihydroxy octadecenoic acid	$C_{18}H_{34}O_5$	5.1	329.2335	0.129	16.9	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H]^-$	331.2490	1.077	17.2	$[M+H]^+$, $[M+K]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	L
decarboxymethyl ligstroside aglycone	C17H20O5	5.1	303.1238	0.177	5.3	$[M-H]^-$, $[M-H_2O-H]^-$	[M-H]-	305.1394	0.167	11.3	$[M+H]^+$, $[M-H_2O+H]^+$, $[M+K]^+$, $[M+Na]^+$	-	I
naringenin	$C_{15}H_{12}O_5$	5.1	271.0613	0.086	13.4	$[M-H]^-$	$[M-H]^-$	273.0760	-2.464	28.7	$[M+H]^+$	$[M+H]^+$	SL
apigenin	$C_{15}H_{10}O_5$	5.1	269.0459	0.329	13.3	$[M-H]^-$	$[M-H]^-$	271.0605	0.398	4.5	$[M+H]^+$	$[M+H]^+$	S
unknown 5	$C_{21}H_{26}O_9$	5.4	421.1507	0.088	5.8	$[M-H]^-$	$[M-H]^-$	423.1631	0.684	28.1	$[M+H]^+$	-	-
diosmetin	$C_{16}H_{12}O_6$	5.4	299.0558	-0.280	14.9	$[M-H]^-$	$[M-H]^-$	301.0714	0.711	2.5	$[M+H]^+$	$[M+H]^+$	L
hydroxy decarboxymethyl ligstroside aglycone	$C_{17}H_{20}O_6$	5.4	319.1189	-0.417	16.8	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H]^-$	321.1343	0.984	22.3	$[M+H]^+$	-	L
trihydroxy octadecanoic acid	$C_{18}H_{36}O_5$	5.4	331.2488	-0.139	6.5	$[M-H]^-$	$[M-H]^-$	333.2641	-0.026	17.3	$[M+H]^+$, $[M-H_2O+H]^+$	-	L
ligstroside aglycone (six isomers)	$C_{19}H_{22}O_7$	5.4, 5.7, 5.8, 7.1, 7.3, 8.3	361.1293	-0.010	3.4	$[M-H]^-$	$[M-H]^-$	363.1443	0.501	6.4	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	I
methyl decarboxymethyl oleuropein aglycone	$C_{18}H_{22}O_6$	5.8	333.1343	-0.018	4.9	$[M-H]^-$	$[M-H]^-$	317.1385	1.817	11.9	$[M-H_2O+H]^+$	-	L
dehydro oleuropein aglycone	$C_{19}H_{20}O_8$	5.8	375.1087	0.164	21.7	$[M-H]^-$	$[M-H]^-$	377.1243	2.783	18.3	$[M+H]^+$	$[M+H]^+$	L
dihydroxy hexadecanoic acid	$C_{16}H_{32}O_4$	6.1	287.2230	0.184	1.4	$[M-H]^-$	$[M-H]^-$	289.2384	1.106	13.6	$[M+H]^+$, $[M-H_2O+H]^+$, $[M+Na]^+$	-	L
methyl oleuropein aglycone	$C_{20}H_{24}O_8$	6.1	391.1395	0.239	12.6	$[M-H]^-$	$[M-H]^-$	393.1537	-0.841	24.2	$[M+H]^+$	-	L
dehydro ligstroside aglycone	$C_{19}H_{20}O_7$	7.0	359.1138	-0.071	19.9	$[M-H]^-$	$[M-H]^-$	361.1294	1.211	25.1	$[M+H]^+$	-	L
gingerol	$C_{17}H_{26}O_4$	8.0	293.1759	0.114	4.2	$[M-H]^-$	$[M-H]^-$	295.1906	0.324	35.6	$[M+H]^+$	-	Met Frag
monohydroxylated derivative of maslinic acid	$C_{30}H_{48}O_5$	8.0	487.3429	-0.020	4.8	$[M-H]^-$	$[M-H]^-$	489.3536	-1.358	19.2	$[M+H]^+$	-	L
dimethyl oleuropein aglycone	$C_{21}H_{26}O_8$	8.2	405.1558	-0.235	16.3	$[M-H]^-$	$[M-H]^-$	407.1695	0.096	32.3	$[M+H]^+$	-	L
unknown 6	$C_{20}H_{38}O_5$	8.2	357.2641	-0.567	12.1	$[M-H]^-$	$[M-H]^-$	359.2794	-0.084	21.2	$[M+H]^+$, $[M-H_2O+H]^+$, $[M+Na]^+$	-	-

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			m/z	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	*m/z*	Error (mDa)	mSigma	ESI MS signal	APCI MS signal	
unknown 7	$C_{25}H_{36}O_7$	8.2	447.2388	-0.054	30	$[M-H]^-$	$[M-H]^-$	449.2514	-3.351	17.1	$[M+H]^+$	-	-
unknown 8	$C_{26}H_{38}O_7$	8.4	461.2545	-0.029	1.5	$[M-H]^-$	$[M-H]^-$	463.2789	3.377	28.6	$[M+H]^+$	$[M+H]^+$	-
unknown 9	$C_{25}H_{36}O_6$	8.6	431.2448	0.128	3.4	$[M-H]^-$	$[M-H]^-$	433.2571	0.366	24.3	$[M+H]^+$, $[M+Na]^+$	$[M+H]^+$	-
hydroxy octadecatrienoic acid	$C_{18}H_{30}O_3$	8.6	293.2122	-0.027	14.5	$[M-H]^-$	$[M-H]^-$	295.2278	0.007	17.4	$[M+H]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	T
dihydroxy octadecanoic acid	$C_{18}H_{36}O_4$	8.7	315.2516	-2.789	13.9	$[M-H]^-$	$[M-H]^-$	317.2692	0.338	16.4	$[M+H]^+$, $[M-H_2O+H]^+$	-	T
hydroxy octadecadienoic acid	$C_{18}H_{32}O_3$	8.7	295.2277	-0.131	15.2	$[M-H]^-$, $[M-H_2O-H]^-$	$[M-H]^-$	297.2429	-1.337	28.5	$[M+H]^+$	$[M+H]^+$	T
dihydroxy octadecadienoic acid	$C_{18}H_{32}O_4$	8.8	311.2222	-0.241	14.1	$[M-H]^-$	$[M-H]^-$	313.2378	-0.348	15.2	$[M+H]^+$	-	T
hydroxy octadecenoic acid	$C_{18}H_{34}O_3$	8.9	297.2435	-0.062	17.2	$[M-H]^-$	$[M-H]^-$	299.2592	0.297	36.8	$[M+H]^+$	$[M+H]^+$	T
maslinic acid	$C_{30}H_{48}O_4$	8.9	471.3488	0.783	15.4	$[M-H]^-$	$[M-H]^-$	455.3503	-1.355	11.9	$[M-H_2O+H]^+$, $[M+H]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	S
hydroxy octadecanoic acid	$C_{18}H_{36}O_3$	8.8	299.2591	-0.071	1.5	$[M-H]^-$	$[M-H]^-$	301.2742	0.479	23.3	$[M+H]^+$	-	T
linolenic acid	$C_{18}H_{30}O_2$	9.1	277.2174	0.122	13.9	$[M-H]^-$	$[M-H]^-$	279.2321	-0.385	18.5	$[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$	S
betulinic acid	$C_{30}H_{48}O_3$	9.4	455.3538	0.568	6.4	$[M-H]^-$	$[M-H]^-$	457.3668	-0.822	8.3	$[M+H]^+$	$[M+H]^+$	S
palmitoleic acid	$C_{16}H_{30}O_2$	9.6	253.2178	0.427	8.7	$[M-H]^-$	$[M-H]^-$	277.2151	0.677	19.7	$[M+Na]^+$, $[M+H]^+$, $[M-H_2O+H]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	S
oleanolic acid	$C_{30}H_{48}O_3$	9.6	455.3537	-0.314	5.9	$[M-H]^-$	$[M-H]^-$	457.3669	-0.722	5.3	$[M+H]^+$	$[M+H]^+$	S
linoleic acid	$C_{18}H_{32}O_2$	9.7	279.2333	0.469	2.3	$[M-H]^-$	$[M-H]^-$	281.2481	0.526	17.8	$[M+H]^+$	$[M-H_2O+H]^+$, $[M+H]^+$	S
hydroxy eicosanoic acid	$C_{20}H_{40}O_3$	9.9	327.2907	-0.726	18.5	$[M-H]^-$	$[M-H]^-$	329.3050	0.360	14.1	$[M+H]^+$	-	T
palmitic acid	$C_{16}H_{32}O_2$	9.9	255.2333	0.345	10.9	$[M-H]^-$	$[M-H]^-$	257.2475	0.558	13.5	$[M+H]^+$	-	SL
oleic acid	$C_{18}H_{34}O_2$	10.0	281.2497	0.173	12.4	$[M-H]^-$	$[M-H]^-$	283.2638	0.915	19.2	$[M+H]^+$	$[M+H]^+$, $[M-H_2O+H]^+$	S
erythrodiol	$C_{30}H_{50}O_2$	10.2	-	-	-	-	-	443.3868	-0.866	11.9	$[M+H]^+$	$[M+H]^+$	S
uvaol	$C_{30}H_{50}O_2$	11.2	-	-	-	-	-	443.3868	-1.359	15.3	$[M+H]^+$	$[M+H]^+$	S
stearic acid	$C_{18}H_{36}O_2$	10.6	283.2642	0.509	10.2	$[M-H]^-$	$[M-H]^-$	285.2801	-0.340	26.4	$[M+H]^+$	$[M+H]^+$	L
lupeol isomers I and II	$C_{30}H_{50}O$	11.6, 11.9	-	-	-	-	-	409.3835	-0.655	10.3	$[M-H_2O+H]^+$, $[M+H]^+$	$[M-H_2O+H]^+$	L
δ-tocopherol	$C_{27}H_{46}O_2$	12.1	-	-	-	-	-	401.3401	-1.307	25.3	$[M+H-H_2]^-$	$[M+H-H_2]^-$	S
β+γ-tocopherol	$C_{28}H_{48}O_2$	12.7	432.3606	-	-	?	?	415.3563	-0.800	15.6	$[M+H-H_2]^-$	$[M+H-H_2]^-$	S
cycloartenol	$C_{30}H_{50}O$	12.8	-	-	-	-	-	409.3828	0.082	19.5	$[M-H_2O+H]^+$, $[M+H]^+$	$[M-H_2O+H]^+$	L
stigmastadienol	$C_{29}H_{48}O$	13.2	-	-	-	-	-	395.3658	1.184	27.5	-	$[M-H_2O+H]^+$	L
a-tocopherol	$C_{29}H_{50}O_2$	13.4	446.3756	-	-	?	?	429.3713	-1.400	9.3	$[M+H-H_2]^-$	$[M+H-H_2]^-$	S
Δ^5-avenasterol	$C_{29}H_{48}O$	13.6	-	-	-	-	-	395.3676	-0.316	7.9	$[M-H_2O+H]^+$	$[M-H_2O+H]^+$	L
stigmasterol	$C_{29}H_{48}O$	13.8	-	-	-	-	-	395.3661	1.107	20.5	-	$[M-H_2O+H]^+$	S
campesterol	$C_{28}H_{48}O$	14.4	-	-	-	-	-	383.3679	-0.690	11.1	$[M-H_2O+H]^+$	$[M-H_2O+H]^+$	S
citrostadienol	$C_{30}H_{50}O$	14.6	-	-	-	-	-	409.3830	-0.164	9.2	-	$[M-H_2O+H]^+$	L
methylencycloartanol	$C_{31}H_{52}O$	15.2	-	-	-	-	-	423.3977	-0.169	15.9	$[M-H_2O+H]^+$	$[M-H_2O+H]^+$	L
β-sitosterol	$C_{29}H_{50}O$	15.3	-	-	-	-	-	397.3836	-0.715	29.4	-	$[M-H_2O+H]^+$	S

m/z, error and mSigma correspond to the first mentioned signal in the ESI column (when available); adducts are sorted by relative abundance.
Different isomers have been included within the same line of the table, indicating the corresponding Rt of each one. They all have been considered for giving the global numbers regarding annotated compounds.
Meaning of abbreviations used in the ID column: S, standard; I, isolated fraction; SL, spectral library; L, previously reported in literature; T, tentative annotation (previous reports missing).

Table S2. List of compounds identified with GC-APCI-MS.

Compound	M	Rt	*m/z*	Error (mDa)	mSigma	Calculated molecular formula	Signal	Other MS signals						ID
unknown 1	$C_{11}H_{18}O_8$	6.9	**351.1475**	0.430	36.3	$C_{14}H_{27}O_8Si$	$[M-H+TMS+H]^+$	261.0981	$(C_{11}H_{17}O_7)$					-
unknown 2	$C_8H_{10}O_3$	7.1	227.1117	-0.851	17.6	$C_{11}H_{19}O_3Si$	$[M-H+TMS+H]^+$	**137.0630**	$(C_8H_9O_2)$					-
unknown 3	$C_8H_{12}O_3$	7.6	**139.0786**	-0.507	14.4	$C_8H_{11}O_2$	$[M-H+TMS-OTMS+H]^+$							-
vanillin	$C_8H_8O_3$	8	225.0942	-0.203	15.6	$C_{11}H_{15}O_3Si$	$[M-H+TMS+H]^+$	209.0635	$(C_{10}H_{13}O_3Si)$					S
t-cinnamic acid	$C_9H_8O_2$	8.1	221.0987	-0.862	30.9	$C_{12}H_{17}O_2Si$	$[M-H+TMS+H]^+$	**205.0649**	$(C_{11}H_{13}O_2Si)$					S
tyrosol*	$C_8H_{10}O_2$	8.8	282.1466	-1.205	36.2	$C_{14}H_{26}O_2Si_2$	$[M-2H+2TMS]^+$	**193.1058**	$(C_{11}H_{17}OSi)$	179.0894	$(C_{10}H_{15}OSi)$			S
4-hydroxybenzoic acid	$C_7H_6O_3$	9.9	283.1184	-0.009	17.2	$C_{13}H_{23}O_3Si_2$	$[M-2H+2TMS+H]^+$							S
unknown 4	$C_9H_{14}O_3$	11.4, 12.2	**243.1409**	-0.170	31.6	$C_{12}H_{23}O_3Si$	$[M-H+TMS+H]^+$	153.0935	$(C_9H_{13}O_2)$					-
vanillic acid	$C_8H_8O_4$	13.6	**313.1278**	-0.463	28.8	$C_{14}H_{25}O_4Si_2$	$[M-2H+2TMS+H]^+$	223.0643	$(C_{11}H_{15}O_3Si)$	297.0940	$(C_{13}H_{21}O_4Si_2)$			S
hydroxytyrosol*	$C_8H_{10}O_3$	13.7	370.1809	-0.699	21	$C_{17}H_{34}O_3Si_3$	$[M-3H+3TMS]^+$	**281.1392**	$(C_{14}H_{25}O_2Si_2)$	193.0688	$(C_{11}H_{17}OSi)$			S
acetylated hydroxytyrosol*	$C_{10}H_{12}O_4$	14.9	340.1523	-0.703	32	$C_{16}H_{28}O_4Si_2$	$[M-2H+2TMS]^+$	**281.1389**	$(C_{14}H_{25}O_2Si_2)$					I
unknown 5	$C_{13}H_{20}O_7$	15.0/16.0/17.3/19.5	**361.1685**	-0.221	29.1	$C_{16}H_{28}O_7Si$	$[M-H+TMS+H]^+$							-
unknown 6	$C_{12}H_{18}O_8$	15.3	**363.1480**	-0.999	22.3	$C_{16}H_{28}O_7Si$	$[M-H+TMS+H]^+$	273.0982	$(C_{12}H_{16}O_7)$					-
elenolic acid isomer I	$C_{11}H_{14}O_6$	15.4	315.1256	-0.276	1.1	$C_{14}H_{23}O_6Si$	$[M-H+TMS+H]^+$	**225.0768**	$(C_{11}H_{13}O_5)$	283.1002	$(C_{13}H_{19}O_5Si)$			I
elenolic acid isomer II	$C_{11}H_{14}O_6$	16	315.1258	-0.155	5.6	$C_{14}H_{23}O_6Si$	$[M-H+TMS+H]^+$	**225.0771**	$(C_{11}H_{13}O_5)$	283.0999	$(C_{13}H_{19}O_5Si)$			I
unknown 7	$C_{14}H_{24}O_9$	16.5	**553.2686**	-0.664	17.3	$C_{23}H_{49}O_9Si_3$	$[M-3H+3TMS+H]^+$	373.1682	$(C_{17}H_{29}O_7Si)$	283.1189	$(C_{14}H_{19}O_6)$			-
quinic acid	$C_7H_{12}O_6$	16.6	**481.2286**	-0.434	15.3	$C_{19}H_{45}O_6Si_4$	$[M-4H+4TMS+H]^+$	391.1783	$(C_{16}H_{35}O_5Si_3)$	301.1273	$(C_{13}H_{25}O_4Si_2)$	211.0741	$(C_{10}H_{15}O_3Si)$	S
unknown 8	$C_6H_{10}O_6$	17.0, 17.3	**467.2132**	-0.093	8.6	$C_{18}H_{43}O_6Si_4$	$[M-4H+4TMS+H]^+$							-
p-coumaric acid	$C_9H_8O_3$	17.7	**309.1333**	-0.392	4.1	$C_{15}H_{25}O_3Si_2$	$[M-2H+2TMS+H]^+$	237.0910	$(C_{12}H_{17}O_3Si)$	381.1728	$(C_{18}H_{33}O_3Si_3)$	293.1012	$(C_{14}H_{21}O_3Si_2)$	S
unknown 9	$C_{20}H_{38}O_{12}$	17.8, 18.2	**543.2837**	-0.598	17.4	$C_{23}H_{47}O_{12}Si$	$[M-H+TMS+H]^+$	453.2339	$(C_{20}H_{37}O_{11})$					-
elenolic acid isomer III	$C_{11}H_{14}O_6$	17.9	315.1256	-0.188	8.3	$C_{14}H_{23}O_6Si$	$[M-H+TMS+H]^+$	**225.0769**	$(C_{11}H_{13}O_5)$	283.0995	$(C_{13}H_{19}O_5Si)$			I
palmitoleic acid	$C_{16}H_{30}O_2$	19.7	**327.2712**	-0.193	40.1	$C_{19}H_{39}O_2Si$	$[M-H+TMS+H]^+$							S
unknown 10	$C_{13}H_{24}O_{10}$	20	629.3185	-0.063	53.2	$C_{25}H_{57}O_{10}Si_4$	$[M-4H+4TMS+H]^+$	**539.2671**	$(C_{22}H_{47}O_3Si_3)$					-
palmitic acid	$C_{16}H_{32}O_2$	20.1	**329.2868**	-0.287	40.8	$C_{19}H_{41}O_2Si$	$[M-H+TMS+H]^+$							L
ferulic acid	$C_{10}H_{10}O_4$	21.3	**339.1439**	0.153	7.2	$C_{16}H_{27}O_4Si_2$	$[M-2H+2TMS+H]^+$	411.1832	$(C_{19}H_{35}O_4Si_3)$	249.0915	$(C_{13}H_{17}O_3Si)$			S
linoleic acid	$C_{18}H_{32}O_2$	23.8	**353.2867**	-0.248	4.6	$C_{21}H_{41}O_2Si$	$[M-H+TMS+H]^+$	263.2347	$(C_{18}H_{31}O)$					S
oleic acid	$C_{18}H_{34}O_2$	23.9	**355.3023**	-0.432	3.6	$C_{21}H_{43}O_2Si$	$[M-H+TMS+H]^+$	265.2525	$(C_{18}H_{33}O)$					S
linolenic acid	$C_{18}H_{30}O_2$	24	**351.2711**	-0.318	43.5	$C_{21}H_{39}O_2Si$	$[M-H+TMS+H]^+$	261.2213	$(C_{18}H_{29}O)$					S
stearic acid	$C_{18}H_{36}O_2$	24.4	**357.3170**	-2.597	47.9	$C_{21}H_{45}O_2Si$	$[M-H+TMS+H]^+$	267.2684	$(C_{18}H_{35}O)$					L
arachidic acid	$C_{20}H_{40}O_2$	28.5	**385.3490**	-1.145	41.6	$C_{23}H_{49}O_2Si$	$[M-H+TMS+H]^+$	295.2996	$(C_{20}H_{39}O)$					L
decarboxymethyl ligstroside aglycone	$C_{17}H_{20}O_5$	30.4	377.1773	-0.104	7.5	$C_{20}H_{29}O_5Si$	$[M-H+TMS+H]^+$	359.1669	$(C_{20}H_{27}O_4Si)$	**193.1056**	$(C_{11}H_{17}OSi)$			I
dihydroxy hexadecanoic acid	$C_{16}H_{32}O_4$	31.1	**415.3054**	0.415	19.4	$C_{22}H_{47}O_3Si_2$	$[M-3H+3TMS-OTMS+H]^+$	505.3546	$(C_{25}H_{57}O_4Si_3)$	325.2558	$(C_{19}H_{37}O_2Si)$			T
decarboxymethyl oleuropein aglycone	$C_{17}H_{20}O_6$	33.7	465.2110	-0.139	11.4	$C_{23}H_{37}O_6Si_2$	$[M-2H+2TMS+H]^+$	375.1620	$(C_{20}H_{27}O_5Si)$	**193.1057**	$(C_{11}H_{17}OSi)$	281.1386	$(C_{14}H_{25}O_2Si_2)$	I
glyceryl linoleate	$C_{21}H_{38}O_4$	33.9, 34.5	427.3236	-0.864	18.6	$C_{24}H_{47}O_4Si$	$[M-H+TMS+H]^+$	501.3777	$(C_{27}H_{57}O_4Si_2)$	**411.3282**	$(C_{24}H_{47}O_3Si)$			L
ligstroside aglycone isomer I	$C_{19}H_{22}O_7$	35.4	435.1832	-0.184	7.6	$C_{22}H_{31}O_7Si$	$[M-H+TMS+H]^+$	475.1959	$(C_{24}H_{35}O_6Si_2)$	**193.1058**	$(C_{11}H_{17}OSi)$			I
squalene	$C_{30}H_{50}$	35.5	**411.3978**	0.74	18.8	$C_{30}H_{51}$	$[M+H]^+$							L
ligstroside aglycone isomer II	$C_{19}H_{22}O_7$	36.7	507.2225	-0.553	9.6	$C_{25}H_{39}O_7Si_2$	$[M-2H+2TMS+H]^+$	475.1957	$(C_{24}H_{35}O_6Si_2)$	**193.1059**	$(C_{11}H_{17}OSi)$			I

Compound	M	Rt	*m/z*	Error (mDa)	mSigma	Calculated molecular formula	Signal	Other MS signals						ID
δ-tocopherol	$C_{27}H_{46}O_2$	36.9	475.3958	-0.808	15.8	$C_{30}H_{55}O_2Si$	$[M-H+TMS+H]^+$							S
ligstroside aglycone isomer III	$C_{19}H_{22}O_7$	37.6	507.2224	-0.538	5.8	$C_{25}H_{39}O_7Si_2$	$[M-2H+2TMS+H]^+$	**193.1058**	$(C_{11}H_{17}OSi)$					I
oleuropein aglycone isomer I	$C_{19}H_{22}O_8$	38.2	523.2176	-0.132	9.5	$C_{25}H_{39}O_8Si_2$	$[M-2H+2TMS+H]^+$	**281.1384**	$(C_{14}H_{25}O_2Si_2)$					I
β-tocopherol	$C_{28}H_{48}O_2$	38.3	**489.4107**	-1.72	12	$C_{31}H_{57}O_2Si$	$[M-H+TMS+H]^+$							S
γ-tocopherol	$C_{28}H_{48}O_2$	38.5	**489.4108**	-1.627	9.1	$C_{31}H_{57}O_2Si$	$[M-H+TMS+H]^+$							S
oleuropein aglycone isomer II	$C_{19}H_{22}O_8$	38.7	523.2176	-0.223	4.8	$C_{25}H_{39}O_2Si_2$	$[M-2H+2TMS+H]^+$	**281.1384**	$(C_{14}H_{25}O_2Si_2)$					I
oleuropein aglycone isomer III	$C_{19}H_{22}O_8$	39.4	595.2568	-0.259	6.6	$C_{28}H_{47}O_8Si_3$	$[M-3H+3TMS+H]^+$	**281.1389**	$(C_{14}H_{25}O_2Si_2)$					I
apigenin isomer	$C_{15}H_{10}O_5$	39.7	415.1387	-0.471	37.4	$C_{21}H_{27}O_5Si_2$	$[M-2H+2TMS+H]^+$							T
hydroxy oleuropein aglycone	$C_{19}H_{22}O_9$	39.8	611.2517	-0.834	19.7	$C_{28}H_{47}OSi_3$	$[M-3H+3TMS+H]^+$	**281.1387**	$(C_{14}H_{25}O_2Si_2)$					L
oleuropein aglycone isomer IV	$C_{19}H_{22}O_8$	40.2	595.2572	-0.495	7.5	$C_{28}H_{47}O_8Si_3$	$[M-3H+3TMS+H]^+$	**281.1385**	$(C_{14}H_{25}O_2Si_2)$					I
α-tocopherol	$C_{29}H_{50}O_2$	40.7	**503.4254**	-2.48	15.2	$C_{32}H_{59}O_2Si$	$[M-H+TMS+H]^+$							S
apigenin	$C_{15}H_{10}O_5$	40.8	**487.1780**	-0.577	4.2	$C_{24}H_{35}O_5Si_3$	$[M-3H+3TMS+H]^+$	415.1389	$(C_{21}H_{27}O_5Si_2)$	471.1467	$(C_{23}H_{31}O_5Si_3)$			S
luteolin isomer	$C_{15}H_{10}O_6$	42.3	503.1727	-0.961	8.2	$C_{24}H_{35}O_6Si_3$	$[M-3H+3TMS+H]^+$							T
campesterol	$C_{28}H_{48}O$	42.4	473.4147	-2.608	12.9	$C_{31}H_{57}OSi$	$[M-H+TMS+H]^+$	**383.3666**	$(C_{28}H_{47})$					S
stigmasterol	$C_{29}H_{48}O$	42.9	485.4160	-1.339	6.3	$C_{32}H_{57}OSi$	$[M-H+TMS+H]^+$	**395.3669**	$(C_{29}H_{47})$					S
luteolin	$C_{15}H_{10}O_6$	43.1	**575.2124**	-0.871	6.2	$C_{27}H_{43}O_6Si_4$	$[M-4H+4TMS+H]^+$	503.1726	$(C_{24}H_{35}O_6Si_3)$	559.1801	$(C_{26}H_{39}O_6Si_4)$			S
pinoresinol	$C_{20}H_{22}O_6$	43.7	503.2262	-1.69	18.1	$C_{26}H_{39}O_6Si_2$	$[M-2H+2TMS+H]^+$	**485.2165**	$(C_{26}H_{37}O_5Si_2)$					S
b-sitosterol	$C_{29}H_{50}O$	43.8	487.4318	-1.675	25.1	$C_{32}H_{59}OSi$	$[M-H+TMS+H]^+$	**397.3819**	$(C_{29}H_{49})$					S
Δ^5-avenasterol	$C_{29}H_{48}O$	44	485.4159	-1.361	8.4	$C_{32}H_{57}OSi$	$[M-H+TMS+H]^+$	**395.3656**	$(C_{29}H_{47})$					L
acetoxy pinoresinol*	$C_{22}H_{24}O_8$	44.3	560.2255	-0.629	38.03	$C_{28}H_{40}O_8Si_2$	$[M-2H+2TMS]^+$	501.21117	$(C_{26}H_{37}O_6Si_2)$					I
Δ^5-stigmastadienol	$C_{29}H_{48}O$	44.5	485.4162	-1.284	12.7	$C_{32}H_{57}OSi$	$[M-H+TMS+H]^+$	**395.3656**	$(C_{29}H_{47})$					L
cycloartenol	$C_{30}H_{50}O$	44.6	499.4318	-1.3	6.3	$C_{33}H_{59}OSi$	$[M-H+TMS+H]^+$	**409.3817**	$(C_{30}H_{49})$					L
methylencycloartanol	$C_{31}H_{52}O$	45.5	513.4473	-1.269	6.3	$C_{34}H_{61}OSi$	$[M-H+TMS+H]^+$	**423.3977**	$(C_{31}H_{51})$					L
eythrodiol	$C_{30}H_{50}O_2$	46.1	497.4163	-1.099	40.7	$C_{33}H_{57}OSi$	$[M-H_2O-H+TMS+H]^+$	**407.3680**	$(C_{30}H_{47})$					S
citrostadienol	$C_{30}H_{50}O$	46.1	499.4314	-1.549	6.8	$C_{33}H_{59}OSi$	$[M-H+TMS+H]^+$	**409.4382**	$(C_{30}H_{49})$					L
uvaol	$C_{30}H_{50}O_2$	46.6	497.4165	-0.801	35.6	$C_{33}H_{57}OSi$	$[M-H_2O-H+TMS+H]^+$	**407.3671**	$(C_{30}H_{47})$					S
oleanolic acid	$C_{30}H_{48}O_3$	47	**511.3961**	-0.489	8.1	$C_{33}H_{55}O_2Si$	$[M-H+TMS]^+$	601.4452	$(C_{36}H_{65}O_3Si_2)$					S
betulinic acid	$C_{30}H_{48}O_3$	47.2	**601.4448**	-1.823	4.3	$C_{36}H_{65}O_3Si_2$	$[M-2H+2TMS]^+$	511.3954	$(C_{33}H_{55}O_2Si)$					S
ursolic acid	$C_{30}H_{48}O_3$	47.6	**511.3958**	-0.781	18.8	$C_{33}H_{55}O_2Si$	$[M-H+TMS]^+$	601.4471	$(C_{36}H_{65}O_3Si_2)$					S
maslinic acid I	$C_{30}H_{48}O_4$	48	617.4401	-0.492	15.7	$C_{36}H_{65}O_4Si_2$	$[M-2H+2TMS]^+$	**527.3902**	$(C_{33}H_{55}O_3Si)$	509.3799	$(C_{33}H_{53}O_2Si)$	599.4296	$(C_{36}H_{63}O_3Si_2)$	S
maslinic acid II	$C_{30}H_{48}O_4$	49.4	527.3909	-0.541	13.3	$C_{33}H_{55}O_3Si$	$[M-H+TMS+H]^+$	**509.3799**	$(C_{33}H_{53}O_2Si)$	599.4296	$(C_{36}H_{63}O_3Si_2)$			S

* Compounds detected as $[M-nH+nTMS]^+$. MS Signals with the highest relative abundance are presented in bold letters.
In this table, the isomers are included by using different lines, since in some cases the achieved MS information was slightly different.
Meaning of abbreviations used in the ID column: S, standard; I, isolated fraction; L, previously reported in literature; T, tentative annotation (previous reports missing).

Table S3. Distribution of the determined metabolites in the eight evaluated samples (all the given values are % referred to the richest sample regarding each analyte).

	Leaves	Wood	Skin	Pulp	VOO	Dehydro VOO	Seed	Seed Oil
gallic acid	100	64	0	0	0	1	0	0
protocatechuic acid	3	6	100	1	0	1	0	0
eudesmic acid	10	9	14	10	73	100	11	66
gentisic acid	0	56	0	0	0	100	0	0
4-hydroxybenzoic acid	100	31	19	20	0	0	0	0
4-hydroxyphenylacetic acid	100	48	24	69	12	7	10	1
vanillic acid	0	14	100	12	77	37	0	19
syringic acid	0	0	97	0	0	100	0	0
homovanillic acid	0	0	100	59	31	43	0	24
vanillin	0	0	100	0	56	76	0	42
caffeic acid	18	85	67	100	0	0	38	19
p-coumaric acid	23	4	100	22	64	70	5	3
verbascoside	89	100	0	107	0	0	14	0
sinapic acid	0	0	0	0	0	100	0	79
ferulic acid	27	40	16	44	100	77	4	25
t-cinnamic acid	0	0	0	0	12	100	12	72
Phenolic acids and aldehydes	56	38	100	44	67	84	12	48
quinic acid	61	62	46	100	8	1	1	0
citric acid	57	100	49	76	0	0	96	0
aesculin	2	100	0	1	0	0	0	0
aesculetin	2	100	1	1	0	0	0	0
Organic acids and coumarins	62	100	49	94	5	0	40	0
3,4-dihydroxyphenylglycol	32	16	26	47	100	11	14	0
oxydized hydroxytyrosol	73	54	53	100	36	5	18	0
hydroxytyrosol glucoside	25	100	3	95	0	1	33	0
hydroxytyrosol	29	43	5	100	7	20	14	3
tyrosol glucoside	6	44	7	100	0	1	72	0
tyrosol	8	3	33	58	91	100	6	23
phenylethyl primeveroside	100	34	3	32	1	0	12	0
hydroxytyrosol acetate	0	100	80	0	25	87	4	21
gingerol	45	77	82	69	90	100	89	66
Simple phenols and derivatives	31	83	10	100	8	12	39	4
hydroxy decarboxymethyl elenolic acid	42	3	82	100	8	17	12	0
acyclodihydroelenolic acid hexoside	24	16	47	100	1	0	22	0
decarboxylated form of hydroxy elenolic acid	15	7	100	65	0	74	10	9
dihydro oleuropein	3	10	0	100	0	0	1	0
oleoside/secologanoside	82	100	39	45	0	0	4	0
elenolic acid glucoside	31	24	16	100	0	1	28	0
decarboxymethyl elenolic acid	63	71	100	51	93	64	2	51
hydroxy oleuropein	85	100	4	22	0	0	15	0
oleuropein glucoside	48	66	0	41	0	0	100	0
hydroxy elenolic acid	2	1	100	36	11	45	1	0
nuzhenide	1	1	1	4	0	0	100	2
cafselogoside	1	3	1	100	0	0	3	0
desoxy elenolic acid	11	3	3	30	27	5	100	18
comselogoside	1	0	6	100	1	0	1	0
10-hydroxy oleuropein aglycone	100	27	11	79	12	2	0	0
hydroxytyrosol acyclodihydroelenolate	0	0	0	100	0	0	0	0

	Leaves	Wood	Skin	Pulp	VOO	Dehydro VOO	Seed	Seed Oil
oleuropein	46	100	1	61	0	0	8	0
hydroxy decarboxymethyl oleuropein aglycone	6	100	21	48	42	59	1	0
elenolic acid	9	1	5	100	11	40	1	1
lucidumoside C	100	71	2	27	0	0	32	0
ligstroside	30	100	11	0	0	1	97	0
decarboxymethyl oleuropein aglycone	52	100	4	98	86	55	1	0
elenolic acid methylester	23	5	76	54	17	100	0	0
oleuropein aglycone	16	17	0	100	6	1	0	0
decarboxymethyl ligstroside aglycone	9	4	0	9	100	35	1	0
hydroxy decarboxymethyl ligstroside aglycone	100	10	0	58	11	91	0	0
ligstroside aglycone	12	10	1	100	79	14	5	1
methyl decarboxymethyl oleuropein aglycone	16	18	4	67	100	5	2	1
dehydro oleuropein aglycone	0	34	2	84	100	16	3	1
methyl oleuropein aglycone	0	72	21	100	0	19	6	0
dehydro ligstroside aglycone	0	0	0	14	100	31	3	3
dimethyl oleuropein aglycone	11	14	0	100	0	0	0	0
Secoiridoids and derivatives	28	31	8	100	20	14	10	1
luteolin diglucoside	100	0	0	0	0	0	0	0
cyanidin 3-O-glucoside	0	100	0	0	0	0	1	0
gallocatechin	100	30	3	13	0	0	17	0
rutin	100	76	2	23	0	0	1	0
luteolin 7-O-rutinoside	100	8	8	23	0	0	0	0
quercetin 4'-O-glucoside	9	100	0	0	0	0	0	0
luteolin 7-O-glucoside	100	34	9	22	0	0	0	0
apigenin 7-O-rutinoside	100	7	13	12	0	0	1	0
luteolin 4'-O-glucoside	100	19	4	4	0	0	0	0
taxifolin	15	100	0	0	0	0	0	0
apigenin 7-O-glucoside	100	21	14	20	0	0	0	0
chrysoeriol 7-O-glucoside	100	4	1	0	0	0	0	0
luteolin glucoside isomer	100	13	8	0	0	0	0	0
dihydrokaempferol	0	100	0	0	0	0	0	0
luteolin	100	16	91	29	46	5	0	0
quercetin	46	100	7	1	4	1	0	0
naringenin	3	100	7	1	28	4	0	1
apigenin	15	5	39	7	100	16	1	0
diosmetin	100	13	34	1	97	19	1	0
Flavonoids	100	72	24	15	22	3	7	0
hydroxypinoresinol	1	4	100	1	7	5	0	2
syringaresinol	11	22	100	12	70	68	10	34
pinoresinol	4	10	100	1	61	29	7	23
acetoxypinoresinol	0	0	68	0	65	0	23	100
Lignans	2	6	100	1	21	12	2	9
azelaic acid	1	0	100	0	2	2	0	4
hydroxy decanoic acid	0	0	7	0	0	0	0	100
trihydroxy octadecadienoic acid	4	2	100	0	1	0	1	0
trihydroxy octadecenoic acid	0	0	100	0	0	1	0	0
trihydroxy octadecanoic acid	0	0	100	0	0	1	0	0
dihydroxy hexadecanoic acid	1	0	100	0	0	2	0	0
hydroxy octadecatrienoic acid	65	13	60	1	97	71	5	100
dihydroxy octadecanoic acid	1	1	21	1	1	1	1	100

	Leaves	Wood	Skin	Pulp	VOO	Dehydro VOO	Seed	Seed Oil
hydroxy octadecadienoic acid	2	1	100	1	26	27	4	70
dihydroxy octadecadienoic acid	0	0	66	1	6	15	1	100
hydroxy octadecenoic acid	1	3	0	1	10	11	2	100
hydroxy octadecanoic acid	8	15	4	27	88	13	19	100
linolenic acid	19	10	11	6	100	67	7	26
palmitoleic acid	4	4	0	8	70	100	9	61
linoleic acid	0	1	100	6	15	5	5	2
hydroxy eicosanoic acid	3	1	0	1	29	29	1	100
palmitic acid	64	100	0	55	18	7	52	10
oleic acid	27	29	0	100	65	12	65	11
stearic acid	0	100	0	79	0	0	44	0
Fatty acids and derivatives	1	1	100	1	4	5	1	21
monohydroxylated derivative of maslinic acid	31	5	100	1	4	9	1	8
maslinic acid	10	6	100	0	1	8	0	1
betulinic acid	38	100	4	0	1	9	0	1
oleanolic acid	45	23	100	0	1	19	0	1
uvaol & erythrodiol	47	32	100	0	90	46	0	1
Pentacyclic triterpenes	22	13	100	0	2	12	0	1
δ-tocopherol	0	0	0	0	32	65	0	100
β- & γ-tocopherol	16	0	0	19	61	89	26	100
α-tocopherol	72	11	0	50	100	33	8	88
Tocopherols	67	10	0	48	100	44	12	96
lupeol	0	0	0	0	17	100	0	0
lupeol isomer	0	0	0	0	16	100	0	0
cycloartenol	0	0	0	58	35	100	0	0
stigmastadienol	0	0	0	0	29	100	0	0
Δ^5-avenasterol	6	0	0	21	42	100	2	20
stigmasterol	0	0	0	0	57	100	0	0
campesterol	0	0	4	10	22	28	16	100
citrostadienol	0	0	0	0	6	36	8	100
methylencycloartanol	0	0	0	17	19	100	0	14
β-sitosterol	13	10	5	23	25	76	25	100
Sterols	10	7	4	21	30	100	19	89

Each chemical class was determined in the most favorable coupling (maximum number of identified compound and good ionization rate avoiding saturation in any matrix): organic acids, coumarins and phenolic compounds (phenolic acids and aldehydes, simple phenols, secoiridoids, flavonoids and lignans) in LC-ESI-MS$^{(-)}$; fatty acids and derivatives as well as triterpenic acids in LC-APCI-MS$^{(-)}$; and triterpenic alcohols, tocopherols and sterols in LC-APCI-MS$^{(+)}$.

Conclusions

Conclusiones

In the Doctoral Thesis reported in this dissertation, several analytical methodologies have been developed and successfully applied to perform different metabolomic studies in matrices derived from the olive tree (different types of oils and plant tissues). This section aims to list in a concise way the most relevant conclusions:

1. Different methodologies have been developed and optimized for the targeted determination of two families of bioactive compounds (triterpenic and phenolic compounds) in the selected matrices.

   - In relation to **triterpenic acids and alcohols**, LC methodologies have been optimized for their simultaneous determination in plant tissues (leaves, skin and olive pulp) and olive oils, in 6 and 13 minutes, respectively. In **Chapter 1** three kinds of detection systems (DAD, ESI-IT MS and APCI-QTOF MS -using positive and negative polarity in MS-) have been used to carry out the quantification of the aforementioned analytes, obtaining statistically equivalents results. In **Chapter 2**, ultrasound-assisted extraction with methanol was selected as optimum olive oil sample treatment; moreover, it was demonstrated that the major triterpenic acids could be accurately quantified by injecting the oil diluted in acetone into the LC-MS system. The results obtained through LC-MS were compared with those provided by a GC-MS method (considered as a reference), and statistically significant differences between them were not found.

   - As far as **phenolic compounds** are concerned, in **Chapter 3**, the exhaustive optimization of a LC-FLD method for the characterization of the olive oil phenolic profile was carried out. The results obtained after applying the developed method to the analysis of virgin olive oil samples were compared with those provided by the LC-MS reference method finding adequate correlation between both strategies. In **Chapter 4**, the individual quantification of phenolic compounds by LC-MS (using a large number of standards, some of them not commercially available) was compared with the quantitative data offered by global methods (Folin-Ciocalteu assay, COI HPLC-DAD method and an approach based on the hydrolysis of complex phenols followed by HPLC-DAD detection). We looked for equivalence factors among all of them and evidenced the great variability regarding results expression of phenolic content that can be found in literature. Moreover, we highlighted the need for consensus regarding the conditions of the health claim associated to hydroxythrosol and derivatives. Finally, in **Chapters 5** and **6**, LC-MS methods were successfully applied to the characterization of Argentinean olive oil and Moroccan olive leaves, respectively. Argentina and Morocco are considered as emerging oil producing powers and, until now, studies focused on the deep assessment of the phenolic profile of their olive oils or leaves (from different varieties) had been scarcely reported.

2. "Multi-class" methodologies have been optimized for the simultaneous characterization of olive oil minor compounds in a single analysis through different platforms (LC-MS and GC-

MS). The developed methods allowed the determination of more than 40 metabolites, belonging to different chemical families (phenolic and triterpenic compounds, free fatty acids, tocopherols and sterols) (**Chapter 7**). These methodologies (which enlarge the metabolome coverage) enabled the differentiation of virgin olive oils according to the olive variety from which they were produced (by quantifying 41 analytes in **Chapter 8**) and the authentication of its geographical origin (through an untargeted approach in **Chapter 10**). In both cases, the use of chemometrics allowed finding potential varietal and origin markers, respectively.

3. A LC-MS "multi-class" method has been applied to achieve the characterization of new products obtained from olives through a novel processing system that aims to reduce the generation of by-products from the olive industry (**Chapter 9**). The proposed process involved fruit stoning, pulp dehydration and subsequent pressing to obtain a new type of olive oil and olive flour. The influence of the dehydration temperature on the composition of the resulting products was assessed and the concentration of phenols, triterpenes and tocopherols in conventional olive oil and in the oil obtained by applying the novel procedure was compared. The high content of bioactive compounds found in the new products made them potential ingredients for food or cosmetic industries.

4. "Multi-class" methods have been applied (LC-ESI MS, LC-APCI MS, GC-APCI MS) to reveal the metabolic profile of eight matrices derived from the olive tree, including plant tissues (leaves, stems, skin, pulp and olive seed) and oils (olive seed oil, virgin olive oil and olive oil obtained from stoned and dehydrated fruits) (**Chapter 11**). The complementarity of the different analytical platforms coupled to MS was demonstrated and their suitability to determine each family of metabolites was systematically evaluated. The use of standards, databases and specialized software allowed the identification of more than 150 metabolites in the selected matrices, which also underwent a detailed semi-quantitative analysis.

Trying to formulate the general conclusions in a very concise way, it is possible to say that in the work that is included in this report, the use of different sample preparation protocols has been combined with targeted and untargeted approaches based on chromatography (LC and GC) coupled to several detection systems (MS having a leading role) with several purposes: to offer repeatable and reliable analytical alternatives with certain advantages over classical methodologies to determine minor compounds from interesting olive matrices; to propose methods to make possible the determination of a large number of compounds within a single analysis; to characterize new olive matrices whose employment could has great interest and impact in the future; and to devise strategies and statistical models that allow to discriminate between oil samples and olive leaves based on their variety or geographical origin, identifying possible markers. In other words, this Doctoral Thesis establishes a strong link between Food Metabolomics and the olive oil industry, where analytical innovation could represent a clear support for the best resolution of classic problems of the sector and a way to ensure its progress and evolution.

All the achievements of this dissertation are, from our point of view, very interesting for the sector and the whole industry related to the olive grove. However, it is more than evident that there is a lot of work to be done. It seems pertinent to conclude, mentioning some of the issues that we would like to address in the near future:

- to complete some of the studies presented herewith by using NMR; the structure and identity of some of the compounds that have been described at the moment as "unknown" could be elucidated;
- to evaluate the potential of hydrophilic interaction chromatography (HILIC) or monolithic columns of adequate functionality to continue expanding the coverage of the metabolome of olive matrices and improving the analytical performance and the analysis time in the determination of minor compounds;
- to apply specific extraction systems based on the use of molecular imprinted polymers (MIPs) or functionalized nanoparticles to extract compounds of interest (oleuropein from olive leaves, some compounds with interesting bioactivity, etc.) with high selectivity;
- to carry out studies regarding the *in-vitro* evaluation of the antidiabetic, neuroprotective and anticancer properties of the phenolic and triterpene compounds of the oil and to continue delving into their bioavailability and metabolism (using both urine and plasma samples),
- to apply some of the most powerful methods developed within this Thesis to characterize the minor compositional profile of other oils whose use and consumption are spreading in recent years (argan, avocado, prickly pear oil, etc.), as well as to comprehensively assess how the culinary process of frying affects the most relevant compounds belonging to the oil unsaponifiable fraction;
- in the same way, those analytical tools could be applied to the study of the Verticilosis of the olive tree, caused by the fungus *Verticillium dahliae*. It would be of great interest to evaluate how this fungus affects the profile of secondary metabolites of different varieties of olive tree; that would identify the most resistant cultivars and, thus, to render profitable all the efforts previously made in programs dedicated to genetic improvement.

En la Tesis Doctoral recogida en esta memoria, diversas metodologías analíticas han sido desarrolladas y aplicadas con éxito para llevar a cabo distintos estudios metabolómicos en matrices derivadas del olivo (distintos tipos de aceites y tejidos vegetales). Esta sección pretende recoger de manera concisa las conclusiones alcanzadas más relevantes.

2. Se han desarrollado y optimizado metodologías para la determinación *targeted* de dos familias de compuestos bioactivos presentes en las matrices seleccionadas como objeto de estudio: compuestos triterpénicos y fenólicos.

   - Respecto a los **ácidos y alcoholes triterpénicos**, se han puesto a punto metodologías LC para su determinación simultánea tanto en tejidos vegetales (hojas, piel y pulpa de aceituna) como en aceite de oliva en 6 y 13 minutos, respectivamente. En el **Capítulo 1** se ha corroborado la capacidad de tres tipos de detectores (DAD, ESI-IT MS y APCI-QTOF MS –usando polaridad positiva y negativa en MS–) para llevar a cabo la cuantificación de los citados analitos, consiguiendo resultados estadísticamente equivalentes. En el **Capítulo 2** se ha optimizado el tratamiento de muestra cuando la matriz a estudiar es aceite de oliva (extracción asistida por ultrasonidos con metanol) y se ha demostrado que los ácidos mayoritarios también pueden ser cuantificados de forma exacta inyectando directamente en el sistema el aceite diluido en acetona. Los resultados obtenidos mediante LC-MS se han comparado con el método GC-MS (considerado como referencia), no encontrando diferencias significativas entre los mismos.

   - En cuanto a los **compuestos fenólicos**, en el **Capítulo 3** se ha realizado la optimización exhaustiva de un método LC-FLD para la determinación del perfil fenólico del aceite de oliva. Los resultados obtenidos tras la aplicación del método desarrollado al análisis de muestras de aceite de oliva virgen se han comparado con los datos alcanzados aplicando el método LC-MS de referencia, observando una más que adecuada correlación entre ambas estrategias. En el **Capítulo 4** se ha comparado la cuantificación individual de fenoles por LC-MS (empleando un elevado número de patrones, algunos de ellos no disponibles comercialmente), con los datos cuantitativos ofrecidos por métodos globales para la determinación de fenoles en aceite de oliva (el método de Folin-Ciocalteu, el método HPLC-DAD del COI y una estrategia basada en la hidrólisis de fenoles complejos seguida de detección HPLC-DAD). Se han buscado factores de equivalencia entre todos ellos pero, sobre todo, se ha puesto de manifiesto la gran variabilidad que puede encontrarse en bibliografía a la hora de expresar los resultados de contenido fenólico y se ha remarcado la necesidad de consenso en cuanto a las condiciones de la declaración de propiedades saludables asociada al hidroxitrosol y sus derivados. Por último, en los **Capítulos 5** y **6**, métodos LC-MS se han aplicado con éxito para la caracterización de muestras de aceite de oliva argentinas y hojas de olivo marroquíes, respectivamente. Argentina y Marruecos están considerados como potencias productoras de aceite en pleno estadio emergente y, hasta el momento, no contaban con estudios focalizados en la evaluación profunda el perfil

fenólico de sus aceites u hojas de olivo (provenientes distintas variedades).

3. Se han optimizado metodologías "multi-class" para conseguir la caracterización simultánea de compuestos minoritarios del aceite de oliva en un solo análisis mediante distintas plataformas analíticas (LC-MS y GC-MS). Los métodos desarrollados han permitido la determinación de más de 40 metabolitos, pertenecientes a distintas familias químicas (compuestos fenólicos y triterpénicos, ácidos grasos libres, tocoferoles y esteroles) (**Capítulo 7**). También se ha demostrado el potencial de este tipo de metodologías de amplia cobertura del metaboloma de muestras oleícolas, para diferenciar aceites de oliva vírgenes en función de la variedad de aceituna a partir de la que se han elaborado (mediante cuantificación de 41 analitos en el **Capítulo 8**) o de su origen geográfico (a través de una aproximación *untargeted* en el **Capítulo 10**). En ambos casos, el uso de herramientas estadísitcas ha permitido encontrar potenciales marcadores varietales y de origen geográfico.

4. Se ha aplicado un método LC-MS "multi-class" para conseguir la caracterización de nuevos productos obtenidos de la aceituna mediante un innovador sistema de procesado que pretende reducir la generación de subproductos de la industria oleícola (**Capítulo 9**). El proceso propuesto incluye el deshuesado del fruto, deshidratado de la pulpa y posterior prensado para obtener un nuevo tipo de aceite de oliva y lo que se ha denominado como "harina de aceituna". Se ha estudiado la influencia de la temperatura de deshidratado en la composición de los productos obtenidos y se ha comparado la concentración de fenoles, triterpenos y tocoferoles en el aceite de oliva convencional y el obtenido aplicando el novedoso procedimiento. El alto contenido de compuestos bioactivos encontrado en los nuevos productos les aporta un gran valor como ingredientes en la industria alimentaria y cosmética.

5. Se han aplicado métodos "multi-class" (LC-ESI MS, LC-APCI MS, GC-APCI MS) para revelar el perfil metabólico de ocho matrices derivadas del olivo, incluyendo tejidos vegetales (hojas, tallos, piel, pulpa y semilla de aceituna) y aceites (aceite de semilla de olivo, aceite de oliva virgen y aceite de oliva obtenido de frutos deshuesados y deshidratados) (**Capítulo 11**). Se ha puesto de manifiesto la complementariedad de las distintas plataformas analíticas acopladas a MS y evaluado la idoneidad de cada una de ellas para el análisis de distintas familias químicas detectadas. El uso de patrones, bases de datos y de un software especializado ha permitido la identificación de más de 150 metabolitos en las matrices seleccionadas, que se han sometido además a un detallado análisis semi-cuantitativo.

Tal y como se ha puesto de manifiesto y tratando de formular las conclusiones generales de modo aún más conciso, en el trabajo que se recoge en esta memoria, se ha combinado el empleo de distintos sistemas de preparación de muestra con aproximaciones *targeted* y *untargeted* basadas en cromatografía (LC y GC) acoplada a varios sistemas de detección (teniendo MS un papel protagonista) con diversos fines: ofrecer alternativas analíticas repetibles y fiables con ciertas ventajas respecto a las metodologías clásicas para determinar compuestos minoritarios en

matrices oleícolas de interés; proponer métodos que permitan determinar un gran número de compuestos en un solo análisis; caracterizar nuevas matrices oleícolas cuyo empleo podría tener gran interés e impacto en el futuro; e idear estrategias y modelos estadísticos que permitan discriminar entre muestras de aceites y hojas de olivo en base a la variedad o al origen geográfico, identificando posibles marcadores. En otras palabras, esta Tesis Doctoral establece un estrecho vínculo entre la Metabolómica de alimentos y la industria oleícola, donde la innovación analítica podría representar un claro apoyo para la mejor resolución de problemas clásicos del sector y una vía para asegurar su progreso y evolución.

Todos los logros de esta tesis son, desde nuestro punto de vista, muy interesantes para el sector y la industria relacionada con el olivar. Sin embargo, es más que evidente que queda mucho trabajo por hacer. Nos parece pertinente concluir, mencionando algunas de las temáticas que nos gustaría abordar en un futuro próximo:

- completar algunos de los estudios aquí recogidos empleando RMN, de modo que se pueda elucidar la estructura e identidad de algunos de los compuestos que han quedado descritos por el momento como "desconocidos";
- evaluar el potencial de la cromatografía de interacción hidrofílica (HILIC) o de columnas monolíticas de funcionalidad adecuada para seguir ampliando la cobertura del metaboloma de matrices oleícolas y mejorando las prestaciones analíticas y el tiempo de análisis en la determinación de compuestos minoritarios;
- aplicar sistemas de extracción específicos basados en el uso de polímeros de impronta molecular (MIPs) o nanopartículas funcionalizadas para extraer compuestos de interés (oleuropeína de hojas de olivo, algún compuesto con interesante bioactividad, etc.) con alta selectividad;
- realizar estudios que continúen con la evaluación *in-vitro* de las propiedades antidiabéticas, neuroprotectoras y anticancerígenas de los compuestos fenólicos y triterpénicos del aceite y seguir ahondando acerca de la biodisponibilidad y metabolismo de los mismos (utilizando tanto muestras de orina como de plasma);
- aplicar algunos de los potentes métodos desarrollados para caracterizar el perfil composicional minoritario de otros aceites cuyo uso y consumo se está extendiendo en los últimos años (aceite de argán, aguacate, higo chumbo, etc.), así como para evaluar exhaustivamente cómo afecta el proceso culinario de fritura a los compuestos más relevantes de la fracción insaponificable;
- de igual modo, esas herramientas analíticas podrían aplicarse al estudio de la Verticilosis del olivo, causada por el hongo *Verticillium dahliae*. Sería de gran interés evaluar cómo afecta dicho hongo al perfil de metabolitos secundarios de distintas variedades de olivo, lo que permitirá identificar las más resistentes y así rentabilizar esfuerzos en los programas dedicados a la mejora genética.

*Óleo pintado por **Sita Pancorbo Cobo** que ilustra un paisaje de la provincia de Jaén*