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FACULTAD DE CIENCIAS

DEPARTAMENTO DE QUÍMICA ANALÍTICA

OBTENCIÓN DE PRODUCTOS DE ALTO VALOR AÑADIDO A PARTIR DE DESECHOS DE LA VID Y DE LA INDUSTRIA VINÍCOLA

OBTAINMENT OF HIGH-ADDED VALUE PRODUCTS FROM VINE RESIDUES AND WINEMAKING WASTE

María del Pilar Delgado de la Torre

Córdoba, 2015

TITULO: *OBTENCIÓN DE PRODUCTOS DE ALTO VALOR AÑADIDO A PARTIR DE DESECHOS DE LA VID Y DE LA INDUSTRIA VITIVINÍCOLA. OBTAINMENT OF HIGH-ADDED VALUE PRODUCTS FROM RESIDUES AND WINEMAKING WASTE*

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TÍTULO DE LA TESIS: OBTENCIÓN DE PRODUCTOS DE ALTO VALOR AÑADIDO A PARTIR DE DESECHOS DE LA VID Y DE LA INDUSTRIA VINÍCOLA

DOCTORANDA: MARÍA DEL PILAR DELGADO DE LA TORRE

La doctoranda María del Pilar Delgado de la Torre cursó brillantemente los estudios de Máster en Química Fina Avanzada, obteniendo excelentes calificaciones en todas las materias. Su trabajo fin de máster se publicó en la revista Journal Agricultural and Food Chemistry, la segunda en el área de "Agriculture Multidisciplinary" y situada en el primer decil del área de "Food Chemistry and Technology".

El tema de la tesis se enmarca en las líneas de investigación del grupo "Plataformas analíticas en metabolómica/proteómica y aprovechamiento de residuos agroalimentarios", de código PAIDI FQM-227. La tesis supone un paso hacia el aprovechamiento integral de residuos agroalimentarios, tal como propugna la convocatoria Horizonte 2020 en el apartado "Waste".

En la investigación realizada se ha trabajado con desechos poco explotados y de enorme abundancia en nuestra región: Los sarmientos de vid y las lías de vinificación, con los que se ha llevado a cabo un estudio exhaustivo tanto de su composición como de su aplicación; abriendo así una vía para su aprovechamiento que puede resultar de gran interés. Al mismo tiempo, la investigación que ha desarrollado la doctoranda le ha permitido adquirir una sólida formación científica y un adiestramiento exhaustivo en la instrumentación analítica actual, especialmente en el manejo de los espectrómetros de masas en sus diferentes versiones (tanto los de tiempo de vuelo como los de triple cuadrupolo), utilizando equipos de última generación.

El trabajo realizado por la doctoranda ha dado lugar a la redacción de 9 artículos (7 de ellos publicados y 2 enviados para su publicación), 1 capítulo de libro ya publicado y 6 comunicaciones científicas en congresos nacionales e internacionales.

Por todo ello los directores consideran que la investigación realizada y recogida en esta Memoria reúne los requisitos necesarios en cuando a originalidad, innovación y calidad, por lo que autorizan la presentación de la Tesis Doctoral de María del Pilar Delgado de la Torre.

Córdoba, 6 de abril de 2015

Fdo.: M. D. Luque de Castro

Fdo.: Feliciano Priego Capote

OBTENCIÓN DE PRODUCTOS DE ALTO VALOR AÑADIDO A PARTIR DE DESECHOS DE LA VID Y DE LA INDUSTRIA VINÍCOLA

Los Directores,

Fdo. María Dolores Luque de Castro
Catedrática del Departamento de
Química Analítica
Universidad de Córdoba

Fdo. Feliciano Priego Capote
Doctor Contratado Ramón y Cajal del
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Universidad de Córdoba

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

Fdo. María del Pilar Delgado de la Torre
Licenciada en Química

María Dolores Luque de Castro, Catedrática, y **Feliciano Priego Capote**, Doctor Contratado Ramón y Cajal, ambos del Departamento de Química Analítica, Facultad de Ciencias, Universidad de Córdoba, en calidad de Directores de la Tesis Doctoral presentada por la Licenciada en Química María del Pilar Delgado de la Torre, con el título "Obtención de productos de alto valor añadido a partir de desechos de la vid y de la industria vinícola",

CERTIFICAN:

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

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, 6 de abril de 2015.

Fdo. María Dolores Luque de Castro

Fdo. Feliciano Priego Capote

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

1. Informes favorables de dos doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:
    - Prof. Dra. Dr Anne-Sylvie Fabiano Tixier, Groupe de Reserche en Eco-Extraction de Produits Naturels (GREEN) — Alimentari Université d'Avignon, France.
    - Prof. Dr. José Ricardo Pérez Correa, Ingeniería Química y Bioprocesos, Pontificia Universidad Católica de Chile, Chile.
2. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a otro centro de Enseñanza Superior de otro país:
    - Prof. Dr. Andrea Versari, Dipartimento di Scienze e Tecnologie Agro — Alimentari University of Bologna, Italia.
3. La defensa de parte de esta Memoria se realizará en una lengua diferente a la materna: Inglés.
4. Estancia de tres meses en un centro de investigación de otro país:
    - Rowett Institute of Nutrition and Health, Dietary Metabolites and Human Health unit, directed by Dr. Wendy Russell, Aberdeen, Scotland, United Kingdom.

Agradecimientos

En primer lugar, quiero transmitir mi más sincero afecto y agradecimiento a mis directores de Tesis, que con su fantástica dedicación y su enorme calidad científica han sabido guiarme desde el kilómetro cero hasta el final del camino. A la Catedrática María Dolores Luque, por ver en mí ese algo que brillaba y saber sacarlo a flote, transmitiéndome durante el viaje ese intenso entusiasmo por la ciencia y por las cosas bien hechas. Por su maravillosa capacidad de trabajo y organización, atendiéndonos a todos con extraordinaria rapidez. Por sus magníficos aportes que han contribuido notablemente a la mejora de todo lo que pasaba por sus manos y por hacer posible que mi formación haya sido tan extensa y completa. Al Doctor Feliciano Priego, por ser siempre tan accesible y tan agradable. Por su excelente visión estratégica del trabajo, sorteando siempre con pericia cualquier inconveniente y por su paciencia cada vez que he necesitado alguna explicación. Por sus brillantes aportes y sus buenos consejos. Ambos con vuestra experiencia y dedicación habéis contribuido enormemente no solo a mi formación, sino también a mantenerme motivada y dispuesta a sacar lo mejor de mí. Gracias por vuestra gran profesionalidad.

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Chuck, nuestro inesperado agregado mejicano, fuente de conocimiento en casi cualquier área y con quien da gusto conversar durante horas. A Antonio Mena y María, que han pasado de ser "los nuevos" a estar ya a mitad del camino y perfectamente integrados en el grupo. Cómo pasa el tiempo!!! Por esas conversaciones con María sobre cine y música. Y por los buenos ratos viendo como Antonio pica a Ángela en la sobremesa (siempre de buena manera y desde el cariño), y especialmente por ese tesoro que Antonio se trajo a nuestro grupo, Mayte, con la que tantas risas nos hemos echado. A las más nuevas, Asun y María del Mar con las que también hemos pasado muy buenos momentos, cuchicheando en la comida sobre cualquier cosa, desde temas de trabajo hasta asuntos de la prensa rosa. Y a los que ya no están aquí, que no por eso son menos importantes, a Chema, que se fue "a medias", y que gracias al delicado estado de salud del TOF lo tenemos entre nosotros cada dos por tres. A Bea, que también aprovecha para volver a vernos cuando puede y con la que después de tanto tiempo se puede hablar como si nos hubiésemos visto el día de antes. A Miguel (y Cristina) con su perol anual en los Villares que ya es tradición tanto como los "veroneos". A Sole, que sigue apuntándose a nuestras salidas y días de piscina. Y a Mara, que aunque esté tan lejos tiene su rinconcito en nuestros corazones. A Antonia, que siempre viene a contarnos sus historias (Y ahora a enseñarnos fotos de su Teresita). Y Sara Pinzi, como no, esa chiquilla que siempre tiene una sonrisa para todo el mundo y te atiende como si se le fuese la vida en ello.

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A nuestros vecinos de pasillo, Jose, que en realidad es como un apéndice del FQM-227, con todos esos almuerzos y buenos momentos, y con quien puedo compartir mis notas de humor más friki y absurdo, además de nuestro contrabando privado de películas selectas, no es que yo tenga buen gusto cinéfilo, es que el tuyo es excelente!! (Guiño, guiño). María José, nuestra doctora más rockera, y que más de una duda nos ha resuelto. María e Isa, siempre dispuestas a una buena charla, aunque últimamente sólo nos veamos de tesis en tesis.

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For all of you:

Should auld acquaintance be forgot,
and never brought to mind.
Should auld acquaintance be forgot,
and auld lang syne

For auld lang syne, my jo,
for auld lang syne,
we'll tak a cup o' kindness yet,
for auld lang syne.

To my lovely housemates, Corine Van der Ploeg, Cavit Arslan (and his dictionary) and Thulile Swagne. It is impossible to forget those friendly chats freezing in the kitchen while we cooked dinner. And especially to Corine and Cavit, always willing to plan a new excursion to nearby cities, castles, villages, christmas parades, museums, ceilidhs….any excuse was good enough!

To the Rowett Ph.D students I had the opportunity to know: William Buosi and Christine McCartney, it is a pity we didn't have the opportunity to do more excursions together, I really enjoyed the Crathes castle day. Joanna Kaniewska, Eva Rosenkranz and Gemma Nadal, for being so friendly and counting with me for all the ceilidhs (God, I love ceilidhs!!!). Andrew Chappell, keep on lifting man!!!! I will keep on commenting your FB publications. Christopher Knowles, we met at the end of my stay, but you are also part of my Scottish experience, and that Christmas lunch day was great!!! After all we keep on chatting by FB from time to time.

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Índice

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OBJETIVOS

La investigación que se recoge en esta Memoria de Tesis se incluye en la segunda de las líneas en las que trabaja el Grupo al que pertenece la doctoranda "FQM-227, Plataformas analíticas en metabolómica/proteómica y aprovechamiento de residuos agroalimentarios", cuya segunda posición en las líneas del Grupo no implica menor importancia o dedicación que a la primera.

El ***objetivo genérico*** de la tesis fue doble. En primer lugar obtener una información analítica lo más completa posible sobre desechos de la vid y de la industria vinícola que habían suscitado poco o nulo interés hasta la fecha: Los sarmientos y las lías de vinificación. La segunda parte de este objetivo genérico fue aprovechar los desechos de la forma más racional posible que se dedujera de su composición y características analíticas. De este doble objetivo genérico derivaron lo siguientes ***objetivos concretos*** que se han organizado en 3 bloques o secciones en función sus características: Bloque I, en el que se recogen los estudios analíticos sobre sarmientos y su comparación con virutas de roble; Bloque II, dedicado a establecer las bases para la aplicación industrial de los sarmientos como sustitutos/potenciadores de las virutas de roble en el proceso de envejecimiento de vinos; Bloque III, en el que aparecen los estudios sobre la composición de las lías, sus compuestos de mayor interés y la variabilidad de concentración de ellos según el cultivar. La aplicación industrial de los compuestos de interés en esta materia prima queda en manos de las industrias del ramo competente, especialmente de las de colorantes. Cada bloque se ha dividido en capítulos en función de la materia abarcada en cada uno de los artículos publicados o en vías de publicación.

El Bloque I está compuesto por 5 artículos, cuyos objetivos concretos fueron:

-Adquirir una formación suficiente sobre las vías de obtención de los compuestos de interés a partir de las materias primas consideradas. Un estudio bibliográfico en profundidad para conseguir esa formación dio lugar a un capítulo de libro multiautor, que constituye también el Capítulo 1 de esta Memoria.

-Comparar métodos basados en diferentes técnicas de extracción (más propiamente de lixiviación) para obtener compuestos de interés existentes en sarmientos de forma efectiva y rápida. Las ventajas e inconvenientes de los 3 métodos desarrollados dieron lugar a la publicación recogida como Capítulo 2.

-Conocer las semejanzas y diferencias de composición entre los sarmientos pertenecientes 18 cultivares de vid y 5 variedades de virutas de roble a través de los perfiles de sus extractos obtenidos mediante cromatografía líquida–detección de masas (LC–TOF/MS). Los estudios quimiométricos apropiados completaron la información que se recoge en el Capítulo 3.

-Evaluar de forma comparativa los componentes de los extractos de sarmientos y de virutas de roble obtenidos mediante líquidos sobrecalentados, utilizando como herramienta analítica un cromatógrafo de gases conectado a un detector de masas de trampa de iones (GC–IT/MS) y con el tratamiento quimiométrico de los datos obtenidos fue el objetivo cuyo resultado se muestra en el Capítulo 4.

-Establecer semejanzas y diferencias en los perfiles de volátiles de los sarmientos y los de las virutas de roble utilizando las muestras sólidas en un dispositivo de espacio de cabeza calentado a diferentes temperaturas (entre 120 y 200 ºC) y diferentes tiempos de tostado fue otro de los objetivos concretos, de cuyo estudio se da información en el Capítulo 5.

El Bloque II recoge los resultados de un estudio de envejecimiento de vinos tintos en presencia de diferentes tipos tanto de sarmientos como de virutas de roble, a distintos tiempos de envejecimiento. La actuación de un panel de cata y el uso de equipos analíticos adecuados proporcionaron la información prevista en los objetivos en este caso, que fueron los siguientes:

-Conocer los datos proporcionados por un excelente panel de cata, tratarlos y presentar los resultados de la actuación del panel fue el objetivo concreto en este caso, tal como se refleja en el Capítulo 6.

-Ratificar/desmitificar/ampliar la información proporcionada por el panel de cata mediante el correspondiente estudio analítico utilizando un equipo GC–IT/MS constituyó un objetivo clave que se recoge en el Capítulo 7.

El Bloque III, destinado al estudio de lías, constituye una auténtica revelación sobre este desecho de la industria vinícola, tan poco estudiado y con tanta riqueza. Los objetivos planteados y conseguidos en este caso fueron los que siguen:

-Realizar una identificación tentativa de los componentes de las lías procedentes de 18 bodegas, tanto de la fracción sólida y seca como del vino embebido en el sólido. Un equipo constituido por un LC y un MS en modo de alta resolución se utilizó para este estudio que recoge el Capítulo 8, mientras que en el Capítulo 9, y tras un estudio mediante LC–TOF/MS de las lías secas y el vino embebido en ellas, se identificaron los compuestos representativos y se realizó un análisis estadístico para constatar el grado de similitud de ambas fracciones con vistas a una explotación conjunta de ambas o separada.

-Llevar a cabo un estudio más concreto y exhaustivo de los componentes considerados de mayor interés en las lías (a saber: Antocianidinas, proantocianidinas y antocianinas). Para ello, un acoplamiento de una estación automatizada de extracción en fase sólida con un LC y un MS proporcionó una adecuada selectividad, mientras que la ausencia de patrones comerciales para muchos de estos compuestos se suplió con la aplicación de métodos de adquisición basados en la versatilidad del triple cuadrupolo para confirmar su presencia.

La formación de la futura doctora, que constituye el ***objetivo último*** de toda tesis doctoral, ha incluido la realización del máster en “Química Fina” con el número de créditos correspondientes, y en paralelo con éste y con la investigación recogida en la parte principal de la Memoria, se ha pretendido una

formación más amplia de la doctoranda mediante la realización de otras actividades que se recogen como anexos, tales como:

Investigación simultánea con la tesis, también relacionada en cierto modo con la tesis, realizada en colaboración con el Departamento de Genética, que ha dado lugar a la publicación de un artículo; mientras que la colaboración con otros miembros del Grupo ha resultado en la redacción de un artículo que se ha enviado para su publicación a la revista Journal of Agricultural and Food Chemistry. Ambos constituyen el Anexo I.

La asistencia a conferencias nacionales e internacionales, con presentaciones orales y en cartel, con un total de 6 comunicaciones, que se recogen como Anexo II.

OBJECTIVES

The research in this PhD Book is included within the second of the working lines of the Group to which the PhD student belongs: "FQM-227, Analytical platforms in metabolomics/proteomics and exploitation of agrofood residues". The second position of this line in the two of the Group does not involve a smaller importance than the first.

The ***general objective*** was double; in a first step the objective was to obtain analytical information as complete as possible on the byproducts and waste from vine and the winery industry that had so far been considered of scant or nil interest: vine-shoots and vinification lees. The second part of this general objective was to take profit from these byproducts and waste in the most rational way by taking into account their composition and analytical characteristics. This dual general objective generated the following ***specific objectives***, organized into three sections as a function of their characteristics: Section I, which encompasses analytical studies on vine-shoots and comparison with oak chips: Section II, devoted to establishing the basis for the industrial application of vine-shoots as substitutes/enhancers of oak chips in the wine ageing process; Section III, which contains the studies on lees composition, the high-priced compounds they contain and the variability in concentration depending on the cultivar. The industrial application of the target compounds in this raw material is in the hands of specific industries, particularly those devoted to colorants. Each section is divided into chapters corresponding to the articles, published or in the process of being published.

Section I is constituted by 5 publications, the specific objectives of which were as follows:

-To acquire enough training on the ways to obtain the target compounds from the given raw materials. An in-depth study of the literature provided both appropriate training and a chapter in a multiauthor book that also constitutes Chapter 1 of this PhD Book.

-To compare methods based on different extraction (better expressed as leaching or lixiviation) techniques to obtain interesting compounds in vine-shoots in an effective and fast way. The advantages and disadvantages of the three proposed methods gave place to a publication that constitutes Chapter 2.

-To know the similitudes and differences in composition among vine-shoots from 18 vine cultivars and 5 varieties of oak by using the extracts to obtain

their profiles by liquid chromatography–mass detection (LC–TOF/MS). The appropriate chemometric studies completed the study in Chapter 3.

-To make a comparison between the components of the extracts from vine-shoots and oak chips obtained by superheated liquids, using as analytical tool a gas chromatograph coupled to an ion-trap mass detector (GC–IT/MS), with subsequent chemometric treatment of the obtained data was the objective, the results of which are in Chapter 4.

-To establish the common and differentiating aspects of the volatile profiles of vine-shoots and oak chips by using a headspace device that was heated at different temperatures (between 120 and 200 ºC) and different toasting times was other of the specific objectives, as shows Chapter 5.

Section II encompasses the results of a study on red wine ageing in the presence of different types of either vine-shoots or oak chips, subjected to different ageing times. An expert panel and the appropriate analytical equipment provided the required information to fulfil the objectives that, in this case, were as follows:

-To know the information provided by an excellent expert panel, subject the data to the proper treatment and present the results thus obtained, as shown in Chapter 6.

-To assess/demystify/amplify the information provided by the expert panel by the appropriate analytical study using GC–IT/MS equipment was a key objective that constitutes Chapter 7.

Section III, devoted to study vinification lees, constitutes a real finding on this byproduct of the wine industry, which has so far been scantly studied and that contain an enormous wealth. The planned and achieved objectives in this case were as follows:

-To carry out a tentative identification of the components of lees from 18 wineries both in the solid fraction and imbibed wine. LC–TOF/MS equipment in high resolution mode was used for this study that constitutes Chapter 8, while Chapter 9 contains the LC–TOF/MS study for identification of significant compounds and the statistical study to know the degree of similarity of both fractions for a potential either joint or separate exploitation.

-To develop a more especific and exhaustive study of compounds of high interest in lees (namely, anthocyanidins, protoanthocyanidins and anthocyanins). With this aim, coupling of an automatized station for solid-phase

extraction, an LC and an MS provided the appropriate selectivity, while the absence of commercial standards for most of these compounds was overcame by using data-dependent methods taking advantage of the versatility of the triple-quad mass detector.

The formation of the future PhD, ***final objective*** of a Doctoral Thesis, has also included the master on "Fine Chemistry", in which the PhD student developed the mandatory courses. Also, the necessary steps to fulfill the requirements to achieve the mention to the International Doctorate were developed by the PhD student. In parallel to the above mentioned tasks and to the research in the main part of the Book, an additional training of the PhD student has been sought by development of other activities summarized below as annexes.

Research simultaneous with that of the Thesis, developed in collaboration with the Department of Genetics, also related at some extent with the body of this thesis, giving place to the publication of an article; while the collaboration with other members of the Group has resulted in a manuscript sent for publication to the Journal of Agricultural and Food Chemistry. Both constitute Annex I.

Attendance to national and international meetings and conferences, with oral or poster presentations, yield a total of 6 communications, which are under Annex II.

Introducción

Introduction

This introduction section is intending to offer an overview of the two main topics of the research that constitutes this PhD Book: characterization of residues and qualitative and quantitative analysis of compounds with potential interest in these residues.

1. General trends to exploitation of residues/waste

Traditionally, most crops have been mainly used to obtain a single, high valued product, as is the case of olive oil, cider or wine, in which a major part of the plant or the fruit is considered as residues of nil or low value; most times highly contaminant, and, thus, undesirable. This is the case of alperujo or alpechín, residues of the olive oil industry. The present remarkable growth of waste generation is a consequence of the increased generation of marketable products. Agro-industrial wastes are solids or liquids generated from transformation of primary products or by industrialization; materials no longer useful to the process that generated them, but they could be used or transformed into products with economic value of social and commercial interest.

Frequently, the terms by-product, waste, and residue are used interchangeably, although there is a conceptual difference among them. In fact, a "by-product" is a well known secondary product resulting from an industrial process, and generally useful at some degree, marketable and with a certain added-value. The term "residue" is applied to materials that may or may not have commercial value, because they are rare or generated at low levels. However, some residue constituents, even at low proportion, may confer an interest to the material. From this point of view, the terms "by-product" and "residue" could be used as synonyms, but not the term "waste", which is referred to as materials

with no commercial value, or no interest for use in any process; therefore, "wastes" are considered trash and are disposed of.

In general, the characteristics of agro-industrial wastes are varied, and they depend on the raw material and the process by which they are generated; however, all agricultural wastes share one main feature: the content of organic matter, composed by different percentages of cellulose, lignin, hemicellulose and pectin. As they are mainly composed by organic matter, in practice they are called "organic waste". Within this category are also included other wastes such as muds from treatment plants, sewage, as well as the household waste and municipal solid waste. As a result of the increase in waste generation, thousands of tons of residues are burnt or discharged to dumps, rivers, etc., with the corresponding damage to the ecosystem. This is the reason why national and international policies are being implemented to punish illegal practices in this field.

In the wine industry, and according to the European Council Regulation (EC) 479/2008 on the common organization of the wine market, grape marcs and wine lees must be sent to alcohol distilleries to yield exhausted grape marcs and a liquid waste known as vinasse. However, it is very common that small wine-producers do not obey this rule, and generate a residue that is a mixture of grape marcs and wine lees together with grape stalks that are treated as organic waste. Also, the usual aerobic depuration of the winery effluents, vinasse and wastewaters, generates other solid waste, known as winery-sludge *(1)*.

According to the Spanish law 22/2013, on National general budget, the industrial waste must be recycled, valorized or disposed of in order to prevent environmental contamination. Many wineries choose the waste disposal option — despite it involves an added cost to the company— instead of using any of the cost-effective options as recycling or valorization of the residues. Moreover, the waste disposal practices imply that companies must bear the cost of the disposal fee imposed by the corresponding authorities, since discharges to public waters are taxed with a disposal fee, used to finance the study, control and protection of

the side-effects of throwing the wastes on a river basin or a land field. Generally, the amount of this fee depends on the waste volume and the price established for the specific waste. For example, in Spain, the basic price per cubic meter is fixed as 0.01202 € for urban wastewaters and 0.03005 € for industrial wastewaters. However, the price per cubic meter may be increased as a function of the toxicity of the waste and an additional disposal fee may also be applied, depending on the local authorities. The companies must also contribute with an additional fee to support the authority investments to avoid contamination. Thus, the disposal fee that the companies must pay to the authorities may be significantly higher (*e.g.* 230 €/month/m^3/h, plus a variable fee that depends on the type and quantity of alcohol produced by a winery). However, in spite of the costs linked to waste emissions to the environment, companies have traditionally opted for the so called "polluter pays", due to the low fines imposed to the pollutant companies. For instance, during the period 1985–2000, the fines charged to the companies for unauthorized discharges on the environment were less than 10000 €, usually around 3000 €.

There is a current concern about the environment that has led authorities to implement economically viable options for recycling or valorizing wastes. Also, the costs of waste disposal and penalties for breaking the law have increased considerably, reaching 40000 € and imprisonment sentences. Additionally, these fines are occasionally combined with the obligation to decontaminate the affected areas. Thus, "polluter pays" is becoming a non profitable practice for companies.

This is the reason why a rational exploitation of undesirable wastes is each time more desirable. In this way, the valorization of a material considered as a waste (and therefore far from being a source of richness and involving a high cost for the industry) can be converted into a source of richness. For decades, agro-industrial residues have been the focus of attention of researchers worldwide, because some of their components may be raw materials to obtain products of interest; a situation that keeps on prevailing today and is expected to continue in the future. A suitable technology to transform organic wastes and

Table 1. Costs associated to residue production in the wine industry in Spain: fines and discharge fees.

Concept	Disposal fee/fine	Reference
Payment to the municipal authorities for treatment of vinasse from alcohol distilleries	230 €/month/m^3/h + variable fee dependent on the type of alcohol and quantity produced	Judgement of the Superior Court of Castilla-La Mancha No. 253/1997 (Division of Administrative Litigation) of June 10
Infraction of the Coastal Law for vinasse spillage	3000 €	High Court of Justice of Andalusia, Granada No. 1180/1998 (Division Contentious-Administrative) of September 14
Unauthorized discharge of wastewater caused by the break of a vinasse pond	34.716,44 € for unauthorized spillage	Magistrates' Court (Contentious-Administrative Section 1) of 14 January 2000
Unauthorized discharge of wastewater of a winery industry	6.010,12 € for unauthorized spillage	High Court of Justice of Castilla La Mancha No. 208/2005 (Division of Administrative Litigation, Section 2) of July 20
Unauthorized discharge of vinasse wastewater to adjacent land	1.803,04 € for unauthorized spillage	Magistrates' Courts of Albacete No. 46/1999 (Section 1) of March 24
Unauthorized discharge of vinasse to a river course	1.800,00 € for unauthorized spillage + withdrawal of the accumulated vinasse and clearance of the river	High Court of Justice of Castilla y León No. 486/2000 (Division of Contentious-Administrative) of March 25
Imposition of the disposal fee	18.805,67 €	Central Economic and Administrative Tribunal of April 28, 1993
Unauthorized discharge of vinasse to a river course	36.529,52 € for unauthorized spillage	High Court of Justice of Madrid No. 458/2002 (Division Contentious-Administrative) of April 23
Unauthorized discharge of vinasse to a river course	9.321,94 €	High Court of Justice of Valencia No. 1228/2002 (Division of Contentious-Administrative) of September 25
Unauthorized discharge of vinasse	3.000,00 Euros	High Court of Justice of Castilla y Leon No. 789/2000 (Division of Contentious-Administrative) of April 28
Unauthorized discharge of vinasse	9.000,00 €	High Court of Justice of Extremadura No. 850/2009 (Division of Contentious-Administrative) of September 29
Unauthorized discharge of vinasse	43.253,95 €	Magistrates' Courts (Division of Contentious-Administrative) of September 25, 2006
Unauthorized discharge of vinasse	30.000,00 €	High Court of Justice of Extremadura No. 393/2008 (Division Contentious-Administrative) of May 15
Unauthorized discharge of vinasse	36.060,73 € + 18 months imprisonment	Magistrates Courts of Barcelona of November 15, 2000
Unauthorized discharge of vinasse	3.005,06 €	High Court of Justice of Castilla y León No. 476/2004 (Division Contentious-Administrative) of November 26

by-products into profitable products generates economic and environmental benefits, taking advantage of the full potential of these materials and not just as animal feed or final disposal to landfills. Among the most significant areas that would take benefits from this integral exploitation of residues we can lead towards different types of industries within the food (human and animal), pharmaceutical and chemical industries by obtaining compounds of interest from wastes, such as phenols, vitamins, colorants, aromatic compounds, etc. Some of these are widely known, but some others are waiting for their identification and exploitation by the industry to develop new and competitive products to be introduced into the market.

2. Generation of residues in the wine industry

Wine production is one of the most important agricultural activities over the world. About 50.5 millions hL of wine were produced in Spain in 2013, thus being the largest wine producer according to the OIV, followed by France and Italy. Also, Spain is the country with the largest area in the world dedicated to vineyards, with approximately 1.2 million hectares, comprising about 15% of the total agricultural production in the country. However, the wine industry is not only characteristic of Mediterranean countries as Spain or France, but also a competitive industry in countries such as Italy, Germany, The United States, Australia, Chile, Argentina and South Africa *(2)*. For example, in Brazil, a country with a long tradition of winemaking, the quantity of grapes processed in 1 year is in the order of tons (1752.5 tons of Chardonnay grapes were processed in 2007) *(3)*.

Vine can be considered as a mixture of vivacious plant and bearing tree that completes an annual cycle of fruits production. In fact, the vinification process is a seasonal activity mainly performed during autumn and involves all the steps carried out during the elaboration of wine from grapes *(4)*. The winemaking process produces different residues (**Figure 1**) characterized by a

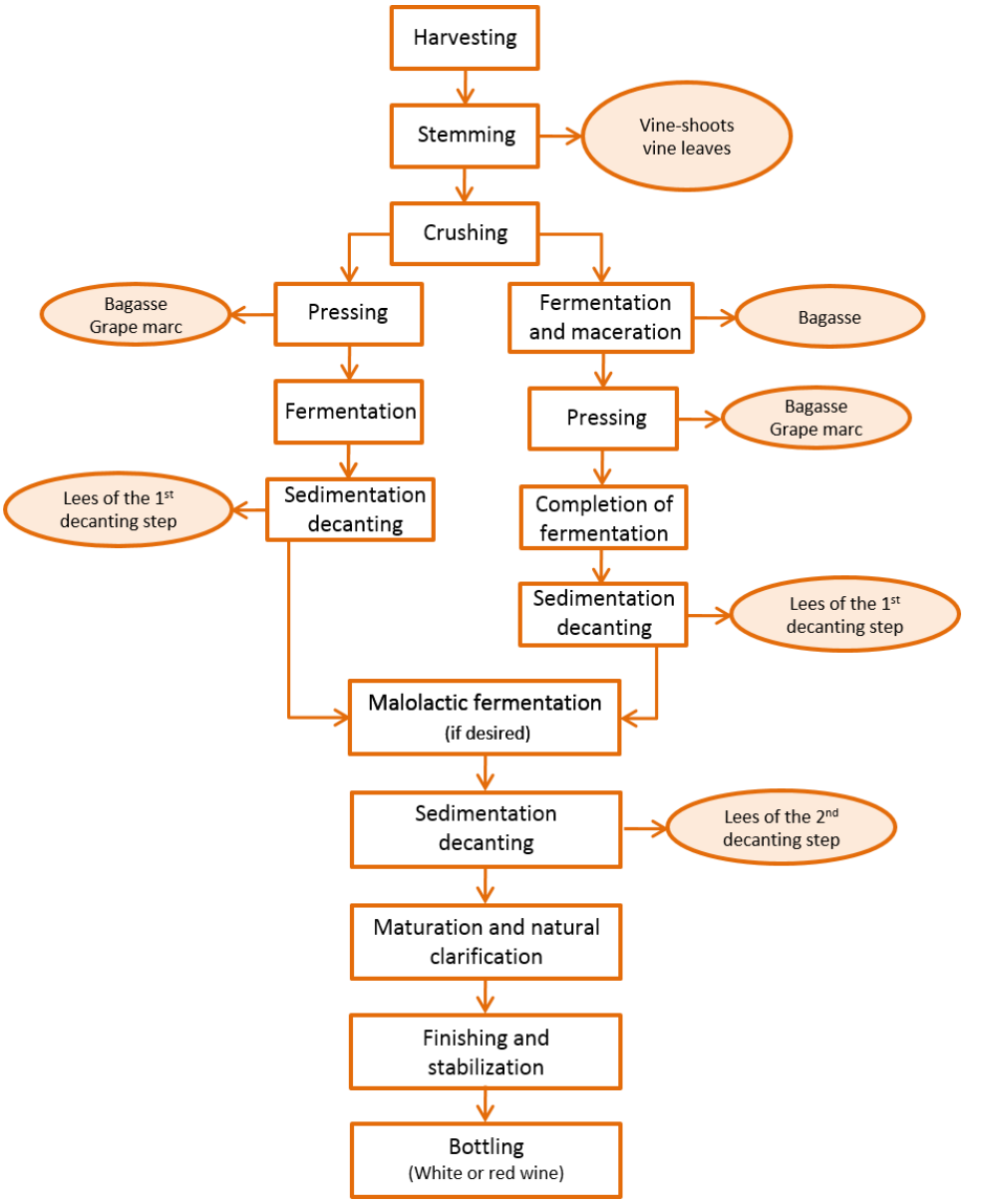

Figure 1. Diagram of the vinification process for white and red wine.

high content of biodegradable compounds and suspended solids *(5)*. In general, the agrofood residues from plant cultivation and vinification consist of plants remains such as vine-shoots and vine leaves, and sediments formed during clarification, bagasse from pressing, which is formed by grape skins, grape seeds and grape stems, and wine lees, which result from different decanting steps, depending on the winemaker procedures. The wastewater generated from vinification are rich in sugars and ethanol, organic acids, alcohol, esters and phenols. In fact, wine lees and wastewater may exert phytotoxic effects on the environment *(6–8)*. The physico-chemical characteristics of the wastewater from this industry depend critically of the time of the year when they are generated and the grape used for vinification. In general, these residues are highly biodegradable, have a high organic content (organic acids, sugars, alcohol, pectins, proteins, etc.), and a slightly acid pH. The volume of waste generated is around 20% of total wine production *(9)*.

3. Classification of residues

Residues from the winemaking industry can be classified attending to their source; hence, wastes can be differentiated between by-products derived from the vineyard and those from the winemaking process.

3.1 By-products from the vineyard

There are two by-products from vineyard cultivation: vine leaves and vine-shoots, both equally underexploited.

- ✓ Vine leaves: this material has experienced an unequal exploitation depending on their state. Currently, some high end cosmetics include in their composition extracts made with vine leaf sprouts, with the consequent damage to the plant. Green leaves are used, both fresh and salted, as a culinary item in some

countries such as Greece and Turkey *(10)*. Furthermore, fresh leaves have been used as a treatment for hypertension, diarrhea, bleeding, varicose veins, inflammation and diabetes, and it seems that they exert a protective effect on the liver *(11)*. Moreover, red vine leaf extracts are used in the pharmaceutical industry to obtain effective drugs against circulatory disorders *(12–14)* and could be used in the manufacture and synthesis of chemical intermediates with pharmaceutical interest *(15)*.

Aditionally, there is an aspect of vine leaves that has not been exploited so far, as are senescent leaves. It is well known that, in this state, vine leaves have a color ranging from yellow to red and brown, depending on the cultivar; which reveals the presence of flavonols, anthocyanins/anthocyanidins and carotenoids, with very different characteristics. Thus, vine senescent leaves should be considered as a potential source of natural colorants.

- ✓ Vine-shoots: the huge amount of vine-shoots produced every year has led to a growing interest on exploitation of this residue by turning it into a valuable product. In fact, the trimmings from vine-shoots from the wine industry are usually burnt in the field, thus releasing cancerous compounds such as polycyclic aromatic hydrocarbons and greenhouse gases. The potential, more lucrative use of this material is represented by two applications: recovery of natural constituents, and bioconversion into useful products. In order to decrease the environmental impact produced by this residue, some authors have proposed its use as a source of sugars, which could then be converted into a variety of products *(16–29)*.

  Most research on vine-shoots has been focused on the production of paper pulp *(30)* and ethanol, the former requiring in-depth research to improve production as vine-shoots provide

pulp of lower quality than other agricultural residues such as wheat straw. Moreover, collection and transportation costs are high, and their seasonal production spans a rather short period. Still, vine-shoots may be an effective alternative raw material for paper in places with abundant vineyards, as is the case with the Castilla–La Mancha region in Spain, where about 600000 Ha are used to grow vineyards. Assuming an average density of 1 700 shots/Ha and an average wood trimming weight of 1 kg/shoot, roughly 1 million tones of shoots are produced each year *(31)*.

The composition of vine-shoots is characterized by three main fractions; cellulose, hemicellulose and lignin, where the content of holocellulose (mixture of cellulose and hemicellulose) is around 68%. This percentage is lower than in other non-wood raw materials (*e.g*. wheat straw, sunflower stalks, cotton stalks), but similar to that in pine and higher to that in olive trimmings. On the other hand, the content of lignin in vine-shoots is around 20% (dry weight), similar to that in eucalyptus and non-wood raw materials *(31)*. Lignin, a well-known component of secondary cell walls, is a high-molecular-mass randomly crosslinked polymer due to the lack of enzymatic control, that is built up by oxidative coupling of three major C6-C3 (phenylpropanoid) units (monolignols), namely, *trans-p*-coumaryl (4-hydrocycinnamyl), coniferyl (4-hydroxy-3-methoxy-cinnamyl, forming guayacyl units), and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl, forming syringyl units), which form a randomized structure in a three-dimensional network inside the cell wall. The content and structure of lignin differs among species, among mutants of species, among internodes of a culm, and among isolation methods. For example, wood contains 10–30% lignin, meanwhile wheat straw comprises 14–17% lignin, depending on its morphological origin *(32)*.

As lignin can be hydrolyzed to release aromatic phenolic compounds such as low-molecular mass alcohols, aldehydes, ketones or acids, vine-shoots are suitable to be used as a phenols source. In addition, they could play a key role in the oenological field to improve wine quality, since they could determine to a large extent their color, flavor and aroma, acting similarly to wine aging either in contact with oak chips or in oak barrels *(33)*. In fact, the most studied low-molecular mass phenols in aged spirits are lignin-derived phenols such as vanillin and syringaldehyde, present in smoke and smoke flavorings. Lignin oligomers or intermediate- and high-molecular mass polyphenols from lignin are considered the main phenols in old spirits and wine *(32)*.

It is well known that the excellent antioxidant properties of phenolic compounds have promoted an active research on raw materials for their extraction. Their abundance, and their special richness in phenolic compounds, make exploitation of vineyard waste highly interesting in economic terms as this can be used as a raw material to obtain products of a high-added value for application in the nutraceutical, cosmetics, pharmacological, oenological, and food additive industries; thus, an increased number of dietary supplements for disease prevention obtained from grape skins has been placed in the market in recent years *(33,34)*. These achievements, added to the alternative proposals to diminish the environmental impact, would allow the placement of vine market in a competitive position and these wastes should be considered as by-products.

The health benefits of phenolic compounds are ascribed to their antioxidant and free radical scavenging properties *(33,35,36)*. Phenols can be obtained from vine-shoots and leaves in two compatible ways, namely: (a) by extracting the phenols that are not constituents of lignin, and (b) by degrading lignin to obtain

low-molecular weight phenols. At present, the former choice is being developed by some industries, where extracts rich in resveratrol and viniferins (vineatrol) from young vine-shoots and leaves are obtained. These phenols have so far not received enough attention despite their potential health benefits as natural antioxidants and their ability to act as efficient free-radical scavengers. Phenol extracts have proved to be effective in reducing proliferation of leukemic cells *(37)*, and potentially useful against epilepsy *(38)*. In addition, their high antioxidant and free radical scavenging power makes them potentially useful in the cosmetic and food-additives industries, as previously commented, but also in tanning and dyeing for leather *(39)*, and in the production of smoke flavorings *(40)*. Another advantage of phenols, according to recent studies, is that some foods containing phenol compounds are capable of preventing aging and diseases such as atherosclerosis, diabetes and inflammatory processes *(41)* as a result of the antioxidant properties of phenols in general and flavonoids in particular.

Besides, some other ways of vine-shoots exploitation such as production/extraction of phenols *(32,42)*, volatile compounds *(32)*, activated carbon for wine treatment *(29,43),* lactic acid *(19-22,44,45),* biosurfactants *(19-22,44)*, xylitol *(24,26)*, food additives *(26)*, ferulic and coumaric acids *(28,32,46)*, production of leather dyes *(47)* and production of smoke flavorings *(33,48,49)* have been investigated.

3.2 By-products derived from the winemaking process

Grape production worldwide is about 70 million tones, of which 80% is dedicated to winemaking. It is considered that Spain contributes with 18–20% to world production.

However, there is a poor exploitation of by-products from the vinification process, that in no case give rise to products with a high-added value, despite the variety and the characteristics of some of their components. A distinction in this case must be made between grape skins, grape seeds and wine lees.

- ✓ Grape skins: this residue has different characteristics, being its composition highly dependent on the vine cultivar, and especially on the grape color, being the most appreciated the red wine varieties. Grape skins have a significant content in monomeric and polymeric molecules, such as anthocyanins, flavan-3-ols, flavonols, dihydroflavonols, hydroxyl cinnamoyltartaric acids, hydroxybenzoic acids and hydroxystilbenes *(50)*. This varied composition makes grape skins a potential source for isolation of natural compounds *(51)*. Generally, red grapes posses higher crude protein, fat and ash content than white grapes *(52,53)*. They also have a higher content of total extractable pectins, neutral sugars, condensed tannins, dietary fiber and resistant proteins. On the other hand, skins from white grape varieties are characterized by a significantly higher content in soluble sugars, uronic acid and Klason lignin than red skins *(54)*.

  The most important fraction from a nutraceutical point of view is that formed by phenolic compounds, which, in general terms, are endowed with a high antioxidant capacity and are found at higher concentrations in skin and seeds than in the pulp of grapes *(55,56)*. Also, as white skin grapes do not synthesize anthocyanins, total phenols in these grapes is also lower than in the red varieties *(52)*.

  Currently, the grape pomace, which is constituted by grape skins and grape seeds, together with low-quality wines and vinification lees, are destined to distilleries to produce alcohol

after the winemaking process. The grape marc, or grape pomace, is subjected to a cleaning process with water, where a clean pomace and a pomace extract, called pickax, are produced *(57)*. Following extraction, the clean pomace, completely depleted in sugar, is subjected to fermentation as a preparation step for alcohol distillation and isolation. The resulting residue is discarded, tough a small part is valorized as a source of tartatic salts, very abundant in grape skins *(51),* and also of phenolic compounds *(58)*.

Up to date, most research made on these materials has used the skins directly from fresh red grapes *(59),* which involves a high cost of the raw material; however, some studies made with skins from the red wine vinification wastes have demonstrated their high content in a number of dyes that makes them a profitable source to obtain natural colorants *(58)*. Also, extractable compounds from this raw material are a variety of aromas and uncolored phenolic compounds that posses a high antioxidant capacity and are found at higher concentrations in the skins and seeds than in the pulp of grapes *(58–60)*.

- ✓ Grape seeds: this material is the most studied and exploited residue from the winemaking industry, due to the wide variety of compounds it contains. It is estimated that around 38–52% of the grape volume is constituted by grape seeds, which give an overall idea of the high amount of this residue annually generated. Grape seeds have a high content in phenols, mainly flavonols, proanthocyanidins and condensed tannins; content that has promoted numerous studies on grape seeds *(61)*. In fact, proanthocyanidins have proved to provide astringency and flavor to wines, and have also been recognized by their beneficial health effects (such as anti-inflammatory, cardioprotective, radioprotective, anti-hyperglycemic, anti-tumor and antigenotoxic effects),

as well as for prevention and healing of cataracts *(62)*. This is the reason why these compounds are commercialized as nutritional supplements in some countries such as US, Japan, Australia and Korea. In some other countries as Brazil, these compounds are currently used for treatment of dislipidemy *(63)*, and also as antioxidants for prevention of cardiovascular diseases. Recently, Peng *et al*. used phenolic extracts from grape seeds to improve the antioxidant quality of bread *(63)*, and Ferreira *et al*. demonstrated the efficiency of these compounds as free radical scavengers *(62)*. These findings have promoted current research about grape seeds focused on the potential of these raw materials to obtain antioxidant compounds *(64)*.

In addition, the lipid fraction of grape seeds has a high commercial value, although it has been only partially exploited. Presently, grape seed oil is gaining popularity as a culinary oil, since it is made 90% of poly- and mono-unsaturated fatty acids, particularly linoleic acid (58–78%) *(65),* followed by oleic acid (3–15%). Also, this oil contains low amounts of saturated fatty acids (10%), which confer to grape seed oil a high nutritive value as edible oil. It also exhibits properties for prevention of thrombosis, dilation of blood vessels and regulation of autonomic nerves *(62),* and it has been associated to inhibition of cardiovascular diseases by down-regulation of low-density lipoprotein cholesterol *(66)*. Besides, grape seed oil contains tocopherols, which are of high interest because of their antioxidant properties *(67)*.

However, with the exception of grape seed flour, the residue from grape seed oil has not received much attention yet, but this residue may be a potential source for extraction of natural antioxidants and other bioactive compounds *(68)*. Indeed, among the variety of compounds detected in grape seeds, it is

worth emphasizing the family of phenolic compounds found in concentrations ranging from 5 to 8%, depending on the cultivar *(69)*. These phenolic compounds are mainly simple phenols, such as gallic acid, flavonoids including monomeric flavan-3-ols, catechin, epicatechin, gallocatechin, epigalloca-techin and epicatechin 3-O-gallate, as well as procyanidin di-mers and trimers *(70,71)*. Grape seed extracts have demonstrated to have antioxidant properties, promoting youthful skin with higher elasticity and flexibility due to their oxidation inhibition effect by bonding collagen *(70)*.

However, despite the healthy properties of the compounds found in grape seeds, the residues from wine production are still mainly destined to the alcohol industry and thereafter discarded after exhaustion of grape seeds through the alcohol isolation process.

- ✓ Wine lees: this residue, also known as dregs, is defined by the EEC regulation no. 337/79 either as the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following filtration or centrifugation of this product. Wine lees are produced in different decanting steps during the vinification process, and are constituted by liquid and solid lees. Eventually, this semisolid residue is centrifuged and dehydrated prior to storage, then being known as wine lees cake, whereas the liquid phase usually is discarded to waste. Depending on the manufacturer, the wine lees cake is processed for alcohol and tartaric salts isolation or treated as an organic residue. Traditionally, aging over lees has been a process almost exclusively applied to some white and sparkling wines; however, ageing over lees is at present a common practice in red wine production, since it endows wine with interesting organoleptic

properties, such as reduction of astringency and an increase in color stability. This practice has also a positive influence on the mouthfeel and body of wines, as well as on their aroma *(72,73)*.

Wine lees are so far the less studied by-product from the winery industry, where most attention has been set on the study of wine itself, and also on that of grape skins and seeds. Lees are mainly composed by microorganisms, particularly yeasts produced during alcoholic fermentation and, in a less proportion, by phenolic compounds, bacteria, the remnants from plant cells, inorganic matter and tartaric salts, which are deposited during alcoholic fermentation *(74,75)*. Nevertheless, the composition of wine lees is subjected to a strong variability and depends on the winery procedure. Once the alcoholic fermentation has ended, the autolysis of wine yeast starts. The latter step is characterized by the degradation of cell walls and releasing of some enzymes and mannoproteins, amino acids and precursors of volatile compounds into wine as well as some phenolic compounds and sulphur compounds adsorbed on wine lees *(76,77)*. According to Chassagne *et al.* this adsorption phenomenon is directly linked to the degree of autolysis, ethanol concentration, pH of wine and temperature *(78)*. Differences in the concentration of volatile compounds in cells and musts were found by Zea *et al.* when they used different yeast strains *(79–81)*.

Up to date, wine lees have been mainly exploited as a source of tartaric acid and calcium tartrate *(74)*. Attempts to use them for feeding kettle were discarded in view of the extremely poor nutritional value of this residue *(82,83),* probably owing to either the high amounts of phenolic compounds associated with proteins, which render the proteins non assimilable, or to the presence of toxic elements, which accumulate in yeast lipids. The use of wine lees as a biosorbent for removal of undesirable

compounds from wine has also been proposed *(84,85),* after the report on their ability to retain part of the volatile compounds that oak wood transfers to wine. In this way, wine is prevented from having an excessive, unpleasant woody flavor, as well as from the impact of undesirable compounds such as toxins, pesticides, antifoaming and volatile compounds as 4-ethylphenol and 4-ethylguaiacol, which have been described as responsible for the associated horsy, medicinal and spicy aroma of wines *(84–86)*.

The scarce literature on wine lees composition has reported the phenolic content and antioxidant activity of this residue *(87,88)*. Thus, Pérez and Luque de Castro *(87)* and other researchers *(89,90)* have extracted phenolic compounds from wine lees and determined the antioxidant activity of the extracts. From the compositional point of view, some published research has been focused on the pigment content of red wine lees, since their intense red coloration suggests a high anthocyanin and anthocyanidin content *(87,88)*. Apart from this, most published articles on wine lees are focused on their positive effect on the overall bouquet of wine aged over lees *(91,92)*.

Liquid lees or imbibed wine, (the wine obtained from centrifugation of wine lees) contain a high quantity of tartaric acid, which can be extracted and commercialized *(90,93,94)*.

Moreover, a recent business has emerged based on exploitation of winemaking residues, since food lipid antioxidants or dietary supplements are being isolated from grape seed and grape skin extracts *(95–99)*. Extracts from wine production are also used in cosmetic and pharmaceutical products as active ingredients as is the case with hair and skin protection products *(100,101)*. The range of interesting compounds obtained from the residues of

winemaking has a positive economic impact, since they contribute to valorization of these residues. However, a long road is still ahead, as the volume of residues generated by the wine industry and not reused is particularly high. In this sense, wine lees are one of the most unexploited residues of the oenological industry.

4. Analytical tools in the characterization of wine residues

The detection, identification and quantification of large numbers of compounds that are present in wine residues at different concentrations require optimization of the operating conditions. Different analytical tools can be used in each step of the general scheme of an analytical process (**Figure 2**) that starts with sampling and goes through sample preparation, detection and data treatment. As data treatment is to be described in the next point, this section will be mainly focused on the analytical tools employed in the sample preparation and detection steps during the experimental development of this Doctoral Thesis.

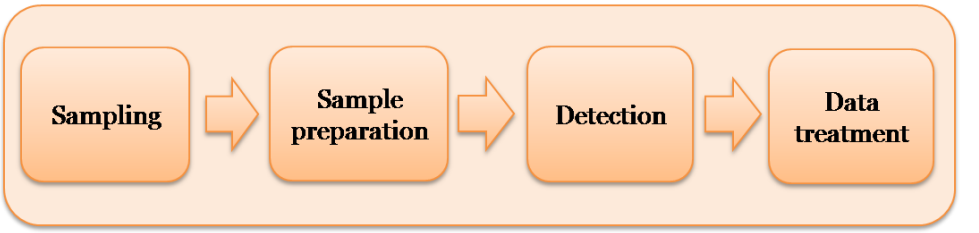

Figure 2. General workflow of the analytical process.

4.1. Analytical tools for sample preparation

As sampling involves the selection of the most suitable material for the aim of the analysis, it should seek to obtain representative analytical samples and ensure their preservation. Sampling is usually a key step, since significant

variations in chemical and physical properties or in the concentration of interesting compounds must be avoided. Although direct analysis is an ideal option pursued for characterization of liquid and solid samples, it is an infrequent option mostly linked to fingerprinting analysis, so most analytical methods involve a sample preparation protocol.

Sample preparation comprises extraction of compounds into a suitable solvent, preconcentration, clean up and/or derivatization, if needed. In all cases, these steps must be compatible with the detection step and their complexity varies with the nature of the sample and the used technique.

Extraction is a critical step of the analytical process because it directly affects to the quality and reliability of the final results. The selectivity will depend on the aim of the study; thus, while targeted analysis requires complete and highly selective extraction, profiling analysis does not need strict fulfillment of these aspects.

The extraction protocol is mainly conditioned by the target sample, which, in the case of solids, is performed by solid–liquid extraction (more properly named as leaching or lixiviation). Extraction is usually based on traditional techniques such as maceration, Soxhlet or Folch extraction, which are typically ascribed to time-consuming protocols and often lead to non-reproducible results and both low selectivity and extraction yields. These extraction techniques are frequently linked to a high consumption of organic volatile solvents. On the other hand, modern extraction techniques (energy-assisted extraction techniques) have numerous advantages over traditional extraction, such as higher efficiency, less solvent consumption, easy automation, more economical and with lower impact on the environment and human health. The most important modern energy-assisted extraction techniques are microwave- and ultrasound-assisted extraction (MAE and USAE, respectively), superheated liquid extraction (SHLE), and supercritical fluid extraction (SFE).

- Microwave assisted extraction: microwave energy is a non-ionizing radiation that causes molecular motion by migration of ions and

rotation of dipoles without changing the molecular structures if the temperature is not high enough. Therefore, non-polar solvents such as hexane or toluene are transparent to microwave energy, so they require addition of polar co-solvents. MAE is an efficient extraction technique for solid samples, only applicable to thermally stable compounds. It becomes a viable alternative to conventional methodologies due to many substantial advantages over other sample preparation techniques such as shorter extraction times and lower volumes of extractants *(102–104)*.

- Supercritical fluid extraction: SFE has several advantages over conventional and modern extraction techniques, particularly when CO_2 (the most common supercritical extractant) is used. One is that CO_2 is a non toxic fluid, non flammable and non corrosive. Also, the extract after depressurization is obtained as a powder without the need for drying *(105)*. In this sense this is the only technique suitable to obtain this kind of product, which is an added plus to take into account, providing the used CO_2 is endowed with enough purity. However, SFE is limited mainly to non polar and medium polar substances *(106),* and, at the industrial scale, the relatively high purchase and maintenance costs of the required equipment can easily surpass benefits.
- Ultrasound assisted extraction: USAE shares some of the advantages of MAE in terms of enhancing leaching kinetics, reducing extractant volume and facilitating automation. USAE is particularly useful for isolation of thermolabile compounds or for sample matrices where disruption favors considerably the contact between solid and liquid phases and mass transfer as a result. USAE is faster and more efficient than conventional extraction and provides high efficiencies with modest consumption of extractant, which does not require to be polar as in the case of MAE. The main shortcoming of USAE is the potential formation of free radicals during sonolysis of the solvent, which can produce degradation of some labile compounds by oxidation *(107)*.

- Superheated liquid extraction: SHLE is one of the most important contributions to the number of modern extraction techniques, which significantly streamlines sample preparation. Under high temperature and pressure enough to be kept as a liquid an extractant increases dramatically the kinetics of the extraction process: the extraction time and solvent consumption are significantly decreased as a result. Organic solvents, or mixtures of water and organic solvents, have traditionally been used in SHLE, but the present trend focuses on the use of water following the guideline of green chemistry. This is reflected in the number of studies based on the use of SHLE that, from an environmental green perspective, have been developed in the last years. The commonly used term "pressurized hot water" or "subcritical water" generally refers to liquid water that is below its critical point of T_{cr}=647.096 K (374 ºC) and P_{cr}=22.064 MPa, which also encompasses room temperature water. At room temperature and pressure, water is a highly polar solvent with a high dielectric constant (εr=80.1) characterized by the presence of extensive intermolecular hydrogen bonding. However, when the temperature is raised, this parameter decreases rapidly, as do its viscosity and surface tension. The associated increase in diffusivity and density also makes it more useful for extraction. From a practical point of view, water can be easily maintained in the liquid phase at temperatures of up to 250 ºC by keeping its pressure above 10 bar. Under these conditions, its dielectric constant decreases to 27, making its polarity between those of methanol and ethanol at 25 ºC. Water under these conditions exhibits a behavior similar to some organic solvents that are widely used to dissolve a broad range of medium and low polar compounds, and can thus serve as an alternative to traditional organic solvents.

On the other hand, liquid samples are mainly extracted by liquid–liquid extraction (LLE), solid-phase extraction (SPE), solid-phase microextraction

(SPME) or dispersive microextraction. On-line SPE was used in this Doctoral Thesis due to the high reliability of automated strategies for sample preparation, which allows cleaning and preconcentration of the target analytes without operator intervention. In addition, the platform used (Prospekt, Spark Holland), in contrast to other semiautomatic or automatic platforms, performs solid phase extraction at high pressures, which facilitates hyphenation with the chromatographic step. Among the many advantages of this system, it can be highlighted the reduction of the extraction time with respect to the manual option, since the number of steps is reduced, as shown in **Figure 3**, and no losses of analytes or alteration by contact with atmosphere are produced with on-line SPE, since it is a closed system. Finally, as analytes elution is performed passing the chromatographic mobile phase through the SPE cartridge, the totality of compounds retained on the sorbent are eluted and pass to the chromatographic column for their individual separation and quantitation. LLE was also used for sample preparation prior to GC–IT/MS analysis as an easy sample preparation technique without the need of further derivatization due to the nature of the samples used.

The necessity for additional sample preparation steps is marked by the analytical platform that follows the extraction step. As an example, the low volatility of many metabolites makes mandatory derivatization prior to GC–IT/MS analyses, which can be easily performed by silylation *(108)*.

4.2. Analytical tools to implement the detection step

The selection of the appropriate detection technique depends on the final purpose of the study. In targeted analysis, and after selective sample treatments, optical detection techniques such as UV/visible molecular absorption —using conventional or diode array detectors—, fluorescence molecular emission, or electrochemical techniques such as voltammetry, potentiometry or conductimetry can be used. Less economically accessible techniques are mass spectrometry (MS) and nuclear magnetic resonance (NMR). MS is endowed with

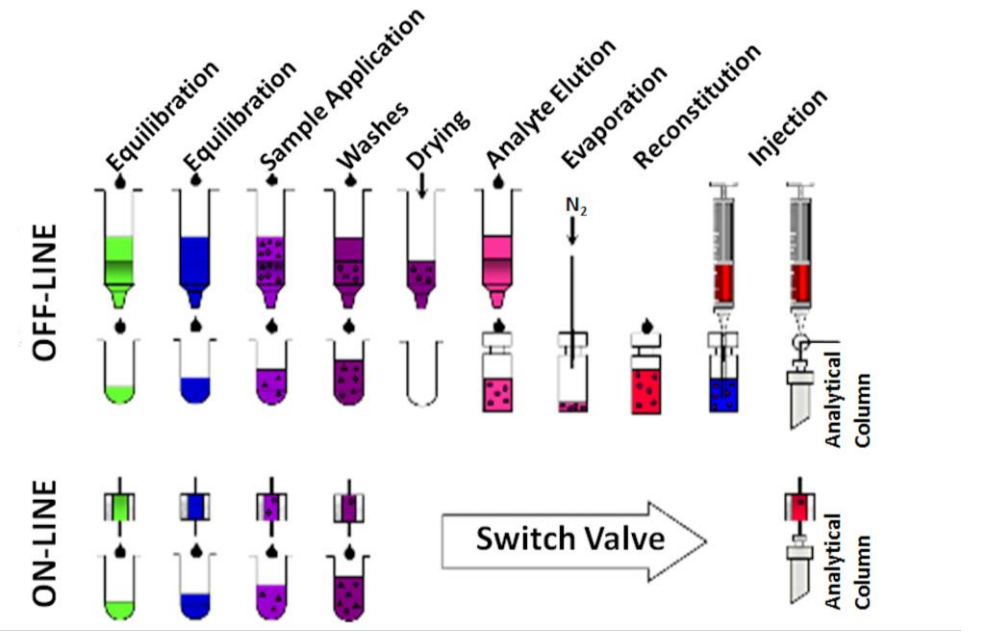

Figure 3. On-line solid phase extraction *vs* the manual option (off-line).

high sensitivity and selectivity, which makes of this technique a suited option in qualitative and quantitative analysis. NMR is characterized by a high identification power when simple samples are analyzed and also by its capability to obtain fingerprints representative of samples composition. Finally, it is worth mentioning infrared spectroscopy (IRS) that is clearly more economical than MS and NMR but it is featured with a low identification power. It is especially suited to obtain fingerprints but the information that can be deduced from analysis is restricted as compared to NMR.

MS is the most recommended technique for untargeted analysis thanks to its sensitivity and resolution. It usually requires to be hyphenated to a high resolution separation technique such as GC or LC for proper separation of the target components *(109,110)*. While GC–MS is particularly appropriate for analysis of volatile (or easily converted into volatile) organic compounds, LC–MS is highly applicable to the analysis of a wider range of polar and mid-polar compounds.

Benefits and downsides of MS are depicted in **Table 2**. As can be seen in the table, one of the strongest points of MS is sensitivity. In fact, MS allows performing quantitative analyses with high selectivity and sensitivity and the potential to identify compounds with high mass accuracy. It should be taken into account that sensitivity in MS is affected by both ionization and type of detector. Quantitation requires suitable internal standards with similar ionization and fragmentation efficiencies. Concretely, quantitative information on a metabolite can be obtained by the peak it provides in one of the three following ways: (i) by integrating it against a reference sample of the same compound (this requires the identity of the compound to be known and compared between separate runs, which can introduce error); (ii) by comparing the relative ratios of a set of peaks across a series of spectra; and, (iii) by addition to the sample of a stable-isotope of the metabolite of interest. Identification of compounds is often expedited by comparison to internal standards and searching against mass spectral libraries. Fragmentation of compounds under certain applied voltages is very reproducible, thus MS/MS measurements are highly selective.

Although MS measurements are fast, the overall run time depends on the previous chromatographic step, which may vary from minutes to hours. However, combination with chromatographic or electrophoretic equipment reduces the complexity of mass spectra due to compound separation in a time dimension, besides delivering additional information on the physico-chemical properties of metabolites. Among their weak points, MS-based techniques usually require sample preparation, which can cause losses of the target analytes. Automated sample preparation on line with either the chromatograph or the mass detector allows full automation of analyses.

MS is currently the most reliable platform for identification as there are a wide variety of libraries with MS and MS/MS information. Nevertheless, global profiling may be limited by the ionization type, due to the fact that specific compound classes may be discriminated by the ionization efficiency.

Table 2. Strengths and weaknesses of MS analysis.

Mass Spectrometry	
STRENGTHS	*WEAKNESSES*
✓ Excellent resolution (improved by separation).	
✓ Accurate *m/z* measurements ideal for identification of metabolites. ✓ Structural information throughout fragmentation patterns (MS^n).	
✓ Highly sensitive in its different modes. ✓ Very selective (especially in MS^n).	× Detection depends on ionization efficiency.
	× Destructive, particularly complicated for analysis of valuable samples.
✓ Low sample volume (0.5 to 500 µL).	× Limited to liquid samples or extracts from solid samples.
✓ Suitable for quantitative targeted analysis, global profiling and fingerprinting.	× Quantification requires chemically-related internal standards
✓ High throughput.	× More efficient when coupled to separation techniques.

Mass spectrometry is based on the differential displacement of ionized molecules through vacuum by applying an electrical field. Simplistically, a mass spectrometer consists of an ion source, a mass analyzer, a detector and a data collection system. Molecules from the sample are inserted to the ion source, where they are ionized. The ions, which are in the gas phase, are separated according to their mass-*to*-charge (*m/z*) ratio in the mass analyzer and finally detected.

There are different types of ionization sources to generate the gas-phase ions, electron impact ionization and chemical ionization being the most commonly used in GC–MS, and electrospray ionization and atmospheric pressure chemical ionization the most frequently employed in LC–MS. For the purpose of the research in this PhD book, electrospray ionization analyzers have been used coupled to LC, and electron impact ionization coupled to GC. Electrospray ionization (ESI) is particularly useful following LC separation as ionization occurs at atmospheric pressure. Polar and ionic compounds are the

best ionized by this type of source. In ESI, the chromatographic eluate is sprayed from a metal or fused silica capillary. An electrospray is achieved by raising the potential on the spray capillary to 4 kV in positive or negative ionization modes. The resulting spray of charged droplets is directed toward a counter-electrode at a lower electrical potential, where the droplets lose solvent leading to ionic species into the gas phase. The counter-electrode contains an orifice through which ions are transmitted into the vacuum chamber of the mass spectrometer, traversing differentially pumped regions via skimmer lenses *(111)*. Electron impact ionization (EI) is produced by interaction of energetic electrons and in gas phase molecules to induce their ionization and fragmentation, yielding radical cations. Once the sample has been ionized, it is transported to the mass analyzer via an electric or magnetic field. The most relevant mass detectors used to develop the research that constitutes this Thesis are here briefly described:

i) Triple quadrupole mass spectrometer (QqQ)

The triple quadrupole MS consists of an ion source followed by ion optics that transfer the ions to the first quadrupole, as shown in **Figure 4** *(112)*. This unit is formed by four parallel rods to which specific direct current and radio frequency voltages are applied. The rods filter out all ions except those of one or more particular *m/z* values as determined by the voltages applied. The applied voltage is variable, so that ions with other *m/z* values are allowed to pass through. Afterwards, selected ions reach a collision cell where they are fragmented. The collision cell is typically called the second quadrupole, but it is generally a hexapole, filled with an inert gas such as nitrogen or argon. The fragment ions formed in the collision cell are then sent to the third quadrupole for a second filtering stage to enable to isolate and examine multiple precursors to product ion transitions. This is called *selected reaction monitoring (SRM)* mode. Since the fragment ions are pieces of the precursor, they represent portions of the overall structure of the precursor molecule.

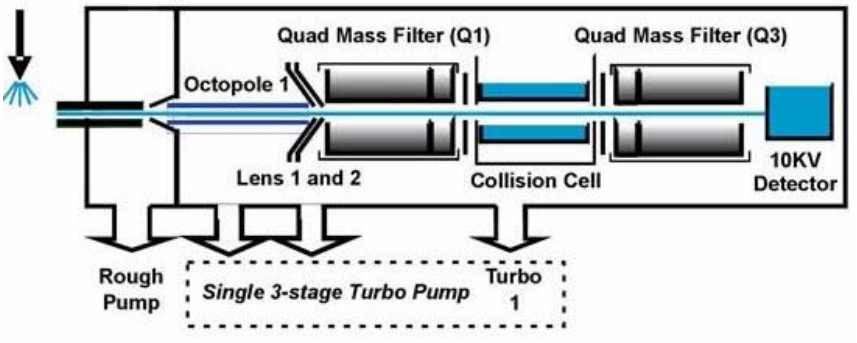

Figure 4. General scheme of a triple quadrupole mass spectrometer.

Due to the low-mass accuracy achieved with respect to other mass analyzers, triple quadrupole spectrometers are preferably used for targeted analyses, as they allow quantitation with high sensitivity and selectivity in SRM mode, presently the most sensitive operational mode for the triple quadrupole MS instrument.

ii) Quadrupole-time of flight (QTOF) mass spectrometer

The QTOF mass analyzer is based on the same configuration as the QqQ in which the last quadrupole is replaced by an acceleration tube as mass analyzer, (usually in orthogonal configuration) to filter out ions according to the equation of kinetic energy *(113)*. The QTOF can operate either in MS mode with the TOF as scanning tool by taking benefit from the high mass accuracy or in MS/MS mode for structural elucidation.

This hybrid mass analyzer offers better selectivity than a triple quadrupole, meanwhile sensitivity is considerably lower. On the other hand, thanks to its great mass accuracy (below 2 ppm) highly reliable identification can be achieved, thus allowing its use for global profiling.

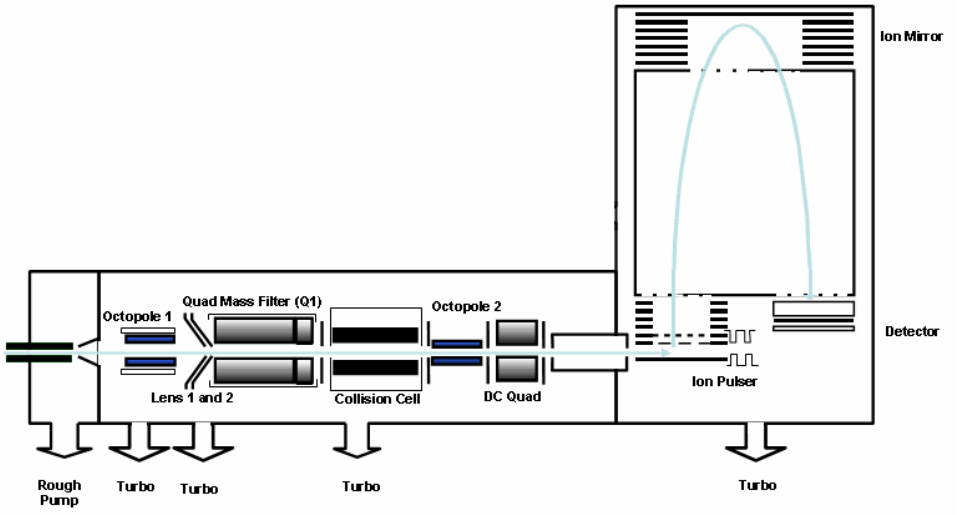

Figure 5. General scheme of a quadrupole time-of-flight mass spectrometer.

iii) Ion trap (IT)

The ion trap is the three dimensional analogue of the linear quadrupole mass filter. In this device, ions are subjected to electric and magnetic forces, not only in two dimensions as in the simple quadrupole, but also in the third dimension, thus trapping ions. This feature gives the ion trap the possibility of tandem mass spectrometry experiments and even of performing multiple stage mass spectrometry (MS^n).

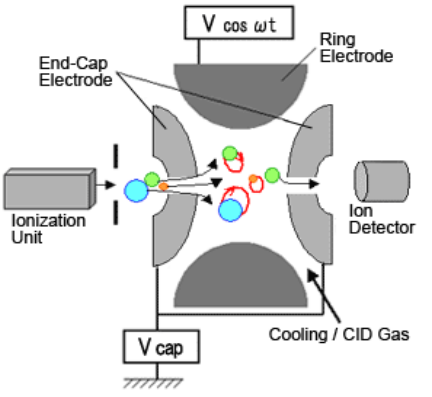

Figure 6. General scheme of an ion trap mass spectrometer.

Figure 6 shows the diagram of the ion trap device *(114),* composed by a ring electrode and two end-cap electrodes. As can be seen in the diagram, the ions trapped describe orbits inside the trap, and the voltage applied in the end-caps (V cap) enables fragmentation of the ions by collisions with the helium damping gas rather than ejection. So, this voltage is used to induce resonance excitation and resonance ejection. The potential applied to the ring electrode determines the range of *m/z* values that can be trapped and the modification of this voltage produces ejection of the ions for their detection.

5. Data analysis

As MS analyses typically generate large amounts of data that complicate interpretation of the results, statistical considerations play a vital role in the whole process of an experiment. These considerations directly affect the data output quality, analysis and subsequent interpretation. The statistical strategies currently used in data treatment are selected according to the experiment design, being slightly different for targeted and untargeted analysis (**Figure 7**).

5.1. Statistical analysis for targeted analysis

Determination of target compounds often requires the use of chemometric methods to develop the optimized analytical methodology. Usually, the best conditions for the determination of the target compounds are explored through statistical procedures such as response surfaces or screening designs *(115–117)*. The rest of statistical procedures used in the development of a new methodology for targeted analysis are mainly based on simple statistics, such as simple regression, used to obtain calibration curves for quantification of compounds, or simple calculations to characterize the analytical method (reproducibility and repeatability).

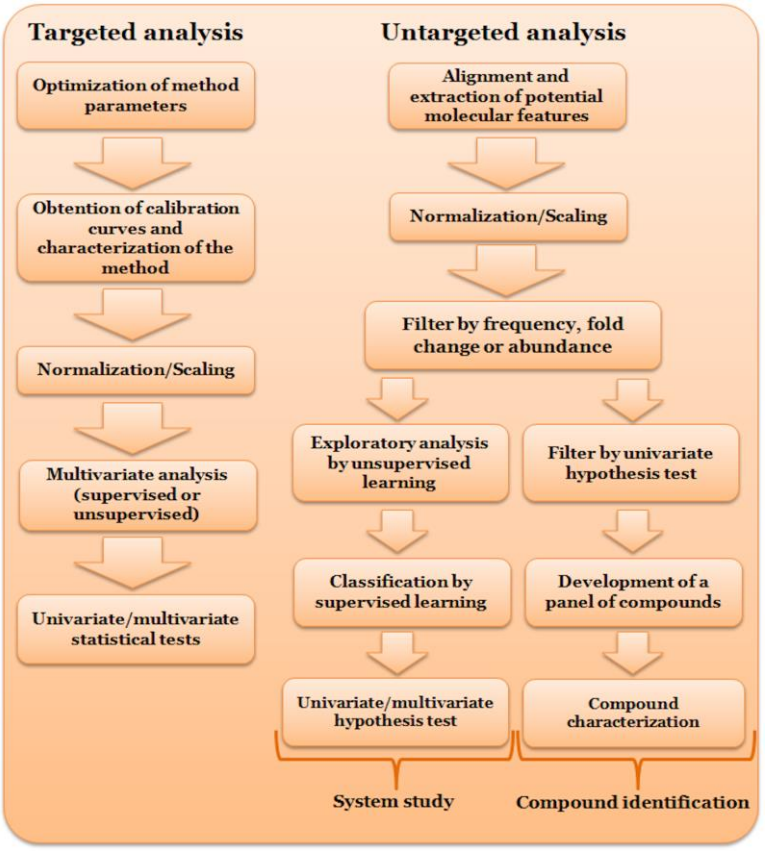

Figure 7. General statistical workflow for targeted and untargeted analyses.

Common pre-processing strategies as normalization or scaling are generally used prior to interpretation of the results or further statistical studies. These are:

- Normalization: the main objective of this approach is the withdrawal of the unwanted systematic bias in ion intensities between measurements, while retaining the characteristic sample variation. Different normalization techniques can be applied, from normalization using a single standard to logarithmic transformation and more complex alternatives

such as probabilistic quotient normalization and quantile normalization *(118)*.

- Scaling: these methods are data pre-treatment approaches that divide each variable by a different factor in order to treat all variables equally, regardless of their intensity. One of the most common factors employed is the mean of each variable, although Z-transform can also be used in case of combining data obtained from different sources or methodologies *(119)*.

5.2. Statistical analysis for untargeted analysis

Untargeted analysis is used to cover the maximum number of compounds by one or more analytical platforms. For this purpose, generic experimental conditions are used for sample analysis and, commonly, a separation step is required prior to detection. After data acquisition, two steps are required: alignment of the chromatograms and extraction of the potential compounds (commonly called molecular features or molecular entities) *(120)*. However, each step implies high complexity owing to the number of factors that need to be considered (removal of adducts, peak shape, removal of noise, etc.). This is the reason why different informatics tools have been designed in the last years.

In fact, this kind of analysis generates large multivariate data sets, which make necessary to take advantage from reliable and robust approaches to obtain relevant information from the vast amount of generated data. Therefore, application of filters is necessary to facilitate further statistical analysis by reducing the number of molecular entities. This procedure assures that only molecular entities with high intensity, high significance with respect to a factor of interest, or entities appearing only with a certain frequency in the samples pass the filter. Different filters have been created to fulfill the different requirements:

- Fold change analysis: this tool compares the level of each potential molecular feature among the groups under study and retains only the entities that show a preset change.
- Filter based on univariate hypothesis test: in this case, only entities with *p*-value below 0.05 or 0.01 pass the filter.
- Filter by Volcano plot: this approach is a combination of analysis of variance and fold change analysis.
- Filter by abundance: this tool allows removing all potential molecular features below certain abundance.
- Filter by frequency: the application of this filter eliminates entities present below a certain percentage of samples within each group.

A common strategy uses different statistical analyses to study the influence of a certain factor on untargeted analysis (extraction method, variety, time, origin, etc.) and how it affects to the molecular entities. Common chemometric tools *(122,123)* such as principal component analysis (PCA) *(124)* are used with visualization and exploratory analysis purposes, whereas univariate statistical tests such as the Student's *t*-test are used to identify the relevant variables after exploratory analysis.

- Exploratory analysis by unsupervised learning: unsupervised methods attempt to analyze a set of observations without measuring or observing any related outcome. As there is not specified class label or response, the data set is considered as a collection of analogous objects. Unsupervised learning uses procedures to find natural partitions of patterns, to facilitate the understanding of the relationship between the samples and to highlight the variables responsible for these relationships. By providing means for visualization, unsupervised learning aids in the discovery of unknown but meaningful categories of samples or variables that naturally fall together. The success of such approaches is most times subjectively evaluated by the interpretability and usefulness of the results

with respect to a given problem. PCA is the most common unsupervised method to the detriment of others such as hierarchical cluster analysis, often used as a starting point for analysis. It is an orthogonal transformation of multivariate data first formulated by Pearson *(125),* and mostly used for exploratory analyses by extracting and displaying systematic variations. The approach attempts to uncover hidden internal structures by building principal components describing the maximal variance of the data *(126)*. This method represents a very useful tool for displaying purposes as it provides a low-dimension projection of the data (*i.e.,* a window into the original K-dimensional space) by transformation into a new coordinate system. The basic concept relies on areas of signal variance in the data where underlying phenomena can be observed. This principle leads to a focus on a small number of uncorrelated independent signals that explain a large part of the total variance in a compact and insightful manner. In practice, PCA builds hyperplanes in the original space that are linear combinations of the original variables and describes the data in a least squares view. The inspection of PCA scores and loadings plots highlights the relationships among the distribution of samples that may reveal grouping, trends or outliers and the corresponding variables. Moreover, more effective data analyses can be performed on the reduced dimensional space, such as clustering, pattern recognition or classification. The vast majority of metabolomics studies involve PCA as a first exploratory step *(127–133)*.

- Classification by supervised multivariate analysis: supervised learning considers each object with respect to an observed response and includes regression and classification problems depending on the output type under consideration (*i.e.,* a numerical value in the first case and a class label in the second). This classification aims at producing general hypotheses based on a training set of examples that are described by several variables and identified by known labels corresponding to the existing classes. The task is to learn the mapping from the first to the last.

Numerous techniques based either on statistics or on artificial intelligence have been developed for this purpose. Within the variety of supervised statistical techniques, which includes decision trees, artificial neural networks and support vector machines, the most employed is partial least squares (PLS). This technique is particularly adapted to situations where fewer observations (N) than measured variables (*e.g.*, detected features, K) are available. Its use has become very popular thanks to its ability to deal with many correlated and noisy variables forming megavariate data structures (K>>N) *(134,135)*. PLS builds a low dimensional sub-space based on linear combinations of the original X-variables and makes use of the additional Y information by adjusting the model to capture the Y-related variation in X. A PLS-based classification therefore has the property that it builds data structures with an intrinsic prediction power by maximizing the covariance between the data and the class assignment. The decomposition relies on latent variables that are computed sequentially to provide a good correlation with the remaining unexplained fraction of Y. In the context of classification, PLS discriminant analysis (PLS-DA) is performed to sharpen the partition between groups of observations, in such a way that a maximum separation among classes is obtained. The model can then be analyzed to understand which variables carry the class-separating information *(136)*. PLS-DA was demonstrated to be a potent tool for data classification *(137)*.

- Univariate hypothesis testing: the Student's *t*-test, the one-way analysis of variance or the non-parametric equivalent can be used to identify statistical differences between distinct classes of samples *(138)*. The predictive power of each variable is assessed by finding statistically significant differences between the mean intensity values of a given signal, of which the calculated *p*-value is a straightforward indicator. Such procedures are easily understandable but their use is rather limited in dealing with thousands of highly correlated variables. False positives

(type I error) are likely to occur when performing multiple comparisons. Procedures such as the Bonferroni of the Benjamini correction have been introduced to address this issue *(139)*. The vast majority of metabolomics-dedicated software provides statistical hypothesis testing *(140,141)*.

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HERRAMIENTAS Y EQUIPOS ANALÍTICOS

Analytical tools and analytical equipment

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

1. Muestras

En la investigación realizada se han utilizado tres tipos de muestras; desechos de la industria vitivinícola como sarmientos de vid y lías de vinificación, productos comúnmente usados en el proceso de envejecimiento del vino como las virutas de roble y vinos envejecidos utilizando sarmientos de la vid y virutas de roble.

En los estudios de comparación de métodos de extracción de la fracción polar y de la de polaridad media, así como en los de comparación y caracterización de la fracción volátil y aromática (Capítulos 2 a 5) se utilizaron sarmientos de vid de distintas variedades procedentes de Sierra de Segura (Jaén). Además, se utilizaron virutas comerciales de roble de distintas variedades, especificadas con más detalle en cada capítulo. Las muestras de sarmientos de vid y las de virutas de roble se utilizaron también en el envejecimiento de vinos, que posteriormente se compararon atendiendo a su perfil aromático y sensorial (Capítulos 6 y 7).

En el último bloque de esta Tesis, dedicado a la caracterización de las lías de vinificación, se usaron muestras de lías de vino tinto procedentes de bodegas españolas de diferentes áreas geográficas, especificadas con más detalle en cada capítulo.

2. Sistemas no automáticos para la preparación de muestra

Se describen a continuación los sistemas de tipo discontinuo utilizados para la preparación de muestra:

Dispositivo de microondas. Se utilizó un digestor comercial Microdigest 301 fabricado por Prolabo, basado en microondas focalizadas, para la extracción de los compuestos fenólicos de los sarmientos de la vid (Capítulo 2), así como para la extracción de las lías sólidas para su posterior análisis (Capítulos 8 a 10).

Dispositivo de ultrasonidos. Existen dos dispositivos básicos en el laboratorio analítico para aplicar ultrasonidos: Los baños y las sondas. Aunque los baños se utilizan con mayor frecuencia debido a su menor precio y omnipresencia en el laboratorio, aumentando así su acción, y de no sufrir el fenómeno de fatiga con el tiempo, proporcionando, por tanto, una mayor reproducibilidad de los resultados, particularmente en tratamientos largos.

La sonda de ultrasonidos utilizada fue una Branson 450 digital, que permite la selección de la amplitud de la radiación así como el modo de aplicación, continuo o discontinuo. Esta sonda se utilizó para acelerar el proceso de extracción de los compuestos fenólicos de los sarmientos de la vid. Para ello la sonda se introdujo directamente en un recipiente que contenía la muestra sólida y el extractante, tal como se detalla en el Capítulo 2.

Equipo de extracción mediante líquidos sobrecalentados. La extracción con etanol–agua a temperaturas altas y presión suficiente para mantener el extractante en estado líquido se llevó a cabo con un extractor diseñado y construido en el laboratorio con los siguientes elementos: Un horno, una bomba de alta presión (Hitachi L-6200A) para impulsar el extractante a través del sistema, una cámara de extracción de acero inoxidable, una válvula de alta presión para permitir el paso de extractante y llenar el sistema o para purgarlo

una vez terminada la extracción, un restrictor (para mantener la presión en el sistema) y tubo de acero inoxidable para construir la zona de transporte y la de calentamiento previo del extractante. Una descripción más detallada del extractor se encuentra en el Capítulo 1. Este extractor se utilizó para la obtención de extractos polares y de polaridad media a partir de sarmientos de vid y de virutas de roble (Capítulos 2 a 4).

Molino de bolas. Se utilizó un molino Restch MM301 para triturar los sarmientos de la vid y las virutas de roble hasta conseguir un tamaño homogéneo de partícula de 40 mesh (0.42 mm de diámetro) (Capítulos 2 a 7).

Agitador eléctrico. Para favorecer la extracción líquido—líquido en el análisis de extractos de sarmientos de vid y virutas de roble, así como en el análisis de vinos, se utilizó un agitador eléctrico MS2 minishaker (Capítulos 4, 6 y 7).

Los distintos extractos obtenidos con los dispositivos descritos se centrifugaron mediante una centrífuga Selecta Mixtasel-BL para separar, en cada muestra tratada, los residuos sólidos del extracto, que se llevó a sequedad en un Rotavapor Büchi R-200 para la posterior reconstitución del residuo en el disolvente adecuado.

3. Sistemas automáticos para la preparación de muestras

En el Capítulo 10 de la Memoria se recoge el uso de un sistema automático para la preparación de muestra en análisis orientado (targeted analysis). Este sistema permite llevar a cabo de manera reproducible, totalmente automatizada —y a veces con drástica reducción del volumen de muestra y reactivos— esta etapa crucial del proceso analítico, que es una de las principales fuentes de error de los métodos de análisis cuantitativo.

La preparación de la muestra mediante extracción en fase sólida de los compuestos de interés se realizó con un equipo automático comercial, el denominado Prospekt-2, que se usó para la preparación de la muestra en el análisis de antocianinas, antocianidinas y proantocianidinas. Este sistema, además de trabajar en modo dinámico, permite la elución directa con la fase móvil cromatográfica o con un pequeño volumen de disolvente que se incorpora a la fase móvil. Trabajar a alta presión posibilita la conexión en línea de este sistema con el conjunto cromatográfo/detector, consiguiéndose así la automatización completa del método analítico. El sistema Prospekt-2 está compuesto por tres módulos: Un muestreador (MIDAS), un dispensador que trabaja a alta presión (high-pressure dispenser, HPD) y que, con auxilio de un conjunto de válvulas, permite realizar todas las etapas de que consta el proceso de extracción en fase sólida; y un sistema automático de cambio de cartucho (automatic cartridge exchange, ACE). Todo el proceso de extracción se realiza a alta presión; lo que lo diferencia de otros sistemas automáticos de extracción en fase sólida.

En los Capítulos 5 a 7 de la Memoria se recoge el uso del horno de un automuestreador de espacio de cabeza para tostar los sarmientos de vid y las virutas de roble simulando el proceso industrial. De esta forma se lleva a cabo de manera reproducible el tostado de la muestra para su análisis inmediato (Capítulo 5) o para su posterior uso en el envejecimiento de vinos (Capítulos 6 y 7). Se usó un automuestreador de espacio de cabeza Agilent 7694E (que está compuesto por tres módulos, un muestreador, un horno para calentar la muestra a temperatura controlada en el interior de un vial sellado y una línea de transferencia termostatizada para la inyección directa de la muestra gaseosa en el cromatógrafo de gases.

Instrumentación analítica

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

En los Capítulos 2 y 3, en los que se recoge el análisis de extractos fenólicos de sarmientos de vid y virutas de roble, obtenidos mediante diversas técnicas de extracción, se utilizó un cromatógrafo de líquidos Varian ProStar, equipado con una bomba ProStar 240, un detector de diodos en fila ProStar 330 y un automuestreador ProStar 410, para la determinación individual de compuestos fenólicos. La columna analítica fue una C18 Inertsil ODS-2 (250 mm de longitud × 4.6 mm de diámetro interno, 5 micras de tamaño de partícula).

Para los estudios de la composición de la fracción volátil y aromática de los sarmientos de vid y las virutas de roble, y su comparación con fines enológicos (Capítulos 4 y 5), así como para la comparación del perfil volátil de vinos envejecidos utilizando sarmientos de vid y virutas de roble (Capítulos 6 y 7), se empleó un cromatógrafo de gases Varian CP-3800 acoplado a un detector de masas de trampa iónica (Varian Saturn 2200) que, dependiendo del estudio, se utilizó acoplado a un automuestreador Varian 8400, o a un automuestreador de espacio de cabeza (Agilent 7694E). La columna analítica utilizada fue una columna capilar de sílice Factor Four VF-5 de Varian (30 m de longitud × 0.25 mm de diámetro interno, 0.25µm de espesor de la película). Se utilizó el software de Varian de control de sistema e integración de señales (Star Chromatography Workstation 6.0) para la recogida y el tratamiento de los datos.

En el Capítulo 10 se recoge el análisis orientado de antocianinas, antocianidinas y proantocianidinas, en el que la separación mediante LC y posterior detección por MS en tándem triple cuadrupolo se llevó a cabo con un cromatógrafo Agilent 1200 Series LC–TOF equipado con una bomba binaria, un

desgasificador, un automuestreador y un compartimento de columna termostatizados, y un espectrómetro de masas Agilent 6410 de triple cuadrupolo con una fuente de ionización por electrospray (ESI). El software Agilent MassHunter Workstation se usó para la toma de datos y el análisis cuali- y cuantitativo.

Para la investigación recogida en los capítulos dedicados a la obtención del perfil global de extractos de sarmientos y de virutas de roble (Capítulo 3) y extractos de lías de vinificación (Capítulos 8 y 9), se utilizó un equipo Agilent 1200 Series LC acoplado a un detector de masas de tiempo de vuelo (TOF) de alta resolución, Agilent 6540. Las columnas para la separación cromatográfica fueron siempre C18 (fase reversa) con diferentes dimensiones en función de la aplicación. En todos los casos se usó el software MassHunter para la adquisición de espectros y el análisis cualitativo.

4. Técnicas quimiométricas

De acuerdo con la importancia actual de la quimiometría en química analítica, en esta Tesis Doctoral se utilizaron extensamente herramientas quimiométricas para el tratamiento de datos, que implicaron distintos programas informáticos según el objetivo:

a) Alineamiento de entidades moleculares en análisis no orientado (profiling):

   - Por un lado se empleó la combinación de dos softwares de Agilent: Qualitative Workstation y Mass Profiler Professional (Agilent). El primero permite extraer las entidades teniendo en cuenta aductos e isótopos, mientras que el segundo posibilita el alineamiento de las entidades potenciales.

- Por otro lado se usó el software Mass Profinder, de Agilent, que permite realizar las etapas de extracción de entidades (teniendo en cuenta aductos e isótopos) y el alineamiento en una única etapa.

b) Análisis estadístico:

- Statgraphics. Permite realizar distintos análisis estadísticos uni- y multivariantes, así como el diseño y evaluación de modelos de cribado (screening) y superficies de respuesta.
- Mass Profiler Professional. Permite la aplicación de diferentes algoritmos de análisis estadístico:
  - El análisis de varianza (ANOVA) para evaluar la influencia de las variables en estudio en las muestras.
  - El análisis no supervisado mediante componentes principales (PCA), que permitió detectar agrupamientos entre muestras.
  - El análisis de cluster para encontrar similitudes entre muestras teniendo en cuenta la abundancia de sus perfiles.
  - El análisis mediante diagramas de Venn para conocer el grado de similitud entre dos clases de muestras.

5. Bases de datos

Aunque queda un gran camino por recorrer, existen varias bases de datos a disposición del usuario que contienen información para la identificación y caracterización de muchos de los compuestos presentes en diversas matrices biológicas y vegetales. Entre ellas, las bases de datos 'Metabolites and Tandem MS Database' (METLIN), 'PlantCyc', 'MassBank' y 'NIST' se han empleado para la identificación de compuestos presentes en extractos de las muestras a partir de los datos espectrales obtenidos mediante espectrometría de masas tal como se

muestra en los Capítulos 3, 4, 5, 8 y 9 de esta Memoria. Como complemento a las bases de datos existentes, se han utilizado bases de datos preparada con compuestos de interés para su identificación en las muestras en estudio.

Existen otras bases de datos que, si bien no están constituidas por datos espectrales experimentales, contienen información útil sobre la rotura *in silico* de las distintas familias de compuestos, como es el caso de MetFrag. Esta base de datos se empleó, según se recoge en los Capítulos 8 y 9 de esta Memoria, como ayuda a la identificación de compuestos en base a los datos espectrales obtenidos por espectrometría de masas.

PARTE EXPERIMENTAL

Experimental part

SECTION I

Characterization and comparison of vine-shoots and oak chips

En esta Sección I de la Memoria se recoge en primer lugar una publicación —un capítulo de libro— que ha contribuido a la formación teórica de la doctoranda en dos aspectos fundamentales: i) En la interpretación crítica de la investigación publicada sobre una técnica de lixiviación utilizada en esta investigación, la extracción con líquidos sobrecalentados. ii) En la puesta al día sobre lo que se ha publicado en la materia en cuestión, de forma que adquirió una base sólida sobre la que soportar la investigación a desarrollar.

Las dos publicaciones que se recogen en los Capítulos 2 y 3 tienen una temática común —la fracción polar y de polaridad media de extractos de sarmientos de la vid y de virutas de roble—, pero abarcan aspectos distintos que han justificado el desdoblamiento. En la primera se comparan diferentes técnicas de extracción asistidas por energías auxiliares, estableciendo cuál es la óptima para compuestos de interés desde el punto de vista enológico y que se encuentran presentes en los sarmientos de la vid, para su subsiguiente cuantificación en los extractos mediante LC–DAD. En la segunda se aplica la técnica de extracción óptima a sarmientos de vid y a virutas comerciales de roble y se analizan los extractos mediante LC–TOF/MS para su comparación estadística y la identificación de los principales compuestos mediante una base de datos personal.

Los Capítulos 4 y 5 tienen también una temática común: El estudio de la composición tanto de extractos de sarmientos de la vid y de virutas de roble como de la fracción de espacio de cabeza generada durante el tostado de las muestras. En ambos casos el análisis se realizó mediante GS–MS y la posterior comparación de los datos mediante técnicas quimiométricas puso de manifiesto la gran similitud entre ambas muestras y la posibilidad de usar los sarmientos de la vid con fines enológicos.

Section I of this PhD Book is devoted, first, to a publication —a book chapter— that has contributed to the theoretical learning of the PhD student in two essential aspects: i) The critical interpretation of the research published about a lixiviation technique used in this research, superheated liquid extraction. ii) The update on what has been published on the matter, to acquire a solid base on which the research to be developed can be supported.

Chapters 2 and 3 share a common subjet —the polar and mid-polar fraction of extracts of vine-shoots and oak chips—, but they encompass different aspects that justify the division. In Chapter 2, extraction techniques assisted by different types of energy are compared to determine what of them is the optimum for extraction of compounds of interest from the oenological point of view, which are present in vine-shoots; subsequently, they are quantified by LC–DAD. In Chapter 3, the optimum extraction technique is applied to vine-shoots and commercial oak chips and the obtained extracts are analyzed by LC–TOF/MS for statistical comparison and identification of the main compounds by application of a personal data base.

Chapters 4 and 5 also have a common subject: the study of the composition of extracts from vine-shoots and oak chips and the headspace fraction generated during toasting of the samples; then analyzed by GC–IT/MS and compared by application of chemometric tools, thus demonstrating the high similarity between both types of materials and the feasibility of vine-shoots to be used with oenological purposes.

Chapter 1:

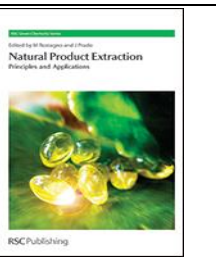
Accelerated solvent extraction

The Royal Society of Chemistry

"Natural Product Extraction"

Chapter 5: Accelerated Liquid Extraction

2013



Accelerated solvent extraction

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Accelerated solvent extraction

Feliciano Priego-Capote and María del Pilar Delgado de la Torre

Table of contents

1. Introduction

Over the last decades, an approach called accelerated solvent extraction (ASE) has competitively emerged for treatment of solid samples by using a liquid phase at high pressure and/or temperature, but below its critical point. This approach is considered an efficient way to increase automation, which is one of the pursued goals in the preparation of solid samples, but it also may shorten process times and reduce the amount of solvent required for sample preparation of solids.

The term “accelerated solvent extraction” was originally coined by Dionex Corporation, which patented the technique and used it as the basis of its commercial devices (1). In fact, the exclusive use of this term in the earliest years was largely the result of the sole commercially available extractor for this purpose being that manufactured by Dionex. At about the same time, Hawthorne, whose group was studying the applicability of water at high pressure and temperature as solvent, named the process “subcritical water extraction” (2). With time, however, other alternative names such as “pressurized liquid extraction” (PLE), “pressurized hot solvent extraction” (PHSE), “high-pressure solvent extraction” (HPSE), “subcritical solvent extraction” (SSE) and “superheated solvent extraction” (SHSE), among others, have gradually replaced ASE, a commercial designation that is not related to the actual physicochemical foundations of the technique. High pressure is not the most salient feature in the ASE process. In fact, most often the only purpose of raising the pressure is to keep the solvent in the liquid state, and pressure rarely exerts an effect over the extraction process. On the other hand, the term “subcritical state” is a wide term since any solvent at temperature and pressure below the critical point would be in this state, even at ambient conditions, and is therefore inappropriate as well. For these reasons, it is recommended to use the generic term “superheated solvent extraction” despite its scant utilization by the scientific community, which has widely accepted ASE supported on the distribution of the commercial devices. Both ASE and SHSE are used interchangeably in this chapter.

Referring to the basic principle of this sample preparation technique, it is worth mentioning that extraction with an aqueous or organic solvent at a high pressure and/or temperature can be done in static regime, dynamic regime — by continuously circulating the solvent through the sample — or in a combined mode of both operation modes (static–dynamic approaches). The basic principles of each operational SHLE mode as well as the main steps for development of both are discussed. The different devices designed for static and dynamic SHLE are also reviewed. In the final sections, a comparison of SHLE *versus* other competing extraction techniques and the applicability of SHLE for isolation of

natural products are evaluated with special emphasis on the diversity of raw materials (leaves, roots, flowers, wood, fruits, vegetables, etc.) and on the different groups of compounds that can be extracted.

2. Static accelerated solvent extraction (static ASE)

Static ASE is the less versatile of the two extraction modes in terms of flexibility and possibility of coupling to other steps of the analytical process, since it is performed in a closed system. Nevertheless, static ASE is by far the most widely used mainly as a result of the availability of commercial extractors from Dionex (series 100, 200 and 300, and the new versions 150 and 350). Although these systems can be used in the static and dynamic modes, they are preferentially operated under static conditions. The static mode is usually selected to avoid dilution of the extract as the transfer equilibrium governing the extraction process is basically displaced under superheated conditions.

2.1. Steps involved in the static ASE process

A common practice in extraction from a solid, applicable prior to static ASE, is to pre-treat it in some way depending on its physicochemical properties. Thus, pre-treatment usually involves grinding to reduce particle size and sieving to isolate a homogeneous fraction of solid particles. In cases of solid–liquid samples centrifugation or filtration, drying (storing samples at moderate temperature for 24–54 h) or freeze-drying is usually required. The last two operations are quite critical, since moisture present in biological samples may detract from the extraction efficiency, particularly for organic non-polar solvents. As in Soxhlet extraction, the addition of sodium sulfate or an alternative desiccant such as Extrelut particles is recommended in handling large amounts of water.

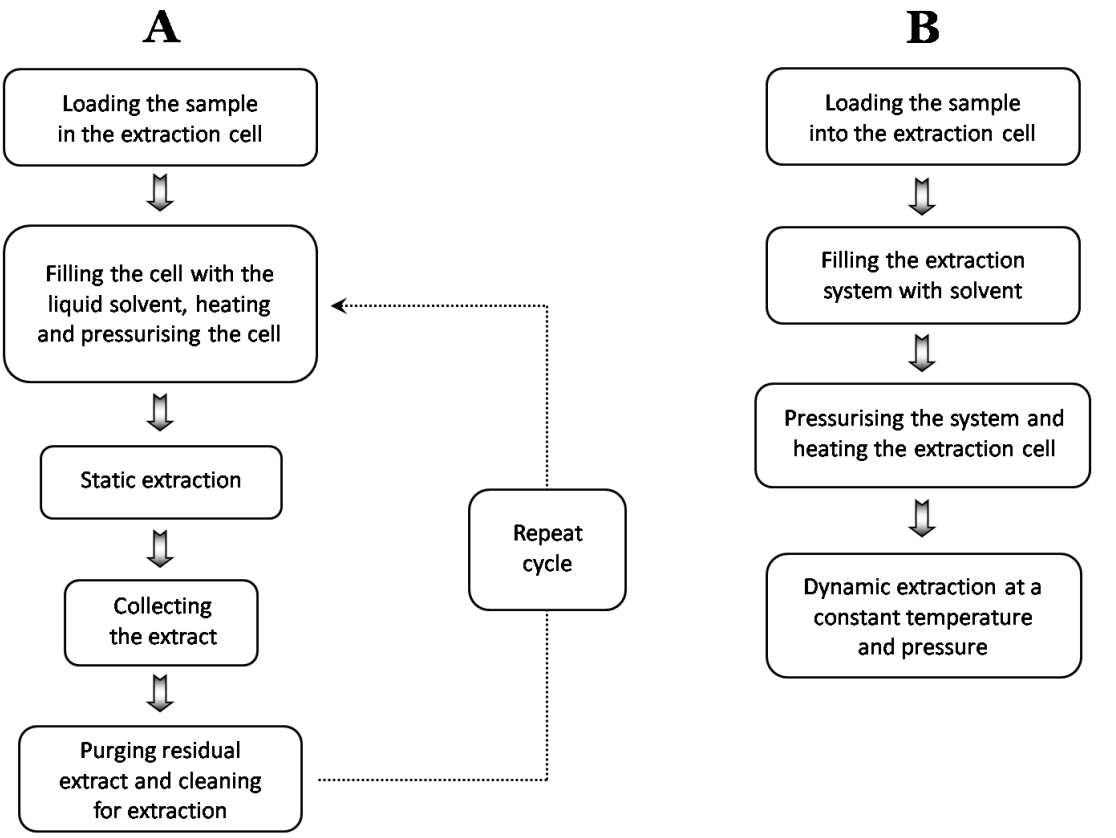

Figure 1. Scheme of the main steps involved in: (A) ordinary static and (B) dynamic ASE procedures.

Figure 1.A schemes the five steps usually involved in a static ASE process, namely:

(1*) Loading the sample into the extraction cell*. The metal frit of the extraction cell is suggested to be covered with a cellulose filter or a small amount of celite in order to prevent clogging at the outlet of the cell. To avoid dead volumes in the extraction cell the sample can be mixed with an inert matrix (e.g. diatomaceous earth, anhydrous sodium sulfate, glass fibre, high-density glass beads, sand, hydromatrix) to ensure proper sample–solvent contact and to reduce solvent consumption. Active materials such as Al_2O_3, silica or Florisil can also be employed for specific purposes. These materials allow the extraction cell to be filled up but also to perform in situ clean-up by the retention of the target

compounds once extracted or, on the contrary side, by the retention of undesired compounds. One other operation that can be carried out in situ during extraction of analytes is derivatisation by adding a suitable reagent in the extraction cell. The extraction–derivatisation combination is another interesting possibility as many derivatisation protocols are developed under high temperature, and this can be a way to increase sensitivity and/or selectivity.

(2) *Filling the cell with solvent, heating and pressurising the cell*. Once the cell is loaded with sample and the end caps of the cell tightened, this is filled with a solvent of suited composition. At this point, there are two possibilities: to preheat the extraction cell before filling it with solvent or to heat it after filling with solvent. The latter is the preferred option by ASE users. The cell is frequently positioned in a vertical position to ensure that the system is completely filled with liquid solvent without air bubbles. Once the operational temperature is selected, the extraction system is thermostated at a constant pressure and equilibrated. Usually, 5 min is enough to equilibrate the system at the desired temperature and pressure.

(3) *Static extraction*. This step is performed after pressure and temperature equilibration, for a preset time during which the analytes are released from the solid matrix and transferred to the solvent by diffusion and solubilisation. The role of pressure is later defined.

(4) *Collecting the extract*. The transfer step begins immediately after static extraction is finished. The pressure valve is opened and the extract flows to the collection vial. In general, cooling the vial is not required as it does not seem to influence the recovery or the precision of the process.

(5) *Purging residual extract and cleaning for a new extraction*. This step entails circulating fresh solvent or an additional inert gas — N_2, for example — through the cell to remove residual extract in it. The use of an inert gas avoids diluting the extract that, in any case, could be concentrated by evaporation, if required. A “rinse-solvent” volume corresponding to 60 % of the empty

extraction cell has proved effective to avoid carry-over between consecutive extractions for most applications.

2.2. Static ASE commercial and laboratory-designed devices

The basic equipment required to implement static ASE is quite simple since it consists of the seven basic components depicted in **Figure 2.A**, namely: (1) a reservoir for the fresh solvent; (2) a high-pressure pump; (c) a thermostated unit (for instance, an electrically heated oven) with thermal control for placing the extraction cell; (d) a stainless steel extraction cell where the solid–liquid extraction takes place; (e) a back-pressure regulator for controlling the system pressure; (f) a cylinder of inert gas (usually nitrogen) for purging the system after extraction; and (g) a vial to collect the extract.

These components are backbone of an SHLE system; which is not technically complex. Concerning commercial models, only three static ASE systems, all from Dionex, were available until approximately the early XXI century, namely the ASE 100, 200 and 300 (3) The main differences between them are, (a) the number of samples that can be simultaneously processed (1, 24 and 12 for ASE 100, 200 and 300, respectively); (b) the capacity of the sample cell (10–100 mL for ASE 100, 1–33 mL for ASE 200, and up to 100 mL for ASE 300); (c) the amount of sample to be processed (between 10 and 100 g); and (d) the maximum pressure they can withstand (100 bar for the ASE 100 and 300 models, and 200 bar for the ASE 200). Two current models (ASE 150 and ASE 350) were developed later as extended versions of the ASE 100 and 300, mainly differing from their older counterparts in specific technological advances to improve their performance.

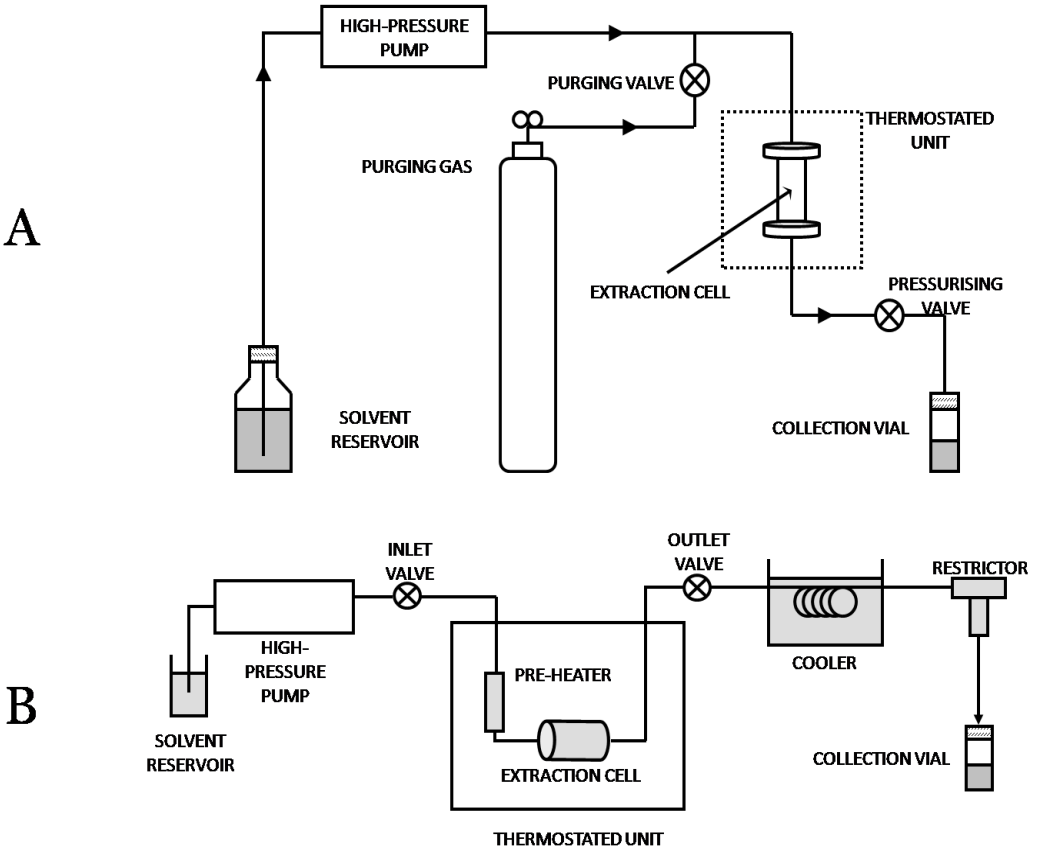

Figure 2. Configurations for development of static (A) and a dynamic (B) ASE procedures. (Reproduced with permission of Elsevier, Ref. Luque de Castro, M.D.; Luque-García, J.L. *Acceleration and Automatition of Solid Sample Treatment*; Elsevier, Amsterdam, 2002, pp. 245 and 261).

Figure 3 shows a picture of the two devices currently commercialized by Dionex. The revised oven designs ensure uniform heating, and precise replicate extractions as a result; Dionium™ cells allow extracting from acid to basic matrices and under extreme pH conditions; new flow-through operational capabilities allow in-line filtration; and faster pumps (up to70 mL/min) accelerate and allow scaling up the process.

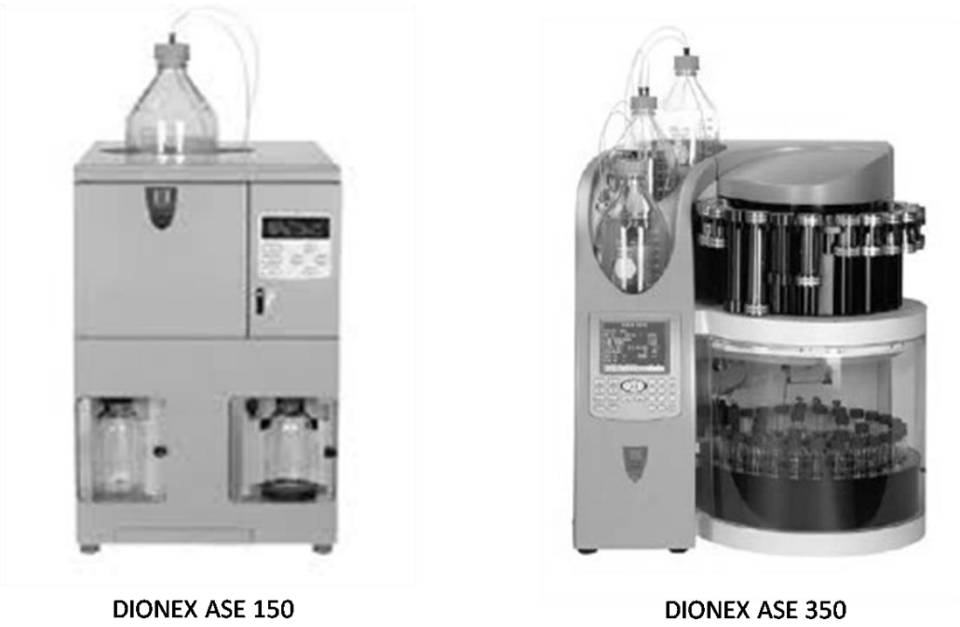

Figure 3. ASE® systems from Dionex.

Other custom-made configurations have been the starting point for subsequent industrial development of processes intended to replace their conventional counterparts, which is one of the main benefits of SHLE. One example is the case of the extraction of non-volatile (4) and volatile (5) components of wood, of great interest to the winery industry. The conventional process to obtain extracts from wood, which is based on recirculation of ethanol–water mixtures through a bed of chips of oak wood and vine-shoots at ambient temperature and pressure for 8–10 h, can be substituted by superheated extraction at 180 °C and 0.3 MPa for 50 min. The resulting extracts are richer in aroma compounds than the conventional extracts, which could have a favourable impact on the composition of wines and spirits. Also, comparing qualitatively and semi-quantitatively the composition of extracts obtained under certain conditions allows preparing tailor-made extracts (6).

Taking into account the fundamentals of the static mode, this extraction approach can be technically scaled up to semi-industrial and industrial applications. Semi-industrial devices for extraction with volumes of 250 mL are commercialized by Buchi and FMS (Fluid Management System), among others. Concerning extraction systems for industrial purposes, they can be easily constructed using a pressurized extraction tank with a temperature control unit. These types of extraction devices are designed according to the industrial application and mainly used for extraction of fragrances, cosmetics or in the food industry.

3. Dynamic accelerated solvent extraction (dynamic ASE)

Dynamic ASE is employed to take benefit from the continuous contact between the solid matrix and fresh solvent. This favours the displacement of the transfer equilibrium and, therefore, the leaching process. The main limitation of this operational mode is the dilution effect by continuous flow of clean solvent, which makes mandatory to implement subsequent concentration steps prior to characterisation of natural products, usually at low concentration levels. Unlike static ASE, there is no commercially available equipment for implementing dynamic ASE. This is the reason for the relative paucity of developed applications. In fact, dynamic operation facilitates coupling to other dynamic systems designed for preconcentration, filtration, chromatographic separation, derivatisation and detection, among the most important.

3.1. Steps involved in the dynamic ASE process

Most steps involved in dynamic ASE are similar to those of the static approach (**Figure 1.B**), except for a few subtle differences, particularly at the final stages of the process. Thus, the steps involved in the dynamic ASE are as follows:

(1) *Loading the sample into the extraction cell.* Similarly as in the static mode, the sample can be mixed with a dispersant, if required. Dispersant agents in this mode are more important than in the static ASE since continuous circulation of the solvent in the same direction increases sample compaction. Additional materials such as surfactants, silica or sorption discs can be placed in the cell to facilitate transfer of the compounds from the solid to either the micellar medium or to the sorbent in order to concentrate the target compounds.

(2) *Filling the extraction system with solvent*. The circuit is filled with solvent propelled by a high-pressure pump or a similar device. The extraction cell should be mounted vertically in the oven, with the solvent flowing from top to bottom so the extracted compounds are immediately swept from the cell.

(3) *Pressurising the system and heating the cell at a preset temperature and constant pressure*. Before the oven temperature is raised up to the programmed value, the system is pressurized by using an outlet valve. In this way, the flowing solvent — as the valve is closed during this time — produces the overpressure required to maintain the solvent in liquid state at high temperature in the extraction system.

(4) *Dynamic extraction at constant temperature and pressure*. Once the preset temperature is reached and stabilised, the outlet valve is partially opened and the liquid phase is continuously circulated through the system for the optimised period (dynamic extraction time). At the same time the extract is cooled by circulation through a coil in a water bath, and then collected at the outlet of the extraction system.

After extraction, the cell is washed with an appropriate solvent at a high flow rate in order to avoid carry-over. No purging of the system with a gas after extraction is required in this mode.

In many cases, the extraction protocols combine dynamic and static operational modes. For this purpose, an inlet valve is installed between the high-pressure pump and the thersmostated extractor. The operation mechanism of

this combined approach is based on the following steps: (i) the inlet valve is closed once the system is pressurised and the high-pressure pump stopped; (ii) the oven temperature is raised up to stable value; (iii) the system is maintained under a static regime with both valves closed for a preset time and; (iv) finally, the valves are opened and the pump works again to keep the solvent flowing during the dynamic extraction period. Several studies including one by Pérez-Serradilla *et al.* (7) have shown a combination of static and dynamic ASE for extraction of natural products. In this particular example, the application of the dual approach resulted in substantially improved sequential extraction of phenol compounds and fatty acids from olive pomace. This lab approach could be scaled up to give place to industrial equipment destined to valorise a semisolid residue generated in the extraction of olive oil.

3.2. Dynamic ASE laboratory-designed devices

The lack of commercial extractors for dynamic ASE has led to its implementation in laboratory-built designs similar to those for static ASE. The basic elements are shown in **Figure 2.B**, namely: (1) a reservoir for storing the liquid solvent; (2) a high-pressure pump or similar device for propelling the solvent to the extraction cell; (3) a thermostated system such as an electrically heated oven to reach and keep the desired temperature; (4) a pre-heater located prior to the extraction cell for ensuring that the solvent is at the required temperature when reaching the extraction cell; (5) an extraction cell for holding the sample; (6) an inlet valve (if static extraction is combined with the dynamic mode) and an outlet valve to pressurise the system and combine static and dynamic modes; (7) a cooling unit located out of the thermostated system; (8) a restrictor for keeping the pressure within the system at the preset level so that the solvent is maintained in the liquid state at the operating temperature; and (9) a vial for collecting the extract.

In dynamic ASE, the propulsion system can be a dual piston pump or a syringe pump, in any case high-pressure devices. The former delivers a

continuous supply of solvent (limited by the barrel size in syringe pumps) and allows easy solvent change-over, while syringe pumps deliver a non-pulsating flow. All tubing and elements of a dynamic ASE extractor must be made of stainless steel to avoid corrosion by solvents (particularly acid or alkaline aqueous solutions) used at high temperatures. Special alloys such as hastelloid, which affords working temperatures close to or above 500 °C, can also be used, although the use of these specific materials would increase considerably the cost of the equipment and has limited application to natural products until the present time.

Sequential extraction of polar and non-polar compounds from the same sample with different solvents requires minimal technical changes of the dynamic ASE system. The modifications are aimed at the passage of a gas stream through the sample chamber to remove solvent residues before the next solvent is circulated (8). This configuration allows sequential extraction of complex matrices with a predefined order of solvents with different chemical properties (polar, non-polar, acid, alkaline, micellar media, etc.).

In relation to industrial extraction systems, the dynamic approach is not scaled up owing to its technical operational mode. Industrial procedures are mostly based on batch extraction.

4. Coupling ASE to other steps of the analytical process

One of the benefits of ASE when compared to conventional extraction as well as supercritical-fluid extraction (SFE) is the possibility of coupling to other steps of the analytical process such as filtration, preconcentration, derivatisation, chromatographic separation or detection. Static ASE as implemented in commercial equipment is rarely coupled to other steps of the analytical process. In general, when static ASE is pretended to be coupled to subsequent steps, the extractor has been custom-made due to the compact design

of the commercial models which preclude adaptation (9). **Figure.4** shows one of the most complex coupled configurations involving static ASE on-line connected to filtration, preconcentration and gas chromatography–mass spectrometry (GC–MS) detection (10). This configuration, designed for an environmental application, can also be implemented for isolation of natural products. Superheated liquid extraction is carried out as previously described in the static mode. The extract is collected in the reservoir (ER), and an exact volume of the extract is isolated in the loop of the injection valve IV1 and filtrated by action of peristaltic pump PP1. Once the loop is filled, the extract is led to the minicolumn MC using water as carrier. The target compounds are retained in the solid sorbent packed in the minicolumn with removal of interferences. Simultaneously, the loop of IV3 is filled with acetonitrile for subsequent elution of the target compounds. Air is used as carrier in this step to avoid dilution of the eluted compounds, which are collected in a vial (V) for analysis by GC–MS/MS.

Although the preferred choice for characterisation of natural products is the utilization of off-line protocols, there are some examples in the literature dealing with the coupling of static ASE to other steps of the analytical process. These examples involve mainly coupling of a commercial Dionex extractor to devices such as a commercial liquid handling system (ASPEC) or liquid chromatographs. These couplings have been implemented to determine dianthrones in St. John's wort (11) by coupling ASE to solid phase extraction (SPE), and to characterise proanthocyanidins and other phenolic compounds in brewing processes by coupling ASE, SPE and liquid chromatography coupled to diode array detection (LC–DAD) or liquid chromatography coupled to mass detection (LC–MS) detection (12). One other characteristic example is that proposed by Zhang *et al.*, who designed an approach coupling a commercial ASE device to a high-performance counter-current chromatograph. This configuration was tested for extraction and determination of caffeoylquinic acids in *Hypericum perforatum L. (*13).

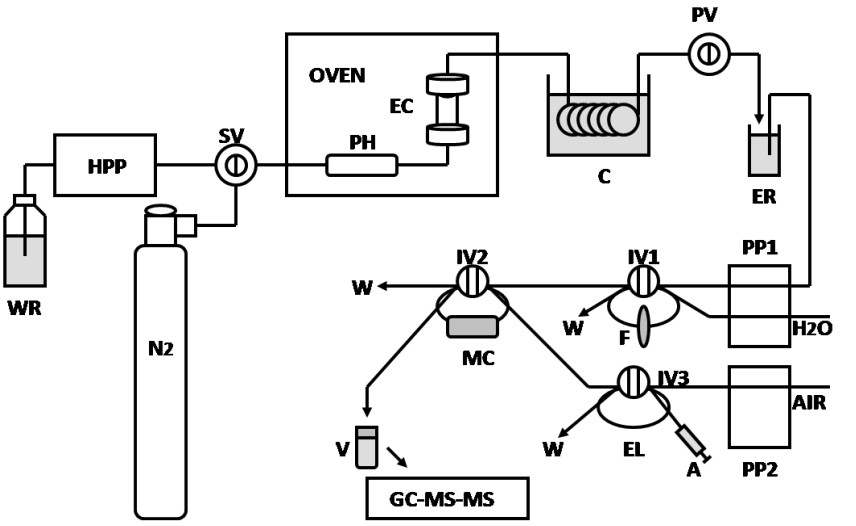

Figure 4. Schematic diagram to illustrate the coupling of static ASE to filtration, preconcentration and chromatographic separation–mass spectrometry detection. WR, water reservoir; HPP, high-pressure pump; SV, switching valve; PH, preheater; EC, extraction chamber; C, cooler; PV, back-pressure valve; ER, extract reservoir; PP1 and PP2, peristaltic pumps; IV1, IV2 and IV3, injection valves; F, filter, W, waste; EL elution loop; A, acetonitrile; MC, minicolumn; V, vial.

As mentioned, the greatest drawback of dynamic ASE is the dilution of target compounds in the extract, which requires subsequent concentration (usually by batch liquid–liquid extraction, solid-phase extraction or simply evaporation). On the other hand, the versatility of the dynamic mode relative to the static mode can be used to circumvent dilution problems, but also to automate and/or facilitate other steps of the analytical process such as filtration, derivatisation, chromatographic separation and detection. Despite the examples in the literature of on-line configurations based on dynamic ASE coupled to other steps, these have not been applied to characterise natural products (9,14).

5. Parameters affecting performance in ASE

Performance of ASE is influenced by variables that contribute to the transference of compounds from the sample matrix to the bulk solvent, such as

temperature, pressure, type of solvent and its characteristics (polarity, volume and — if dynamic mode is used — flow-rate), matrix composition, sample size and extraction time. These parameters are briefly discussed in this section.

5.1. Temperature

Temperature is the most important parameter influencing the kinetics of mass transfer from the sample matrix to the liquid solvent in ASE and, therefore, it is crucial to succeed in the leaching process. It is well-known that physical properties of solvents are modified at high temperature. One example is found in **Figure 5** for water at a constant pressure of 24 MPa.(15). The dielectric constant of water decreases by increasing temperature. As a result, the solubility of water in organic solvents increases at high temperature. This is especially interesting in cases where the extraction efficiency of organic solvents at low temperature and pressure is decreased, since they are excluded from water-sealed pores in the sample matrix which contain the target compounds. The increased solubility of water in organic solvents at high temperature favours mass transfer from pores to the organic solvent. Apart from that, the solubility of water is similar to that of methanol at 200 ºC and 24 MPa, to that of acetone at 300 ºC or even to that of hexane at 500 ºC. Therefore, water can be used as solvent for extraction of non-polar compounds at temperature above 200 ºC. Modelling the dependence of the solubility on the temperature of an ideal solvent allows estimating the optimum extraction temperature for a given application.

The use of high temperature during leaching exerts a favourable effect on efficiency through increased diffusion rates. It is difficult to model the effect of temperature on diffusion rate, especially in multi-component systems. In most cases, diffusion rates are estimated to increase by a factor of 2–10 on raising the temperature from 25 ºC to 150 ºC, which undoubtedly enhances the leaching kinetics (16). Nevertheless, temperature is a key factor to be optimised in ASE because a high temperature does not always guarantee increased extraction efficiency. In certain cases, increased temperature can promote the formation of

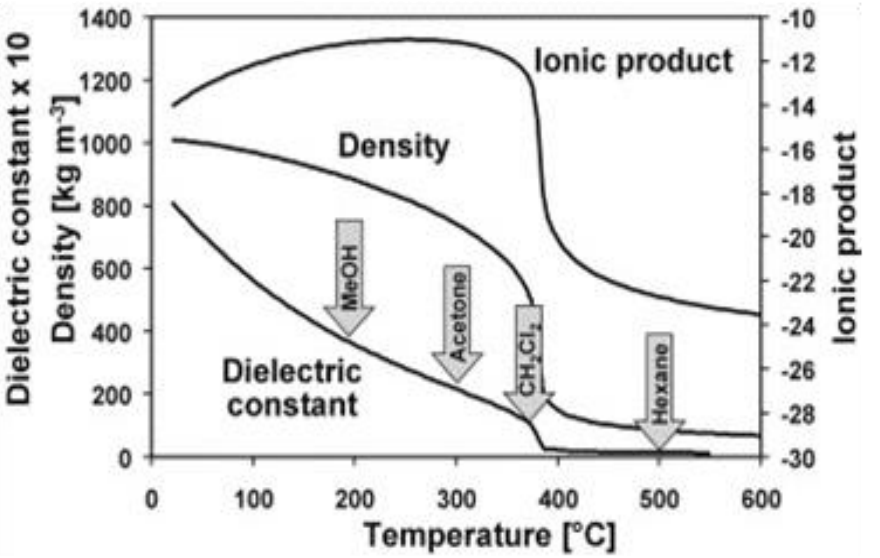

Figure 5. Physical properties of water at a pressure of 24MPa *vs* temperature. Dielectric constants of typical organic solvents at room temperature are indicated.

adverse effects such as degradation of thermolabile compounds or enhancement of secondary reactions that could influence other steps of the analytical process and/or the quality of the final product. The operation conditions during SHLE can also favour hydrolysis reactions when polymeric matrices are extracted. A particular case taking benefits from this principle is the extraction of lignocellulosic materials such as wood for isolation of lignin monomers (coniferyl and syringyl monomers), which is carried out at 180 ºC (17). The resulting extracts are enriched in monomers when SHLE is performed at high temperature. With these premises, the temperature is only limited when the extraction conditions lead to target compounds degradation by chemical conversion to advanced reaction products. In general, the extraction temperature for isolation of natural products is between 100 ºC and 200 ºC, being most of the applications developed within the range 160–180 ºC.

Temperature affects equilibriums occurring at solid surfaces. In fact, they alter strong solute–matrix interactions due to van der Waals forces, hydrogen

bonding and dipole attractions. Thermal energy can overcome cohesive (solute–solute) and adhesive (solute–matrix) interactions by decreasing the activation energy required for desorption.

Concerning the effect of temperature on solvents, increased temperatures decrease their viscosity. This effect facilitates solvent penetration through matrix particles and, therefore, enhances extraction. By way of example, the viscosity of 2-propanol decreases 9-fold as the temperature is raised from 25 to ºC 200 ºC (16). Apart from that, increased temperature also decreases the surface tension of the solvent, thereby allowing it to better access the sample matrix and to form solvent cavities more easily. Both changes improve contact of the compounds with the solvent and hence the extraction efficiency.

5.2. Pressure

A minimum pressure is required in ASE to maintain the solvent in the liquid state and avoid phase transitions. As an example, 2 MPa is sufficient to keep *n*-hexane (atmospheric boiling point of 68.7 ºC) in the liquid state at 209 ºC. The minimum pressure required to keep solvents in liquid state for a given temperature can be estimated from defined equations (16). Usually the influence of pressure on the leaching process is null and for this reason overpressure is not necessary. However, in some cases system pressure can be a key variable to displace the system equilibrium. Thus, a high pressure may favour leaching of compounds trapped in matrix pores by forcing the solvent into matrix areas that would normally not be accessible under atmospheric conditions.

Overpressure may also benefit the time required to fill the extraction cell with the solvent, especially with samples of small particle size that increase compactness in the cell (18). On the other hand, increased pressure can induce changes in the sample by decreasing active surface, which leads to reduced leaching efficiency for compounds in some types of samples (19).

No significant changes in most extraction processes of natural products due to pressure have been detected using laboratory-made ASE systems. For this reason, the pressure is frequently set below 1 MPa. However, protocols developed for commercial ASE devices recommend extraction pressures close to 12 MPa.

5.3. Type of solvent

SHLE can be used with a wide range of solvents, except those with auto-ignition temperatures within 40–200 ºC (*e.g.* carbon disulphide, diethyl ether, 1,4-dioxane) or with low polarity in the eluotropic series (*e.g.* *n*-hexane) (16) Also, strong bases and acids should be avoided as solvents on account of their corrosiveness, which is enhanced with increased temperature and pressure, causing damage to the fluidic system.

The static extraction mode uses preferentially non-toxic non-residual organic solvents such as ethanol or acetone, but dichloromethane, acetonitrile, hexane as well as mixtures of them have also been used. When the natural products to be extracted are destined for food industry or pharmaceutical applications, non-toxic solvents are selected.

Water is also a quite usual solvent, alone or in mixtures, for isolation of polar and mid-polar compounds in ASE (20) for a variety of samples including foods (21) and plants (22), but also for compounds of widely variable polarity (23–26) The use of modifiers occasionally improves leaching. Water can be modified with organic solvents such as methanol, acetone or acetonitrile in low proportions (usually less than 5 %) in order to decrease its dielectric constant — and hence its polarity — without drastic increase of temperature (27).

Weak acids and bases can be used when the solvent pH plays a decisive role on the leaching efficiency. Micellar media and ionic liquids are also used in certain applications to favour leaching efficiency. One example is the study proposed by Choi *et al.* for extraction of ginsenoids from medicinal plants by using Triton X-100 micellar media (28). Micellar ASE enhanced the extraction

efficiency compared to the use of water, leading to results similar to those provided by pure methanol. Therefore, surfactant media can replace organic toxic solvents such as methanol, which is of great interest in the case of natural products. On the other hand, ionic liquids (ILs) also seem to favour leaching kinetics by displacing the system equilibrium. Although few examples are in the literature, the first evidences of these benefits have recently been published, as the method for extraction of organic acid preservatives from glace fruits (29). However, cautions must be taken on the use of ILs in dealing with intake of compounds extracted with them as their long-time effects are unknown.

5.4. Solvent to feed ratio

The amount of solvent required for efficient leaching strongly depends on the extraction mode. Static SHLE usually involves using less than 15 mL of solvent for sample sizes ranging from 1 to 5 g. Obviously, bigger extraction cells can be used for extraction of higher amount of material. Once the extraction equilibrium is reached, the compounds extracted are rapidly collected by flushing the extraction cell with solvent and an inert gas; as a result, the matrix contains a residual amount of the original solutes, which depends on its partition equilibrium. When new solvent is added, the partition is slightly displaced, and as a consequence more solutes are solubilised. To complete the mass transfer several extraction cycles may be needed in unfavourable cases.

In the dynamic ASE, the solvent is continuously circulated through the extraction cell, so the volume that contacts the sample is a function of the flow-rate and extraction time. If the extraction efficiency does not change with the flow-rate, then extraction is limited neither by solubility nor by the equilibrium of mass transfer between the matrix and the solvent, so the rate-determining step of the process is diffusion inside the solid particles. In this case, the extraction rate can be increased by raising the extraction temperature. However, if the limiting factor is the solubility of the target compound, doubling the flow rate or the amount of solvent used will double the amount of solutes extracted over the same range; if the solutes undergo several re-adsorption/desorption steps during

elution from the extraction chamber, doubling the flow rate will also double the extraction rate.

Flow rates reported for most SHLE methods described in the literature range from 0.5 mL/min to 3.0 mL/min for protocols involving sample size ranging from 1 to 5 g. Rates below 0.5 mL/min are not recommended because they can easily cause blockage of the restrictor used to maintain the pressure in the system; also, rates above 3 mL/min provide diluted extracts.

5.5. Sample composition

The sample matrix has a critical influence on extraction of target compounds. Solid samples can differ significantly in physicochemical properties and matrix composition. However, the influence of sample composition is scarcely considered in experimental strategies for optimization. This parameter can be only evaluated by comparison of extraction protocols for the same group of compounds carried out with different types of samples. As an example, the temperature used for extraction of phenolic compounds depends strongly from the sample composition. Thus, phenolic compounds have been extracted from olive leaves at 140 ºC (30) while the same compounds required 200 ºC for extraction from olive pomace (31). It is worth mentioning that both protocols were optimized with the same extraction system.

5.6. Particle size

Particle size is important parameter that affects extraction efficiency. The influence of particle size depends on the mass transfer mechanism that determines the efficiency in ASE. Obviously, if the ASE efficiency is determined by diffusion, it can be substantially increased by decreasing particle size up to a limit, below which the efficiency is decreased owing to technical limitations. Other variables with a direct influence on ASE efficiency include sample aging, moisture and presence of dispersant agents. Particle size is not frequently

included in optimization studies. In studies in which this variable is taken into account the common particle size is below 0.4 mm (4–6).

5.7. Extraction time

Extraction time in ASE is very short as compared to conventional solid–liquid extraction techniques such as Soxhlet or maceration, and depends on the mass transfer phenomenon that determines the extraction rate. In the static mode, 5–20 min often suffices to ensure quantitative extraction of the target fraction. This is not the case for complex matrices of polymeric structures as wood, in which extraction should be continued over 30–60 min to release monomer compounds with interest for preparation of smoke liquid flavouring (6). However, extraction in the static mode is not always quantitative, especially with a single step, since the species to be extracted partition between the solvent and the matrix; as a result, the process is more or less quantitative depending on the partition coefficient for the system in question. In this case, a kinetics study would allow setting the optimum extraction time to isolate the fraction of interest.

Concerning the dynamic mode, where the solvent is continuously circulated through the sample, the leaching process is generally more efficient since the sample is continuously brought into contact with fresh solvent. Dynamic extraction time usually range from 5 min to 30 min, although special applications could demand for longer extraction times in unfavourable cases of complex samples.

6. Comparison of ASE with other extraction techniques

The exploitation of natural products isolated from different animal and vegetal sources is a growing trend due to the number of fields interested in these products. The bioactivity of natural products is gaining attention of industries for production of drugs, cosmetics, nutraceuticals or foods, among others. Taking into account the relevance of sample preparation in this scenario it is worth discussing the suitability of ASE *versus* other solid–liquid extraction techniques presently used to isolate natural products. Prior to entering into discussion, some general aspects are briefly described to introduce the development of modern extraction techniques.

Classical extraction techniques such as maceration, Folch or Soxhlet extraction are typically ascribed to time-consuming protocols and often lead to non-reproducible results, low selectivity and/or low extraction yields. Consequently, repeated extraction cycles are necessary in most cases to obtain high extraction efficiencies. All these aspects constitute crucial reasons to explain why their implementation in the industry or in studies involving the analysis of a great number of samples is not an easy issue. Apart from these aspects, “green” extraction techniques are gaining attention over traditional extraction techniques for isolation of natural products taking into account that they are frequently used for human consumption. Conventional extraction techniques, frequently using high volume of organic solvents, involve potential danger, not only for the environment but for the laboratory personnel who suffer the consequences of a continuous exposition to a toxic atmosphere.

With the aim of circumventing these negative aspects, exhaustive investigation has been carried out for more than thirty years to develop new techniques that should fulfil the following aims in comparison to classical techniques: higher efficiency, less solvent consumption, ease of automate, more economical and with lower impact on the environment and human health. The

result of this exhaustive research has been modern extraction techniques which have widely proved the previously cited benefits. The most important modern extraction techniques are microwaves- and ultrasound-assisted extraction (MAE and UAE, respectively) and supercritical fluid extraction (SFE), in addition to ASE. In the light of their characteristics, all of them can give place to "green extraction protocols" and more efficient processes.

The importance of natural products in the last years has promoted numerous studies aimed at comparing the extraction efficiency of classical and modern techniques (26,32–39). Taking into account the wide acceptance of ASE for isolation of natural products, it is one of the techniques most frequently involved in these studies. One notable example in which different extraction techniques were compared is the study of Dawidowicz *et al.* who compared ASE with steam distillation, Soxhlet, SFE and headspace solid-phase microextraction (SPME) for isolation of essential oil components from *Thymus Vulgaris* L. ASE extracts were characterised by the highest yield of essential oil components providing similar results to steam distillation, which is recognised as the standard technique for extraction of essential oils in aromatic plants (35). Additionally, the ASE protocol required the shortest extraction time (10 min versus 20 min for SFE and 3 h for steam distillation and Soxhlet).

One other representative study was developed by Luque-Rodríguez *et al.*, in which commercial extracts obtained from skins of red grapes by maceration protocols were qualitatively and quantitatively compared to extracts obtained by ASE (40). This study enabled to conclude that ASE extraction at laboratory level allows obtaining extracts richer in certain families of phenolic compounds than the commercial ones. Indeed, spectrophotometric data demonstrated that the proportion of total flavanols with respect to total anthocyans and phenols was much higher in ASE extracts, as well as the percentage of monomeric anthocyanins in total anthocyans (34.4 % in ASE *versus* 17.4 % and 20.6 % in two different commercial extracts, respectively).

In other cases the extraction efficiency is replaced by other parameters related to the bioactivity of the extracts. Hossain *et al.* reported that extracts from spices as sage (*Salvia officinalis L.*), basil (*Ocimum basilicum L.*) and thyme (*Thymus vulgaris L.*) obtained by ASE had a higher antioxidant capability than extracts isolated by conventional solid–liquid extraction (41). SFE by using CO_2 as solvent has been the technique competing with ASE due to the fundamentals of both techniques. CO_2 is a non-toxic, non-flammable and non-corrosive fluid, which is especially suited to obtain natural products for human consumption. One other benefit is that CO_2-SFE is the only technique that allows obtaining a powder without the need of drying (42). However, it is mainly limited to non-polar and medium polar substances due to the non-polar character of CO_2 (43). One other limitation is the high acquisition and maintenance costs of SFE equipment as compared to ASE, the fundamentals of which support the scaling up of the extraction process for implementation at an industrial level.

Accelerated liquid extraction is generally performed with organic solvents (32,35) or in aqueous solutions (41). The current trend is to optimise protocols by using organic solvents compatible with human consumption such as ethanol or acetone. These solvents can be easily removed at very low trace level. Nevertheless, in the last years there is a trend in ASE for the use of water as solvent to convert protocols in completely “green” methods. This is reflected in the considerable number of studies focused on the use of ASE from an environmental perspective proposed in the last years (44,45). In addition, due to ASE fundamentals, it can also be used to extract compounds exhibiting low solubility in the solvent selected. One recent example has been reported by Euterpio *et al.* in the extraction of curcumin from the *turmeric rhizome* using SHLE by adjusting the pH of water as solvent (46).

Water is a highly polar solvent with a high dielectric constant (ε_r = 80.1) at room temperature and pressure, characterised by the extensive presence of intermolecular hydrogen bonding. However, when the temperature is raised, its permittivity coefficient rapidly decreases as well as its viscosity and surface

tension. The associated increase of diffusivity and density under these conditions also makes water especially suited for extraction. From a practical point of view, water can be easily maintained in the liquid state at temperatures up to 250 ºC by keeping the pressure above 5 MPa. Under these conditions, its dielectric constant decreases up to 27; this means that its polarity index is between those of methanol and ethanol at 25 ºC. Therefore, water under these conditions exhibits similar behaviour to some organic solvents that are widely used to dissolve a broad range of medium and low polarity compounds, and can thus serve as an alternative to traditional organic solvents (see **Figure 5**).

SFE has been successfully used to extract phenolic compounds from grape pomace using CO_2 modified with methanol (47–49) or ethanol as co-extractant (50). Some other studies have compared SFE to ASE as that carried out by Herrero *et al.* in 2010, which revealed that bioactive extracts isolated from rosemary plants using ASE reported not only higher extraction efficiencies (up to 38.6 % for ASE working under 200 °C and 10 MPa for 20 min *versus* 6.5 % for SFE under 40 °C, 10 MPa and 300 min with 7 % of methanol as a modifier of CO_2), but also higher antioxidant capabilities measured by the DPPH radical scavenging method (18.2 ± 0.1 μg/mL EC_{50} for ASE *versus* 12.1 ± 0.0 μg/mL EC_{50} for SFE) (34).

In addition to the benefits discussed above, ASE has a number of other advantages over other modern techniques. Notably, the use of high pressure may facilitate extraction from samples in which the solutes are trapped in the pores of the sample matrix. The high pressures used may force water into areas of the matrices that would not be accessible to solvents under atmospheric pressure.[1] Moreover, the reduced risk of contamination with exogenous chemicals is an attractive feature, especially when preparing extracts for human consumption (in cosmetics, drugs, foods, etc.). One other complementary factor is that ASE is an ideal choice for the extraction of non-stable compounds such as colorants. The absence of light and air in the extraction chamber reduces significantly degradation and oxidation of these compounds during extraction (51).

One other competing alternative with ASE is the assistance of leaching by microwaves, which have been deeply investigated and applied in analytical chemistry during the last decade to accelerate sample digestion and chemical reactions, and to enhance leaching of solutes from different solid matrices. Microwave energy is a non-ionising radiation that causes molecular motion by migration of ions and rotation of dipoles without changing the molecular structures if the temperature is not too high. Therefore, non-polar solvents such as hexane and toluene are not affected by microwave energy, so it is necessary to add polar additives. MAE is an efficient extraction technique for solid samples and is applicable only to thermally stable compounds, which, in certain way, is common to ASE. Similarly to ASE, MAE has became a viable alternative to conventional methodologies due to many substantial improvements over other sample preparation techniques such as reduced extraction time and lower amount of solvent (52–54). There are numerous studies comparing the extraction efficiency of ASE and MAE for isolation of natural products. Recently, Taamalli *et al.* have evaluated the leaching efficiency of MAE and ASE together with SFE and conventional solid–liquid extraction for recovering phenolic compounds from Tunisian olive leaves (55). The evaluation was supported on the analysis of the extracts by LC–ESI–TOF/MS and LC–ESI–IT/MS^2. Higher extraction yields were obtained for ASE followed by MAE using the Folin-Ciocalteu test, while phenolic profiles showed a larger number of phenolic compounds in the extracts obtained using MAE followed by the conventional method. In general, MAE and conventional methods were the best choice for extracting more polar compounds such as oleuropein derivatives, apigenin turinoside and luteolin glucoside. On the other hand, SFE and ASE were more efficient to extract compounds with less polarity such as apigenin, luteolin or diosmetin.

Other comparative studies have revealed the superiority of ASE over MAE as in that carried out by Delgado de la Torre *et al.*, who have recently compared UAE, MAE and ASE to obtain extracts enriched in bioactive compounds from vine-shoots (17). This study clearly supported ASE as the best extraction technique for isolation of phenols followed by UAE, for which the

global determination test based on the Folin-Ciocalteu reagent was used. **Figure 6** shows the results of extraction efficiency provided by the three extraction techniques in terms of hydroxymethylfurfural and total phenolic concentrations. SHLE led to extracts with higher concentration of total phenols but also with higher concentration of hydroxymethylfurfural formed by degradation of lignocellulosic material.

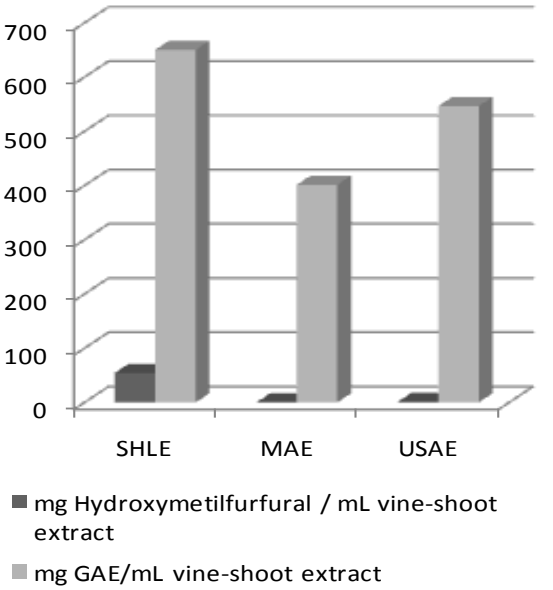

Figure 6. Concentration of hydroxymethylfurfural (mg/mL) and total phenolic content expressed as mg equivalent to gallic acid per mL of vine-shoot extract obtained by the Folin-Ciocalteu method obtained by SHLE, MAE and UAE approaches.

One other auxiliary energy used to assist solid–liquid extraction is ultrasound. Ultrasound-assisted extraction shares some of the advantages of MAE in terms of enhancing leaching kinetics, reducing solvent volume and possibility of automation. However, UAE is particularly useful for isolation of thermolabile compounds or for sample matrices where disruption favours considerably the contact between solid and liquid phases. In this sense, UAE competes with ASE thanks to the working temperatures reached in UAE, most times at (or close to) ambient conditions. Apart from these aspects, a common

aspect to ASE is the low cost of the extraction process thanks to the simplicity of the equipment required (56). The main shortcoming of UAE *versus* ASE is the potential formation of free radicals generated by sonolysis of the solvent, which can produce degradation of some labile compounds by oxidation (57). A comparison of the feasibility of ASE *versus* UAE was carried out by Fojtová *et al.* who applied these extraction methods to walnut-tree leaves prior to GC–MS analysis for quantification of terpenes (58). The efficiency of ASE performed with *n*-hexane at 150 °C and 15 MPa in three cycles of 5 min was superior to that of UAE at room temperature using *n*-hexane for 1 h (198.7 μg/g total terpenes *versus* 59.2 μg/g, respectively). Nevertheless, the relative distribution of particular terpenes changed depending on the extraction method, *i.e.*, the relative concentration of β-pirene and limonene was higher for UAE. On the other hand, the final extracts obtained by ASE were found to be clean enough for direct analysis by GC–MS without need for any pretreatment. This is a great benefit since when working with volatile compounds, every additional handling of samples increases the risk of losses.

7. Applications of ASE for the isolation of natural products

Since SHLE was born around 1995, its number of applications has increased exponentially. At the beginning, SHLE arose mainly as a preparation technique to replace conventional techniques based on traditional protocols. In the first period, the trend on leaching applications was primarily marked by a competing technique such as SFE. In fact, US Environmental Protection Agency (EPA) introduced SFE as sample preparation technique in several official methods in the environmental field. However, SFE has not fulfilled the initial expectations at present while ASE has growing to achieve an unpredicted impact at early years. Nowadays ASE has consolidated its position in some fields, mainly

environmental and food analysis, but also it has covered the area of natural products which is characterised by a high number of application fields.

The evolution of ASE in the area of natural products can be charted with bibliographic surveys of publications using "ASE", "PLE" or "SHLE" as key terms. Figure 7.A shows the number of papers published from 1995 to 2011 found in three databases, namely, Scifinder research tool (which retrieves information contained in databases produced by Chemical Abstracts Service, MEDLINE database and CAplus database). The number of ASE applications grew rapidly from 1999 to 2007. Then, the situation was stabilized until 2010 with a new increase. Figure 7.B shows a distribution of the number of papers according to the name given to the technique. This graph confirms that although ASE is a widely extended name from a commercial point of view, the term "pressurised liquid extraction" is more accepted in the field of natural products in the last years. Nevertheless, the term "pressurised liquid extraction" is not well founded.

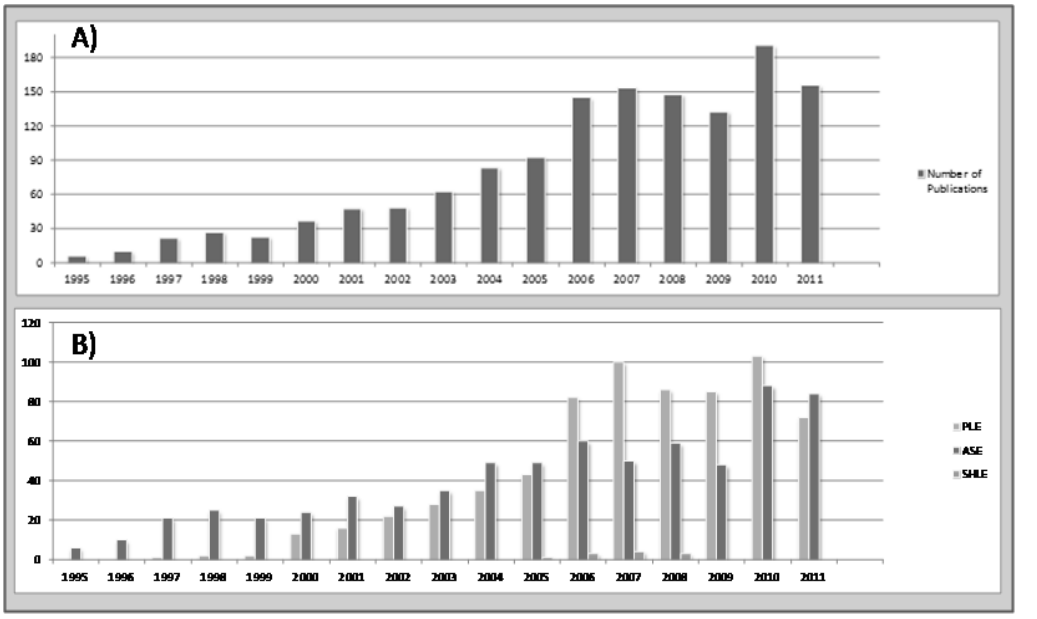

Figure 7. (A) Number of publications involving the utilisation of ASE for extraction of natural products in the period 1995-2011. (B) Distribution of the number of publications according to the nomenclature adopted by the authors.

The wide applicability of ASE in the area of natural products is linked to the diversity of samples extracted with this solid–liquid extraction technique. Vegetal material such as wood, leaves, branches, flowers, fruits, gum, vegetables, agriculture residues, among others, have been treated by ASE. This section has been organised from the perspective of the type of compounds extracted. Thus, three subsections are reviewed to discuss ASE applications for extraction of lipids, volatile compounds and polar compounds. Due to the variability of compounds that could be qualified as polar, the potential application of the isolated natural products will be considered. **Table 1** summarises the protocols used for extraction of different compounds with special emphasis on operational conditions and preparation of extracts, if required, prior to analysis.

7.1. Lipids

Lipidic natural products constitute one of the main compound classes extracted by ASE. Numerous studies have been proposed with common methodological aspects in the extraction protocols. One particular case is that for extraction of total lipids from food for preparation of nutritional supplements. A recent example is the study by Zhou *et al.* who used ASE for extraction of phospholipids from various food matrices, namely: soybeans, egg yolk, calf brain and ox liver (59). The protocol was based on the use of 2:1 chloroform–methanol (v/v) at 10 MPa and 140 ºC for 5 min. These conditions led to recovery of over 96 % phospholipids in each type of food by one single extraction step. The Folch method, a conventional approach for isolation of lipids, required up to four successive extractions to obtain similar leaching efficiency values.

The main application field in the extraction of lipids is the isolation of bioactive compounds such as saponins, liposoluble vitamins, terpenoids, sterols, etc. One example is the research developed by Zhao *et al.* who extracted bioactive compounds as saponins together with fatty acids from a Chinese medicine plant (*Ziziphus jujube,* Suanzaoren) (60). The extraction protocol in this case was based on a 95:5 methanol–ethyl acetate (v/v) solution and the conditions used

were 140 ºC and 8.2 MPa for 15 min. In this type of applications, the stability of the fraction of interest is crucial. Thus, in the extraction of vitamin E isomers from seeds and nuts developed by Delgado-Zamarreño *et al.* two extraction cycles were required since the optimum temperature for extraction was set at 50 ºC (61).

The main limitation of these applications is the need for organic solvents owing to the lipidic character of these compounds. In this sense, the use of non-toxic organic solvents such as ethanol or acetone should be promoted. Denery *et al.* tested different solvent compositions for isolating carotenoids and kavalactones from green algae (62). Among these solvents it is worth mentioning those consisting of acetone, ethanol, 7:3 acetone–ethanol (v/v) and 1:3 methylene chloride–methanol (v/v) mixtures. The methylene chloride–methanol mixture provided the best extraction results by monitoring efficiency for isolation of total pigments, astaxanthin and lutein.

In cases where different families of lipids are co-extracted, the fractionation scheme proposed by Poerschmann *et al.* can be used (63). Essentially, the protocol is based on a two-step extraction process using 9:1 *n*-hexane–acetone (v/v) at 50 ºC to obtain neutral lipids followed by 1:4 chloroform–methanol (v/v) at 110 ºC to obtain polar lipids. The extraction process was combined with an in-cell fractionation using silica-based sorbents (silicic acid or cyanopropyl silica) placed at the outlet of the extraction cell. Thermally pre-treated sorbents were appropriate to ensure clear-cut boundaries between neutral lipids and phospholipids. The proposed protocol is superior to the approaches commonly used which consist of an exhaustive lipid extraction followed by off-line fractionation using SPE, in terms of fractionation efficiency, time and solvent consumption.

Table 1. Characteristics of ASE protocols used for analysis of natural products.

Sample	Compounds	Pre-treatment	ASE				Post-treatment	Technique	Ref.
			Solvent	T (°C)	Cycles	Extraction time (min)			
Pharmacologically active compounds									
Ginseng	Gingenosides	Dried sample	Water + TritonX	50–120	1	10	Not required	LC–UV	28
Rumex nepalensis Spreng. Roots	Naphtalene and Anthraquinone	Not required	MeOH	60	1	10	Concentration	LC–DAD	33
Trifolium L.	isoflavones	Dried sample	MeOH, MeOH-water (75:25, v/v)	75–125	4	5	Concentration	LC–DAD	86
Medicinal plants and health supplements	Gingenosides	Sample between sand layers	MeOH	140	Dynamic mode (1 mL/min)	20	Not required	LC–DAD	87
Ginger	Gingerol-related compounds	Dried sample	Bioethanol/water (70%)	100	1	5	Not required	LC–MS	88
Medicinal plants	Aristolochic acids	Sample/sand	MeOH	120	Dynamic mode (1.5 mL/min)	20	Not required	LC–DAD	90
Kava root	Kavalactones	Not required	Water	175	Dynamic mode (1 mL/min)	20–40	LLE	GC–FID	92
Plant leaves	Glycosides	Not required	Water	100	Dynamic mode	30	LLE	MEKC–DAD	93
Medicinal plants	Aristolochic acids	Sample/sand	MeOH	120	Dynamic mode (1 mL/min)	20	Not required	CZE–UV	94
Medicinal plants	Alkaloids	Sample/sand	MeOH	120	Dynamic mode (1 mL/min)	20	Not required	CZE–UV	95
Coca leaves	Cocaine, benzoylecgonine	Sample/sand (1:3, w/w)	MeOH	80	Dynamic mode (1 mL/min)	10	Concentration	GC–MS	96
Medicinal plants	Alkaloids	Sample/sand	Water or water/ethanol	95–140	Dynamic mode (1 mL/min)	40	Concentration	LC–UV	97
Natural health products	Ephedrine, pseudoephedrine and metabolites	Sample/Ottawa sand	Water/3% MeOH	90	3	5	Not required	FI–MS	98

Grape seeds and skins	9 phenols	Sample/ sea sand	MeOH	100/150	3	10	Not required	LC–DAD	103
Grapes	6 phenols	Sample + LiChrolut EN sorbent layer	Two extractions (1) Water 40 °C, 150 atm (2) MeOH 100°C, 40 atm	100	3	10	Not required	LC–DAD	104
Aromatic plant (sage)	Phenolic diterpenes, phenolic acids	Sample/sea sand	Water	100	Dynamic mode (1 mL/min)	60	SPE	LC–MS	105
Rosemary leaves	Phenolic diterpenes, flavonoids	Not required	Water	25-200	Dinamic mode (1 mL/min)	30	Freeze-drying	LC–MS, LC–DAD	106
Carob pods (Mediterranean leguminosae)	41 phenols	Sample/DE (1:2, w/w)	Acetone/water (1:1, v/v)	60	2	5	SPE	LC–UV LC–MS	107
Tea leaves and grape seeds	Flavanols	Sample/sea sand	MeOH	130	2	5	-	LC–DAD	108
Microalga	Polyphenols	Not required	Hexane, light petroleum, ethanol, water	115/170	1	9, 15	Concentration	MEKC–DAD	109
Soybean food	Isoflavones	Florisil/sand	Two extractions: (1) Hexane (2) 60% MeOH/ 0.3%FA	100	2	5	Concentration	LC–ED	110
Anatolia propolis	13 phenols	Not required	Ethanol/water/HCl (75:20:5, v/v/v)	40	3	15	Not required	LC–DAD	111
Essential oils									
Thymus vulgaris L.	Essential oils	Air-dried	n-hexane, DCM, ethyl acetate and distilled water	20–175	1	5-30	LLE	GC–MS	10
Chinese medicine	Essential oils	Not required	Water	160	Dynamic mode (1 mL/min)	5	(HS)-LPME	GC–MS	32
Mate tea leaves	Essential oils	Air-dried	N-hexane, toluene, DCM,	100	1	10	Not required	GC–MS	38

Medicinal plants	Three glycosides	Sample/sand	Water + Triton-100	95	Dynamic mode (1 mL/min)	40	SPE	LC–MS	99
Fruit	Furanocoumarins	Sample/neutral glass	MeOH	100	1	10	Concentration	LC–DAD	100
Chinese herbal medicine	Rutin and quercetin	Dried sample	1-Butyl-3-methylimidazolium (1M)	125	1	5	Not required	LC–CL	101
Phenolic compounds									
Malt	Proanthocyanidin	Sample/DE	Acetone/water (4:1, v/v)	60	2	10	Automated SPE	LC–UV LC–MS	12
Rosmarinus officinalis	Bioactive phenolic compounds	Air dried	Ethanol, water	100/200	1	20	Concentration	LC–DAD LC–MS	32
Red grape skins	Anthocyanins and other phenols	Dried sample	Ethanol/water (1:1)	120	1	30	Not required	LC–DAD LC–MS	40
Sage (*Salvia officinalis L.*), basil (*Ocimum basilicum L.*) and thyme (*Thymus vulgaris L.*)	Total phenolic content	Dried sample	32–88% methanol	66–129	1	10	Filtration	LC–DAD	41
Tunisian olive leaves	37 phenols	Ground under liquid N_2	Ethanol	150	1	20	Not required	ESI-TOF/MS ESI-IT/MS^n	55
Vine-shoots of *Vitis vinifera*	14 phenols	Dried sample	Ethanol/water (80%)	240	1	60	LLE	LC–DAD	56
Golden apple	12 phenols	Sample/DE (1:1, w/w)	MeOH	40	2	5	Concentration	LC–DAD	75
Soybeans	Isoflavones	Freeze-dried sample/sand	Ethanol/water (70:30, v/v)	100	3	7	Not required	LC–DAD LC–MS	85
Cider apple	16 phenols	Freeze-dried sample/DE (1:1, w/w)	MeOH<	40	2	5	Not required	LC–DAD	102

			ethyl acetate, acetone and MeOH.						
Origanum onites	Essential oils	Air-dried	Water	100/175	Dynamic mode (2 mL/min)	30	SPE	GC–TOF/MS	47
Medicinal plant (fennel)	Monoterpenes, oxygenates	Not required	Water	150	Static-dynamic mode (2 mL/min)	30 +20	LLE	GC–FID GC–MS	77
Rosemary	Terpenes, oxygenates	Not required	Water	150	Dynamic mode (2 mL/min)	30	LLE	GC–FID	112
Peppermint	Oxygenates, carbophyllene	Air dried sample	Water	125-150	Dynamic mode (1 mL/min)	20	LLE	GC–MS	113
Majoram leaves	Terpenes, pinenes alcohols	Not required	Water	150	Dynamic mode (2 mL/min)	15	LLE	GC–FID GC–MS	114
Laurel	Essential oils	Not required	Water	150	Static-dynamic mode (2 mL/min)	15 + 25	LLE	GC–FID GC–MS	115
Savory and peppermint	Terpenes, oxygenates	Air dried sample	Water	100-175	Dynamic mode (2 mL/min)	12-40	LLE	GC–FID GC–MS	116
Oregano	11 Oregano oil compounds	Not required	Water	125	Dynamic mode (1 mL/min)	24	LLE	GC–FID GC–MS	117
Lemon grass	Essential oils (neral, geranial, geraniol, limonene)	Not required	Hexane, DCM, Acetone, MeOH	40	3	10	Not required	GC–FID	118
Thymbra spicata L.	Essential oils	Not required	Water	150	Dynamic mode (2 mL/min)	30	SPE	GC–TOF/MS	119
Lime peel	Essential oils	Not required	Water/MeOH or ethanol	130	Static-dynamic mode (1 mL/min)	5 + 15	LLE	GC–FID GC–MS	120
Chinese medicin	Essential oils	Not required	Water	150	Dynamic mode (1 mL/min)	5	SPME	GC–MS	121
Fat matter									
Alperujo	Phenols and fatty acids	Dried at 70°C for 24h.	MeOH/water (80%)	200	Static-dynamic	10–13	LLE	GC–MS	7

Piper gaudichaudianum Kunth	Terpenes, fatty acids and Vitamin E	Air dried	Ethanol, pentane	85–150	1	10	Concentration	GC–MS	37
Egg yolk, ox liver, calf brain and soybean	Total lipids and glycerofosfolipids	Freeze drying	$CHCl_3/CH_3OH$ (2:1, v/v)	120/150	4	5	LLE	LC–ELSD	59
Ziziphus jujuba	Saponins and fatty acids	Powdered sample/diatomaceous earth (1:1)	MeOH/ethyl acetate (95:5)	140	1	15	Not required	LC–ELSD	60
Egg containing food	Oxysterol	Sample/celite	Hexane/isopropanol (3:2, v/v)	60	2	8–10	Concentration	GC–MS	122
Dairy products	Fat	Sample/celite	Hexane, DCM, MeOH, petroleum ether, acetone, ethanol, isopropanol	80–120	2	8–10	Concentration	Gravimetric	123

Superheated liquid extraction has also been used for specific applications to study or predict biological processes. One example is the use of SHLE for simultaneous isolation of certain families of lipids (straight-chain lipids, plant sterols and terpenoids) from sandy soil profiles under Corsican pine material (64). These compounds can be used as vegetation tracers, based on the principle that plant-specific combinations of lipids are preserved in soils and can act as biomarkers to identify past vegetation compositions. The solvent in this case was a 93:7 dichloromethane–methanol (v/v) mixture at 75 ºC, 6.9 MPa and 20 min as processing time.

7.2. Volatile compounds

Superheated liquid extraction has frequently been used for isolation of volatile compounds from plants and foods. Among the great number of studies on SHLE extraction of aromatic volatile compounds it is worth mentioning those dealing with compounds contributing to aroma fractions or food flavour. A particular study is that reported by Cincchetti *et al.* in 2009, who proposed a method based on ASE for the authentication of natural vanilla flavors in foods by using detection techniques based on isotopic ratio distributions (65).

Wood material has also been a vegetal source used for isolation of volatile compounds. Vichi *et al.* (66) and Natali *et al.* (67) focused their research on the study of volatile and semivolatile components extracted from oak wood chips. Both studies used ASE to obtain the extracts following the same extraction method: 5 g of sample, 15 mL dichloromethane as solvent, leaching temperature of 150 ºC, pressure of 20 MPa, extraction time of 7 min. Similar results were obtained in characterisation by both studies with slight differences as the presence of solerone and two C-13 norisoprenoids identified by Natali *et al.* or the identification for the first time in oak wood of ten lignin dimmer derivatives by Vichi *et al.* A key conclusion from both studies was that the toasting degree was the variable with stronger influence on composition of wood extracts. Also focused on the use of ASE with wood is the study published in 2011 by

Dawidowicz *et al.* for isolation of essential oil. This study also monitored the extraction yield as a function of the purge time during the ASE procedure (68). Longer purge times led to losses of volatile organic compounds.

Volatile compounds usually correspond to secondary metabolites present at low concentration. The optimisation of the extraction step was focused on them. Numerous examples dealing with optimisation of SHLE to obtain profiles from secondary metabolites can be found in the literature. Thus, two representative examples are those of Cho *et al.*, who worked with *Angelica* roots (69), and Liu *et al.*, who used *Nigella sativa* seeds (70), both herbaceous plants commonly used for medicinal purposes. Both extraction procedures were similar with certain differences associated to the sample matrix. Thus, both protocols used *n*-hexane as leaching solvent, but higher temperature and pressure (100 ºC and 10 MPa) were applied by Liu *et al.* due to the nature of seeds, a more complex material to be extracted than plant material used by Cho *et al.* (80 ºC and 7 MPa).

The volatile fraction from tobacco has been studied using ASE by Vial *et al.*, who used the extracts for discrimination among different varieties of tobacco products. For this purpose the extracts were analysed by GC×GC–MS (71). Previously, Shen *et al.* applied ASE to different varieties of tobacco for extraction of terpenoids and sterols prior to characterisation of both fractions (72). The ASE-based method exhibited better reproducibility and extraction yields than classical methods.

7.3. Polar compounds

Most of applications dealing with ASE and natural products are focused on the extraction of polar compounds. Due to the great variety of these applications in terms of chemical properties of compounds isolated and the diversity of sample matrices, they are distributed depending on the final use of the extracted components as antioxidants, essential oils, nutraceuticals or drugs.

7.3.1. Antioxidants

Antioxidant compounds are gaining popularity in the last years thanks to their beneficial properties for human health, but also as food preservatives and dietary supplements. Attending to the potential uses of antioxidants in the clinical and food fields, strong efforts have been made in the last years to obtain antioxidants from a wide range of natural sources, mainly from waste materials from the agrofood industry. Thus, extensive research has been carried out in the Mediterranean countries to isolate antioxidants from residues of the olive oil industry. Taking into account that these extraction protocols are pretended to be implemented at industrial level, ASE has been one of the preferred options for solid–liquid extraction of antioxidants. Different materials from Olea europaea have been characterised because of the high content of antioxidant phenolic compounds. Japón-Luján *et al.* optimised an ASE method for isolation of extracts enriched in oleuropein and other bioactive phenols such as verbascoside, apigenin-7-glucoside and luteolin-7-glucoside from olive leaves (30). Under optimal working conditions, complete extraction without degradation of the target compounds was achieved in 13 min. The same authors worked in 2007 with olive pomace, a semisolid residue from the olive oil industry, and used ASE to obtain potent antioxidants such as hydroxytyrosol together with other olive phenols. The extractions were carried out with ethanol–water mixtures, which provided an added value to the extraction protocols due to compatibility of extracts for human consumption (31).

Residues from the wine industry have also been extensively studied using ASE for isolation of phenolic compounds. Luque-Rodríguez *et al.* proposed a method for extraction of phenols from grape skins by ASE using ethanol–water mixtures (40). Higher extraction efficiencies were obtained by ASE for certain families of phenols such as anthocyanins, total phenols and flavanols than those provided by conventional solid–liquid extraction based on maceration. The same authors also proposed ASE as extraction technique for isolating phenolic compounds and derivatives from vine-shoots of Vitis vinifera (73). This research

proved the significance of the extraction conditions on the qualitative profile of the compounds extracted. The extracts obtained under operation conditions that maximised the concentration of total phenols were especially rich in low molecular mass compounds from lignin degradation (e.g. vanillin, syringaldehyde), while those extracts obtained at low temperature were richer in phenolic acids, particularly those formed by hydrolysis of tannins. This research supports the great chemical variability of antioxidants obtained depending on the ASE extraction protocol. Ju *et al.* have studied the effects of the solvent and temperature on the extraction efficiency of anthocyanins and total phenols from dried red grape skin (74). They revealed the significance of both variables on the qualitative profile of the extracts. As example acidified water extracts obtained at 100 ºC reported the highest levels of monoglucoside phenols; acidified methanol extracts obtained at 60 ºC provided the highest levels of total anthocyanins while at 120 ºC the extract contained the highest level of total phenols.

Phenolic extracts have also been obtained from diverse materials such as apple peel and pulp (75) or rosemary (Rosmarinus officinalis) (34). Herrero *et al.* have compared the phenolic extracts obtained from rosemary using various extraction techniques and they concluded that SHLE was the most efficient alternative in terms of extraction yield, antioxidant activity and total concentration of phenols (34).

7.3.2. Essential oils

Essential oils are very appreciated thanks to their applications in pharmaceutical, cosmetic and food industries. For this reason, it is of great interest to characterise essential oil components isolated from different varieties of aromatic plants. In order to preserve the stability of essential oils, high-efficient, fast, simple and automatable methods are demanded for preparation of extracts. The most common techniques for the isolation of essential oils have been classical steam distillation and maceration. However, the loss of volatile compounds that contribute to the quality of essential oils, low extraction

efficiency and degradation effects are common in methods based on classical extraction techniques. That is the reason why other alternatives such as ASE have been implemented for preparation of essential oils.

The number of studies found in the literature supports the superiority of ASE for the extraction of essential oils *versus* conventional alternatives. In fact, a number of studies compare qualitatively and quantitatively the essential oils obtained using ASE with those obtained using other approaches. One example is the research carried out by Tam *et al.*, who extracted pharmacological essential oils from *Cyperus rotundus* using three different techniques, hydrodistillation, SFE and ASE (76). The last exhibited the highest extraction efficiency for α-copaene, cyperene, β-selinene, β-cyperone and α-cyperone, while SFE reported the best selectivity for extraction of β-cyperone and α-cyperone.

Despite the interest of ASE application for extraction of essential oils began practically since this technique was developed, the operating conditions for isolation of essential oils is still subject of study. In 2000 Gámiz-Gracia *et al.* proposed a continuous extraction method using bidistilled water as solvent for the isolation of essential oil from fennel (*Foeniculum vulgare*), a medicinal plant traditionally used for the treatment of several stomach affections and obesity (77). The authors compared their ASE-based method to others based on traditional extraction techniques proving the superiority of ASE in terms of rapidity, efficiency and cleanliness. Water was also used as solvent by Ozel *et al.* for isolation of essential oils from *Thymbra spicata*, where the optimized extraction was run under 150 ºC and 3 MPa for 30 min (78).

On the other hand, Schaneberg *et al.* compared four different procedures for extraction of essential oils from *Cymbopogon citrates* (lemon grass), demonstrating the superiority of ASE in general terms. Apart from that, the composition of the extract was modified as demanded. Dichloromethane extracts contained the highest concentration of marker compounds such as geraniol, limonene, neral and citronellal (13 %), while hexane extracts contained the highest concentration of citral (75 %) (79).

7.3.3. Nutraceuticals and drugs

The term “nutraceutical” was first coined in 1989 by the Foundation for Innovation in Medicine to define “any substance that may be considered a food or part of a food, and provides medical or health benefits, including the prevention and treatment of diseases”. Nutraceuticals do not only maintain, support and normalise any physiologic or metabolic function, but can also potentiate, antagonise, or otherwise modify these functions (80,81). In general, nutraceuticals may include dietary fibers, different types of phenolic compounds and antioxidants, polyunsaturated fatty acids, amino acids, proteins and minerals. Therefore, this concept is not referred to a specific family of compounds.

In the last years, ASE is becoming more and more popular for extraction of nutraceuticals. Among them, isoflavones is one of the most studied groups of compounds due to their widely recognized health benefits against menopausal problems as well as their possible preventive role in breast and prostate cancer, osteoporosis and cardiovascular diseases (82–84). The critical point in the extraction of isoflavones is to avoid degradation since some isoflavone derivatives are particularly sensitive to hydrolysis. Superheated liquid extraction has faced up this limitation with excellent results. Rostagno *et al.* have proved the absence of degradation in the ASE extracts of isoflavones from freeze-dried soybeans. For this purpose, samples were analysed by LC–DAD and LC–MS in a well-planned stability study involving both spiked and real samples (85). Complementarily, Bajer *et al.* proved the efficiency of ASE for isolation of isoflavones from various plants (*Matricaria recutita*, *Rosmarinus officinalis*, *Foeniculum vulgaris* and *Agrimonia eupatoria L.*) (36). In another study, Zgórka *et al.* compared ASE to other extraction techniques for leaching of phytoestrogenic active isoflavones from clover species (*Trifolium L.*) (86).

Apart from these compounds, numerous applications based on ASE for extraction of nutraceuticals can be found in the literature. Lee *et al.* employed an ASE dynamic approach for extraction of ginsenosides from *Panax ginseng* and

American ginseng as well as from health supplements (87). The extraction process was carried out at 10 MPa and 120 ºC for 20 min. In a recent research, Hu *et al.* optimised an SHLE procedure for isolation of gingerols from *Zingiber officinale* Roscoe using ethanol as solvent *versus* water to increase the extraction efficiency (88).

Superheated liquid extraction has been massively used for isolation of natural products from medicinal plants with pharmacological purposes. Most of these applications are focused on extraction protocols to isolate target compounds with known pharmacological effects such as those by Ong for extraction of glycyrrhizin from *Radix glycyrrhizae (*89) or that for isolation of berberine and aristolochic acids from different medicinal plants (90). Other final purpose of extraction is the identification of bioactive components to explain the pharmacological effects of well-known medicinal plants. One example is the study carried out by Lao *et al.* who optimised an ASE method for isolation of bioactive components (ferulic acid, ligustilide and other phthalides such as butylidenephthalide) from *Angelica sinensis* (Danggui), a well-known Chinese medicine plant (91). These active components were identified by GC–MS as a first test to elucidate the proved pharmacological effects of the plant.

8. Case study

As a test to evaluate the efficiency of SHLE for isolation of natural products, this approach was applied to characterization of vine-shoots, an agricultural residue obtained in wine-producing countries. The extraction efficiency was assessed by measurement of total phenolic compounds using the Folin–Ciocalteu test and by analysis of phenolic extracts by LC–DAD. The vine-shoot variety selected for this study was Pedro-Ximénez due to the geographical importance of this variety in the area where the study was developed. Extraction tests were performed with 1 g of dry material.

8.1. Optimization of the main variables involved in SHLE

The influence of the main variables involved in the leaching process was estimated with a multivariate study. These variables were the percentage of ethanol, temperature and extraction time, while the response variable was the yield of phenolic compounds extracted from vine-shoots in a time as short as possible. The tested ranges and the selected values are shown in **Table 2** as well as a detailed information about the designs used and the results obtained. The applied pressure (100 bar) was high enough to guarantee the liquid state of the ethanol–water mixtures in all instances. A complete factorial design was selected for the first approach. The results showed that the three variables had significant positive effects; therefore, the highest value of each variable was chosen as the lowest value in a second complete factorial design. The analysis of the data obtained in the second design showed that the only significant variable was the temperature; the percentage of ethanol and extraction time had negative and positive effects, respectively. According to its effect, 80% ethanol (v/v) was selected. However, in the case of the time, the most reasonable option was to select the shortest (60 min), because the increase of efficiency for longer times was not significant. Under these conditions, higher temperatures (270 and 300 ºC) were tested, thus increasing the amount of total phenolics extracted. Nevertheless, two trends were also detected, which made inadvisable the use of temperatures above 240 ºC: the strong increase of the burnt wood smell of the extract and the decrease in the concentration, or even the disappearance of groups of phenolic compounds such as phenolic aldehydes. Therefore, 240 ºC was the temperature selected for further studies.

8.2. Influence of extraction pH

The influence of pH on the yield of the process under the selected conditions was also investigated by a univariate approach. Thus, extractions were carried out adjusting the pH of the extractant at 1, 3, 5, 7, 9, 11, and 13. **Figure 8** revealed that the yield of phenolic compounds enormously increased with pH decreased. This result can be ascribed to the fact that processing of lignocellulosic

materials with acidified water facilitates the breakage of ether linkages in lignin, especially under high temperature conditions, generating a great number of low molecular weight phenols. Nevertheless, fast corrosion of capillary tubes of the system was observed after only a few extraction cycles at pH 1. Consequently, a minimum pH of 3 was used, for which no trace of corrosion was detected after numerous extractions.

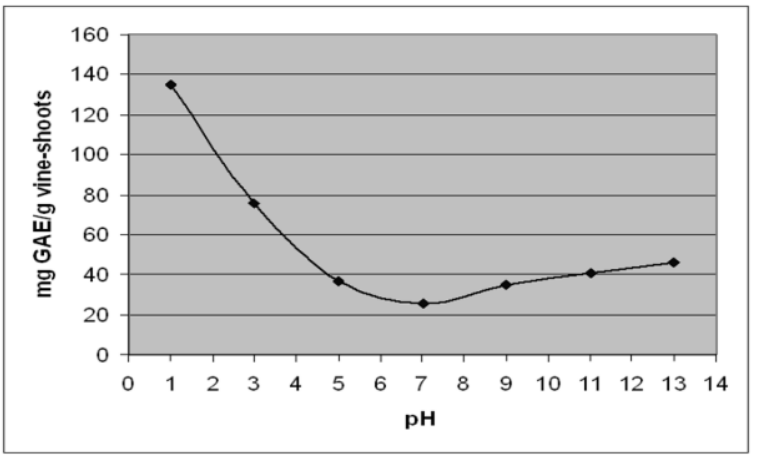

Figure 8. Influence of pH on the efficiency of superheated liquid extraction of phenolic compounds from vine-shoots using 80% ethanol (v/v), 240 ºC and 60 min as extraction parameters. Response variable is the total phenol content measured by the Folin-Ciocalteu test and expressed as mg equivalent of gallic acid/g vine-shoots.

8.3. Comparison of SHLE with MAE and UAE for extraction of vine-shoots

The extraction efficiency of SHLE was compared with that provided by protocols based on MAE and UAE. In both cases 1 g of milled vine-shoots was placed into the extraction vessel with 20-mL 80% (v/v) aqueous ethanol at pH 3. In the MAE process, the vessel was positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 140 W irradiation power for 5 min, after which the solid residue was removed by centrifugation prior to analysis of the extract. For the UAE process, the ultrasonic probe was immersed into the extraction mixture for sonication at 280 W irradiation power

for 10 min with a duty cycle of 70% (0.7 s/s irradiation cycles). Similarly, the extract was isolated by centrifugation prior to analysis.

The leaching efficiencies of the three extraction methods were compared in terms of concentration of total phenols estimated by the F–C test. Attending to the results, SHLE provided the highest concentration of phenolic compounds expressed as μg of gallic acid per gram of initial solid vine-shoots (3252 μg/g versus 2732 and 2007 μg/g obtained with UAE and MAE, respectively). Therefore, SHLE seems to be the suited strategy for extraction of phenolic compounds from vine-shoot, which can be considered a potential vegetal source to obtain this valuable fraction.

9. Conclusions: benefits and limitations of ASE for isolation of natural products

As final conclusion of this chapter it can be said that ASE is one of the most competitive solid–liquid extraction techniques for isolation of natural products. The foundations of the technique, its high-efficiency for leaching, its ease of scaling-up applications to industrial level, its automatability and its adaptation to the "green" concept contributes to the selection of ASE as one of the main techniques for extraction of natural products. The development of new instrumental configurations with high versatility is demanded to improve the possibilities of this technique. Replacement of toxic organic solvents is a pending goal.

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Chapter 2:

Comparison of accelerated methods for the extraction of phenolic compounds from different vine–shoots cultivars

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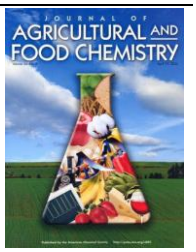

Comparison of accelerated methods for the extraction of phenolic compounds from different vine-shoots cultivars

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Comparison of accelerated methods for the extraction of phenolic compounds from different vine-shoots cultivars

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Abstract

Most research on extraction of high-priced compounds from vineyard/wine by-products has traditionally been focused on grape seeds and/or skins as raw materials. Vine-shoots can represent an additional source to those materials, the characteristics of which could depend on the cultivar. A comparative study of hydroalcoholic extracts from 18 different vineyard cultivars obtained by superheated liquid extraction (SHLE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (USAE) is here presented. The optimal working conditions for each type of extraction have been investigated by using multivariate experimental designs in order to maximize the yield of total phenolic compounds, measured by the Folin–Ciocalteu method, and control hydroxyl-methylfurfural because of the organoleptic properties of furanic derivatives but toxic at given levels. The best values found for the influential variables on each extraction method were 80% (v/v) aqueous ethanol at pH=3, 180 ºC and 60 min for SHLE; 140 W and 5 min microwave irradiation for MAE; and 280 W, 50% duty cycle and 7.5 min extraction for USAE. SHLE reported better extraction efficiencies as compared to the other two approaches supporting the utility of SHLE for scaling-up the process. The extracts were dried in a rotary evaporator, reconstituted in 5 mL methanol, and finally subjected to liquid–liquid extraction with n-hexane to remove non-polar compounds that could complicate chromato-

graphic separation. The methanolic fractions were analyzed both by LC–DAD and LC–TOF/MS and the differences in composition according to the extraction conditions were studied. Compounds usually present in commercial wood extracts (mainly benzoic and hydroxycinnamic acids and aldehydes) were detected in vine-shoot extracts.

1. Introduction

Tons of agricultural waste and by-products from the agrofood industry with no economic value are produced every year all over the world. Some examples of these materials from the Mediterranean basin are olive-trimmings, vine and olive leaves, wine-lees and vine-shoots. Most of these materials have traditionally been used mainly as a heating source or cast upon the ground to rot. However, these uses have drawbacks associated with transportation costs and environmental contamination.

Spain is the country with the largest area in the world dedicated to vineyards, with approximately 1.1 million hectares, being the third wine-producer country, following France and Italy. Thus, the huge amount of vine-shoots produced every year has led to a growing interest in exploitation of this residue. Most research on vine-shoots has been focused on the production of paper pulp and ethanol, the former requiring in-depth studies to improve production as vine-shoots provide pulp of lower quality than other agricultural residues such as wheat straw *(1)*. Some other ways of vine-shoot exploitation, such as tanning and dyeing of leather *(2)*, production/extraction of phenols *(3,4)*, volatile compounds *(5)*, activated carbon for wine treatment *(6)*, lactic acid *(7,8)*, biosurfactants *(7)*, ferulic and coumaric acids *(9)* and production of smoke flavorings *(10–12)* have been investigated.

The composition of vine-shoots is characterized by three main fractions: cellulose, hemicellulose and lignin, where the content of holocellulose

is around 68%, and that of lignin around 20% (dry weigth). Lignin, a well-known component of secondary cell walls, is a high-molecular-mass crosslinked polymer, which is built up by random oxidative coupling of three major C6–C3 (phenylpropanoid) units (monolignols) due to the lack of enzymatic control. These units [namely, *trans-p*-coumaryl (4-hydrocycinnamyl), coniferyl (4-hydroxy-3-methoxy-cinnamyl, forming guayacyl units), and sinapyl (3,5-dimethoxy-4-hydroxycinnamyl, forming syringyl units)] are characterized by a phenolic structure.

As lignin can be hydrolyzed to release aromatic phenolic compounds such as low-molecular mass alcohols, aldehydes, ketones or acids, vine-shoots can be a suitable phenolic source. The abundance and richness of vine-shoots make their exploitation highly interesting in economic terms as this raw material can be very useful to obtain products of a high-added value in the nutraceuticals, cosmetics, pharmacological, oenological and food additive industries. Thus, the phenol extracts from vine-shoots have proved their effectiveness in animals by reducing proliferation of leukemic cells *(13)*, against epilepsy *(14)*, and for prevention of aging and diseases such as atherosclerosis, diabetes and inflammatory processes *(15)* as a result of the antioxidant properties of phenols and their ability to act as efficient free-radical scavengers.

Conventional methods for the extraction of phenolic compounds from solid samples have traditionally been based on stirring. Presently, the use of auxiliary energies such as microwaves or ultrasound has provided dramatic acceleration of the extraction process *(16, 17)*. Also, superheated liquids are an attractive alternative for extraction, with two fundamental advantages over conventional techniques, namely: (a) raising the temperature above the boiling point of the solvent (but keeping it in liquid state by increasing the pressure as required) increases the diffusion rate, solubility and mass transfer of the compounds, and decreases the viscosity and surface tension of the solvent. These changes improve the contact of the compounds with the solvent and enhance extraction, which can then be done more rapidly and with less solvent

consumption as compared with conventional methods. (b) The absence of light and air significantly reduces both degradation and oxidation of the target compounds during extraction *(18)*. Toxic solvents such as methanol–water mixtures *(5,19)* have traditionally been reported for the extraction of phenols from vine by-products; nevertheless, the increased human use of these compounds makes mandatory the development of methods based on non-toxic extractants such as ethanol–water mixtures.

Taking into account the content of cellulose and hemicellulose in vine-shoots, furan derivatives are a family of compounds to be taken into account. The extraction temperature can enhance degradation of sugars released from vine-shoots wood and promote the formation of furans *(20)*. The contribution of two furanic compounds such as furfural and hydroxymethylfurfural (HMF) to flavoring in food processes based on heating is well-known *(21)*. Most of the research facilities around the world, including US-FDA, have examined furans, not only as flavor compounds, but also as novel harmful substances in food that undergo a thermal treatment. The European Food Safety Authority (EFSA) has articulated that furan is obviously carcinogenic in rats and mice, probably due to the combination of a genotoxic mechanism *(22,23)* and hepatotoxicity *(24)*. From a safety perspective and for food quality assurance, HMF legal limits have been already issued for some foodstuffs. In the particular case of concentrated rectified grape must, the EC Regulation No. 1493/99 sets up a limit of 25 mg/kg *(25)*. With these premises, this fraction should be minimized for a proper exploitation of vine-shoot extracts.

Based on this background, the present study was aimed at: (i) demonstrating the feasibility of using vine-shoots to obtain extracts with high phenolic content, (ii) comparing the suitability of extraction techniques such as superheated liquid extraction (SHLE), ultrasound-assisted extraction (USAE) and microwave-assisted extraction (MAE) for isolation of nutraceutical extracts, (iii) showing the variability of vine-shoot cultivars in terms of concentration of monitored phenols.

2. Experimental

2.1. Samples

Vine-shoots of different *Vitis vinifera* cultivars were sampled in Sierra de Segura (Spain). The studied cultivars were: Airén, Baladí, Bobal, Cabernet Franc, Cabernet Sauvignon, Charnonnay, Garnacha Tinta, Garnacha Tintorera, Malbec, Mazuelo, Merlot, Montepila, Moscatel, Pedro Ximénez, Petit Verdot, Sauvignon Blanc, Syrah and Tempranillo that constitute a mixture of traditional and new cultivars in Spain. All of them had been cultivated under the same conditions of soil, climate, hydric regime, etc. The samples were taken in autumn, after leaf-fall, by making a selection of ten similar stocks of each cultivar. A piece of 10 cm of vine-shoot at the hight of the firt leaf bud was taken in all cases. All species were dried for 72 h at 35 ºC, milled to get a homogeneous 40-mesh particle size (less than 0.42 mm diameter), and kept at –20 ºC until use.

2.2. Reagents

Ethanol (96% v/v) PA from Panreac (Barcelona, Spain) and distilled water were used to prepare the different ethanol–water mixtures. Methanol (HPLC grade) and phosphoric acid (85%, v/v) (both supplied by Panreac) were used to prepare the HPLC mobile phases. Deionized water (18 MΩ·cm) was obtained from a Millipore (Bedfore, MA, USA) Milli-Q plus system, and *n*-hexane (LiChrosolv, Merck, Darmstadt, Germany) was used for liquid–liquid extraction.

Fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1[3*H*],9'[9*H*]-xanthen]-3-one) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Fluka (Buches, Switzerland). Folin–Ciocalteu (F–C) reagent, sodium carbonate, gallic acid and AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) were from Sigma. Calibration curves were run for 5-hydroxymethylfurfural (5-hydroxymethyl-2-furancarboxaldehyde) and for the following phenols: (+)-Catechin, C6 phenols; pyrogallol (1,2,3-

trihydroxy-benzene) and pyrocatechol (1,2-dihydroxybenzene), C6-C1 phenols; aceto-vanillone (1-(4-hydroxy-3-methoxyphenyl)-ethanone, vanillin (4-hydroxy-3-methoxybenzaldehyde), guaiacol (2-methoxyphenol), and gallic (3,4,5-trihydroxybenzoic acid), protocatechuic (3,4-dihydroxybenzoic acid), *p*-hydroxybenzoic, vanillic (4-hydroxy-3-methoxybezoic acid) and syringic (4-hydroxy-3,5-dimethoxybenzoic acid) acids, C6-C3 phenols; coniferaldehyde (4-hydroxy-3-methoxycinnamaldehyde), sinapaldehyde (4-hydroxy-3,5-dimethoxycinnamaldehyde) and *p*-coumaric (4-hydroxycinnamic acid), ferulic (4-hydroxy-3-methoxycinnamic acid) and sinapic (4-hydroxy-3,5-dimethoxycinnamic acid) acids. Standards were acquired from Sigma–Aldrich (St.Louis, USA), and *p*-cresol (1-hydroxy-4-methylbenzene) was used as external standard.

2.3. Apparatus

Vine-shoots were milled with a ball grinder (Restch MM301, Haan, Germany). Superheated liquid extractions were performed by a laboratory-made dynamic extractor *(12),* consisting of the following units: a) an extractant supplier; b) a high-pressure pump (Shimadzu LD-AC10) that propels the extractant through the system; c) a switching valve placed next to the pump to develop static extractions when switched off; and d) a stainless steel cylindrical extraction chamber (550 mm × 10 mm i.d., 4.3 mL internal volume) where the sample is placed. This chamber is closed at both ends with screws whose caps contain cotton-made filters to ensure the sample is not carried away by the extractant; e) a restriction valve to maintain the desired pressure in the system; f) a cooler made of a stainless steel tube (1 m length, 0.4 mm i.d.) and refrigerated with water; g) a gas chromatograph oven (Konix, Cromatix KNK-2000) where the extraction chamber is placed and heated.

A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used to enhance microwave assisted extraction, and a Branson 450 digital sonifier (20 kHz, 450 W, duty cycle —fraction of time

ultrasound irradiation is applied/second— equipped with a cylindrical titanium alloy probe (12.7 mm in diameter) were used for MAE and USAE. An R-220 rotary evaporator from Büchi (Flawil, Switzerland) working with a 50-mL balloon flask was used to concentrate the liquid extracts.

The absorbance of the extracts was monitored by a Spectrometer Termo Spectronic Helios Gamma (Waltham, MA, USA). Fluorimetric monitoring of ORAC assay was performed by an F-2500 Hitachi fluorescence spectrophotometer (Pleasanton, Canada) equipped with a 10-mm pathlength cuvette.

Shaking and centrifugation of the extracts were carried out by an MS2 Minishaker (IKA, Germany) Vortex and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

Individual separation of phenolic compounds and carbohydrate derivatives was carried out by a liquid chromatograph (LC) consisting of a ProStar 410 autosampler equipped with a 0.5 mL sample loop (Varian, Palo Alto, California, USA) connected on-line with a liquid chromatograph pump (Varian, 240 pump). A 330 Varian diode array detector (DAD) was used for monitoring the chromatographic eluate at the optimal wavelength for each analyte. Data processing was carried out using the Star Chromatography Workstation version 5.52 software running on a personal computer.

The Polyview-2000 software (Varian) was used both for characterization of the spectra and assessment of peak purity. This software allows examination and analysis of spectra, including plots of purity parameter, setting of absorbance ratios and determination of the maximum absorbance. Determination of the purity of chromatographic peaks, recalculation of the peak at different wavelengths, and integration parameters, which allow exchange signal-to-noise ratio in a diode array data file, are also provided by this software.

Statgraphics Centurion XV Version 15.1.02 for Windows was used for multivariate analysis of generated data.

2.4. Superheated Liquid Extraction

One g of milled vine-shoots was placed into the extraction cell that was inserted into the gas chromatograph oven. Then, a relative high flow rate (7 mL/min) was used for 1 min to fill the cell rapidly. To ensure the absence of air inside the extraction cell, the restrictor valve was kept opened until the first drop of extractant appeared at its end. At that moment, the restrictor valve was closed and when the desired pressure was reached, the switching valve was closed, the pump was turned off and the oven was switched on. During temperature rising, the switching valve had to be opened at short intervals to prevent the pressure from surpassing the working value. Once the selected temperature and pressure were reached, static extraction was performed for a preset time. Finally, the oven was switched off, the chamber was cooled below the boiling point of ethanol and then, the switching valve and the restrictor valve were switched to enable new extractant to flow through the cell and flush out the extract. The extractant used was 80% (v/v) ethanol in water at pH 3, and the extraction time was 1 h.

2.5. Microwave-Assisted Extraction

One g of milled vine-shoots was placed into the extraction vessel with 20-mL 80% (v/v) ethanol in water at pH 3. The vessel was positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 140 W irradiation power for 5 min, after which the solid residue was removed by centrifugation prior to analysis of the extract.

2.6. Ultrasound-Assisted Extraction

One g of milled vine-shoots was placed into the extraction vessel with 20-mL 80% (v/v) aqueous ethanol at pH 3. The ultrasonic probe was immersed into the extraction mixture for sonication at 280 W power for 7.5 min with a duty cycle of 70% (0.7 s/s application cycles). After that, the extract was isolated by centrifugation prior to analysis.

2.7. Determination of Total Phenols by the F–C Method

The amount of total phenolic compounds was measured by the F–C method using gallic acid as calibration standard. The calibration curve was carried out with solutions of 100, 200, 300, 400, 500 and 600 mg/L of this compound ($y = 0.0009x + 0.0081$, $R^2 = 0.9978$). A 0.5-mL aliquot of extract, 10-mL distilled water, 1-mL F–C reagent and 3-mL Na_2CO_3 (20%, w/v) were mixed, made to 25 mL with distilled water and heated at 50 ºC for 5 min. After heating, the samples were kept at room temperature for 30 min and, finally, the absorbance was measured at 765 nm against a blank solution containing distilled water instead of extract. The concentration of phenolic compounds thus obtained was multiplied by the dilution factor of the extract volume and divided by the amount of vine-shoots used. The results were expressed as equivalent to milligrams of gallic acid per g of vine-shoots extract (mg GAE/g vine-shoots).

2.8. Determination of the Antioxidant Capacity by the ORAC (Oxygen Radical Absorbance Capacity) Assay

The antioxidant capacity of the extracts was measured following the ORAC assay, based on inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds such as [2,2'-azobis(2-amidino-propane) dihydrochloride] (AAPH). In addition to AAPH as a peroxyl radicals generator, fluorescein as a fluorescent probe (acting as a target for the peroxyl radicals generated by AAPH, which quench the fluorescein emission), and Trolox as an antioxidant standard were used. All solutions were prepared in phosphate buffer (10 mM, pH 7.4). Excitation and emission wavelengths were set at 485 and 520 nm, respectively. 0.625 mL of diluted extract, blank, or Trolox calibration solution (12.5, 6.25, 3.13 and 1.56 μM final concentrations) were mixed with fluorescein solution (3.75 mL, 10 nM) for 30 min at 37 ºC without shaking. Then, the AAPH solution (0.625 mL, 240 mM) was added to the mixture and the fluorescence was monitored every 5 min for 85 min.

2.9. Treatment of Extracts

The extracts from SHLE, MAE or USAE were dried in a rotary evaporator, then reconstituted in 5 mL methanol (methanolic fractions, MF). The extracts were subjected to liquid–liquid extraction with *n*-hexane (10 mL, 5 min shaking and 6 min centrifugation at 855 g) to remove non-polar compounds, which could complicate the chromatographic separation. For obtainment of the aqueous fraction (AF) of the extracts, 2 mL of MF were subjected to rotary evaporation up to a final volume of 200 μL. Finally, this fraction was taken up to a volume of 650 μL with the chromatographic mobile phase A and filtered using a 0.45 μm pore size filter before injection into the chromatograph.

2.10. LC–DAD Analysis

The separation of analytes was performed on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain) using an injection volume of 20 μL and a flow rate of 1 mL/min. A mobile phase A consisting of 0.2% (v/v) phosphoric acid aqueous solution and a mobile phase B consisting of methanol were used. The gradient method was as follows: from 96% to 82% mobile phase A in 20 min, held for 20 min, from 82% to 74% mobile phase A in 24 min and from 74% to 50% mobile phase B in 9 min. The analytes were identified by comparing both their retention times and UV spectra with those of the corresponding standards and quantified by interpolation in the corresponding calibration curves. The absorption wavelengths were set at 260 nm for ellagic acid, at 280 nm for hydroxybenzoic acids, catechin and phenolic aldehydes; at 320 nm for hydroxycinnamic acids, and at 360 nm for hydroxycinnamic aldehydes.

2.11. LC–TOF/MS Confirmatory Analysis

The separation of analytes was performed on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain) using an injection volume of 20 μL and a flow rate of 1 mL/min. A mobile

phase A consisting of 0.2% (v/v) phosphoric acid aqueous solution and a mobile phase B consisting of methanol were used. The gradient method was as follows: from 96% to 82% mobile phase A in 20 min, held for 20 min, from 82% to 74% mobile phase A in 24 min and from 74% to 50% mobile phase B in 9 min. The analytes were identified by comparing both their retention times and UV spectra with those of the corresponding standards and quantified by interpolation in the corresponding calibration curves. The absorption wavelengths were set at 260 nm for ellagic acid, at 280 nm for hydroxybenzoic acids, catechin and phenolic aldehydes; at 320 nm for hydroxycinnamic acids, and at 360 nm for hydroxycinnamic aldehydes.

3. Results and discussion

3.1. Extraction Protocols for Isolation of Polar Compounds

Three different extraction protocols were tested for isolation of interesting compounds from vine- shoots because of their potential applicability associated to their nutraceutical properties. Optimization of the three extraction protocols was carried out with Pedro Ximénez cultivar vine-shoots because of its geographical relevance in the area where the study was developed.

Solid–liquid extraction was the critical step in the sample preparation scheme for selective separation of the target compounds. The three extraction approaches were SHLE to take benefits from solvent properties in superheated state, and MAE and USAE to take benefits from the assistance of auxiliary energies to enhance the leaching process. The extractant composition in the case of SHLE was set according to previous studies reported in the literature *(3, 12, 17)*. Ethanol–water mixtures (60:40, v/v) acidified at pH 3 were used as extractant media also in the case of MAE and USAE to compare the leaching efficiency with that of SHLE. Apart from the extractant composition, the main variables involved in each leaching technique were optimized to evaluate their incidence on the leaching efficiency. These variables were temperature for SHLE,

irradiation power for both MAE and USAE, and duty cycle (defined as the fraction of a second during which ultrasonic energy is applied) only for USAE. The extraction time was also optimized in the three extraction protocols. The ranges studied were set according to the literature and preliminary experiments. The ranges of study for each type of extraction and the optimal values are shown in **Table 1.** Two parameters were selected as independent response variables: the concentration of total phenols estimated by the F–C method that should be maximized and the concentration of 5-hydroxymethylfurfural estimated by chromatographic analysis to evaluate the degradation of carbohydrates, which should be minimized.

Table 1. Ranges and optimum values of the variables studied by the different extraction methods.

Extraction method	Variable	Range	Optimum value
USAE	Power (W)	280–40	280
	Duty cycle (%)	70–30	50
	Time (min)	5–15	7.5
MAE	Power (W)	140–60	140
	Time (min)	3–15	5
SHLE	Temperature (ºC)	240–160	180
	Time (min)	5-90	60

In the case of SHLE, the pressure was not included in the multivariate optimization due to its null effect on extraction. Thus, this variable was simply set at a value high enough (10 bar) to assure the liquid state of the ethanol–water mixtures during extraction. As **Figure 1** shows, extraction tests at 160 and 180 ºC did not report statistical differences in the concentration of total phenols measured by the F–C method, while there was a significant increase in the response of the F–C method provided by the extract obtained at 200, 220 and 240 ºC. However, extraction temperatures from 200 to 240 ºC involved a significant burnt wood smell that was indicative of qualitative alteration of the

extract. Additionally, the concentration of hydroxymethylfurfural in the extract reached the maximum level at temperature above 200 ºC.

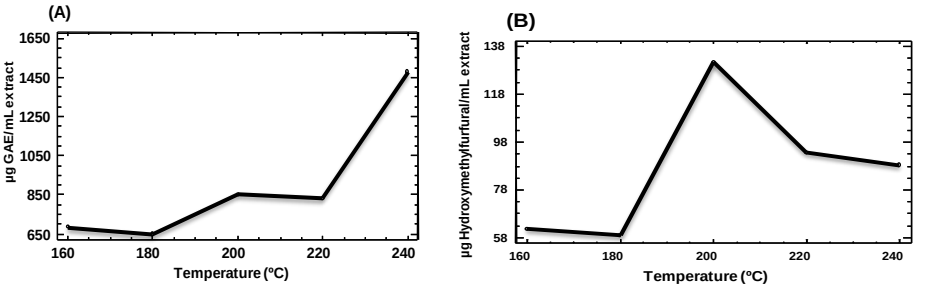

Figure 1. Influence of temperature on both the concentration of phenolic compounds in SHL extracts estimated by the F–C method (A) and **t**hat of hydroxymethylfurfural calculated after chromatographic separation by LC–DAD (B).

Attending to these results, maximum concentration of phenolic compounds in the extract with minimum level of hydroxymethylfurfural was attained in the range 160–180 ºC. Temperatures lower than 160 ºC yielded extracts with reduced total phenolic concentration (data no shown). Attending to these results, two kinetics experiments were carried at 160 and 180 ºC with extraction times from 5 to 90 min. In the SHLE developed at 180 ºC a plateau of the efficiency was reached after 60 min (95% confidence level, data no shown). On the other hand, leaching at 160 ºC for 60 min yielded extracts with lower concentration of compounds such as coniferaldehyde, sinapic acid or sinapaldehyde (see **Supplementary Fig. 1**). Therefore, 180 ºC was selected as optimum extraction temperature.

Surface response designs were applied to optimize USAE and MAE. The microwave power had a positive effect on MAE at short extraction times but it was practically null at 15 min, as shows the surface response in **Figure 2.A** (95% confidence level). At high irradiation power (140 W, the maximum without ejections) the extraction time was not an influential variable for times longer than 5 min. The shorter extraction time at which a constant total concentration of

phenol compounds was obtained (5 min) was selected for subsequent experiments.

Both power and duty cycle presented positive effects on USAE, as can be seen in **Figure 2.B**; by contrast, the increase of the extraction time proved to have an opposite influence, as the total concentration of phenols in the extract was decreased a 70% from 5 to 15 min; consequently, the highest values of power (280 W), and duty cycle (70%) were chosen together with 7.5 min as extraction time.

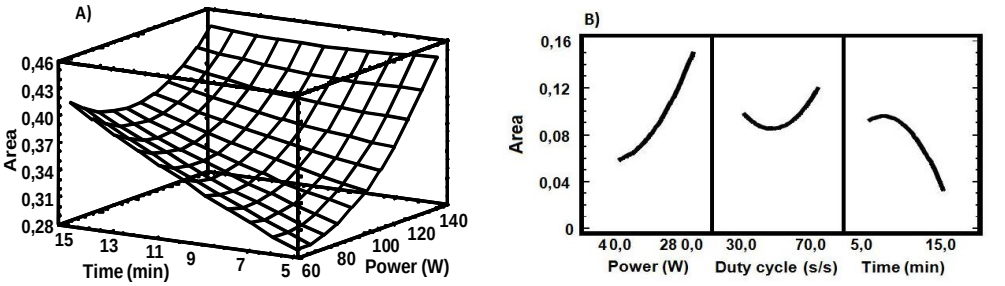

Figure 2. Influence of the main variables involved on MAE (A), and USAE (B) of phenolic compounds from vine-shoots.

The effect of both types of energy (microwaves and ultrasound) on the extraction of the target compounds clearly differ from that exerted when the target compounds were extracted from other raw materials, such as olive-tree leaves *(19, 26)*, vine-lees *(15, 27)* or alperujo *(12, 28)*.

Once the three extraction protocols were optimized, their leaching efficiencies were compared in terms of concentration of total phenols estimated by the F–C method and that of hydroxymethylfurfural. Attending to these results, SHLE provided the highest concentration of phenolic compounds expressed as μg of gallic acid per gram of initial solid vine-shoots (n=3, p=0.00022), 650.4 μg/mL, versus 546.4 and 401.4 μg/mL obtained with USAE and MAE, respectively (see **Figure 3**). Concerning hydroxymethylfurfural, SHL extracts

were the only containing significant levels of this furanic aldehyde, while MAE and USAE extracts reported very low concentrations. The concentration of hydroxymethylfurfural in the SHL extract at 180 ºC for this cultivar was below 60 μg/mL, while this level surpassed 130 μg/mL at 200 ºC, as shown in **Figure 1.B**. Under controlled conditions, furanic derivatives are flavor correctors, which is quite interesting for oenological applications. For this reason, SHL extracts seems to be more interesting than those provided by USAE and MAE from an organoleptic point of view.

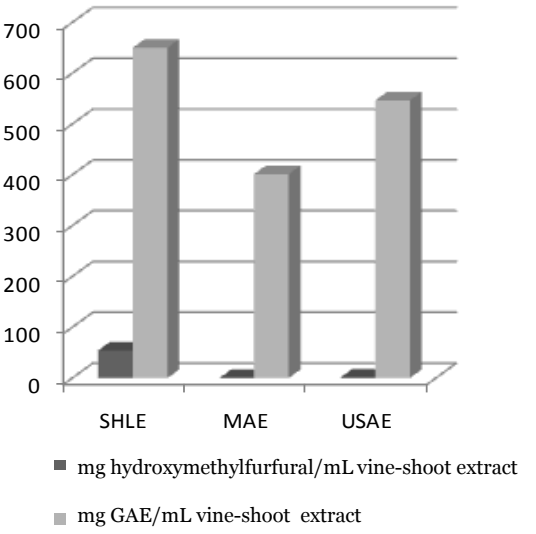

Figure 3. Hydroxymethylfurfural content (μg/mL) and total concentration of phenols expressed as μg equivalent to gallic acid per mL of vine-shoot extract obtained by the Folin–Ciocalteu method for the three extraction approaches**.**

3.2. Comparison of the Antioxidant Potential of Different Vine-Shoot Cultivars

After selection of SHLE as the most suited approach for isolation of phenols, SHLE was applied to eighteen vine-shoot cultivars. The purpose was to compare the antioxidant potential of the different cultivars with a view to further application to qualitative characterization. As **Figure 4** shows, the total

concentration of phenols was quite homogeneous among the different vine-shoots cultivars, corresponding the highest values to Chardonnay, Montepila, Petit Verdot and Tempranillo (ranging between 5775.55 and 4864.44 μg GAE/g of vine-shoots for Chardonnay and Tempranillo, respectively) and the lowest to Pedro Ximénez and Baladí, with 3323.70 and 3401.70 μg, respectively.

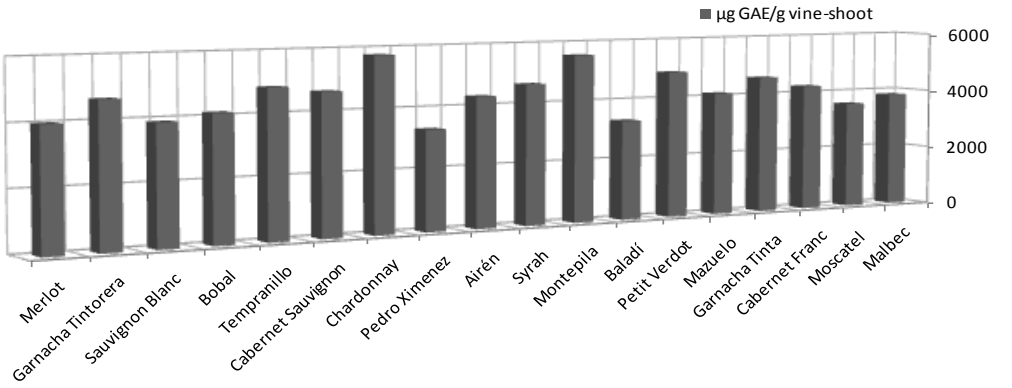

Figure 4. Total concentration of phenols in the SHL extracts from different vine-shoot cultivars expressed as μg GAE/g vine-shoots obtained by the F–C method.

The ORAC assay was applied to compare the antioxidant capacity of the SHLE extracts from the different vine-shoot cultivars. The high antioxidant power of the vine-shoot extracts demanded for dilution of the extracts to detect a significant kinetic decay. Thus, the extracts were 1:10 diluted prior to measurement of ORAC activity. **Figure 5** shows the kinetic curves for five vine-shoot varieties representative of the different varieties under study. As can be seen, the curves are characterized by a slight decay along the ORAC experiment. By calculating the difference between the fluorescence signal at the beginning of the experiment and after 85 min, a calibration curve of this parameter versus the concentration of Trolox was plotted fitting a second-grade polynomial function ($y = -0.3274x^2 + 6.9431x + 64.071$; $R^2 = 0.9904$). The ORAC antioxidant activity of the vine-shoot extracts was estimated by the calibration curve resulting in a

capacity ranging from 5.7 to 6.8 μM equivalents of Trolox for Airen and Chardonnay varieties, respectively (data not shown). These results are quite consistent with those provided by the F–C method since Chardonnay vine-shoots was the variety reporting the highest antioxidant capacity and, at the same time, that with the highest total phenolic content.

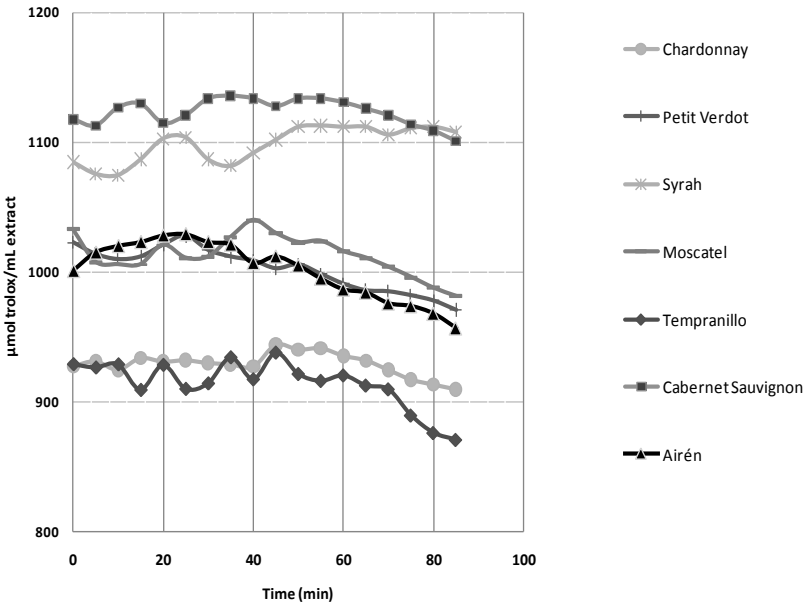

Figure 5. Comparison of the antioxidant capacity of different vine-shoot SHL extracts measured by the ORAC assay

3.3. Composition of Extracts from Different Vine-Shoot Cultivars

Vine-shoots from eighteen cultivars were extracted by SHLE under the selected operation conditions to evaluate the content of interesting compounds from the oenological and nutraceutical points of view. The same panel of compounds (composed by the standards described under "Materials and Methods") was determined in SHL extracts. **Figure 6** shows a DAD chromatogram at 260 nm corresponding to a vine-shoots extract from Chardonnay cultivar with identification of interesting compounds. **Table 2** lists the concentrations of representative compounds in extracts from different

varieties of vine-shoots. The identity of these compounds was confirmed by LC–TOF/MS in high resolution. For this purpose, SHL extracts and standards were analyzed. Identification of target compounds was supported on the chromatograms corresponding to monoisotopic masses and retention times. Search parameters were mass accuracy cut-off below 10 ppm and a peak spacing tolerance of 0.0025 *m/z* plus 7 ppm. Retention times, formulae, experimental and theoretical masses, and errors, expressed as ppm and obtained by accurate mass measurements of monitored compounds, are shown in **Table 3**. Hydroxymethylfurfural, was found in all extracts. This is a degradation product from hexoses, with the highest concentrations of this furanic aldehyde responsible of light creamy toast and toffee flavor found in the varieties Baladí and Montepila (729 and 662 μg/g, respectively). The rest of the cultivars could be discriminated attending to the hydroxymethylfurfural concentration in the extracts from their vine-shoots (below and above 200 μg/g) with a 95% confidence level. Thus, extracts from Tempranillo, Airén, Cabernet Sauvignon, Chardonnay and Pedro Ximénez vine-shoots were characterized by a high content of hydroxymethylfurfural ranging from 208 to 456 μg/g. On the other hand, the resting varieties provided a content ranging from 40.0 to 183 μg/g for Syrah and Bobal, respectively.

One of the most characteristic phenolic compounds found in the extracts from all studied cultivars was gallic acid, a final product in the hydrolysis of ellagitannins that contributes to the astringency character of wines. Gallic acid concentration was in the range 55–570 μg/g for Syrah and Cabernet Sauvignon varieties, respectively. Pyrogallol, formed from gallic acid decarboxylation, was in all cultivars covering a wide concentration range: from 1.2 mg/g for Syrah to 12.3 mg/g found in Cabernet Sauvignon cultivar. The similarity between concentrations of pyrogallol and gallic acid in each cultivar demonstrated that both compounds are connected through a biochemical pathway. This similarity was not found in the case of protocatechuic acid and pyrocatechol (decarboxylated product of protocatechuic acid). Thus, the concentration of protocatechuic acid ranged from 40 μg/g in Syrah extract to 302

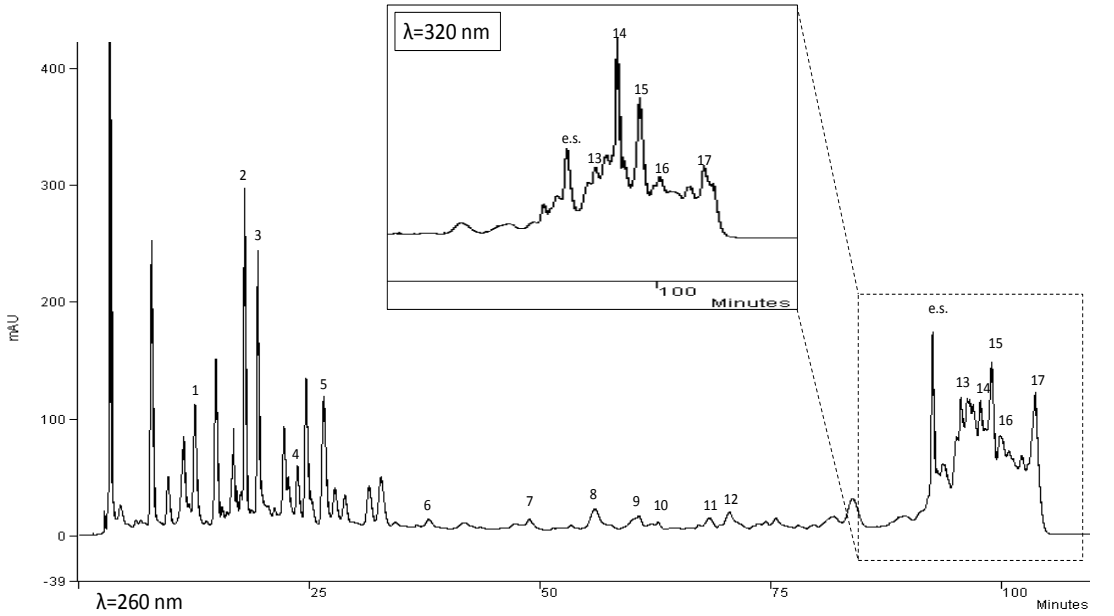

Figure 6. DAD chromatogram at 260 and 320 nm corresponding to a vine-shoots extract from Chardonnay cultivar with identification of interesting compounds. Peaks: 1, pyrogallol; 2, gallic acid; 3, hydroxymethylfurfural; 4, pyrocatechol; 5, protocatechuic acid; 6, hydroxybenzoic acid; 7, catechin; 8, vanillic acid; 9, guaiacol; 10, vanillin; 11, syringic acid; 12, acetovanillone; 13, coumaric acid; 14, ferullic acid; 15, coniferaldehyde; 16, sinapic acid; 17, sinapaldehyde; e.s. *p*-cresol.

μg/g for Cabernet Sauvignon; while pyrocatechol ranged from 17 μg/g in Bobal to 232 μg/g in Merlot. Catechin, the building block for tannins synthesis, was found at significant concentrations from 0.4 to 7.4 mg/g in Cabernet Franc and Baladí, respectively. This high concentration is indicative of an important effect of the extraction process on the hydrolysis of tannins. Acetovanillone was detected in all varieties of vine-shoots. Other compounds with organoleptical incidence were coniferaldehyde and sinapaldehyde. The former was found in all cultivars but in Syrah, in concentrations ranging from 10 to 40 μg/g, while sinapaldehyde was found in all extracts in concentrations ranging from 2 to 476 μg/g.

Table 2. Concentration and limit of detection of a panel of selected compounds in SHL extracts from different cultivars of vine-shoots, expressed as μg/g vine-shoots (n=3 analytical replicates).

Compound / Cultivar	Pg	G. Ac	Hf	Py	P.Ac	H.Ac	C	V. A	G	V	S. Ac	Av	C. Ac	F. Ac	Cf	S.Ac	S
Tempranillo	5816	269	386	9	215	21	6735	Nd	62	13	Nd	35	31	23	12	241	301
Airén	5661	262	311	52	118	Nd	589	3	55	9	22	28	Nd	32	39	1464	2
Cabernet Sauvignon	12307	570	456	38	302	Nd	6233	Nd	169	31	26	41	Nd	34	16	56	207
Baladí	4961	229	729	120	182	28	7467	65	19	32	1	15	28	45	13	2283	10
Cabernet Franc	7475	346	153	33	215	19	405	Nd	33	Nd	88	19	31	34	16	63	130
Syrah	1206	55	40	49	40	Nd	1824	8	8	7	0.6	5	Nd	12	Nd	104	2
Bobal	3629	167	183	17	139	19	1982	Nd	5	Nd	5	18	12	41	29	112	106
Chardonnay	5522	255	208	40	129	19	512	68	39	4	22	1	25	9	35	26	19
Garnacha Tintorera	4708	217	170	45	136	25	1764	4	2	18	2	30	23	33	23	161	476
Garnacha Tinta	1504	69	128	25	106	95	1315	61	41	36	4	39	32	650	15	44	197
Malbec	6477	300	79	116	217	Nd	1033	19	12	11	18	17	25	68	23	1537	74
Mazuelo	5254	243	179	57	293	44	5141	Nd	19	24	5	79	20	59	10	178	120
Merlot	5208	241	127	232	111	13	1048	13	16	39	11	25	30	36	31	78	46
Montepila	6746	312	662	44	197	23	4914	28	38	53	25	10	12	356	16	77	89
Moscatel	3435	158	115	135	93	23	3624	11	13	6	22	11	28	42	40	38	51
Pedro Ximénez	2361	109	247	66	49	12	1133	10	26	13	4	20	26	74	37	1247	81
Petit Verdot	6793	314	124	91	155	23	2843	152	18	18	18	10	27	10	38	158	49
Sauvignon Blanc	5148	238	183	182	150	70	1167	28	38	30	11	6	18	528	40	324	112
L.O.D.	0.8548	0.5415	0.5704	0.5742	0.6051	0.5682	1.4430	0.0739	0.0922	0.6340	0.4918	0.4229	0.6045	0.1822	2.2824	3.1373	0.9259

ND: not detected; Pg: pyrogallol; G.Ac: gallic acid; Hf: hydroxymethylfurfural; Py: pyrocatechol; P.Ac: protocatechuic acid; H.Ac: p-hydroxybenzoic acid; C: catechin; V.A: vanillic acid; G: guaiacol; V: vanillin; S. Ac: syringic acid; Av: acetovanillone; C. Ac: coumaric acid; F. Ac: ferulic acid; Cf: coniferaldehyde; S.Ac: sinapic acid; S: sinapaldehyde.

From an oenological point of view, the concentration of representative phenols in extracts from vine-shoots makes foreseeable their use to improve wine quality through oxidation/reduction reactions, since they could determine to a large extent its color, flavor and aroma, acting similarly to wine-aging either in contact with oak chips or in oak barrels *(13)*. In relation to health benefits attributed to phenolic compounds, the nutraceutical interest of these extracts should be evaluated.

Acknowledgements

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Table 3. Confirmatory analysis of monitored compounds by LC–TOF/MS in vine-shoot extracts.

Compound	Retention time (min)	Ion	Theoretical mass	Formula	Experimental mass	Error (ppm)
5-Hydroxymethylfurfural	10.1	(M-H)-	126.0317	$C_6H_6O_3$	126.0309	-6.09
Gallic acid	12.8	(M-H)-	170.0215	$C_7H_6O_5$	170.0202	-7.62
Ferulic acid	17.5	(M+CHO_2)-	194.0579	$C_{10}H_{10}O_4$	194.0584	2.62
Pyrocatechol	20.1	(M-H)-	110.0368	$C_6H_6O_2$	110.0359	-8.18
Protocatechuic acid	20.1	(M-H)-	154.0266	$C_7H_6O_4$	154.0256	-6.46
Pyrogallol	21.0	(M+H)+	126.0317	$C_6H_6O_3$	126.0314	-2.6
Guaiacol	21.4	(M-H)-	124.0524	$C_7H_8O_2$	124.0518	-5.09
Vanillic acid	26.3	(M+H)+	168.0423	$C_8H_8O_4$	168.0412	-6.51
Syringic acid	26.8	(M+H)+[-H_2O]	198.0528	$C_9H_{10}O_5$	198.051	-9.08
4-Hydroxybenzoic acid	27.0	(M+CHO_2)-	138.0317	$C_7H_6O_3$	138.0323	4.56
Catechin	27.8	(M-H)-	290.079	$C_{15}H_{14}O_6$	290.0803	4.43
o-Coumaric acid	28.2	(M-H)-	164.0473	$C_9H_8O_3$	164.0474	0.32
Sinapic acid	36.0	(M+H)+	224.0664	$C_{11}H_{12}O_5$	224.0685	-9.24
Vanillin	37.4	(M+CHO_2)-	152.0473	$C_8H_8O_3$	152.0486	8.22
Acetovanillone	38.47	(M+H)+	166.0630	$C_9H_{10}O_3$	166.0625	-2.92
Coniferaldehyde	42.1	(M+CHO_2)-	178.063	$C_{10}H_{10}O_3$	178.0647	9.49
Sinapaldehyde	44.8	(M-H)-[-H_2O]	208.0736	$C_{11}H_{12}O_4$	208.0742	3.11

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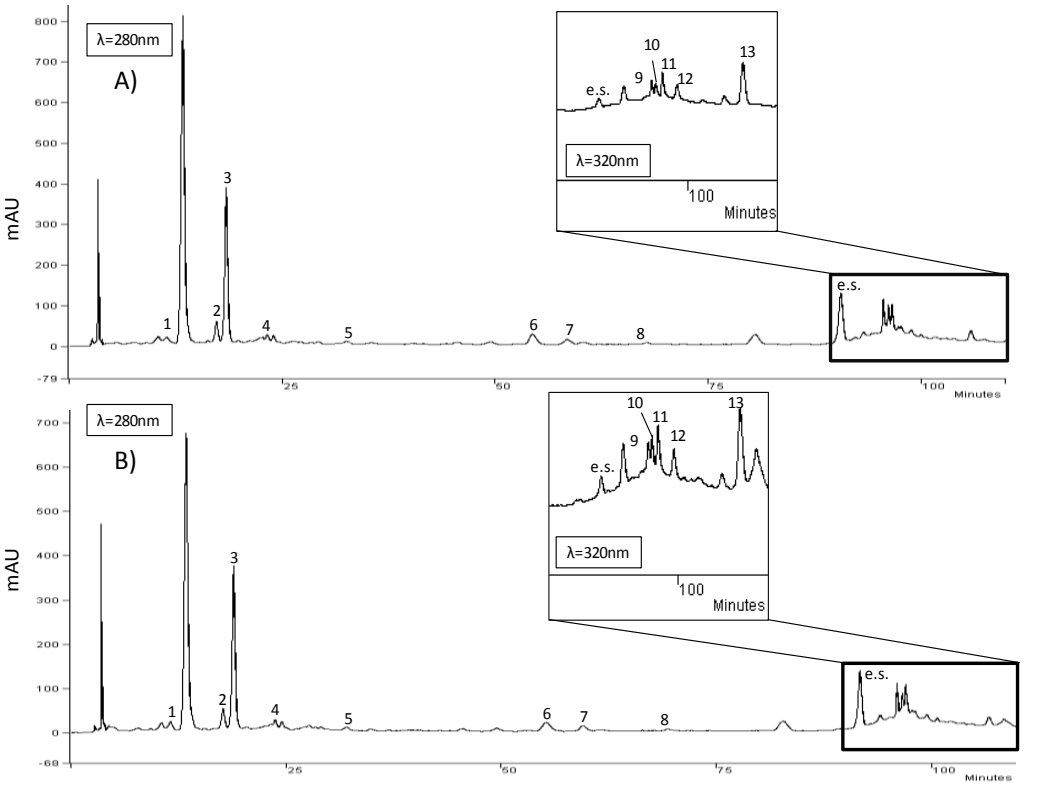
Supplementary material



Supplementary Figure. 1. DAD chromatograms at 280 and 320 nm corresponding to a SHL extracts from Pedro-Ximenez vine-shoots at 160 ºC (A) and 180 ºC (B) with identification of interesting compounds. Peaks: 1, pyrogallol; 2, gallic acid; 3, hydroxymethylfurfural; 4, pyrocatechol; 5, protocatechuic acid; 6, catechin; 7, vanillic acid; 8, vanillin; 9, coumaric acid; 10, ferullic acid; 11, coniferaldehyde; 12, sinapic acid; 13, sinapaldehyde., e.s., p-cresol.

Chapter 3:

Evaluation of the composition of vine–shoots and oak chips for oenological purposes by superheated liquid extraction and high–resolution LC-TOF/MS analysis

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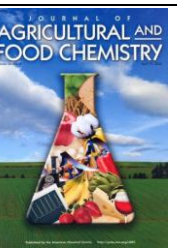

Evaluation of the composition of vine-shoots and oak chips for oenological purposes by superheated liquid extraction and high-resolution LC−TOF/MS analysis

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Abstract

Vine-shoots are characterized in this research and compared to oak chips, frequently employed in the aging of wine or spirits. For this purpose, LC–DAD and LC–TOF/MS analyses of hydroalcoholic extracts from vine-shoots pertaining to 18 different vine varieties and from 5 varieties of oak chips have been carried out. The concentrations of a representative panel of interesting compounds from an oenological point of view have been compared in the extracts, finding similarity patterns for many of them. The analysis by LC–TOF/MS in high accuracy mode has led to the identification of numerous compounds in the hydroalcoholic extracts. The statistical analysis has enabled to identify the vine-shoot varieties providing extracts with more similar composition to that given by extracts from oak chips. Therefore, these vine-shoots varieties are suitable to be proposed as an alternative to the use of oak barrels or oak chips in the aging process of wine and spirits.

1. Introduction

Spain is the country with the largest area in the world dedicated to vineyards, where approximately 1.5 million of tons of vine-shoots are produced every year. This fact has led to a growing interest on exploitation of this agricultural residue with the aim of turning it into valuable products. Most research about vine-shoots has been focused on the production of paper pulp *(1),* ethanol, lactic acid *(2,3)*, methanol, fuels, biomass, biosurfactants *(2)*, and activated carbon for wine treatment *(4)*, extraction of volatile compounds, phenols *(5,6)*, ferulic and coumaric acids *(7)*, among others.

Phenols can be obtained from vine-shoots in two compatible ways, namely: (a) by extracting the phenols that are not constituents of lignin, and (b) by degrading lignin to obtain low-molecular weight phenols. The composition of vine-shoots is characterized by lignin content around 20% (dry weight). As lignin can be hydrolyzed to release aromatic phenolic compounds such as low-molecular mass alcohols, aldehydes, ketones or acids, vine-shoots are suitable to be used as a phenols source. In addition, they could play a key role in the oenological or spirits field to improve quality, acting similarly to wine-aging agents either in contact with oak chips or in barrels *(8)*. In fact, lignin oligomers or intermediate- and high-molecular mass phenols from lignin are considered the main phenols in old spirits and wine *(6, 7)*.

Traditionally, oak barrels have been used for centuries to store and aging wine and other beverages, since the sensory complexity of the substrate subjected to aging is increased as the wood transfers to the liquid phase a series of aromatic compounds *(9)*. Wine aging involves changes in color and organo-leptical properties that are highly appreciated by the consumers *(10)*.

Extraction of compounds of interest from oak barrels depends on the quantity of potentially extractable compounds originally present in the barrel, and on time-related factors, particularly the contact time between the beverage

subjected to aging and wood *(11–13)*. During barrel aging, wine or spirits are partially enriched with lignin-derivative compounds forming complexes that are hydrolyzed to release aromatic aldehydes, which undergo oxidation reactions generating aromatic acids. Thus, the most important phenolic acids and phenolic aldehydes present in these beverages come exclusively from the barrel wood.

Economic grounds have led to the use of alternatives to oak barrels. This is the case of segments, staves, and more common, oak chips *(12,14–17)*, which provide results similar to those obtained by barrel aging for several years *(9)*. The final product is determined, not only by the different characteristics of the added oak wood portions such as their origin *(18)*, size and toasting process, but also by the dosage, period of contact with wine, and possible oxygenation of the aging product *(12)*.

Superheated liquid extraction (SHLE) can be an attractive industrial alternative for isolation of these compounds as it possesses two fundamental advantages over conventional extraction. The first one is ascribed to the fact that raising the temperature above the boiling point of the extractant (but keeping it under liquid state by increasing the pressure as required) increases the diffusion rate, solubility and mass transfer of the compounds and decreases the viscosity and surface tension of the extractant. These changes improve the contact of the compounds with the extractant and enhance mass transfer, which can then be achieved more rapidly and with less solvent consumption as compared with conventional industrial extraction methods. Secondly, the absence of light and air significantly reduces both degradation and oxidation of these compounds during extraction *(19)*.

At this point, and once discussed the importance of wood in general, and lignin in particular, in the aging of wine, the comparison of extracts obtained from oak wood and vine-shoots would provide information about the possibilities of using vine-shoots in the aging process, either as a complement to oak barrels or as a cheaper and available alternative. To this end, the different chemometric tools currently available play a fundamental role in multivariate analysis to treat

the large amount of information provided by analytical techniques such as liquid chromatography–time-of-flight/mass spectrometry (LC–TOF/MS). This powerful analytical arrangement actually provides effective validation of the information obtained by other less complex techniques such as LC–DAD, and, in addition, it would provide new information, such as identification of unknown compounds. Based on this background, the objective of this study was to compare both the metabolic profile of extracts from vine-shoots and oak chips and similarity/dissimilarity patterns among different varieties of vine-shoot and oak wood with the final aim of using these vineyard residues in the aging step.

2. Experimental section

2.1. Samples

Vine-shoots from different Vitis vinifera cultivars were sampled from Sierra de Segura (Spain). The studied cultivars were: Airén, Baladí, Bobal, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Garnacha Tinta, Garnacha Tintorera, Malbec, Mazuelo, Merlot, Montepila, Moscatel, Pedro Ximénez, Petit Verdot, Sauvignon Blanc, Syrah and Tempranillo. Five varieties of oak chips were studied: American blend (toasted), American fresh, French sweet (toasted), French spice and French intense (toasted). All species were dried for 72 h at 35 ºC, then milled to get a homogeneous 40-mesh particle size (less than 0.42 mm diameter).

2.2. Reagents

Ethanol (96% v/v) PA from Panreac (Barcelona, Spain) and distilled water were used to prepare the extractant solution. Methanol (LC–MS grade) and formic acid (MS grade) (both supplied by Panreac) were used to prepare the mobile phases. Deionized water (18 MΩ·cm) was obtained from a Millipore (Bedford, MA, USA) Milli-Q plus system and *n*-hexane (LiChrosolv, Merck, Darmstadt, Germany) was used for liquid–liquid extraction.

Calibration curves were constructed for the following phenols: (+)-Catechin; 5-hydroxymethylfurfural (5-hydroxymethyl-2-furancarboxaldehyde); C6 phenols: pyrogallol (1,2,3-trihydroxybenzene) and pyrocatechol (1,2-dihydroxybenzene); C6-C1 phenols: acetovanillone (1-(4-hydroxy-3-methoxy-phenyl)-ethanone; vanillin (4-hydroxy-3-methoxybenzaldehyde); guaiacol (2-methoxyphenol); and gallic (3,4,5-trihydroxybenzoic acid); protocatechuic (3,4-dihydroxybenzoic acid); *p*-hydroxybenzoic; vanillic (4-hydroxy-3-methoxybezoic acid) and syringic (4-hydroxy-3,5-dimethoxybenzoic acid) acids; C6-C3 phenols: coniferaldehyde (4-hydroxy-3-methoxycinnamaldehyde); sinapaldehyde (4-hydroxy-3,5-dimethoxycinnamaldehyde) and *p*-coumaric (4-hydroxycinnamic acid); ferulic (4-hydroxy-3-methoxycinnamic acid) and sinapic (4-hydroxy-3,5-dimethoxy-cinnamic acid) acids; and *p*-cresol (1-hydroxy-4-methylbenzene); used as external standard, were from Sigma–Aldrich (St.Louis, USA).

2.3. Apparatus

Vine-shoots were milled with a ball grinder (Restch MM301, Haan, Germany). Superheated liquid extractions were performed by a laboratory-made dynamic extractor *(11),* consisting of the following units: a) an extractant supplier; b) a high-pressure pump (Shimadzu LD-AC10) that propels the extractant through the system; c) a switching valve placed next to the pump to develop static extractions; and d) a stainless steel cylindrical extraction chamber (550 mm × 10 mm i.d., 4.3 mL internal volume) where the sample is placed. This chamber is closed at both ends with screws whose caps contain cotton made filters to ensure the sample is not carried away by the extractant; e) a restriction valve to maintain the desired pressure in the system; f) a cooler made of a stainless steel tube (1 m length, 0.4 mm i.d.) and refrigerated with water; g) a gas chromatograph oven (Konix, Cromatix KNK-2000) where the extraction chamber is placed and heated.

Shaking and centrifugation of the extracts were carried out by an MS2 Minishaker (IKA, Germany) Vortex and a Mixtasel (Selecta, Barcelona, Spain)

centrifuge, respectively. An R-220 rotary evaporator from Büchi (Flawil, Switzerland) working with a 50-mL balloon flask was used to concentrate the liquid extracts.

Individual separation of phenolic compounds and carbohydrate derivatives was carried out by an LC consisting of a ProStar 410 autosampler equipped with a 0.5 mL sample loop (Varian, Palo Alto, California, USA) connected on-line with an LC pump (Varian, 240 pump). A 330 Varian PDA detector was used to monitor the chromatographic eluate at the optimal wavelength for each analyte. Data processing was carried out using the Star Chromatography Workstation version 5.52 software running on a personal computer.

The Polyview-2000 software (Varian) was used both for characterization of the spectra and assessment of peak purity. This software allows examination and analysis of spectra, including plots of purity parameter, setting of absorbance ratios and determination of maximum absorbance. Determination of the purity of chromatographic peaks and recalculation of the peak at different wavelengths, and integration parameters, which allow exchange signal-to-noise ratio in a diode array data file, are also provided by this software.

2.4. Extraction of Phenolic Compounds and Treatment of Extracts

Extracts of vine-shoots and oak wood were performed by SHLE as described in *(15)*, where the extractant used was 80:20 (v/v) ethanol–water at pH 3, with an extraction time of 1 h and an extraction temperature of 180 ºC. The extracts were dried in a rotary evaporator and then reconstituted in 5 mL methanol. The reconstituted solutions were subjected to liquid–liquid extraction with *n*-hexane (10 mL, 5 min shaking and 6 min centrifugation at 855 g) to remove non-polar compounds, which could complicate the chromatographic separation. Preconcentration of the methanolic phase was attained by evaporation of 2 mL of methanol to a final volume of 200 µL. Finally, this

fraction was taken up to 650 μL with milli-Q water and filtered using a 0.45 μm pore size filter before injection into the chromatograph.

2.5. LC–DAD Analysis

Separation of the analytes was performed on an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), using an injection volume of 20 μL and a flow rate of 1 mL/min. A mobile phase A consisting of 0.2% (v/v) phosphoric acid aqueous solution and a mobile phase B consisting of methanol were used. The gradient method was as follows: from 96% to 82% mobile phase A in 20 min, held for 20 min, from 82% to 74% mobile phase A in 24 min and from 74% to 50% mobile phase B in 9 min. The analytes were identified both by comparing their retention times and UV spectra with those of the corresponding standards. The absorption wavelengths were set at 260 nm for monitoring ellagic acid, at 280 nm for hydroxybenzoic acids, catechin and phenolic aldehydes; at 320 nm for hydroxycinnamic acids, and at 360 nm for hydroxycinnamic aldehydes.

2.6. LC–TOF/MS Confirmatory Analysis

The analyses, conducted with a view to confirming the identity of the studied compounds, were performed in an Agilent 1200 Series LC system (consisting of a binary pump, a vacuum degasser, an autosampler and a thermostated column compartment) interfaced to an Agilent 6540 UHD Accurate-Mass LC–TOF/MS detector (Palo Alto, USA), equipped with an Agilent Jet Stream Technology electrospray ion source operating in the negative and positive ion modes.

Chromatographic separation was performed using an Inertsil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), kept at a temperature of 25 °C. Mobile phases were water (phase A) and acetonitrile (phase B) both LC–MS/MS grade and with 0.1% formic acid as ionization agent. The LC pump was programmed with a flow rate

of 1 mL/min and the following gradient elution was carried out: from 4% to 18% mobile phase B in 20 min, held for 20 min, from 18% to 26% mobile phase B in 44 min, from 26% to 50% mobile phase B in 26 min and from 50% to 100% phase B in 30 min. The injection volume was 10 µL and the injector needle was rinsed for 5 times with 70% methanol. Furthermore, the needle seat back was flushed for 12 s at a flow rate of 4 mL/min with 70% methanol to clean it.

The operating conditions of the mass spectrometer were as follows: gas temperature, 350 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 35 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3250 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; focusing voltage, 90 V. Data acquisition (2.5 Hz) in both the centroid and profile modes was governed via the Agilent MassHunter Workstation software. The instrument was operated in MS-high accuracy mode. The mass range and detection window were set at *m/z* 100–1100 and 100 ppm, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. To assure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses with the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in positive ion mode; in negative ion mode, ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.000725 (formate adduct of HP-921) were used.

2.7. Data Processing and Statistical Analysis

MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing all data obtained with LC–TOF/MS in full single MS mode. The feature extraction algorithm took into account all ions exceeding 5000 counts with a charge state equal or above to one, and a feature had to be composed of two or more ions to be valid (*e.g.* two ions in the isotope cluster). Within the algorithms employed for full single MS data, ions with identical elution profiles and related *m/z* values

(representing different adducts or isotopes of the same compound) were extracted as molecular features (MFs) or entities characterized by retention time (RT), intensity in apex of chromatographic peak, and accurate mass. Various intensity threshold settings, ranging from 3000 to 15000 counts per second (cps), were tested for the MFs extraction in the whole RT range. Files in compound exchange format (.cef files) were created for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. In the next step, alignment of RT and *m/z* values was carried out across the sample set using a tolerance window of 0.2 min in retention time and 5 ppm mass accuracy. Baseline correction eliminated the contribution of background noise. Stepwise reduction of MFs number was performed based on frequency of occurrence, abundance of the respective MFs in classes and PCA results of the data. MPP software also enabled one-way analysis of variance (ANOVA). The data were mean centered as a data pretreatment to lower relatively large differences among the respective MFs abundances.

3. Results and discussion

3.1. Composition of Extracts from Different Varieties of Vine-Shoot and Oak Wood by LC–DAD Analysis

Eighteen vine-shoot varieties and five types of oak chips were subjected to SHLE under optimum operation conditions to evaluate the content of interesting compounds from an oenological point of view. Oak samples could be grouped as follows: intense oak, sweet oak and spice oak for the French variety and, blend oak and fresh oak for the American variety. Also, three of them were toasted oak chips, particularly American blend, French sweet and French intense. **Table 1** lists the concentrations of a panel of representative compounds in extracts from vine-shoots, which are used in oenology in common with those in oak wood chips.

Firstly, hydroxymethylfurfural, a degradation product from hexoses, was found in all extracts, which reported similar levels of this furanic aldehyde responsible of light creamy toast and toffee flavor in vine-shoots and oak chips, as can be seen in **Table 1**. Taking into account the content of cellulose and hemicellulose in vine-shoots, furanic compounds are a family of compounds to be taken into account. The extraction temperature of 180 ºC foreseeably enhances degradation of sugars released from vine-shoots wood and promotes the formation of furans *(20)*. There is a controversy about the interest of these compounds: the contribution of two furanic compounds such as furfural and hydroxymethylfurfural to flavoring in processed food by heating is well-known *(22); h*owever, several international organisms, such as the Food and Drug Administration in US (US-FDA), have examined furans, not only as flavor compounds, but also as novel harmful substances in food that undergo a thermal treatment. The European Food Safety Authority (EFSA) has articulated that furan is obviously carcinogenic in rats and mice, probably due to the combination of a genotoxic mechanism *(22, 23)* and hepatotoxicity *(24)*. From a safety perspective and for food quality assurance, the EC Regulation No. 1493/99 sets up a maximum limit for hydroxymethylfurfural of 25 mg/kg in concentrated rectified grape must. With these premises, furans content in extracts should be controlled for a proper exploitation of the latter. Anyway, attending to the concentrations of the extracts (**Table 1**), an enrichment on hydroxymethyl-furfural that surpasses legal established limits in spirits or wine would not be a shortcoming.

One of the most characteristic phenolic compounds found in extracts from all varieties of vine-shoots is gallic acid, a final product from the hydrolysis of ellagitannins, responsible for the astringency character of wines, which was found at a concentration similar to that in extracts from oak wood. Pyrogallol, formed from gallic acid decarboxylation, was detected in all extracts with similar concentrations. There was similarity between concentrations of pyrogallol and gallic acid among vine-shoots varieties, demonstrating that both compounds are connected through a biochemical pathway. The same behavior was not found in

the case of protocatechuic acid and pyrocatechol (decarboxylated products of protocatechuic acid). The pyrocatechol/protocatechuic acid pair was more concentrated in extracts from vine-shoots than in those from any oak wood variety except for the American fresh oak variety. The same situation was found for the guaiacol/vanillic acid pair. The concentration of vanillic acid in oak extracts was higher than that found in any vine-shoot variety, except for the French spice oak class. Guaiacol was exclusively detected in two oak varieties: French sweet oak and American fresh oak. The same behavior as guaiacol was found for vanillin, which was slightly more concentrated in oak wood (except French spice oak) than in extracts from vine-shoots (**Table 1**). This compound is of special interest because of its contribution to flavor.

Catechin, the building block for tannins synthesis, was found at significant concentrations, which is indicative of an important effect of the extraction process on the hydrolysis of tannins. The maximum concentration of catechin in extracts from oak wood was found in the American class, with 1.4 and 2.1 mg/g for fresh oak and blend oak woods, respectively.

The rest of phenolic acids reported similar concentrations in extracts from vine-shoots and those from oak wood; therefore, no discrimination was possible attending to the concentration of these compounds. Acetovanillone was detected in all varieties of vine-shoots, while this compound –with high flavor contribution– was only detected in extract from French spice oak chips. Other compounds with organoleptical incidence were coniferaldehyde and sinapaldehyde, detected at lower concentrations in extracts from vine-shoots, particularly in the case of sinapaldehyde, as can be seen in **Table 1.**

3.2. LC–TOF/MS Analysis of Vine-Shoot Extracts

LC–TOF/MS analysis provided a global profile of polar and mid-polar compounds present in extracts by SHLE that represent a characteristic fingerprinting of each variety. Comparison between them enables evaluation of the similarity/dissimilarity patterns among cultivars considering the complete

Table 1. Concentrations of a panel of interesting compounds in extracts by SHLE from oak wood chips and the averaged concentration found for each compound in the different varieties of vine-shoots, expressed as µg/g vine-shoots.

Sample	Pg	G. Ac	Hf	Py	P. Ac	H. Ac	C	V. Ac	G	V	S. Ac	Av	C. Ac	F. Ac	Cf	S. Ac
French intense oak	2618	121	126	81	61	66	154	340	Nd	259	505	Nd	282	52	130	Nd
French sweet oak	5951	275	94	55	82	166	643	298	66	163	316	Nd	232	Nd	282	Nd
American blend oak	999	45	92	29	79	16	1436	143	D	214	178	Nd	236	19	143	Nd
French spice oak	6680	309	144	89	38	138	599	58	Nd	Nd	133	171	83	Nd	202	Nd
Vine-shoots media	6756	313	385	121	171	41	3936	78	86	30	45	21	22	269	26	1155

D: detected, Nd: not detected; Pg: pyrogallol; G.Ac: gallic acid; Hf: hydroxymethyl-furfural; Py: pyrocatechol; P.Ac: protocatechuic acid; H.Ac: hydroxybenzoic acid; C: catechin; V.Ac: vanillic acid; G: guaiacol; V: vanillin; S.Ac: syringic acid; Av: aceto-vanillone; C.Ac:coumaric acid;F.Ac: ferulic acid; Cf: coniferaldehyde; S.Ac: sinapic acid; S: sinapaldehyde.

data set representative of the extracts composition. Additionally, the identification of characteristic compounds for each variety is allowed, which is not accessible by LC–DAD. This information could lead to the selection of a panel of compounds with interest from the oenological point of view, which would enable to select the cultivar varieties for collection of vine-shoots to extract the target compounds. In this study, non-targeted analysis of extracts was per-formed. The reverse-phase chromatographic method selected for this research enabled separation of a wide range of compounds present in the extracts. A representative example is given in **Figure 1** that illustrates the base peak chromatograms (BPCs) corresponding to the analysis in positive and negative ionization modes of extract isolated from Garnacha Tintorera vine-shoots.

As described under Materials and Methods, molecular features or entities (MFs) were extracted to compare vine-shoot cultivars in both ionization modes. The range of MFs extracted for the different varieties encompasses from 90 to 422 for positive ionization mode and from 310 to 1138 for negative ionization mode. Identification of molecular features in both ionization modes was supported on on-line searching of monoisotopic masses corresponding to molecular entities found in the analyses to PlantCyc database. Monoisotopic masses included in this database were searched in the raw data files obtained by the analysis of extracts from vine-shoot varieties. Search parameters were mass accuracy cut-off below 10 ppm, minimum peaks height of 2500 counts on the profile and centroid spectra and a peak spacing tolerance of 0.0025 *m/z* plus 7 ppm. **Supplementary Table 1** lists the compounds identified in the extracts from Garnacha Tintorera, Merlot and Syrah vine-shoot cultivars, thus demonstrating their different composition.

3.3. Qualitative Comparison of LC–TOF/MS Fingerprints Corresponding to Vine-Shoot Extracts

Attending to the complexity of the obtained data sets, a strategy was designed to simplify the dimensionality of the multivariate matrix. The first algorithm employed for this purpose was application of a frequency filter, which

involved the elimination of those molecular features absent in at least 20% of the samples. In this way, comparability of vine-shoot extracts is based on two fractions: one of them common to all extracts, and another one that could be characteristic of each variety. This data mining involved the reduction of the

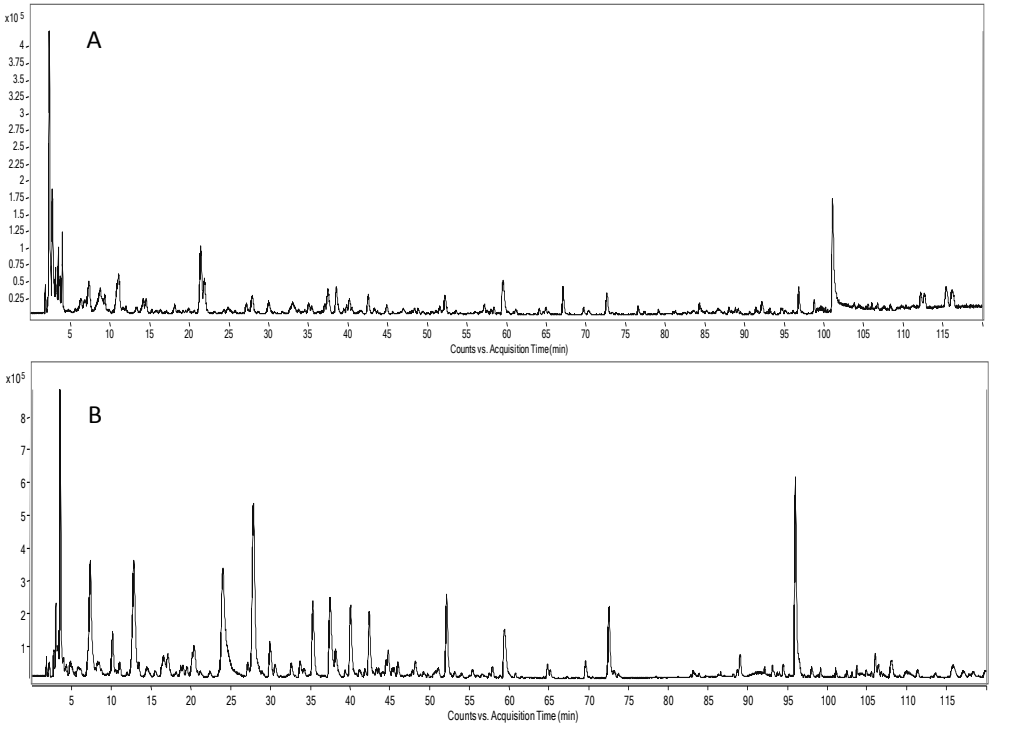

Figure 1. Base-peak chromatograms in positive (A) and negative (B) ionization modes by LC–TOF/MS analysis of an extract by SHLE from Garnacha Tintorera vine-shoot.

molecular features to 68 in the negative mode and 190 in the positive mode as the pretreated data set. After this pretreatment, the data matrix was mean-centered prior to statistical analysis, which was initiated by non-supervised analysis using PCA both to evaluate the distribution of the samples and detect clusters in the new space defined by principal components. **Figure 2** shows the scores plots obtained for data matrices acquired in the positive and negative ionization modes. The variability explained by PC1, PC2 and PC3 was 89.68% and 87.50% in positive and negative ionization modes, respectively. As can be seen, there is a

random distribution of the vine-shoot varieties, but some trends can be observed. There is a main cluster grouping most of the varieties, but also some extracts present differences in their composition. Thus, in the positive ionization mode, extracts from Bobal, Montepila, Syrah, Merlot and Cabernet Franc (B, MP, S, M and CF) provided significant composition differences, whereas the negative ionization mode revealed differences in the composition of extracts from

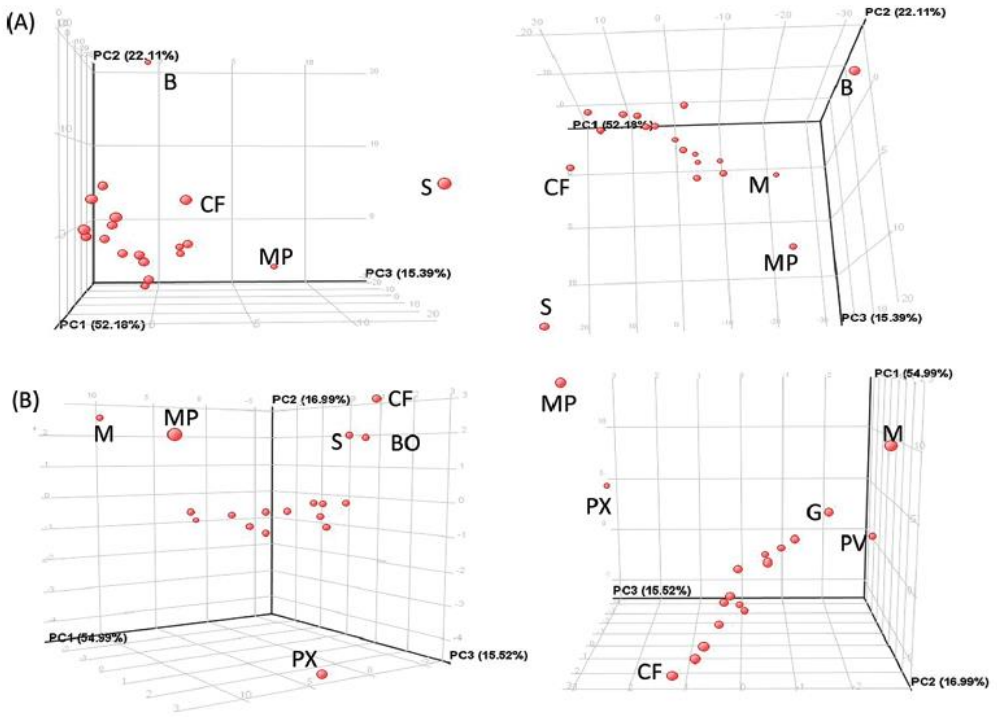

Figure 2. PCA scores plot of the metabolite profiles obtained for different vine-shoot varieties in positive (A) and negative (B) ionization modes. The original data set was filtered by frequency eliminating molecular features non-detected in at least 80% of the samples pertaining to each class.

Montepila, Merlot, Syrah, Bobal and Pedro-Ximénez (MP, M, S, BO, and PX) vine-shoots. The BPCs obtained from these extracts (and their replicates) do not reveal anomalous behaviors that could justify their labeling as laboratory outliers and, for this reason, the discrimination should be linked to their composition.

After this preliminary statistical analysis, the next objective was to apply an ANOVA test to identify molecular features with the highest contribution

to explain the variability observed in PCA graphs. The Mann-Whitney test against zero was employed for estimation, the *p*-value computation was asymptotic and no multiple testing correction was performed. A key parameter to be defined was the confidence level to set the number of features. Thus, the higher the confidence level, the lower number of molecular features. A 95% confidence level involved a reduction from 68 and 190 to 47 and 27 molecular features for the positive and negative ionization modes, respectively. A 98.5% confidence level filtered molecular features to 10 and 11 in the negative and positive ionization modes, while a 99% confidence level reduced them to 8 in global terms.

This test allowed labeling the most significant features contributing to explain the variability visualized for composition of extracts from vine-shoot varieties. **Figure 3** shows the PCA scores plots resulting from the data set obtained after ANOVA with 98.5% confidence level, which limited the number of molecular features to 10. Attending to this panel of compounds, a main cluster was observed with other two additional groupings: one formed by Pedro-Ximénez, Montepila, Merlot, Garnacha Tintorera and Bobal (PX, MP, M, G and B) and, a second one formed by Moscatel, Chardonnay and Syrah (MG, CH and S).

This study can be completed with the PCA corresponding to 99% of confidence level. By analogy to the positive ionization modes, differences in composition were found for extracts from Montepila and Moscatel (MP and MG) varieties and, at a lesser extent, Petit Verdot, Pedro-Ximénez, Garnacha Tintorera and Merlot (PV, PX, G and M) varieties, as can be seen in **Figure 3.** The identification of these molecular features with significant contribution to explain the observed variability reported interesting compounds such as 5-hydroxymethylfurfural, pyrogallol, acetovanillone, coniferyl alcohol, aceto-veratrone, homovanillic acid, syringaldehyde, desapidinol A or ethyl proto-catechuate.

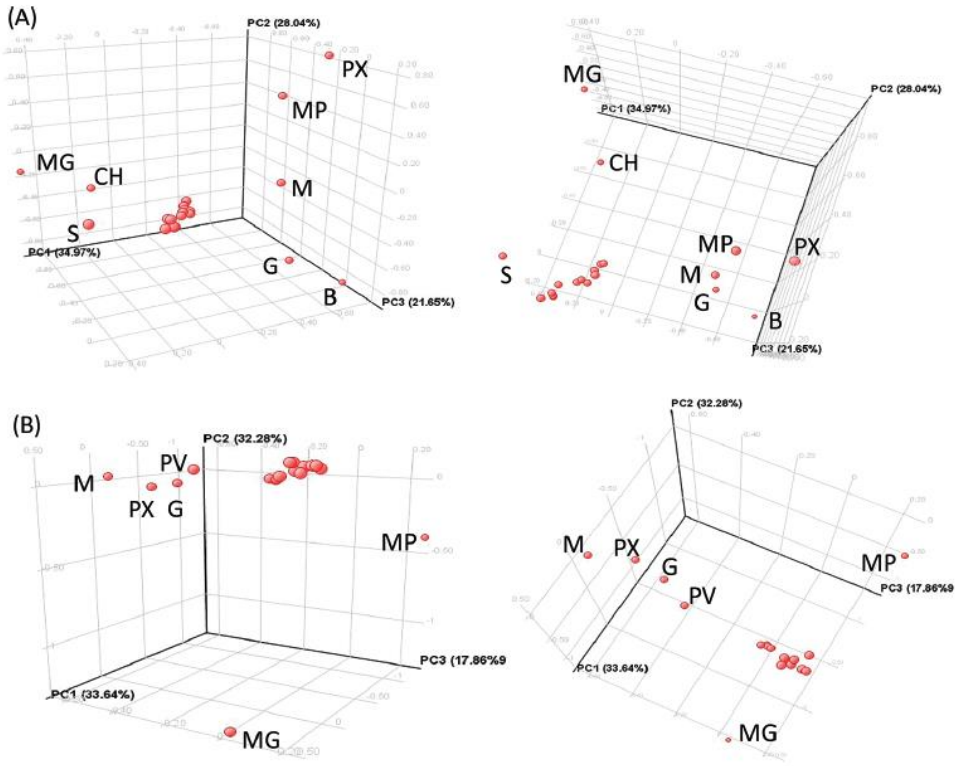

Figure 3. PCA scores plot resulting from the data set obtained in negative ionization mode after one-way ANOVA with (A) 98.5% and (B) 99% confidence level. Moscatel, Chardonnay, Syrah, Pedro-Ximénez, Merlot, Garnacha Tintorera, Baladí, Petit Verdot (MG, CH, S, PX, MP, M, G, B, PV, respectively).

3.4. Comparison of SHL Extracts from Vine-Shoots and Oak Wood Attending to Their LC–TOF/MS Profiles

After comparison of global composition profiles of extracts from different varieties of vine-shoots, the following step was to compare the similarity of these extracts with those obtained from oak wood chips. This study could provide information about those vine-shoot varieties with a more similar composition to that of oak wood as a preliminary test to evaluate the applicability of vine-shoots or their extracts with oenological purposes. Thus, the first statistical analysis with this aim was to filter by frequency the original data set composed by molecular features obtained from TOF analysis from both types of

extracts. The restriction for this type of filter is crucial to ensure comparability. Thus, if the filter limits molecular features to those existing in all extracts of each type of sample, vine-shoots or oak wood chips, the representativeness of each type of sample is ensured. This filter reduced the data set to 69 and 37 molecular features in positive and negative ionization modes, respectively, and provided PCAs such as that illustrated in **Figure 4** for the positive ionization mode. As can be seen, there is a clear discrimination between extracts from oak wood and from vine-shoots.

Taking into account that extracts from two different types of samples are being compared, a less restrictive filter should be employed to estimate the similarity level between both raw materials. Thus, an additional analysis was carried out by filtering the original data set to eliminate those molecular features not present in at least 40% of the extracts from vine-shoots or from oak wood chips. In this case, the original data set was reduced to 647 molecular features in positive and negative ionization modes. **Supplementary Table 2** lists the compounds identified in the extracts from American Fresh, American Blend and French Intense oak varieties, thus demonstrating the varied composition.

The scores graph obtained by principal component analysis of this new data set, shown in **Figure 5**, illustrates a cluster formed by extracts from different vine-shoot varieties with a high similarity to those from oak wood that remain grouped. The varieties were Garnacha Tinta, Malvec, Cabernet Sauvignon, Sauvignon Blanc, Petit Verdot, Pedro-Ximénez, Chardonnay, Garnacha Tintorera, Merlot and Montepila (GT, MA, CS, SB, PV, PX, CH, G, M and MP). According to sample distribution, two red grape varieties such as Garnacha Tintorera and Merlot and a white grape variety such as Montepila reported extracts with the most similar composition to that from oak chips. Therefore, the vine-shoot type in terms of grape color seems not to play a relevant role to yield extracts with similar composition to those provided by oak chips. As can be seen, PC1, PC2 and PC3 explained more than 90% of the variability observed in the scores graph.

This study is the basis to support the valorization of this agricultural residue produced in vineyards. The extracts from this residue have been characterized in this research resulting for some vine cultivars in a composition similar to that of extracts from oak wood chips, which are used with oenological applications.

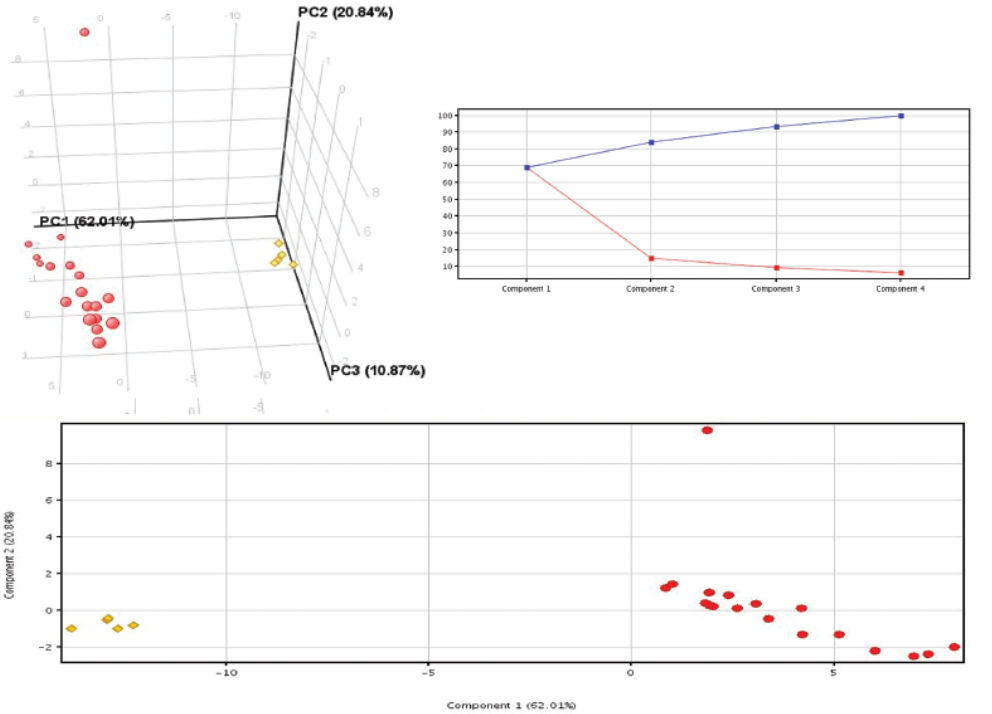

Figure 4. PCA scores plot for the positive ionization mode using the original data set after filtration of those molecular features non-present in all samples belonging to each class: vine-shoots or oak wood. As can be seen, there is a clear discrimination between extracts from oak wood (diamonds) and vine-shoots (circles).

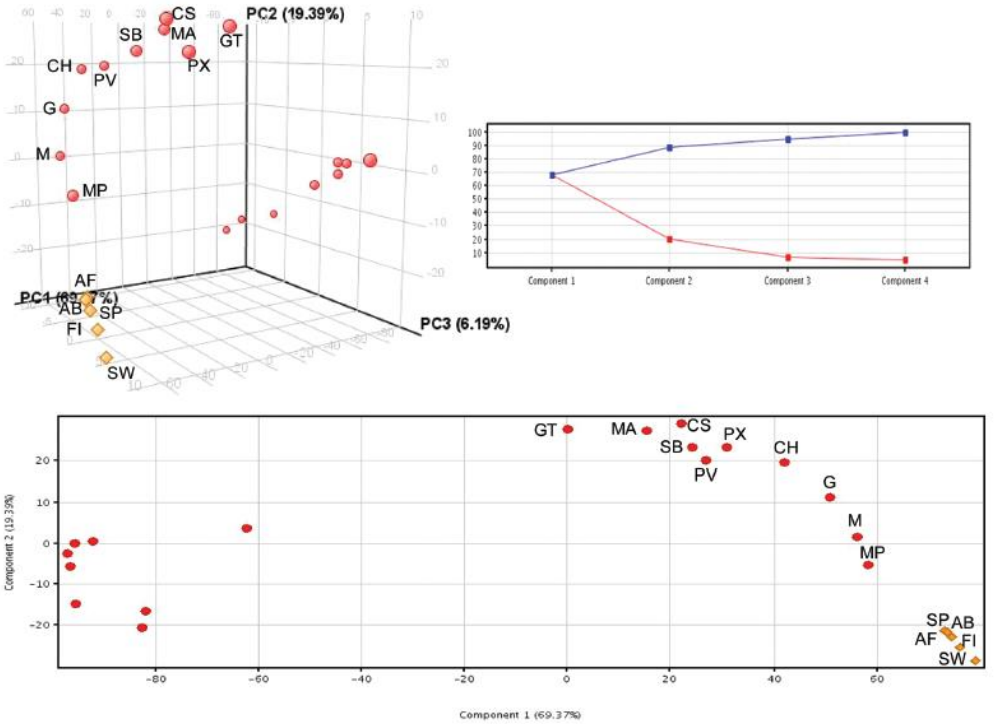

Figure 5. PCA scores plot for negative ionization mode using the original data set after filtration of molecular features non present in 40% of samples belonging to each class: vine-shoots or oak wood. Garnacha Tinta, Malbec, Sauvignon Blanc, Petit Verdot, Cabernet Sauvignon, Pedro-Ximénez, Chardonnay, Garnacha Tintorera, Merlot, Montepila, American Fresh Oak, American Blend Oak, French Spice Oak, French Intense Oak, French Sweet Oak (GT, MA, SB, PV, CS, PX, CH, G, M, MP, AF, AB, SP, FI, SW, respectively).

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Supplementary material

Supplementary Table 1. Compounds identified in the extracts from Garnacha Tintorera, Merlot and *Syrah vine-shoot var*ieties.

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
Garnacha Tintorera						
Pyrogallol	10.156	(M-H)-	125.0242	$C_6H_6O_3$	126.0314	-2.1
5-Hydroxymethylfurfural	10.264	(M+H)+	127.0395	$C_6H_6O_3$	126.0322	3.91
Peonidin-3-glucoside acetaldehyde	10.574	(M+H)+	499.0999	$C_{22}H_{23}ClO_{11}$	498.0926	-0.52
Malvidin-3-glucoside-pytuvic acid	13.076	(M-H)-[-H_2O]	553.0725	$C_{24}H_{25}ClO_{14}$	572.0897	-6.31
Gallic acid	12.802	(M-H)-	169.0145	$C_7H_6O_5$	170.0218	1.73
Fertaric acid	16.279	(M-H)-	325.057	$C_{14}H_{14}O_9$	326.0642	1.41
Epigallocatechin	19.343	(M-H)-	305.0677	$C_{15}H_{14}O_7$	306.075	3.4
Protocatechuic acid	20.185	(M-H)-	153.0186	$C_7H_6O_4$	154.0259	-4.57
Pyrocatechol	20.187	(M-H)-	109.0286	$C_6H_6O_2$	110.0359	-8.25
5-Methyl-2-furancarbaldehyde	20.188	(M-H)-	109.0286	$C_6H_6O_2$	110.0359	-8.2
Mequinol	21.322	(M-H)-	123.0449	$C_7H_8O_2$	124.0522	-2.14
Guaiacol	21.444	(M-H)-	123.0453	$C_7H_8O_2$	124.0526	1.37
Trans-Pterostilbene	22.297	(M-H)-	255.1013	$C_{16}H_{16}O_3$	256.1086	-5.2
2-Furancarboxylic acid, ethyl esther	22.398	(M-H)-	139.0398	$C_7H_8O_3$	140.0473	-0.44
3-Hydroxybenzoic acid	23.97	(M-H)-	137.0248	$C_7H_6O_3$	138.0321	2.88
Procyanidin B2	24.369	(M+H)+	579.1515	$C_{30}H_{26}O_{12}$	578.1441	2.9
Kaempferol	25.21	(M-H)-	285.0423	$C_{15}H_{10}O_6$	286.0496	6.58
Vanillic acid	26.313	(M+H)+	169.0487	$C_8H_8O_4$	168.0412	-6.51
Syringic acid	26.565	(M+H)+	199.0591	$C_9H_{10}O_5$	198.0518	-5.16
3-4-5-Trimethoxyphenol	27.214	(M+H)+	185.0809	$C_9H_{12}O_4$	184.0736	0.29
Ethyl ferulate	27.783	(M-H)-[-H_2O]	203.0714	$C_{12}H_{14}O_4$	222.0885	-3.36
Epicatechin	27.908	(M+H)+	291.0866	$C_{15}H_{14}O_6$	290.0794	1.11
Catechin	27.96	(M+H)+	291.0867	$C_{15}H_{14}O_6$	290.0794	1.4

p-Coumaric acid	28.253	(M-H)-	163.0398	$C_9H_8O_3$	164.0471	-1.63
o-Coumaric acid	28.323	(M-H)-	163.041	$C_9H_8O_3$	164.0483	5.82
4-Hydroxybenzaldehyde	30.576	(M-H)-	121.0291	$C_7H_6O_2$	122.0363	-3.65
Homosyringic acid	33.739	(M+H)+	213.0744	$C_{10}H_{12}O_5$	212.0671	-6.32
Desapidinol A	33.881	(M-H)-	181.0508	$C_9H_{10}O_4$	182.0581	1.19
4-Methylguaiacol	33.885	(M-H)-	137.0604	$C_8H_{10}O_2$	138.0676	-3.2
4-Ethoxyphenol	35.214	(M-H)-	137.0613	$C_8H_{10}O_2$	138.0685	3.29
Lignin	37.376	(M-H)- [-H_2O]	161.0615	$C_{10}H_{12}O_3$	180.0792	2.98
Coniferaldehyde	37.415	(M-H)-	177.0563	$C_{10}H_{10}O_3$	178.0636	3.15
Ethyl homovanillate	38.527	(M+Na)+	233.0792	$C_{11}H_{14}O_4$	210.0899	3.5
Lignin	38.686	(M+Na)+	233.08	$C_{11}H_{14}O_4$	210.0908	7.62
Catechin-O-gallate	41.391	(M+Na)+	465.0795	$C_{22}H_{18}O_{10}$	442.0896	-0.96
Coniferyl alcohol	41.739	(M+H)+	181.0864	$C_{10}H_{12}O_3$	180.0791	2.67
2-Phenylacetaldehyde	41.897	(M-H)-	119.0508	C_8H_8O	120.058	4.37
Piceid	43.747	(M+Na)+	413.1206	$C_{20}H_{22}O_8$	390.1314	-0.19
Sinapaldehyde	44.82	(M-H)- [-H_2O]	189.0569	$C_{11}H_{12}O_4$	208.0742	3.11
Astringinin	46.005	(M-H)-	243.0678	$C_{14}H_{12}O_4$	244.0751	6.37
Ethyl protocatechuate	47.993	(M-H)-	181.0498	$C_9H_{10}O_4$	182.0588	5.11
Sinapaldehyde	48.197	(M+H)+ [-H_2O]	191.0709	$C_{11}H_{12}O_4$	208.0738	1.35
4-Hydroxy-3-methoxyphenilacetic acid	48.198	(M-H)-	181.0505	$C_9H_{10}O_4$	182.0578	-0.64
Astilbin	48.714	(M-H)-	449.1115	$C_{21}H_{22}O_{11}$	450.1187	5.6
Pinoresinol	52.065	(M-H)-	357.1352	$C_{20}H_{22}O_6$	358.1426	2.71
Kaempferol	53.122	(M-H)-	285.0412	$C_{15}H_{10}O_6$	286.0485	2.61
Engeletin	53.664	(M-H)-	433.1164	$C_{21}H_{22}O_{10}$	434.1236	5.39
Kaempferol-3-O-glucoside	55.685	(M-H)-	447.0941	$C_{21}H_{20}O_{11}$	448.1014	1.89
1-Acetoxypinoresinol	56.559	(M+H)+	417.1566	$C_{22}H_{24}O_8$	416.1487	3.79
Diosmetin	57.389	(M-H)-	299.0571	$C_{16}H_{12}O_6$	300.0643	3.18
Viniferin	59.017	(M+H)+	679.1997	$C_{42}H_{30}O_9$	678.1924	5.03
Trans-Viniferin	59.591	(M+H)+	455.152	$C_{28}H_{22}O_6$	454.1447	6.76
1-2-3-4-Tetramethoxy-5-2-propenylbenzene	60.24	(M+Na)+	261.1117	$C_{13}H_{18}O_4$	238.1225	8.36
Quercetin	62.085	(M-H)-	301.0365	$C_{15}H_{10}O_7$	302.0438	3.88
1-Hydroxypinoresinol	67.17	(M-H)-	373.1309	$C_{20}H_{22}O_7$	374.1381	4.23
Trans-Resveratrol	55.823	(M-H)-	227.0709	$C_{14}H_{12}O_3$	228.0782	-1.96

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
2-2-Diethoxyethylbenzene	72.695	(M+H)+	195.1397	$C_{12}H_{18}O_2$	194.1324	8.77
(E,Z)-2,6-Nonadienal	81.427	(M+Na)+	161.0939	$C_9H_{14}O$	138.1047	1.85
Phtalic acid, dibutyl ester	84.367	(M+Na)+	301.1423	$C_{16}H_{22}O_4$	278.1531	4.68
Rutin	97.018	(M+H)+	611.162	$C_{27}H_{30}O_{16}$	610.1547	2.17
Lutein	116.06	(M+H)+	569.4354	$C_{40}H_{56}O_2$	568.4281	0.07
Merlot						
Procyanidin B2	2.969	(M+H)+	579.147	$C_{30}H_{26}O_{12}$	578.1405	-3.26
Astilbin	5.824	(M-H)-	449.111	$C_{21}H_{22}O_{11}$	450.1183	4.7
Pyrogallol	10.147	(M-H)-	125.0237	$C_6H_6O_3$	126.0309	-5.99
Gallic acid	12.842	(M-H)-	169.0145	$C_7H_6O_5$	170.0218	1.52
Malvidin-3-glucoside-pyruvic acid	13.076	(M-H)-[-H_2O]	553.0725	$C_{24}H_{25}ClO_{14}$	572.0897	-6.31
Fertaric acid	16.279	(M-H)-	325.0576	$C_{14}H_{14}O_9$	326.0649	3.34
Epigallocatechin	19.343	(M-H)-	305.0677	$C_{15}H_{14}O_7$	306.075	3.4
5-Methyl-2-furancarbaldehyde	19.462	(M-H)-	109.0292	$C_6H_6O_2$	110.0364	-3.01
Protocatechuic acid	20.144	(M-H)-	153.0193	$C_7H_6O_4$	154.0266	-0.01
Pyrocatechol	20.17	(M-H)-	109.0288	$C_6H_6O_2$	110.0361	-6.23
Guaiacol	21.456	(M-H)-	123.045	$C_7H_8O_2$	124.0523	-1.15
2-Furancarboxylic acid, ethyl esther	22.298	(M-H)-	139.0399	$C_7H_8O_3$	140.0471	-1.41
3-Hydroxybenzoic acid	23.98	(M-H)-	137.0248	$C_7H_6O_3$	138.0321	2.75
Kaempferol	25.248	(M-H)-	285.0403	$C_{15}H_{10}O_6$	286.0476	-0.58
Vanillic acid	26.313	(M+H)+	169.0487	$C_8H_8O_4$	168.0412	-6.51
3-4-5-Trimethoxyphenol	27.202	(M+H)+	185.0815	$C_9H_{12}O_4$	184.0742	3.58
Ethyl ferulate	27.783	(M-H)-	221.0806	$C_{12}H_{14}O_4$	222.0878	-6.19
Epicatechin	27.889	(M+H)+	291.0877	$C_{15}H_{14}O_6$	290.0804	4.53
Catechin	27.937	(M+H)+	291.0876	$C_{15}H_{14}O_6$	290.0803	4.49
p-Coumaric acid	28.253	(M-H)-	163.0398	$C_9H_8O_3$	164.0471	-1.63
o-Coumaric acid	28.323	(M-H)-	163.041	$C_9H_8O_3$	164.0483	5.82
4-Hydroxybenzaldehyde	30.568	(M-H)-	121.0291	$C_7H_6O_2$	122.0364	-3.14
Luteonin-7-O-Glucoside	55.685	(M-H)-	447.0941	$C_{21}H_{20}O_{11}$	448.1014	1.88

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
Homosyringic acid	33.642	(M+H)+	213.0746	$C_{10}H_{12}O_5$	212.0673	-5.35
4-Methylguaiacol	33.853	(M-H)-	137.0619	$C_8H_{10}O_2$	138.0692	8.13
Desapidinol A	33.853	(M-H)-	181.0507	$C_9H_{10}O_4$	182.058	0.29
5-Hydroxymethylfurfural	34.954	(M+H)+	127.0393	$C_6H_6O_3$	126.0321	2.89
4-Ethoxyphenol	35.127	(M-H)-	137.0602	$C_8H_{10}O_2$	138.0675	-4.03
Coniferaldehyde	37.415	(M-H)-	177.0563	$C_{10}H_{10}O_3$	178.0636	3.15
Lignin	38.441	(M+H)+	211.098	$C_{11}H_{14}O_4$	210.0901	4.46
Ethyl homovanillate	38.625	(M+Na)+	233.0799	$C_{11}H_{14}O_4$	210.0907	7.21
Piceid	40.489	(M+H)+	391.1386	$C_{20}H_{22}O_8$	390.1313	-0.36
Catechin-O-gallate	41.391	(M+Na)+	465.0795	$C_{22}H_{18}O_{10}$	442.0896	-0.96
2-Phenylacetaldehyde	41.949	(M-H)-	119.0499	C_8H_8O	120.0572	-2.49
4-Hydroxy-3-methoxyphenilacetic acid	48.169	(M-H)-	181.0499	$C_9H_{10}O_4$	182.0572	-3.86
Sinapaldehyde	48.197	(M+H)+ [-H_2O]	191.0709	$C_{11}H_{12}O_4$	208.0738	1.35
Pinoresinol	52.065	(M-H)-	357.1352	$C_{20}H_{22}O_6$	358.1426	2.71
Trans-Resveratrol	52.748	(M-H)-	227.0718	$C_{14}H_{12}O_3$	228.079	1.71
1-Acetoxypinoresinol	56.559	(M+H)+	417.1566	$C_{22}H_{24}O_8$	416.1487	3.79
Diosmetin	57.389	(M-H)-	299.0571	$C_{16}H_{12}O_6$	300.0643	3.18
Trans-Viniferin	59.488	(M+H)+	455.1515	$C_{28}H_{22}O_6$	454.1442	5.59
1-2-3-4-Tetramethoxy-5-2-propenylbenzene	60.116	(M+Na)+	261.1119	$C_{13}H_{18}O_4$	238.1227	9.07
Quercetin	62.085	(M-H)-	301.0365	$C_{15}H_{10}O_7$	302.0438	3.88
1-Hydroxypinoresinol	67.17	(M-H)-	373.1309	$C_{20}H_{22}O_7$	374.1381	4.23
Syrah						
Pyrogallol	10.018	(M-H)-	125.025	$C_6H_6O_3$	126.0323	4.71
Peonidin-3-glucoside acetaldehyde	10.574	(M+H)+	499.0999	$C_{22}H_{23}ClO_{11}$	498.0926	-0.52
Ethyl ferulate	11.868	(M+Na)+	245.0787	$C_{12}H_{14}O_4$	222.0895	1.11
Gallic acid	12.702	(M-H)-	169.0127	$C_7H_6O_5$	170.02	-8.96
Malvidin-3-glucoside-pytuvic acid	12.793	(M-H)-	571.0834	$C_{24}H_{25}ClO_{14}$	572.0907	-4.48
Fertaric acid	16.137	(M-H)-	325.0553	$C_{14}H_{14}O_9$	326.0626	-3.6

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
Protocatechuic acid	16.414	(M-H)-	153.0189	$C_7H_6O_4$	154.0262	-2.72
Epigallocatechin	18.852	(M-H)-	305.0683	$C_{15}H_{14}O_7$	306.0756	5.37
5-Methyl-2-furancarbaldehyde	20.053	(M-H)-	109.0293	$C_6H_6O_2$	110.0364	-3.88
Mequinol	21.322	(M-H)-	123.0449	$C_7H_8O_2$	124.0522	-2.14
2-Furancarboxylic acid, ethyl ester	22.182	(M-H)-	139.0398	$C_7H_8O_3$	140.047	-2.18
p-Coumaric acid	23.477	(M-H)-	163.0394	$C_9H_8O_3$	164.0467	-4.03
3-Hydroxybenzoic acid	23.848	(M-H)-	137.0238	$C_7H_6O_3$	138.0311	-4.45
Epicatechin	27.712	(M-H)-	289.072	$C_{15}H_{14}O_6$	290.0794	1.23
Coumaric acid	32.896	(M-H)-	295.0472	$C_{13}H_{12}O_8$	296.0544	4.13
4-Ethoxyphenol	33.7	(M-H)-	137.0603	$C_8H_{10}O_2$	138.0675	-3.99
4-Methylguaiacol	34.931	(M-H)-	137.0601	$C_8H_{10}O_2$	138.0674	-5.21
Piceid	40.228	(M-H)-	389.1259	$C_{20}H_{22}O_8$	390.1333	4.81
Catechin-O-gallate	41.043	(M-H)-	441.0848	$C_{22}H_{18}O_{10}$	442.0921	4.83
Epicatechin-O-gallate	41.046	(M-H)-	441.0848	$C_{2_2}H_{18}O_{10}$	442.0921	4.76
2-Phenylacetaldehyde	41.782	(M-H)-	119.0498	C_8H_8O	120.0571	-3.82
Trans-Viniferin	43.165	(M-H)-	453.1362	$C_{28}H_{22}O_6$	454.1433	3.59
Astringinin	45.873	(M-H)-	243.0666	$C_{14}H_{12}O_4$	244.0739	1.49
p-HPEA-EA Ligstroside aglycon	46.015	(M-H)-	361.1283	$C_{19}H_{22}O_7$	362.1356	-2.63
Ethyl protocatechuate	47.993	(M-H)-	181.0498	$C_9H_{10}O_4$	182.0588	5.11
4-Hydroxy-3-methoxyphenilacetic acid	47.998	(M-H)-	181.0516	$C_9H_{10}O_4$	182.0588	5.11
Astilbin	48.714	(M-H)-	449.1115	$C_{21}H_{22}O_{11}$	450.1187	5.6
1-Acetoxypinoresinol	51.354	(M-H)-	415.1394	$C_{22}H_{24}O_8$	416.1466	-1.16
Pinoresinol	51.915	(M-H)-	357.1347	$C_{20}H_{22}O_6$	358.1419	0.86
Kaempferol	53.122	(M-H)-	285.0412	$C_{15}H_{10}O_6$	286.0485	2.61
Engeletin	53.664	(M-H)-	433.1164	$C_{21}H_{22}O_{10}$	434.1236	5.39
Kaempferol-3-O-glucoside	55.685	(M-H)-	447.0941	$C_{21}H_{20}O_{11}$	448.1014	1.89
Luteonin-7-O-Glucoside	55.685	(M-H)-	447.0941	$C_{21}H_{20}O_{11}$	448.1014	1.88
Trans-Resveratrol	55.823	(M-H)-	227.0709	$C_{14}H_{12}O_3$	228.0782	-1.96

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
Diosmetin	57.297	(M-H)-	299.0536	$C_{16}H_{12}O_6$	300.0609	-8.29
Phtalic acid, dibutyl ester	84.284	(M+H)+	279.1607	$C_{16}H_{22}O_4$	278.1519	0.25
Methoxyeugenol	91.507	(M-H)-	193.0898	$C_{11}H_{14}O_3$	194.0971	14.43
1-2-3-4-Tetramethoxy-5-2-propenylbenzene	92.354	(M+H)+	239.1296	$C_{13}H_{18}O_4$	238.1224	7.79
Rutin	97.018	(M+H)+	611.162	$C_{27}H_{30}O_{16}$	610.1547	2.17
Oleanolic acid	98.616	(M-H)-	455.3542	$C_{30}H_{48}O_3$	456.3615	2.47
Lutein	116.06	(M+H)+	569.4354	$C_{40}H_{56}O_2$	568.4281	0.07

Supplementary Table 2. Compounds identified in the extracts from American blend, American fresh and French intense oak varieties.

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
American Blend						
Pyrogallol	10.21	(M-H)-	125.0246	$C_6H_6O_3$	126.0319	1.65
Hydroxymethylfurfural	10.254	(M-H)-	125.0247	$C_6H_6O_3$	126.032	2.21
Gallic acid	12.875	(M-H)-	169.0143	$C_7H_6O_5$	170.0215	0.04
Caffeic acid	15.527	(M-H)-	179.0359	$C_9H_8O_4$	180.0432	5.22
Protocatechuic acid	20.248	(M-H)-	153.0194	$C_7H_6O_4$	154.0267	0.54
5-Methyl-2-furancarbaldehyde	20.253	(M-H)-	109.0285	$C_6H_6O_2$	110.0358	-8.88
Guaiacol	21.302	(M-H)-	123.0445	$C_7H_8O_2$	124.0518	-5.3
3-Hydroxybenzoic acid	23.974	(M-H)-	137.0237	$C_7H_6O_3$	138.0310	-5.33
3-Ethoxy-4-methoxyphenol	26.381	(M-H)-	167.0703	$C_9H_{12}O_3$	168.0777	-5.8
p-Coumaric acid	28.321	(M-H)-	163.0392	$C_9H_8O_3$	164.0465	-5.04
Desapidinol A	33.882	(M-H)-	181.0506	$C_9H_{10}O_4$	182.0578	-0.38
4-Methylguaiacol	33.953	(M-H)-	137.0597	$C_8H_{10}O_2$	138.0670	-7.7
Homosyringic acid	34.421	(M+H)+	213.076	$C_{10}H_{12}O_5$	212.0692	3.65
Lignin	37.308	(M+H)+	211.0969	$C_{11}H_{14}O_4$	210.0899	3.46
4-Hydroxybenzaldehyde	37.569	(M+H)+	123.0445	$C_7H_6O_2$	122.0372	3.44
Ethyl homovanillate	38.372	(M+H)+	211.0968	$C_{11}H_{14}O_4$	210.0896	2.02
Piceid	43.739	(M-H)-	389.1278	$C_{20}H_{22}O_8$	390.1351	9.29
4-Hydroxy-3-methoxyphenilacetic acid	48.162	(M-H)-	181.0506	$C_9H_{10}O_4$	182.0579	-0.22
Pinoresinol	52.988	(M+Na)+	381.1323	$C_{20}H_{22}O_6$	358.1430	3.91
Egallic acid	54.746	(M-H)-	300.9963	$C_{14}H_6O_8$	302.0036	-8.86
1-Acetoxypinoresinol	56.561	(M+H)+	417.1541	$C_{22}H_{24}O_8$	416.1469	-0.6
1-2-3-4-Tetramethoxy-5-2-propenylbenzene	60.297	(M+Na)+	261.111	$C_{13}H_{18}O_4$	238.1218	5.42
Methoxyeugenol	64.426	(M+H)+	195.1024	$C_{11}H_{14}O_3$	194.0948	2.43
1-Hydroxypinoresinol	67.810	(M+H)+	375.1459	$C_{20}H_{22}O_7$	374.1383	4.68
Phtalic acid dibutyl ester	84.327	(M+Na)+	301.1423	$C_{16}H_{22}O_4$	278.1531	4.53

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
American Fresh						
Pyrogallol	10.152	(M-H)-	125.0234	$C_6H_6O_3$	126.0307	-8.12
Gallic acid	12.804	(M-H)-	169.014	$C_7H_6O_5$	170.0217	0.97
Protocatechuic acid	16.545	(M-H)-	153.0185	$C_7H_6O_4$	154.0258	-5.19
5-Methyl-2-furancarbaldehyde	20.187	(M-H)-	109.0295	$C_6H_6O_2$	110.0368	-0.03
p-Coumaric acid	20.259	(M-H)-	163.039	$C_9H_8O_3$	164.0463	-6.61
3-Hydroxybenzoic acid	24.051	(M-H)-	137.0231	$C_7H_6O_3$	138.0304	-9.68
3-Ethoxy-4-methoxyphenol	26.381	(M-H)-	167.0703	$C_9H_{12}O_3$	168.0777	-5.8
Caffeic acid	33.047	(M-H)-	179.0359	$C_9H_8O_4$	180.0432	5.22
Vanillin	33.059	(M-H)-	151.0403	$C_8H_8O_3$	152.0476	1.73
Homosyringic acid	37.09	(M+H)+	213.0739	$C_{10}H_{12}O_5$	212.0667	-8.51
Methoxyeugenol	38.292	(M+H)+	195.1013	$C_{11}H_{14}O_3$	194.094	-1.54
1-Hydroxypinoresinol	38.296	(M-H)-	373.13	$C_{20}H_{22}O_7$	374.1373	1.91
Lignin	38.568	(M+Na)+	233.0798	$C_{11}H_{14}O_4$	210.0906	6.49
1-Acetoxypinoresinol	42.039	(M+H)+	417.1551	$C_{22}H_{24}O_8$	416.1476	1.08
Coniferaldehyde	44.673	(M+H)+	179.0705	$C_{10}H_{10}O_3$	178.0633	1.57
Sinapaldehyde	44.941	(M+H)+	209.0816	$C_{11}H_{12}O_4$	208.0744	3.89
4-Hydroxy-3-methoxyphenilacetic acid	48.099	(M-H)-	181.0512	$C_9H_{10}O_4$	182.0584	2.88
1-3-4-Dihydroxyphenyl-1-propanone	49.433	(M+H)+	167.0709	$C_9H_{10}O_3$	166.0637	4.48
Egallic acid	54.305	(M+H)+	303.0149	$C_{14}H_6O_8$	302.0076	4.37
1-2-3-4-Tetramethoxy-5-2-propenylbenzene	60.297	(M+Na)+	261.111	$C_{13}H_{18}O_4$	238.1218	5.42
p-Hydroxybenzalacetone	61.1	(M+H)+	163.0763	$C_{10}H_{10}O_2$	162.0691	6.59
Piceid	61.739	(M-H)-	389.1278	$C_{20}H_{22}O_8$	390.1351	9.29
Pinoresinol	68.348	(M-H)-	357.1376	$C_{20}H_{22}O_6$	358.1449	9.15
Phtalic acid dibutyl ester	84.351	(M+Na)+	301.143	$C_{16}H_{22}O_4$	278.1538	7.25
French Intense						
Pyrogallol	10.21	(M-H)-	125.0246	$C_6H_6O_3$	126.0319	1.65
5-Hydroxymethylfurfural	10.254	(M-H)-	125.0247	$C_6H_6O_3$	126.032	2.21

Compound	Retention time (min)	Ion	Actual *m/z*	Formula	Experimental *m/z*	Error (ppm)
Gallic acid	12.886	(M-H)-	169.0138	$C_7H_6O_5$	170.0211	-2.47
5-Methyl-2-furancarbaldehyde	20.348	(M-H)-	109.0286	$C_6H_6O_2$	110.0359	-8.18
3-Hydroxybenzoic acid	24.127	(M-H)-	137.0236	$C_7H_6O_3$	138.0309	-5.91
p-Coumaric acid	28.338	(M-H)-	163.0387	$C_9H_8O_3$	164.0465	-5.05
Homosyringic acid	34.48	(M+H)+	213.0764	$C_{10}H_{12}O_5$	212.0693	3.87
Ethyl homovanillate	35.372	(M+H)+	211.0968	$C_{11}H_{14}O_4$	210.0896	2.02
Lignin	35.39	(M+H)+	211.0968	$C_{11}H_{14}O_4$	210.09	3.82
Desapidinol A	37.484	(M-H)-	181.0506	$C_9H_{10}O_4$	182.0578	-0.38
4-Hydroxy-3-methoxyphenilacetic acid	37.534	(M-H)-	181.0508	$C_9H_{10}O_4$	182.0581	1.19
Coniferaldehyde	44.613	(M-H)-	177.0554	$C_{10}H_{10}O_3$	178.0627	-1.84
1-3-4-Dihidroxyphenyl-1-propanone	51.82	(M+H)+	167.0702	$C_9H_{10}O_3$	166.063	-0.2
1-Acetoxypinoresinol	53.969	(M+H)+	417.1541	$C_{22}H_{24}O_8$	416.1469	-0.59
Egallic acid	54.024	(M+H)+	303.0137	$C_{14}H_6O_8$	302.0064	0.45

Chapter 4:

Comparative profiling analysis of woody flavoring from vine-shoots and oak chips

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Comparative profiling analysis of woody flavoring from vine-shoots and oak chips

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Comparative profiling analysis of woody flavoring from vine-shoots and oak chips

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Abstract

Background: Woody liquid flavorings extracted from different varieties of vine-shoots and oak chips have been characterized by GC–MS to compare the profile of compounds foreseeable contributors to the organoleptical properties of wine and spirits aged in oak barrels. Oak chips are frequently added to barrels to accelerate the ageing process, while vine-shoots are produced in high amounts in wine-producing countries.

Results: The extracts were isolated by superheated liquid extraction (SHLE) after optimization of extraction variables. The SHLE protocol was performed using ethanol–water mixtures (pH 3) at 220 ºC for 60 min. Compounds were identified using NIST databases, and the resulting profile was used as data set for qualitative and semiquantitative comparison between extracts obtained from different varieties of vine-shoots and oak chips.

Conclusion: Statistical analysis enabled to demonstrate the similarity between extracts from vine-shoots and oak wood, providing the first study on this subject. The special role of phenols and furanic derivatives has been described. This study is the first stage for characterization of vine-shoots as a by-product with potential to be used in the oenological field.

1. Introduction

Oak barrels have traditionally been used for storage and ageing of wines because of the positive effects of oak wood on key organoleptical properties of wine, such as aroma, odor, color and taste *(1)*. These effects are the result of a spreading chain of physical and chemical transformations *(2)*. which are responsible, for example, of the reduction of wine astringency *(3)*.

Oak wood is mainly composed of three large insoluble polymers: cellulose, hemicellulose and lignin. Additionally, there is a minor fraction of low molecular-weight compounds such as lipids (oils, fats and waxes) and tannins, which are characterized by a high solubility in hydroalcoholic media that justifies the partial transfer to wine when is in contact with oak wood *(4)*. As a result, tannins enhance the oxidizing potential and taste properties of wine *(5–7)*, playing a role in the aging process in oak barrels *(8,9)*.

Thermal treatments of oak wood favor hydrolysis pathways of insoluble polymers, which are degraded leading to raised levels of compounds with direct influence on the organoleptical properties of wine *(10,11)*. Apart from that, a small portion of lignin in contact with wine may undergo slow hydrolysis according to well-known mechanisms *(12,13)*, since wine is a hydroalcoholic solution with a pH around 3.6. The released components are mainly phenolic compounds and derivatives present also as monomers in wood and with a marked sensory standpoint. Aldehydic phenols play a major role in the organoleptic quality of premium quality wine *(14)*. Among them, vanillin is the main aldehyde exerting effects on the aroma of wines aged in oak barrels *(15)*. Syringaldehyde and cinnamic aldehydes such as coniferaldehyde and sinapaldehyde are also present in oak wood, as well as their aromatic acid derivatives. On the other hand, gallic and ellagic acids and various hydrolyzable tannins (condensed with gallic and ellagic acids) from wood can also be extracted

(16). It is worth mentioning the role of small phenols such as eugenol and guaiacol that provide spice and smoke attributes.

The phenolic fraction, including ellagitannins, extracted from wood into the wine during aging depends on the pool of these compounds present in the barrel wood. Their structures and concentrations can be affected by the species of wood and other factors such as geographical origin *(17–19)*, cask size, individual cask history and warehouse conditions, although wood type and wood treatment are the main factors *(20)*. Particularly, the seasoning and toasting processes play critical roles in the tannic composition of oak wood *(18–20)*.

Furans can also be formed by thermal treatment of oak wood due to degradation of monosacharides released by partial hydrolysis of hemicelluloses *(15)*. Among them, furan aldehydes such as furfural possess almond-like taste. However, furan aldehydes and their corresponding hydroxymethyl analogues possess high aroma thresholds and, therefore, their contribution to wine aroma is generally presumed to be limited *(13)*.

Traditional wine ageing is an expensive and time-consuming process owing to the long lasting of wine maturation by using this method, which can range from six months to even twenty years in cases such as ageing of Madeira wine *(23)*. Different techniques have arisen to accelerate the ageing process and turn it into more convenient and economical. A wide extended practice is the addition of oak chips to barrels. This material can be commercially found in different forms, such as cubes, beans, powder, pencil shavings or, simply, granulated pieces or chips. The final product is determined, not only by the different characteristics of the added oak such as their origin *(24),* size or toasting process, but also by the dosage used, period of contact with wine, and possible oxygenation of wine *(25)*. Taking into account the variability of oak woods, it should be interesting to compare the composition of woody flavorings isolated from them with those obtained from other sources. In this sense, vine-shoots from *Vitis vinifera* could be a promising alternative to oak wood taking into

account the amount of this residue produced in wine-producing countries. The concentration of phenolic compounds in extracts from different varieties of vine-shoots by LC–DAD was determined in a recent study *(26)*. For that purpose, superheated liquid extraction (SHLE) was selected as extraction approach according to the method developed by Luque-Rodríguez *et al. (27)*, who developed a method for determination of a panel of representative compounds in vine-shoot extracts. Among these compounds it is worth mentioning phenolic acids such as gallic acid and vanillic acid and aldehydes such as syringaldehyde, and vanillin. However, a comparative profiling analysis of the woody flavorings isolated from vine-shoots and oak wood by GC–MS has not been made yet. The purpose of the present study was to carry out a statistical comparison of the profile of low-molecular weight phenols and furans present in extracts obtained by superheated liquids from different varieties of vine-shoots and oak chips to evaluate the similarity among them. For this purpose, five varieties of French and American oak chips and seventeen varieties of vine-shoots were used.

2. Experimental

2.1. Samples

Chromatographic mobile phase B was prepared in LC–MS grade acetonitrile from Sigma–Aldrich (Madrid, Spain). MS grade formic acid from Scharlab (Barcelona, Spain) was used as ionization agent in LC–QTOF analyses, and deionized water (18 MΩ·cm) supplied by a Millipore Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to prepare the chromatographic aqueous phase. All samples were prepared in LC grade methanol from Scharlab.

2.2. Instruments and Apparatus

Vine-shoots from different *Vitis vinifera* cultivars were sampled from the same plantation located at "Bodega y Viñedos Vallebravo" (Sierra de Segura, Jaén, Spain) in autumn, after leaf-fall, by randomized selection of ten similar stocks of each cultivar. Vine cultivars were selected from the following agronomical varieties: Airen, Baladí, Bobal, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Garnacha Tinta, Garnacha Tintorera, Malbeq, Mazuelo, Merlot, Moscatel, Pedro-Ximénez, Sauvignon Blanc, Syrah and Tempranillo (A, B, BO, CF, CS, CH, GT, G, MA, MZ, M, MO, PX, SB, S and T, respectively). The varieties were chosen because they are widely distributed in Spain. All them were cultivated under the same agronomic conditions in terms of soil properties, climate, hydric regime, etc. Pieces of 10 cm of vine-shoot at the height of the first leaf bud were taken in all cases. All species were dried for 72 h at 30 ºC, milled to get a homogeneous 40-mesh particle size (less than 0.42 mm diameter), and kept at –20 ºC until use.

Samples of five oak chips representing the main types of oak barrels (American blend, American fresh, French sweet, French nobile spice and French intense) (AB, AF, FN, FS and FI, respectively) were provided by Laffort (Guipúzcoa, Spain). As oak chips are commercialized as dried product, they were directly milled following the same protocol as for vine-shoots.

2.3. Reagents

Ethanol (96% v/v) PA from Panreac (Barcelona, Spain) and distilled water were used to prepare the extractant. Deionized water (18 MΩ·cm) was obtained from a Millipore (Bedfore, MA, USA) Milli-Q plus system. LC-grade dichloromethane (supplied by Scharlab, Barcelona, Spain) was used for liquid–liquid extraction. Hydrochloric acid from Panreac was used to acidify both the extract-ant and the extract at the optimum pH.

2.4. Apparatus and Instruments

Vine-shoots and oak chips were milled by a ball grinder (Restch MM301, Haan, Germany). Superheated liquid extraction (SHLE) was performed by a laboratory-made dynamic extractor *(27),* consisting of the following units: a) an extractant supplier; b) a high-pressure pump (Shimadzu LD-AC10) that propels the extractant through the system; c) a switching valve placed next to the pump to develop static extractions; and d) a stainless steel cylindrical extraction chamber (550 mm × 10 mm i.d., 4.3 mL internal volume) where the sample is placed. This chamber is closed at both ends with screws whose caps contain cotton made filters to ensure the sample is not carried away by the extractant; e) a restriction valve to maintain the desired pressure in the system; f) a cooler made of a stainless steel tube (1 m length, 0.4 mm i.d.) and refrigerated with water; g) a gas chromatograph oven (Konix, Cromatix KNK-2000) where the extraction chamber is placed and heated.

Shaking and centrifugation of the extracts were carried out by an MS2 Minishaker (IKA, Germany) Vortex and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

Individual separation of volatile compounds was carried out by a Varian CP 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2200 ion-trap mass spectrometer (Sunnyvalley, TX, USA). The chromatograph was furnished with a Varian CP 8400 autosampler and a Factor Four VF-5ms fused silica capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness) provided by Varian.

2.5. Extraction of Phenolic Compounds and Treatment of Extracts

Extracts from vine-shoots and oak chips were obtained by SHLE as described by Delgado-Torre *et al. (26)*, where 1.5 g of sample was used, the extractant was 80% (v/v) aqueous ethanol at pH 3, with an extraction time of 1 h, and an extraction temperature of 220 ºC. An external standard (3,5-dichloro-

anisole) was added at 10 μg/mL to 3 mL aliquots of each extract, which were adjusted to pH 2.5 using hydrochloric acid and, then, 1 mL of dichloromethane and 2 mL of milli-Q water was added to each. After 10 min stirring in a vortex, the extracts were subjected to 8 min centrifugation at 850 × g. Finally, the organic fraction was isolated and filtered using a 0.45 μm pore-size filter before chromatographic analysis.

2.6. GC–MS Analysis

1.5 μL of the analytical sample was injected into the chromatograph. The injector temperature was fixed at 280 ºC, and the injection was in the split mode, for which the splitter was opened (10:1) for 10 min, then closed. The oven temperature program was as follows: initial temperature 50 ºC (held for 10 min), increased at 3 ºC/min to 80 ºC (held for 2 min), increased at 5 ºC/min to 180 ºC (held for 1 min), followed by a third gradient at 2 ºC/min to 200 ºC, and, finally, increased at 10 ºC/min to 300 ºC (held for 10 min). The total analysis time was 68 min, and 2 min extra time was necessary for re-establishing and equilibrating the initial conditions. The ion-trap mass spectrometer was operated in the electron impact ionization (EI) fixed mode, for which the instrumental parameters were set as follows: filament emission current 70 μA; transfer-line, ion trap and manifold temperatures were kept at 170, 150 and 40 ºC, respectively. The recording window was set between 90 *m/z* and 650 *m/z* and the data were acquired using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment in order to remove the chromatographic background. Additionally, chromatograms were smoothed by application of a Savitzky-Golay filter (5 points). AMDIS GC–MS analysis software was used to transform raw data files into generic files, which were treated with the qualitative software MassHunter.

2.7. Data Processing and Statistical Analysis

MassHunter Workstation software (version 3.01 Qualitative Analysis, Agilent Technologies, Santa Clara, CA, USA) was used for processing all data obtained once they were transformed to AMDIS generic files. The feature

extraction algorithm took into account all single charged ions exceeding 5000 counts with a signal-to-noise ratio (*S/N*) higher than 10. Unique mass was used for peak area calculations while the peak width was set at 0.5 s. The NIST08 electron ionization mass spectral database was used for the spectral search. Tentative identification was supported on correlation between experimental mass and database spectra above 700 in normal search mode. The retention index (*I*) for each compound was calculated using as reference linear hydrocarbons from C10 to C40, which were analyzed by the same chromatographic method. *I* values were calculated on the basis of the retention time. The experimental *I* for each compound was compared with values reported in the literature included in the NIST database, which can be either experimental or theoretical. The cut-off limit for *I* difference was set to 100 units for theoretical reference values, while 30 units was used for experimental reference values.

The elu. and fin. files created for each sample were exported to the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA, USA) for further processing. Within this algorithm, ions with related *m/z* values were extracted as molecular features (MFs) or entities characterized by retention time (RT) and intensity in apex of chromatographic peak. In the next step, alignment of RT and *m/z* values was carried out across the sample set using a tolerance window of 0.2 min retention time.

Baseline correction eliminated the contribution of background noise. Stepwise reduction of MFs number was performed based on frequency of occurrence, abundance of the respective MFs in classes and PCA results of the data. MPP software also enabled partial least squared analysis (PLS) and fold-change. The data were mean centered as a data pretreatment to lower relatively large differences among the respective MFs abundances.

3. Results and discussion

Characterization of extracts from vine-shoots was carried out using SHLE followed by a fractionation protocol with dichloromethane. This enables tentative identification of phenols and furans, potentially transferable to wine in case that vine-shoots were used as material for wine ageing. SHLE was selected as extraction approach according to the results obtained by Delgado de la Torre *et al. (26)* and Luque-Rodríguez *et al. (27)*. In these studies, the influence of the extractant composition, expressed as ethanol concentration in water and pH, temperature and extraction time was studied to maximize the isolation of the target compounds from vine-shoots in a time as short as possible. The optimum extraction conditions for these studies were 220 ºC for 60 min with 80% (v/v) ethanol in a pH 3 aqueous solution. Under these conditions, the extract was dark colored and its odor could be described as from strongly aromatic wood. However, the extraction process has not been evaluated from an overall point of view. For this reason, a new optimization study was planned to evaluate the influence of the main variables on the extraction process.

3.1. Influence of the Extractant pH and Temperature

The first study was focused on the effect of pH on the composition of the extracts from vine-shoots isolated by SHLE. For this purpose, three operation temperatures —120, 170 and 220 ºC— and two pH values —3 and 11— were combined to evaluate the different profiles of compounds. The extractant composition was set at 80% (v/v) ethanol, while the leaching time was programmed for 60 min according to previous studies *(26,27)*. The effect of both variables was assessed by targeting analysis of three representative families of compounds: guaiacyl-type ring metabolites (homovanillyl alcohol, homovanillic acid, propiovanillone, vanillin, coniferaldehyde, acetovanillone, ferulic acid, eugenol, coniferyl alcohol and vanillylacetone), syringyl-type ring metabolites (syringol, syringic acid, sinapaldehyde, siryngaldehyde, homosyringic acid,

acetosyringone and methoxyeugenol), and furanic derivatives (furfural, 5-hydroxymethylfurfural and 2-hydroxymethylfuran). The evolution of each family of compounds can be observed in **Figure 1**, where three trends can be distinguished for the monitored families: 1) their relative concentrations (calculated using the signal of the target compounds/signal of the external standard) were considerably higher at 220 ºC for both tested pHs; 2) the differences between compounds extracted at acid and basic pH were only significant at 220 ºC; and 3) furanic derivatives formed by degradation of carbohydrates were mainly formed at 220 ºC and pH 3. It is worth pointing out that both variables, the extractant pH and the operation temperature, played a decisive role in the isolation of the studied families of compounds since the combination of acid pH and high temperature enhanced the hydrolysis of the polymeric structure of lignin. According to these results, the extraction pH was set at 3.

3.2. Evolution of the Extracts Composition with Temperature

Due to the decisive influence of the extraction temperature on the qualitative composition of SHL extracts, an additional test was developed by extraction at five temperatures: 160, 180, 200, 220 and 240 ºC. The extractant composition was defined according to the previous studies of this research. **Figure 2.A** illustrates the chromatographic profiles reported by analysis of extracts from Pedro-Ximénez vine-shoots at the five extraction temperatures. As can be seen, temperatures below 220 ºC did not result in extracts with a rich composition in compounds foreseeable contributors to the organoleptical properties. On the other hand, the extracts obtained at 220 and 240 ºC were quite similar with subtle differences such as the signal detected at 13 min retention time that corresponded to 5-hydroxymethylfurfural. This chromatographic peak practically disappeared when the extraction temperature was set at 240 ºC. These results were compared to the same tests carried out with oak chips, as shows **Figure 2.B**. In the case of oak wood, significant differences for some chromatographic signals were detected. Thus, there is a high intense

peak eluting at ca. 50 min that corresponded to benzene-1,2,3,4-tetramethoxy-5-(2-propenyl). This peak was clearly increased up to 200 ºC extraction temperature, but it was practically undetected at 220 ºC and definitively no detected at 240 ºC. The opposite situation was observed at 38.5 min retention time. This peak was identified as acetovanillone, extracted at temperature above 200 ºC. One other significant difference was the peak detected at 55 min in the extract from oak chips at 220 ºC. This compound, identified as ethyl oleate, was not detected at other extraction temperatures.

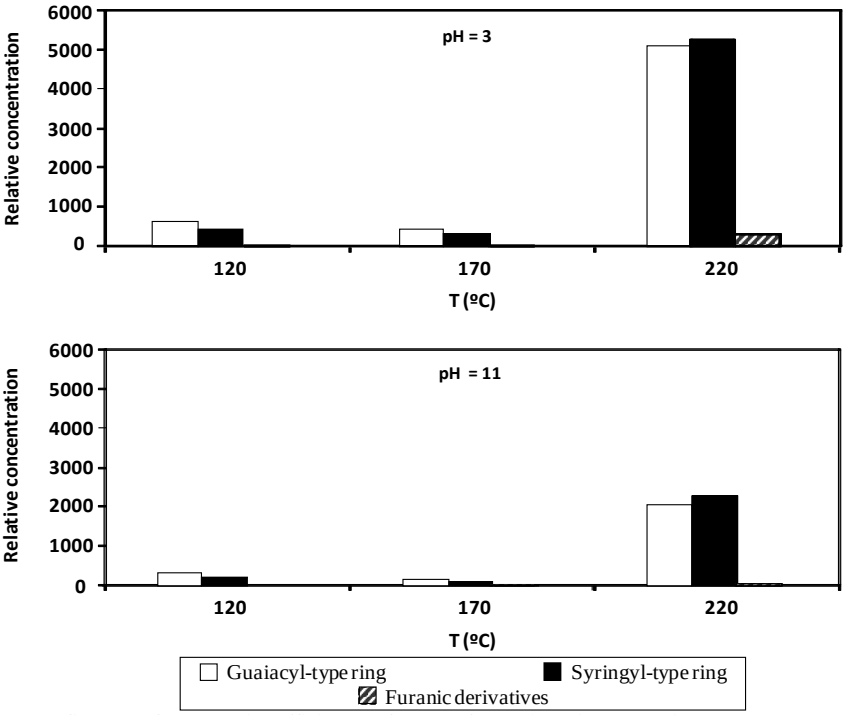

Figure 1. Influence of pH on the efficiency of SHLE from vine-shoots at three temperatures. The response variable corresponds to relative concentration of each family of compounds calculated by addition of the peak areas obtained by GC–MS analysis for a representative group.

Comparing the two sets of experiments it is evident that the extraction temperature should be higher for vine-shoots than for oak chips to obtain a woody flavoring rich in compounds foreseeable contributors to the organoleptical properties. Anyway, a parallel situation could be set at 220 ºC at which both wood materials provided extracts comparable from a qualitative point of view.

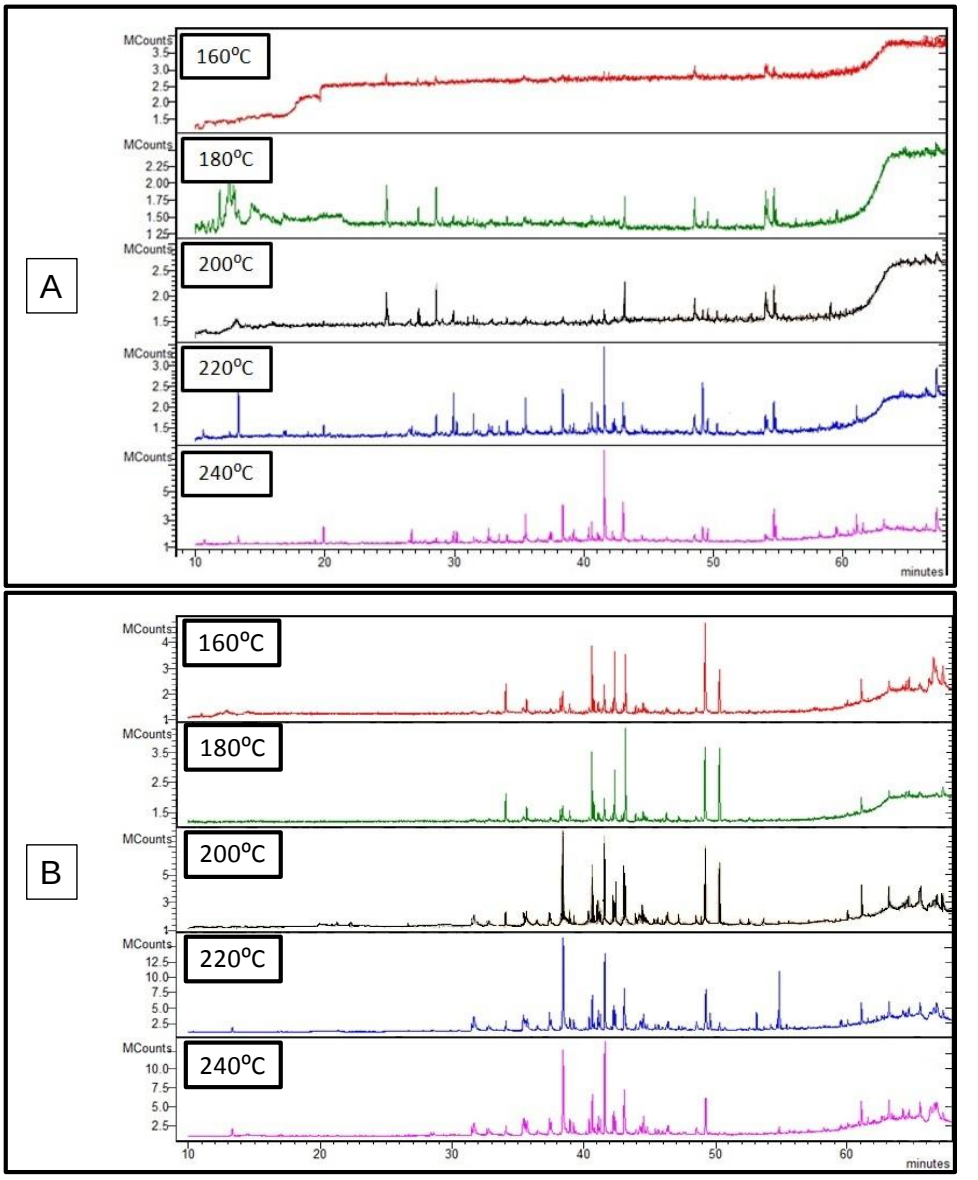

Figure 2. Influence of SHLE temperature on the chromatographic profiles obtained by GC–MS analysis of extracts from Pedro-Ximénez vine-shoots (A) and French Nobile spice oak chips (B).

3.3. Characterization of SHL Extracts from Vine-Shoots and Oak Chips

The extracts from vine-shoots obtained under optimum conditions were compared with those provided by oak chips from different varieties under the

same conditions (220 ºC, 60 min, 80% ethanol pH 3). GC–MS chromatographic profiles revealed differences between both types of wood extracts, but also variability among the different extracts from oak chips and among vine-shoots. **Table 1** lists the compounds detected in the woody flavorings extracted from three varieties of oak chips and vine-shoots. The compounds are classified into different families according to their chemical structure. As can be seen, similar profiles were obtained for phenolic compounds with guaiacyl and syringyl-type structures with certain differences. Thus, vanillin and two characteristic derivatives such as acetovanillone and propiovanillone were detected in all extracts. **Figure 3.A** shows the EIC of vanillin in three extracts of oak chips and three extracts of vine-shoots. These compounds, particularly vanillin due to its low threshold of odor detection, are of critical relevance in the organoleptical properties of wine due to vanilla and toffee flavoring contribution. As can be seen, both types of extracts contained vanillin, although its concentration was generally higher in SHL extracts obtained from oak chips. Nevertheless, vine-shoot varieties such as Chardonnay and Merlot reported levels of this compound similar to those found in some varieties of oak chips. One other compound detected in some extracts from oak chips and vine-shoots was ethyl vanillate, which is the final product for vanillin breakdown in the sequence alcohol>aldehyde>ketone>carboxylic acid>ester. This vanillin ester is responsible for burnt and smoky flavoring, which can produce negative effects on the organoleptical properties of wines or spirits when in excess. **Figure 3.B** illustrates the relative levels of ethyl vanillate in the extracts from oak chips and vine-shoots. It can be seen that this compound was more concentrated in liquid woody flavorings from oak chips than in those from vine-shoots.

Alkyl and alkenyl derivatives of guaiacyl-type phenols such as eugenol, 4-vinylguaiacol and 4-propylguaiacol were also detected in all extracts analyzed, except for eugenol that was not found at quantifiable levels in extracts from AF oak chips. These volatile compounds are characterized by a spicy odor with clove-taste. In contrast to ethyl vanillate, the levels of this volatile compound in extracts from vine-shoots were slightly higher than those detected in extracts from oak

chips (**Figure 3.C**). Similarly to vanillin, ethyl vanillate has a strong incidence on the organoleptical properties of wine or spirits. In the case of extracts from oak chips, one other peak from the same precursor ion and similar spectrum was eluted at 38 min, then identified as isoeugenol a structural isomer of eugenol but with similar organoleptical characteristics.

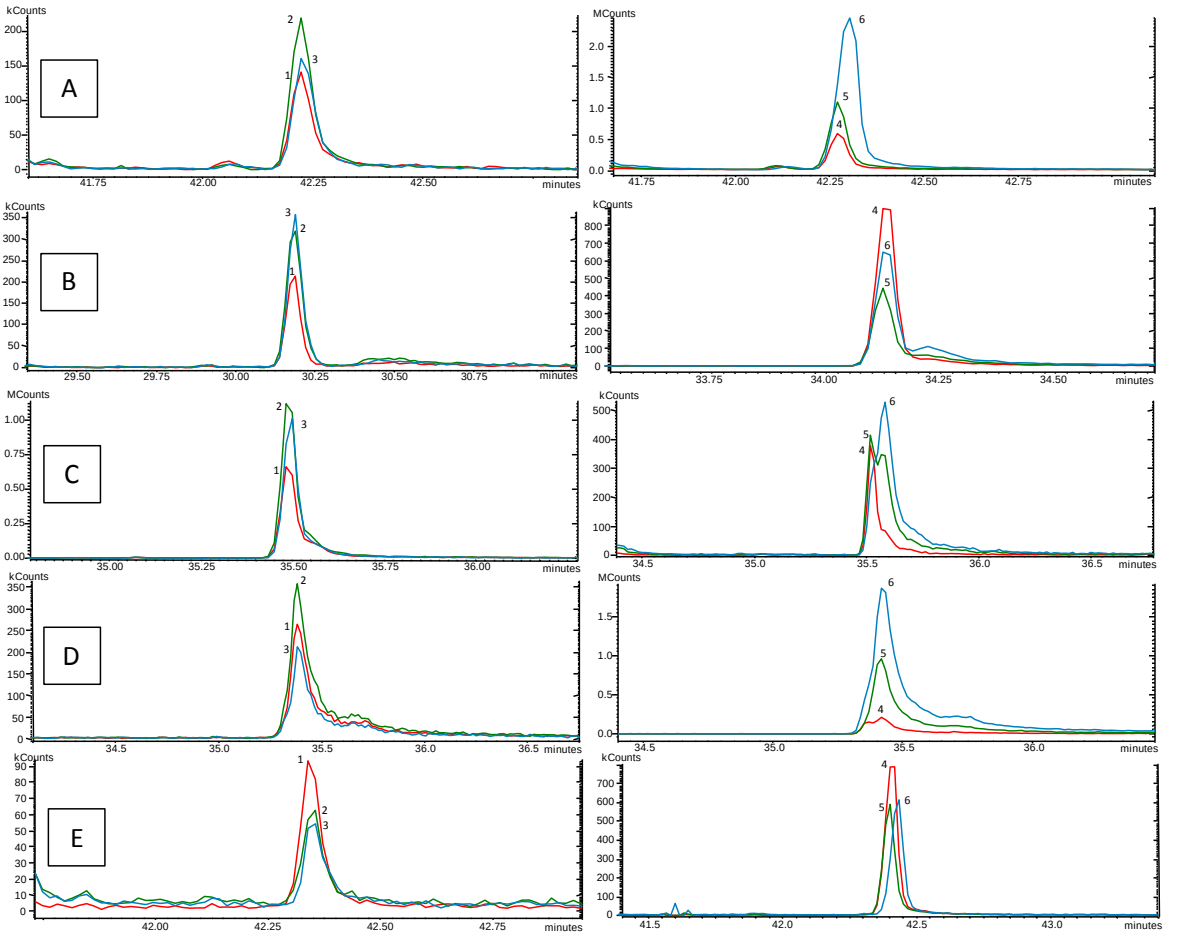

Figure 3. Extracted ion chromatograms obtained by GC–MS analysis of extracts from vine-shoots (left) and oak chips (right) for: (A) vanillin; (B) ethyl vanillate; (C) eugenol; (D) homovanillyl alcohol; (E) coniferaldehyde. (1) Tempranillo; (2) Airen; (3) Malbeq; (4) French Sweet oak; (5) French Nobile Spice oak; (6) French Intense oak.

Table 1. Compounds with oenological and nutraceutical interest in SHL extracts (•) identified by GC–MS. AF= American Fresh oak; AB= American Blend oak; FS= French Sweet oak; CH=Chardonnay vine-shoot; T= Tempranillo vine-shoot; BA=Baladí vine-shoot.

Compounds	Characteristic fragments	RI (Ri)	Formula	AF	AB	FS	CH	T	BA
Phenolic compounds with guaiacyl-type ring (G)									
4-Hydroxy-3-methoxy-methyl ester benzeneacetic acid (methyl homovanillate)	196, 165	1685 (1662)	$C_{10}H_{12}O_4$	•	•	•	•	•	•
4-Hydroxy-3-methoxyphenylethyl alcohol (homovanillyl alcohol)	168, 122, 137	1551 (1545)	$C_9H_{12}O_3$	•	•	•	•	•	•
4-Hydroxy-3-methoxytoluene (methylguaiacol)	138, 123, 95	1229 (1203)	$C_8H_{10}O_2$		•	•	•	•	
2-Methoxy-4-vinylphenol (4-vinylguaiacol)	150, 135, 107	1358 (1321)	$C_9H_{10}O_2$	•	•	•	•	•	•
2-Methoxy-4-propylphenol (4-propylguaiacol)	166, 137, 122	1460 (1402)	$C_{10}H_{14}O_2$	•	•	•	•	•	•
4-Hydroxy-3-methoxyphenylacetic acid (homovanillic acid)	182, 137, 122	1604 (1603)	$C_9H_{10}O_4$	•	•	•	•	•	•
4'-Hydroxy-3'-methoxypropiophenone (propiovanillone)	180, 137, 91	1635 (1582)	$C_{10}H_{12}O_3$	•	•	•	•	•	•
3-Hydroxy-4-methoxybenzaldehyde (vanillin)	151, 123, 109	1349 (1330)	$C_8H_8O_3$	•	•	•	•	•	•
4-Hydroxy-3-methoxycinnamaldehyde (coniferaldehyde)	178, 135, 107	1759 (1741)	$C_{10}H_{10}O_3$	•	•	•	•	•	•
4-Hydroxy-3-methoxyacetophenone (acetovanillone)	166, 151, 123	1576 (1496)	$C_9H_{10}O$	•	•	•	•	•	•
3-(4-Hydroxy-3-methoxyphenyl)-2-propenoic acid (ferulic acid)	194, 179, 133	1735 (1767)	$C_{10}H_{10}O_4$	•	•	•	•	•	•
1,2-Dimethoxy-4-(1,3-dimethoxy-1-propenyl)benzene	238, 209, 161	1782 (1706)	$C_{13}H_{18}O_4$	•	•	•		•	•
4-Hydroxy-3-ethoxybenzaldehyde (ethyl vanillin)(bourbonal)	166, 137, 109	1482 (1491)	$C_9H_{10}O_3$		•	•		•	•
4-(1-Propenyl)-2-methoxyphenol (eugenol)	164, 131, 103	1461 (1386)	$C_{10}H_{12}O_3$		•	•	•	•	•
4-Hydroxy-3-methoxyphenylacetic acid, ethyl ester (ethyl homovanillate)	210, 137	1687 (1669)	$C_{11}H_{14}O_4$			•	•	•	•
2-(2-Methoxy-5-methylphenyl)propanal	178, 149, 117	1349 (1419)	$C_{11}H_{14}O_2$	•		•	•	•	
1-(4-Hydroxy-3-methoxyphenyl)-2-propanone	180, 137, 122, 94	1606 (1538)	$C_{10}H_{12}O_3$	•	•		•	•	•

(acetohomovanillone)									
4-Hydroxy-3-methoxycinnamyl alcohol (coniferyl alcohol)	180, 137, 91	1622 (1653)	$C_{10}H_{12}O_3$	•			•	•	•
1-Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-2-propanone	186, 151, 196	1607 (1541)	$C_{10}H_{12}O_4$				•	•	•
4'-Hydroxy-3'-methoxybenzylacetone (vanillylacetone)	194, 137, 119	1687 (1638)	$C_{11}H_{14}O_3$			•			
1-(p-Methoxyphenyl)-2-phenylethene	210, 165, 152	1777 (1763)	$C_{15}H_{14}O$	•	•	•	•	•	•
Phenolic compounds with syringyl-type ring (S)									
2,6-Dimethoxyphenol (syringol)	154, 139, 111, 92	1495 (1573)	$C_8H_{10}O_3$	•	•	•	•	•	•
3,5-Dimethoxy-4-hydroxybenzoic acid (syringic acid)	198, 183, 127	1808 (1823)	$C_9H_{10}O_5$	•	•	•	•	•	•
4-Hydroxy-3,5-dimethoxycinnamaldehyde (sinapaldehyde)	208, 165, 137, 91	1961 (1909)	$C_{11}H_{12}O_4$	•	•	•	•	•	•
3,5-Dimethoxy-4-hydroxybenzaldehyde (syringaldehyde)	182, 167, 111	1604 (1581)	$C_9H_{10}O_4$	•	•	•	•	•	•
3,4,5-Trimethoxybenzaldehyde	196, 181, 125	1666 (1608)	$C_{10}H_{12}O_4$	•	•		•	•	
3,5-Dimethoxy-4-hydroxyphenylacetic acid (homosyringic acid)	212, 168, 198, 181	1874 (1848)	$C_{10}H_{12}O_5$			•	•	•	•
4-Hydroxy-3,5-dimethoxyacetophenone (acetosyringone)	181, 196,153	1573 (1628)	$C_{10}H_{12}O_4$		•			•	•
2,6-Dimethoxy-4-(2-propenyl)phenol (methoxyeugenol)	194, 91, 119	1659 (1615)	$C_{11}H_{14}O_3$		•	•	•	•	
Other phenolic compounds or benzoic acid derivatives									
5-Tertbutylpyrogallol	182, 167, 121	1653 (1669)	$C_{10}H_{14}O_3$	•	•	•	•	•	•
4-Terbutylphenol	150, 135, 107, 95	1874 (1848)	$C_{10}H_{14}O$	•	•	•	•	•	•
2,3,5,6-Tetramethylphenol	150, 135, 107, 91	1424 (1361)	$C_{10}H_{14}O$	•	•	•	•	•	•
3,4,5-Trimethoxyphenylacetic acid	226, 181, 131	1833 (1817)	$C_{11}H_{14}O_5$	•	•	•	•	•	•
3,5-Bis(1,1-dimethylethyl)phenol	206, 191, 181	1591 (1514)	$C_{14}H_{22}O$	•	•	•	•	•	•
4-Butoxybenzoic acid	194, 138, 121	1697 (1637)	$C_{11}H_{14}O_3$	•	•	•	•	•	•
1-(2,4,6-Trihydroxyphenyl)ethanone	195, 180, 137, 210	1733 (1691)	$C_8H_8O_4$	•	•	•	•	•	•
Acetyl-7-hydroxy-4-methyl-2H-chromen-2-one (3,4-Dimethoxycinnamic acid)	208, 179, 151	1777 (1735)	$C_{11}H_{12}O_4$	•	•	•		•	•
3',5'-Dimethoxyacetophenone	180, 165, 107	1715 (1740)	$C_{10}H_{12}O_3$	•	•	•		•	•
2,5-Dihydroxypropiophenone	166, 137, 109	1576 (1570)	$C_9H_{10}O_3$		•		•	•	•

2,4,6-Tris(1-methyl-1-phenylethyl)phenol	448, 443, 119, 91	3564 (3602)	$C_{33}H_{36}O$	•	•	•		•	
2-(3,5-Dimethoxy-phenyl)-2-methyl-propionaldehyde	208, 180, 137, 149	1629 (1574)	$C_{12}H_{16}O_3$	•	•		•	•	•
1-Methyl-2-(3,4,5-trimethoxyphenyl)ethylamine	224, 182, 167	1746 (1739)	$C_{12}H_{19}NO_3$		•	•	•	•	•
2,6-Bis(1,1-dimethylethyl)-4-(1-methyl-1-phenylethyl)phenol	142, 127, 101	1173 (1123)	C_7H_7ClO		•		•	•	•
p-Chloroanisole	126, 108, 52	1287 (1342)	$C_6H_6O_3$		•		•	•	•
1,2-Benzenediol (pyrocatechol)	184, 169, 142, 111	1529 (1468)	$C_9H_{12}O_4$		•		•	•	•
1,2,3-Benzenetriol (pyrogallol)	154, 125, 111	1461 (1531)	$C_8H_{10}O_3$		•		•	•	•
3,4,5-Trimethoxyphenol	137, 151, 109	1391 (1740)	$C_8H_8O_3$		•		•	•	•
3,5-Dimethoxyphenol	168, 153, 107	1429 (1377)	$C_9H_{12}O_3$	•		•	•		•
1(2,5-Dihydroxyphenyl)ethanone	92, 138, 120	1411 (1371)	$C_7H_6O_3$		•				•
5-Hydroxy-2-(4-hydroxyphenyl)-7-methoxy-4H-1-benzopyran-4-one (Apigenin 4'-dimethyl ether)	92, 120, 151	1281 (1281)	$C_8H_8O_3$						•
1,2,4-Trimethoxybenzene	182, 167, 121	1653 (1669)	$C_{10}H_{14}O_3$	•	•	•	•	•	•
1,3,5-Tris(3-methyl-3-butenyl)benzene	150, 135, 107, 95	1874 (1848)	$C_{10}H_{14}O$	•	•	•	•	•	•
Benzoic acid, ethyl ester	150, 135, 107, 91	1424 (1361)	$C_{10}H_{14}O$	•	•	•	•	•	•
1-(2,3,4-trihydroxyphenyl)-1-decanone	226, 181, 131	1833 (1817)	$C_{11}H_{14}O_5$	•	•	•	•	•	•
o-Hydroxybenzoic acid (Salicylic acid)	206, 191, 181	1591 (1514)	$C_{14}H_{22}O$	•	•	•	•	•	•
o-Hydroxybenzoic acid methyl ester (Methyl salicylate)	194, 138, 121	1697 (1637)	$C_{11}H_{14}O_3$	•	•	•	•	•	•
Furanic compounds									
Furfural	96, 95	902 (831)	$C_5H_4O_2$				•	•	•
Benzofuran	118, 90, 95	971 (1018)	C_8H_6O		•	•	•	•	
Methyl furoate	126, 112, 95	965 (909)	$C_6H_6O_3$		•	•	•	•	•
2-Acetyl-5-methylfuran	124, 109, 95	1095 (1048)	$C_7H_8O_2$		•	•	•	•	•
Furfural diethylacetal	170	1464 (1442)	$C_6H_{14}O_3$	•	•	•	•	•	•
2-Hydroxymethylfuran	99, 98	885 (971)	$C_5H_6O_2$	•			•	•	
5-Hydroxymethyl-2-furaldehyde (5-hydroxymethylfurfural)	96, 125, 109	1312 (1261)	$C_6H_6O_3$			•	•	•	•
Others									
2-Butenedioic acid diethyl ester	127, 99, 173	1238 (1160)	$C_8H_{12}O_4$	•	•	•	•	•	•

n-Hexadecanoic acid	129, 73, 60, 43	1933 (1968)	$C_{16}H_{32}O_2$	•	•	•	•	•	•
Methyl 7,12-octadecadienoate	294, 150, 109, 95	2121 (2093)	$C_{19}H_{34}O_2$	•	•	•	•	•	•
n-Docosanoic acid methyl ester	354, 143, 74, 87	2466 (2475)	$C_{24}H_{48}O_2$	•	•	•	•	•	•
12-Hydroxy-cis-9-octadecenoic acid (ricinic acid)	55, 98, 166, 280	2389 (2337)	$C_{18}H_{34}O_3$	•	•	•	•	•	•
9-Hexadecen-1-ol	222, 96, 82	1839 (1862)	$C_{16}H_{12}O_2$	•	•	•	•	•	•
Nonadecanoic acid, methyl ester	312, 143, 97	2157 (2177)	$C_{20}H_{40}O_2$	•	•	•	•		•
2,3-Dihydroxypropyl ester-9,12-octadecadienoic acid	79, 95, 109	2606 (2689)	$C_{21}H_{38}O_4$	•	•	•	•	•	
Eicosanoic acid	312, 185, 97	2342 (2366)	$C_{20}H_{40}O_2$	•	•	•	•	•	
Hexadecanoic acid ethyl ester	101, 157, 284	1979 (1968)	$C_{18}H_{36}O_2$		•	•	•	•	•
n-Propyl-9,12-octadecadienoate	263, 95, 81, 67	2266 (2292)	$C_{21}H_{38}O_2$		•	•	•	•	•
Nonanal	98, 114, 141, 57	1095 (1104)	$C_9H_{18}O$		•	•	•	•	•
2-Dodecanone	184, 169, 126	1402 (1350)	$C_{12}H_{24}O$		•		•	•	•
Ethyl 9,12-hexadecadienoate	67, 81, 234, 280	1926 (1994)	$C_{18}H_{32}O_2$			•	•	•	•
Z-7-Pentadecenol	272, 211	1802 (1763)	$C_{15}H_{30}O$				•	•	•
2-Hydroxytriethyl ester1,2,3-propanetricarboxylic acid (ethyl citrate)	203, 157, 115	1684 (1627)	$C_{12}H_{20}O_7$		•	•	•		

Note: RI: Calculated retention index; Ri: retention index

One other sub-group of compounds was formed by homovanillic acid and derivatives such as homovanillyl alcohol and acetohomovanillone. These compounds are featured by fruit taste notes with a profile similar to that found for vanillin derivatives. Similarly to vanillin, ester derivatives such as methyl homovanillate and ethyl homovanillate were found in most extracts and, therefore, their concentration should be minimized. As shows **Figure 3.D**, levels of homovanillyl alcohol in SHL extracts from oak chips varied in a wide range of relative concentration. Despite this variation among oak chip varieties, the concentration of this compound in extracts from oak chips was clearly higher than in extracts from vine-shoots. Other group of compounds was formed by coniferyl alcohol, coniferaldehyde and ferulic acid. The aldehyde and acid were detected in the extracts isolated from all varieties, while the alcohol was only detected in the American Fresh variety of oak chip. These compounds are

frequently associated to woody and sweet aromas. Similarly to homovanillic acid derivatives, the concentration of coniferaldehyde in extracts from oak chips was significatly higher than that in extracts from vine-shoots (**Figure 3.E**).

Concerning syringyl-type phenols, compounds such as syringol, syringaldehyde and syringic acid were found both in vine-shoot and oak chip extracts. However, their concentrations were clearly higher in the case of extracts from oak chips, as shows **Figure 4**. One other aldehyde such as sinapaldehyde was also detected in extracts from both types of wood materials. Other compounds found in some varieties of extracts were acetosyringone, homosyringic acid and methoxyeugenol. Similarly to other families, also in this case aldehydes, ketones and acids can be associated to woody and sweet aromas, while other compounds such as syringol and methoxyeugenol are associated to spicy, burnt flavorings. The last volatile compounds are formed when the thermal attack is more persistent. These compounds are characterized by a high concentration for detection odor threshold, usually higher than their concentration in oak wood. For this reason, they do not contribute appreciably to wine aroma. However, new red-orange stable pigments formed by the union of these compounds to flavanols or anthocyans have recently been identified *(28)*.

Concerning furanic compounds, the aroma of which has been described as caramel or burnt sugar *(29),* showed a non significant variability among extracts, probably because these compounds are lost in some steps of sample preparation However, a previous research had demonstrated that furanic compounds are at more significant quantities in vine-shoot extracts than in oak wood extracts *(26)*. Several furans appeared in the first part of the chromatograms, including aldehydes (furfural, 5-methylfurfural, 5-hydroxymethylfurfural), alcohols (2-hydroxymethylfuran) and ester derivatives (2-furoic acid ethyl ester, 2-acetyl-5-methylfuran). Similar levels of 5-hydroxymethylfurfural were detected in extracts from oak chips and vine-shoots (**Figure 5**). The resting furan derivatives were generally more concentrated in extracts from vine-shoots, while furfural was not found in varieties from oak

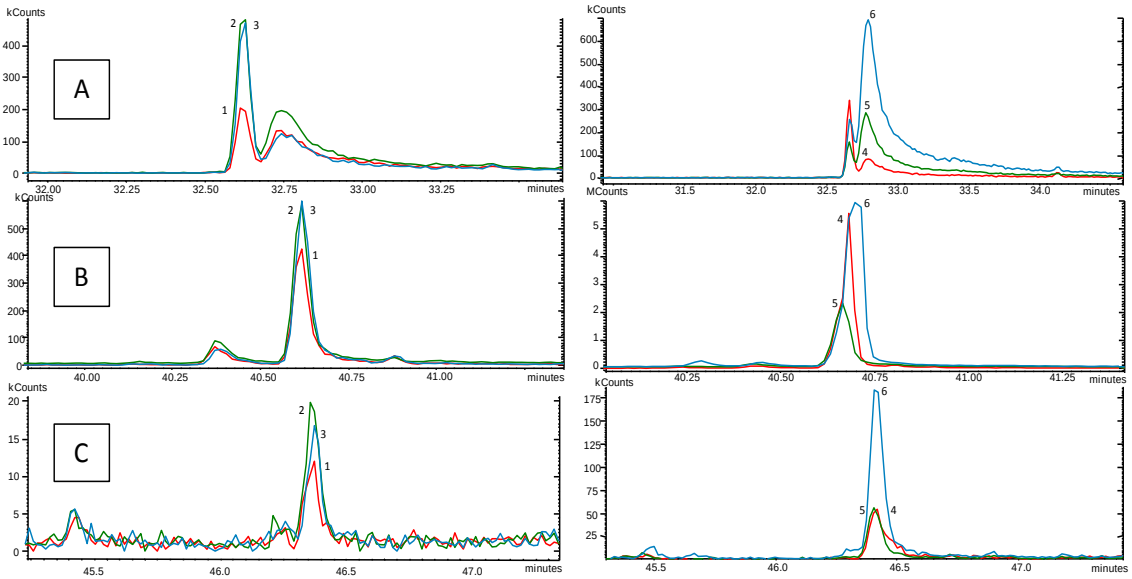

Figure 4. Extracted ion chromatograms obtained by GC–MS analysis of extracts from vine-shoots (left) and oak chips (right) monitoring characteristic ions for: (A) syringol; (B) syringaldehyde; (C) syringic acid. (1) Tempranillo; (2) Airen; (3) Malbeq; (4) French Sweet oak; (5) French Nobile Spice oak; (6) French Intense oak.

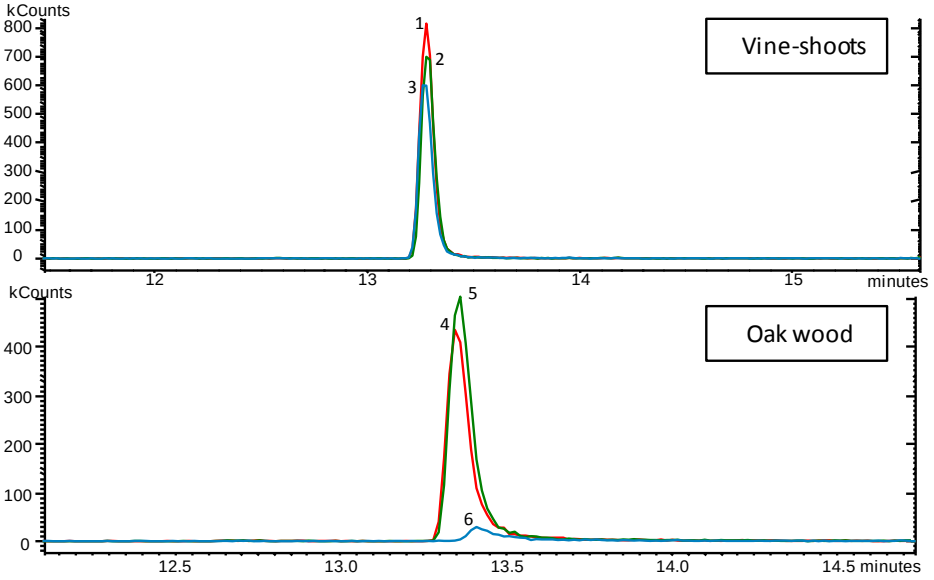

Figure 5. Extracted ion chromatograms obtained by GC–MS analysis of extracts from vine-shoots and oak chips monitoring characteristic ions for 5-hydroxymethylfurfural. (1) Tempranillo; (2) Airen; (3) Malbeq; (4) French Sweet oak; (5) French Nobile Spice oak; (6) French Intense oak.

chips. Finally, it is important to emphasize that oak lactones, characteristic compounds of some wines aged in oak barrels, were not detected in SHL extracts from oak chips.

3.4. Comparison of SHL Extracts from Vine-Shoots and Oak Chips

AMDIS generic files were aligned according to the protocol described in the experimental section. This step allows obtaining a data set composed of the molecular features detected in the extracts from the different varieties of vine-shoots and oak chips. After that, a filtration by frequency step was included to keep in the data set those molecular features detected in a representative number of samples. Frequency filters were applied from two points of view: by considering all samples as one group and by applying separately the filters to each group of samples, vine-shoots or oak chips. The application of this algorithm to the data set should retain those molecular entities detected in at least a preset percentage of samples. Different restriction filters from 60 to 90% were tested to evaluate the variability in the composition of the extracts among the different varieties of both oak chips and vine-shoots. The number of entities that passed the filters is shown in **Table 2.** These results revealed a high variability, not only between extracts from oak chips and vine-shoots, but also among the different varieties studied in each set of extracts. Attending to this variability, two data sets were selected: that formed for molecular entities present in at least 70% of all samples, and a second one formed by entities which were detected in at least 90% of samples pertaining to either vine-shoots or oak chips. The application of both filters led to data sets formed by 42 and 30 entities, respectively. The percentage of the filter considered in the first case was lower than that based on samples grouping because it was applied to the complete sample set, which obviously present higher variability. Similarity between extracts isolated from vine-shoots and oak chips was evaluated by multivariate analysis based on Principal Component Analysis (PCA). **Figure 6.A** reveals the three-dimensional plots of scores obtained by PCA to detect the presence of clusters for each of the studied classes: vine-shoots and oak wood extracts. As can be seen, PCA plots do not

show complete discrimination between the qualitative composition of extracts from both classes. **Figure 6.B** shows the PC1–PC2 two dimensional plots of scores revealing varieties of vine-shoots with a composition more similar to that of oak chips. These varieties were Merlot, Bobal, Pedro-Ximénez, Mazuelo and Baladí, which provided extracts close to those of American oak chips, while the extracts from Cabernet Sauvignon, Sauvignon Blanc and Chardonnay were close to those from French oak chips.

Table 2. Number of molecular entities present in data sets generated after application of both frequency filters: (1) : that formed by entities present in 70% of samples considering one group and, (2) that formed by entities present in 90% of samples discriminating between vine-shoots and oak chips.

Percentage	One group[1]	Two groups[2]
60	80	275
70	42	91
80	24	78
90	10	30

Note: [1]*Entities present in at least a percentage value of all samples in one group*
[2]*Entities present in at least a percentage value of samples discriminating between vine-shoots and oak chips.*

4. Conclusions

Extracts from different varieties of vine-shoots and oak chips have been obtained by SHLE using ethanol–water mixtures. The resulting extracts were analyzed by GC–MS in order to compare their composition of low-molecular weight compounds with special emphasis on phenolic and furanic compounds. A similar composition has been obtained at qualitative terms for extracts obtained from both types of raw materials. The assessment of these preliminary results also enabled to identify those varieties of vine-shoots providing extracts with a more similar composition to those obtained from oak chips. These preliminary results could be considered for future tests intended to evaluate the utilization of vine-shoots in wine ageing.

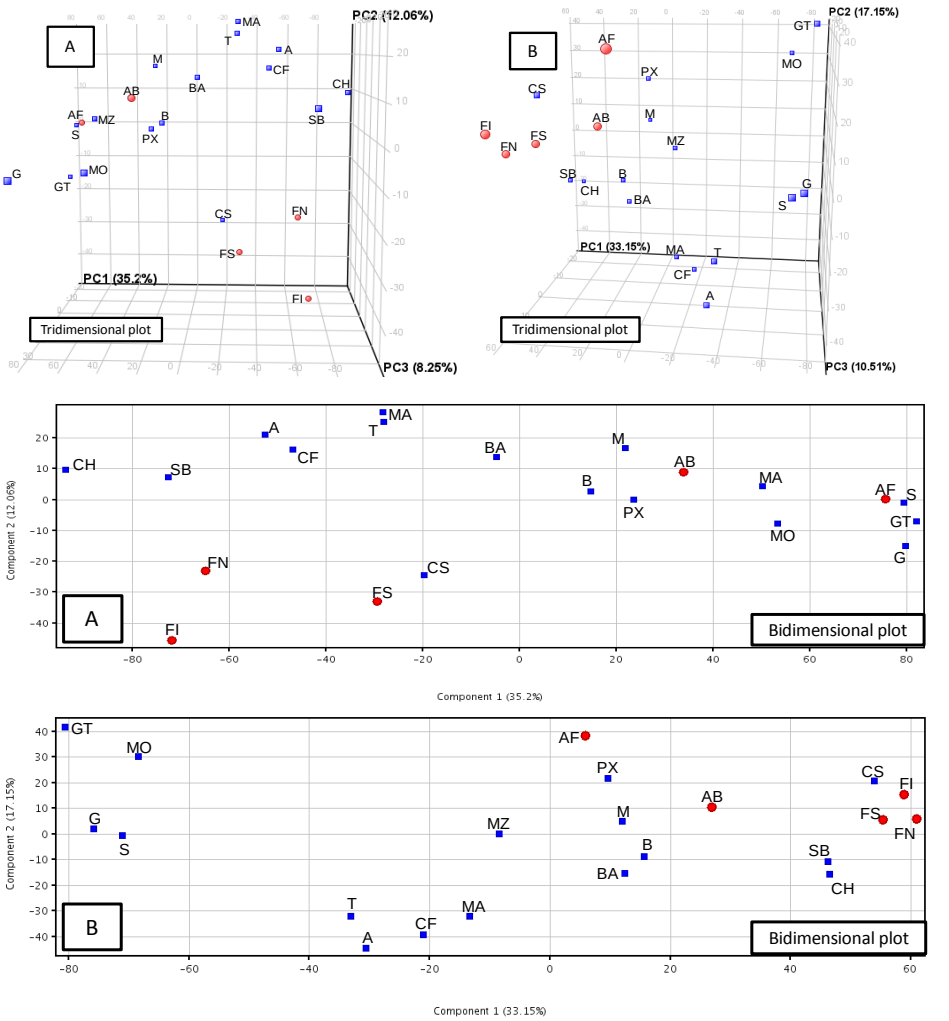

Figure 6. Three-dimensional and bidimensional PCA plots of scores for comparison of extracts from vine-shoots and oak chips with two data sets formed by entities present in 70% of samples considering one group (A), and 90% of samples discriminating between vine-shoots (squares) and oak chips (circles) (B).

Note: Garnacha Tintorera, GT; Moscatel, MO; Garnacha Tinta, G; Syrah, S; Tempranillo,T; Airen, A; Cabernet Franc, CF; Malbeq, MA; Mazuelo, MZ; Baladí, BA; Bobal, B; Merlot, M; Pedro-Ximénez, PX; Sauvignon Blanc, SB; Chardonnay, CH; Cabernet Sauvignon, CS; American Fresh oak, AF; American Blend oak, AB; French Sweet oak, FS; French Nobile Spice oak, FN and French Intense oak, FI.

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Chapter 5:

Comparison of the volatile profile of vine–shoots and oak chips by headspace–gas chromatography–mass spectrometry (HS–GC–MS)

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Abstract

Profiling and identification analyses of the volatile fraction formed by heating vine-shoots or oak chips powder have been obtained by headspace–gas chromatography–mass spectrometry (HS–GC–MS). For this purpose, vine-shoots collected from eighteen varieties of vineyards and five types of oak chips were heated at different temperatures in a headspace autosampler to compare the volatile compounds formed from both types of wood. This strategy allowed obtaining a representative profile of volatile compounds from each sample. Multivariate statistical analysis of the resulting data by application of Venn diagrams and principal component analysis showed the strong influence of the temperature on the composition of the volatile compounds obtained from vine-shoots and oak chips. Finally, identification of the compounds in the volatile fraction of vine-shoots and tested oak chips was carried out. The main families of compounds were identified as a function of both the heating temperature and type of wood. Semiquantitative analysis of compounds with oenological interest in the volatile fraction formed by heating vine-shoots and oak chips was used to evaluate similarities and differences between both materials.

1. Introduction

Traditionally, wine and spirits are aged in oak barrels by virtue of the beneficial characteristics that oak wood transfers to these beverages. Barrel ageing allows color to be stabilized and astringency reduced as a result of oxidation of certain compounds by the atmospheric oxygen transferred through wood pores *(1,2)*. A wide variety of compounds (e.g. phenols, lactones, coumarins, polysaccharides, hydrocarbons, fatty acids, terpenes, carotenoids, steroids, norisoprenoids and furans) is known to be transferred in this process from the wood barrel to wine. Among the transferred compounds, volatile phenols and benzoic aldehydes are particularly relevant since they are responsible for desirable sensory properties of wine and spirits *(3–5)*.

Natural oak wood only contains small amounts of these compounds. For this reason, wood is first modified by a natural or artificial seasoning in which ellagitannins are decreased *(6)*. After drying, the wood is thermally treated by toasting or charring *(7),* the former treatment standing as the most critical for aroma production due to the severe modification of the chemical composition of wood, which involves a significant increase of desirable volatile compounds. Depending on the toasting degree, pyrolysis and hydrothermolysis degrade wood constituents to some extent, not only ellagitannins, which are easily hydrolyzed *(8,9)*, but also polymers such as lignin, cellulose and hemicellulose (the most important polymers in natural oak) *(10)*. Hydrolysis of these polymers leads to toasty flavor compounds such as furfural, 5-hydroxymethylfurfural and derivatives (dried fruit and burned almonds aroma), phenolic aldehydes (responsible for sugar flavor) such as vanillin (vanilla aroma), and a wide variety of volatile phenols (responsible for smoky, medicinal and burnt wood aromas) such as guaiacol, vinylguaiacol and derivatives, or eugenol (spices, cloves character) *(3–5)*. Oak lactones, such as *cis*- and *trans*-β-methyl-γ-octalactone (known as whisky lactones), and nonanoic lactone are present in natural oak, but their contents may either increase or decrease by both seasoning and toasting.

The transferred compounds contribute to the final bouquet of wine and, although they are present at low concentrations (μg/L or less), they exert a strong influence on wine aroma because of their low sensory thresholds.

Numerous oak wood varieties have been studied in depth, being American oak (*Quercus alba*) and French oak (*Q. roburand Q. petraea*) species the most commonly used for oenological purposes *(11)*. American oak is characterized by lower levels of phenols and higher amounts of whisky lactone than European species *(12,13)*. Some studies have also confirmed differences between the two European species *(14)*, with *Q. petraea* possessing characteristics similar to those of American oak, while *Q. robur* is characterized by high levels of ellagitannins, but often negligible amounts of whisky lactones *(13)*. Contribution of *cis*- and *trans*-β-methyl-γ-octolactones to the coconut, woody fresh odor descriptors is dissimilar, the former being the strongest odorant *(15–17)*. Apart from oak wood, there is a present trend to the use of some other types of wood such as that from chesnut, acacia, cherry or mulberry for ageing of wine and spirits. Alternative ageing methods such as addition of either oak or chesnut chips have proven to be profitable for the involved industry by decreasing ageing time. In addition, this alternative has led to an increase in the available types of commercial wine with different flavor characteristics *(2,3,11)*.

Vine-shoots have been characterized as a potential source of phenols usually detected in wines *(18,19)*. The phenolic composition of extracts from vine-shoots was similar to that observed in extracts from oak wood. The aim of this research was to characterize the volatile fraction formed by heating different varieties of vine-shoots and oak chips. The heating temperature was selected according to treatments applied to oak wood. The application of the protocol would allow obtaining a qualitative profile of the volatile fraction from vine-shoots. This fraction was analyzed in eighteen varieties of vine-shoots and compared with that obtained from five commercial varieties of oak chips by using multivariate statistical analysis. For this purpose, a headspace (HS) autosampler–gas chromatograph with an ion-trap mass detector (HS–GC–

IT/MS) approach was used with a double aim: firstly, to develop an in-situ preparation step since homogeneous heating of wood powder can be carried out under controlled temperature and atmosphere in the sealed vial; and, secondly, to propose global "fingerprinting" of the volatile profile formed by heating each sample, based on mass spectrometry since the headspace content is injected into the gas chromatograph where it is separated and transferred to the ionization chamber of the MS where the volatile compounds are fragmented *(20,21)*. Chemometric data treatment of the MS complex data matrix allows comparison and classification of the samples as a function of their composition in volatile compounds *(20)*.

2. Experimental

2.1. Samples

Vine-shoots from different red and white Vitis vinifera cultivars were sampled from the same plantation located at "Bodega y Viñedos Vallebravo" (Sierra de Segura, Jaén, Spain) in autumn 2011, after leaf-fall, by randomized selection of ten similar stocks of each cultivar. Vine shots were selected from the following cultivars: Airen, Baladí, Bobal, Cabernet Franc, Cabernet Sauvignon, Chardonnay, Garnacha Tinta, Garnacha Tintorera, Malbec, Mazuelo, Merlot, Montepila, Moscatel, Pedro-Ximénez, Petit Verdot, Sauvignon Blanc, Syrah and Tempranillo. All of them were from the same plot, cultivated under the same agronomic conditions in terms of soil properties, climate, hydric regime, etc. Piece of 10 cm of vine shoot at the height of the first leaf bud were taken in all cases.

Samples of five untoasted commercial oak chips representing different types of oak barrels used for wine ageing (American Blend, American Fresh, French Sweet, French Spice and French Intense) were kindly supplied by Laffort España S.A. (Guipúzcoa, Spain).

Each vine-shoot sample was dried for 72 h at 30 ºC and milled to produce a homogeneous particle size (less than 0.42 mm diameter), then stored at –20 ºC until use. As oak chips are commercialized as a dried product, they were milled and stored following the same protocol.

2.2. Standards

Vanillin, acetovanillone, 4-ethylguaiacol, syringol, 4-vinylguaiacol, coniferaldehyde, guaiacol, *trans* and *cis* oak lactone, 5-hydroxymethylfurfural, eugenol, acetosyringone and furfural from Sigma–Aldrich (St. Louis, USA) were used as chromatographic standards to confirm identification. The standards mixture of n-alkanes (C10-C40) for performance test of GC systems was from Sigma–Aldrich (St. Louis, USA).

2.3. Apparatus

Vine-shoots and oak chips were milled by a ball grinder (Restch MM301, Haan, Germany) and homogenized by a 40-mesh sieve (particle size less than 0.42 mm diameter). For chromatographic separation, 1.5 g of the given sample was placed in a 10 mL headspace vial, then sealed using a 20 mm aluminium vial cap (Análisis Vínicos, Tomelloso, Spain) with a 20 mm silicone/PTFE septum (Análisis Vínicos). The vials were placed in the headspace rack, where sample heating was carried out at four different temperatures (120, 150, 180 and 200 ºC) to obtain detailed information on the changes produced in the volatile fraction as a function of temperature. Parameters of the headspace device are listed in **Table 1**.

Individual separation of volatile compounds was carried out by a Varian CP 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable-temperature injector and coupled to a Saturn 2200 ion-trap mass spectrometer (Sunny Valley, TX, USA). A 7694E headspace autosampler from Agilent (Palo Alto, CA, USA) and a Factor Four VF-5ms fused silica capillary

column (30 m × 0.25 mm I.D., 0.25µm film thickness, Varian) completed the experimental approach.

Table 1. Headspace parameters for analysis of powder vine-shoots and oak chips.

Heating temperature, ºC	120	150	180	200
Vial temperature, ºC	120	150	180	200
Loop temperature, ºC	130	160	190	200
Transfer line temperature, ºC	145	175	200	200
Sample equilibration time, min	20			
Vial pressurization time, min	0.2			
Loop fill time, min	0.02			
Loop equilibration time, min	0.05			
Injection time, min	0.3			
GC cycle time, min	45			
Loop size, mL	1			
Vial pressure, psi	10			
Shaking	Intense shaking for 5 min			

2.4. GC/MS Analysis

One mL of the headspace content was injected into the chromatograph. The injector temperature was fixed at 180 ºC, and injection was in the splitless mode. The oven temperature program was as follows: initial temperature 50 ºC (held for 5 min) and increased at 6 ºC/min to 200 ºC (held for 31.6 min). The total analysis time took 36.6 min, and 2 min extra time was necessary for re-establishing and equilibrating the initial conditions. The ion-trap mass spectrometer was operated in the electron impact ionization (EI) fixed mode, for which the instrumental parameters were set as follows: filament emission current 70 µA; transfer-line, ion-trap and manifold temperatures were kept at 170, 150 and 40 ºC, respectively. Data acquisition was set between 50 *m/z* and 450 *m/z* using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment to remove the chromatographic background. Additionally, chromatograms were smoothed by application of a Savitzky-Golay

filter (5 points). Automated Mass Spectral Deconvolution and Identification System (AMDIS) v 2.6 software was used to transform raw data files into generic files.

2.5. Data Processing and Statistical Analysis

Mass Profiler Professional (MPP) software package (Version 12.0, Agilent Technologies, Santa Clara, CA, USA) was used for processing all data obtained once they were transformed into AMDIS generic files. The feature extraction algorithm took into account all single charged ions exceeding 5000 counts with a signal-to-noise ratio (S/N) higher than 10. Unique mass was used for peak area calculations, while the peak width was set at 0.7 s. The NIST Mass Spectral Search Program v.1.6d (NIST, Washington, DC, USA) was used for the spectral search (Mainlib and Replib libraries). Tentative identification was supported on correlation between experimental mass and database spectra above 700 in normal search mode. The retention index (I) for each compound was calculated using as reference linear hydrocarbons from C10 to C40, which were analyzed by the same chromatographic method. I values were calculated on the basis of retention times. The experimental I for each compound was compared with experimental or theoretical values reported in the literature included in the NIST database. The cut-off limit for I difference was set to 100 units for theoretical references, while 30 units was the value used for experimental references.

The .elu and .fin files created for each sample by the AMDIS software were exported to the MPP software for further processing. Within this algorithm, ions with related *m/z* values were extracted as molecular features (MFs) or entities characterized by retention time (RT) and intensity in the apex of chromatographic peak. In the next step, alignment of RT and *m/z* values was carried out across the sample set using a tolerance window of 0.2 min retention time and 10 ppm mass accuracy. Baseline correction and data mean-centering were used as data pretreatment.

3. Results and Discussion

3.1. Evaluation of the Heating Conditions

One of the main aims of the present study was the characterization of the volatile fraction detected in the two types of samples, vine-shoots and oak chips. For this purpose, both types of materials were prepared as described above. Before optimization of the main variables involved in the heating process, a variability study was programmed by analyzing consecutively five replicates of one of the samples of each group (oak chips and vine-shoots). This study allowed detecting an experimental variability in the peak areas of 10%, which is clearly below the variability expected among the different samples included in the study.

The first variable studied was the heating time, tested at 6, 20 and 40 min, (**Supplementary Figure 1.A, B** and **C**) to compare the relative concentration of interesting compounds. At this step, the heating temperature was set at 180 ºC. As can be seen, heating for 6 min was not enough to promote the formation of volatile compounds. On the other hand, the most intense chromatographic peaks were obtained for 20 min heating time, since the experiment carried out for 40 min reported a slight decrease in the intensity of the peaks, which could be ascribed to degradation. Longer heating times were also processed but overpressure problems were found; therefore, they were discarded. Consequently, 20 min was selected as heating time.

The influence of temperature was studied by control of the heating unit of the headspace autosampler to check the effect temperature exerts on the volatile profile of the samples. The temperatures selected in this study were 120, 150, 180 and 200 ºC to check the influence of this parameter on the volatile profile. The temperature used for heating oak wood with oenological purposes is around 200 ºC *(2,22)*. **Figure 1.A** illustrates the chromatographic profiles of volatiles obtained by analysis of Cabernet Franc powder vine-shoot at the selected heating temperatures. As can be seen, there is an evolution in the

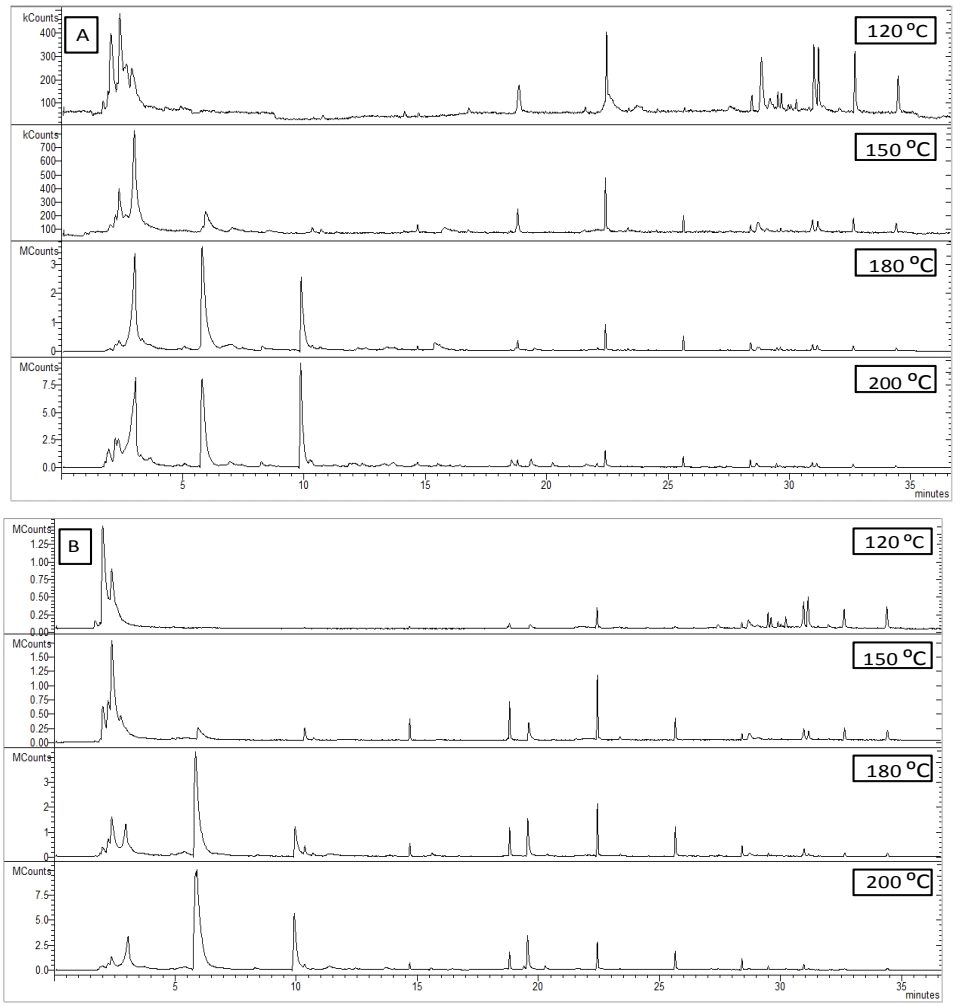

Figure 1. Chromatograms from Cabernet Franc powder vine shoot (A) and American Fresh powder oak chips (B) at different heating temperatures.

chromatographic profile with the heating temperature. The best temperatures were 120 and 200 °C as they provided the highest number of volatile compounds. An increased temperature exerted a contradictory effect on the chromatographic peaks depending on their retention time: in the first part of the chromatogram the peaks increased significantly, while peaks eluting within the second part of

the chromatogram (15–38 min) decreased. **Table 2** shows the volatile profile from heating powder vine-shoots at 200 ºC, which is rich in compounds belonging to very different chemical families such as volatile phenols, furans, phenolic aldehydes and phenolic ketones.

Table 2. Identification of compounds detected in the volatile fraction sampled at 200 ºC in the different varieties of vine-shoots.

Vine shoots 200 ºC						
Compound	**Mass**	**RI (Ri)**	**Formula**	**Characteristic fragments**	**Red wine varieties**	**White wine varieties**
Propylenesulfide	74	538 (562)	C_3H_6S	41, 59, 74	✓	
2-Methylfuran	82	519 (602)	C_5H_6O	82, 53, 39	✓	✓
1,5-Heptadiene	96	766 (715)	C_7H_{12}	55, 81, 67		✓
Furfural	96	723 (831)	$C_5H_4O_2$	96, 95, 39	✓	✓
Isopropylbutylamine	115	801 (859)	$C_7H_{17}N$	58, 72, 100	✓	
Methyl 5-hexenoate	128	890 (874)	$C_7H_{12}O_2$	74, 43, 68		✓
1-(2-Furanyl)ethanone	110	899 (878)	$C_6H_6O_2$	95, 110, 67	✓	✓
2-Furanmethanol	98	860 (885)	$C_5H_6O_2$	98, 69, 81	✓	✓
Methyl (2E,4E)-hexadienoate	126	936 (900)	$C_7H_{10}O_2$	67, 111, 95		✓
2H-Pyran-2-one	96	873 (909)	$C_5H_4O_2$	96, 38, 69	✓	
Methyl-2-furoate	126	961 (909)	$C_6H_6O_3$	95, 126, 67	✓	✓
4-Methylpentanoic acid	116	956 (910)	$C_6H_{12}O_2$	57, 73, 83		✓
2,3-Dihydroxypropanal	90	827 (913)	$C_3H_6O_3$	43, 61, 73	✓	✓
5-Methyl-2-furancarboxaldehyde	110	948 (920)	$C_6H_6O_2$	110, 53, 81	✓	✓
2-Cyclopentene-1,4-dione	96	903 (924)	$C_5H_4O_2$	96, 68, 54		✓
Cyclohexane-2-propenyl	124	865 (926)	C_9H_{16}	83, 55, 53	✓	✓
N-Butyl-tert-butylamine	129	944 (930)	$C_8H_{19}N$	86, 58, 114		✓
2-Hydroxy-2-methylbutanoic acid	118	960 (966)	$C_5H_{10}O_3$	55, 73, 89	✓	
Glycerin	92	903 (967)	$C_3H_8O_3$	61, 43, 31	✓	✓
Methoxymethylbenzene	122	927 (969)	$C_8H_{10}O$	91, 122, 65	✓	✓
(5-Methyl-2-furyl)methanol	112	918 (975)	$C_6H_8O_2$	95, 112, 69	✓	✓
Pyrrole-2-carboxaldehyde	95	912 (988)	C_5H_5NO	95, 66, 50	✓	
2-Furanmethanol acetate	140	978 (1009)	$C_7H_8O_3$	81, 98, 140	✓	✓

2-Acetylpyrrole	109	1066 (1035)	C_6H_7NO	94, 66, 109		✓
2-Pentylfuran	138	997 (1040)	$C_9H_{14}O$	81, 53, 138	✓	
1-Methyl-4-(1-methylethylidene) cyclohexane	138	1058 (1041)	$C_{10}H_{18}$	95, 81, 67		✓
5-Methyl-2-propionylfuran	138	1098 (1067)	$C_8H_{10}O_2$	109, 138, 53	✓	✓
3-Ethyl-2-hydroxy-2-cyclopenten-1-one	126	1103 (1072)	$C_7H_{10}O_2$	126, 83, 55		✓
Guaiacol	124	1088 (1090)	$C_7H_8O_2$	109, 81, 124	✓	✓
6-Nonenal	140	1136 (1112)	$C_9H_{16}O$	55, 67, 81	✓	✓
Phenylethyl alcohol	122	1158 (1136)	$C_8H_{10}O$	91, 122, 65		✓
1-Cyclohexene-1-carboxylic acid	126	1179 (1148)	$C_7H_{10}O_2$	81, 108, 126	✓	✓
2,2'-Difurylmethane	148	1186 (1164)	$C_9H_8O_2$	148, 91, 120	✓	
2,4-Dimethylundecane	184	1106 (1185)	$C_{13}H_{28}$	57, 75, 81	✓	
Pentylbenzene	148	1126 (1191)	$C_{11}H_{16}$	65, 91, 148	✓	
1-Methyl-4-(1-methylethylidene) cyclohexanol	154	1256 (1191)	$C_{10}H_{18}O$	93, 121, 136	✓	
1,3-Octanediol	146	1196 (1221)	$C_8H_{18}O_2$	75, 57, 99		✓
2,2-Dimethyl-3-(2-methyl-1-propenyl) cyclopropane-carboxylic acid	168	1284 (1256)	$C_{10}H_{16}O_2$	123, 81, 168		✓
3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	144	1266 (1269)	$C_6H_8O_4$	144, 101, 55		✓
2,6-Dimethoxyphenol (Syringol)	154	1245 (1279)	$C_8H_{10}O_3$	154, 139, 111	✓	✓
Methylsalicylate	152	1223 (1281)	$C_8H_8O_3$	120, 92, 152	✓	✓
5-Acetoxymethyl-2-furaldehyde	168	1269 (1286)	$C_8H_8O_4$	126, 79, 109	✓	✓
4-Vinylguaiacol	150	1291 (1293)	$C_9H_{10}O_2$	135, 150, 77	✓	✓
Eugenol	164	1352 (1392)	$C_{10}H_{12}O_2$	164, 149, 77	✓	✓
Vanillin	152	1374 (1392)	$C_8H_8O_3$	151, 123, 81	✓	✓
1,9-Nonanediol	160	1388 (1401)	$C_9H_{20}O_2$	55, 68, 82	✓	
3-tert-Butyl-4-hydroxyanisole	180	1390 (1417)	$C_{11}H_{16}O_2$	165, 180, 137	✓	
Acetovanillone	166	1455 (1439)	$C_9H_{10}O_3$	151, 166, 123	✓	✓
2',6'-Dihydroxy acetophenone	152	1442 (1470)	$C_8H_8O_3$	137, 152, 81	✓	✓
Ethyl vanillin	166	1463	$C_9H_{10}O_3$	137, 166, 109	✓	✓

(Bourbonal)		(1491)				
3,4-Dimethoxyphenyl acetone	194	1542 (1507)	$C_{11}H_{14}O_3$	151, 194, 107		✓
Vanillylmethylketone (Guaiacylacetone)	180	1496 (1538)	$C_{10}H_{12}O_3$	137, 180, 122	✓	✓
Cyclohexylidenecyclo hexanone	178	1586 (1551)	$C_{12}H_{18}O$	149, 178, 81		✓
Syringaldehyde	182	1597 (1581)	$C_9H_{10}O_4$	182, 167, 139	✓	✓
Methoxyeugenol	194	1611 (1581)	$C_{11}H_{14}O_3$	194, 91, 119	✓	✓
13-Tetradecenal	210	1612 (1591)	$C_{14}H_{26}O$	55, 67, 98	✓	
3-(4-Hydroxy-3-methoxyphenyl)-2-propenal (Coniferaldehyde)	178	1623 (1599)	$C_{10}H_{10}O_3$	178, 135, 107	✓	✓
1-Hexadecyne	222	1644 (1609)	$C_{16}H_{30}$	81, 67, 55		✓
Tributyl phosphate	235	1629 (1613)	$C_{12}H_{27}O_4P$	99, 155, 125		✓
Acetosyringone	196	1622 (1628)	$C_{10}H_{12}O_4$	181, 196, 153	✓	
2,6-bis(1,1-Dimethylethyl)-2,5-cyclohexadiene-1,4-dione	220	1598 (1633)	$C_{14}H_{20}O_2$	177, 220, 135		✓
2,5-di-tert-Butyl-1,4-benzoquinone	220	1602 (1633)	$C_{14}H_{20}O_2$	205, 220, 177		✓
4-(3-Hydroxy-1-propenyl)-2-methoxyphenol (Coniferyl alcohol)	180	1608 (1653)	$C_{10}H_{12}O_3$	137, 124, 180	✓	
9,12-Tetradecadien-1-ol	210	1677 (1672)	$C_{14}H_{26}O$	68, 81, 55	✓	
Homoprotocatechuic acid	168	1668 (1691)	$C_8H_8O_4$	123, 77, 51	✓	
6,10,14-Trimethyl-2-pentadecanone	268	1755 (1754)	$C_{18}H_{36}O$	58, 71, 250	✓	✓
2-Hexyl-1-decanol	242	1803 (1790)	$C_{16}H_{34}O$	57, 71, 85	✓	✓
2-Methyl-7-octadecyne	264	1866 (1863)	$C_{19}H_{36}$	81, 67, 95	✓	✓
Hexadecanoicacid methylester (Methylpalmitate)	270	1892 (1878)	$C_{17}H_{34}O_2$	74, 57, 85	✓	✓
3,7,11,15-Tetramethyl-1-hexadecen-3-ol (Isophytol)	296	1893 (1899)	$C_{20}H_{40}O$	71, 57, 82	✓	✓
Hexadecanoicacid ethenylester	282	2021 (1968)	$C_{18}H_{34}O_2$	239, 57, 71	✓	
1,2-Benzenedicarboxylic	278	1988 (1972)	$C_{16}H_{22}O_4$	149, 223, 57	✓	✓

acid butyl 2-methylpropyl ester						
Hexadecanoicacidethyl ester (Ethylpalmitate)	284	1978 (1978)	$C_{18}H_{36}O_2$	88, 101, 43	✓	✓
3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	296	2012 (2045)	$C_{20}H_{40}O$	67, 95, 123	✓	✓
9-Octadecen-1-ol	268	2014 (2061)	$C_{15}H_{30}O$	82, 67, 55	✓	
9,12-Octadecadien-1-ol	266	2033 (2069)	$C_{18}H_{34}$	81, 67, 95	✓	✓
Dodecenylsuccinican hydride	266	2109 (2159)	$C_{16}H_{26}O_3$	55, 69, 83	✓	

The results provided by vine-shoots were compared to those from powder oak chips (American Fresh, in this case) subjected to the same heating temperatures, and a similar behavior was observed, as shows **Figure 1.B**. The increase in temperature also provided an increase in the number and intensity of the chromatographic peaks generated by pyrolysis of polysaccharides (furanic aldehydes) or Maillard reaction (furanones and pyranones), and those formed by degradation of lignin (phenolic aldehydes and volatile phenols). **Table 3** shows the volatile profile obtained from powder oak chips toasted at 200 ºC. These results are in agreement with the study reported by Chatonnet *et al. (22)*, who

Table 3. Identification of compounds detected in the volatile fraction sampled at 200 ºC in the different varieties of oak chips.

Oak wood 200 ºC						
Compound	**Mass**	**RI (Ri)**	**Formula**	**Characteristic fragments**	**French oak**	**American oak**
1,1-Dimethyl-2-propynylamine	83	701 (675)	C_5H_9N	51, 68, 82	✓	
Propanoicacid	74	688 (676)	$C_3H_6O_2$	74, 56, 57	✓	✓
3-Penten-1-ol	86	711 (769)	$C_5H_{10}O$	55, 68, 85	✓	✓
Hexanal	100	811 (806)	$C_6H_{12}O$	56, 72, 82	✓	
Furfural	96	812 (831)	$C_5H_4O_2$	96, 95, 67	✓	✓
Pentanoicacid	102	872 (875)	$C_5H_{10}O_2$	60, 73, 87		✓
1-(2-Furanyl)ethanone	110	885 (878)	$C_6H_6O_2$	95, 110, 67		✓
5-Methyl-2-furancarboxaldehyde	110	896 (920)	$C_6H_6O_2$	110, 53, 81	✓	✓

N-Butyl-tert-butylamine	129	952 (930)	$C_8H_{19}N$	114, 58, 34	✓	
Glycerin	92	955 (967)	$C_3H_8O_3$	61, 60, 47	✓	✓
2-Hexenoic acid	114	1016 (982)	$C_6H_{10}O_2$	73, 99, 68		✓
1,5-Pentanediol	104	998 (1004)	$C_5H_{12}O_2$	56, 68, 74		✓
Guaiacol	124	1102 (1090)	$C_7H_8O_2$	109, 81, 124	✓	✓
6-Nonenal	140	1132 (1112)	$C_9H_{16}O$	55, 70, 86	✓	✓
5-Hydroxymethylfurfural	126	1151 (1163)	$C_6H_6O_3$	97, 126, 69	✓	
2-Hydroxybenzaldehyde	122	1186 (1203)	$C_7H_6O_2$	122, 65, 76	✓	✓
4-Methylguaiacol	138	1198 (1203)	$C_8H_{10}O_2$	138, 123, 95	✓	✓
Pentanedioic acid	132	1216 (1231)	$C_5H_8O_4$	86, 55, 114		✓
5-Butyldihydro-4-methyl-2(3H)-furanone (*trans*-oak lactone)	156	1243 (1245)	$C_9H_{16}O_2$	99, 71, 87	✓	✓
5-Butyldihydro-4-methyl-2(3H)-furanone (*cis*-oak lactone)	156	1259 (1245)	$C_9H_{16}O_2$	99, 71, 87	✓	✓
1,2,4-Trimethoxybenzene	168	1257 (1248)	$C_9H_{12}O_3$	168, 153, 125	✓	
2,6-Dimethoxyphenol (Syringol)	154	1262 (1279)	$C_8H_{10}O_3$	154, 139, 111	✓	
4-Vinylguaiacol	150	1292 (1293)	$C_9H_{10}O_2$	150, 135, 107	✓	✓
6-Octen-1-ol, 3,7-dimethylacetate	198	1321 (1302)	$C_{12}H_{22}O_2$	81, 69, 95	✓	
4-Ethylguaiacol	152	1332 (1303)	$C_9H_{12}O_2$	137, 152, 122	✓	
δ Nonalactone	156	1332 (1304)	$C_9H_{16}O_2$	99, 71, 55	✓	
p-Methoxybenzoicacid	152	1366 (1339)	$C_8H_8O_3$	135, 150, 77	✓	
Eugenol	164	1388 (1392)	$C_{10}H_{12}O_2$	164, 149, 77	✓	✓
Vanillin	152	1397 (1392)	$C_8H_8O_3$	151, 109, 81	✓	✓
6-Octen-1-ol-3,7-dimethylpropanoate	212	1376 (1402)	$C_{13}H_{24}O_2$	81, 69, 57		✓
Dihydroeugenol	166	1416 (1402)	$C_{10}H_{14}O_2$	137, 166, 122	✓	
Acetovanillone	166	1428 (1439)	$C_9H_{10}O_3$	151, 166, 123	✓	✓
2-tert-Butyl-4-methoxyphenol	180	1442 (1417)	$C_{11}H_{16}O_2$	165, 137, 180	✓	
2',6'-Dihydroxyacetophenone	152	1455 (1470)	$C_8H_8O_3$	137, 152, 109		✓
Homovanillyl alcohol	168	1588 (1545)	$C_9H_{12}O_3$	137, 168, 122	✓	
Tributylphosphate	266	1587 (1613)	$C_{12}H_{27}O_4P$	99, 155, 57		✓
4-Methyl-2,6-di-tert-	220	1654	$C_{15}H_{24}O$	205, 220, 57		✓

butyl-phenol		(1668)				
1,2-Cyclododecanediol	200	1844 (1815)	$C_{12}H_{24}O_2$	55, 82, 96	✓	
1-Octadecyne	250	1846 (1808)	$C_{18}H_{34}$	81, 55, 67		✓
9-Octadecyne	250	1844 (1828)	$C_{18}H_{34}$	81, 67, 95		✓
2-Methyl-7-octadecyne	264	1892 (1863)	$C_{19}H_{36}$	81, 67, 95	✓	
Hexadecanoicacidethyl ester (Ethylpalmitate)	284	1884 (1878)	$C_{18}H_{36}O_2$	74, 87, 55	✓	✓
3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	296	2077 (2045)	$C_{20}H_{40}O$	81, 95, 123	✓	✓

Note: RI: Calculated retention index; Ri: retention index

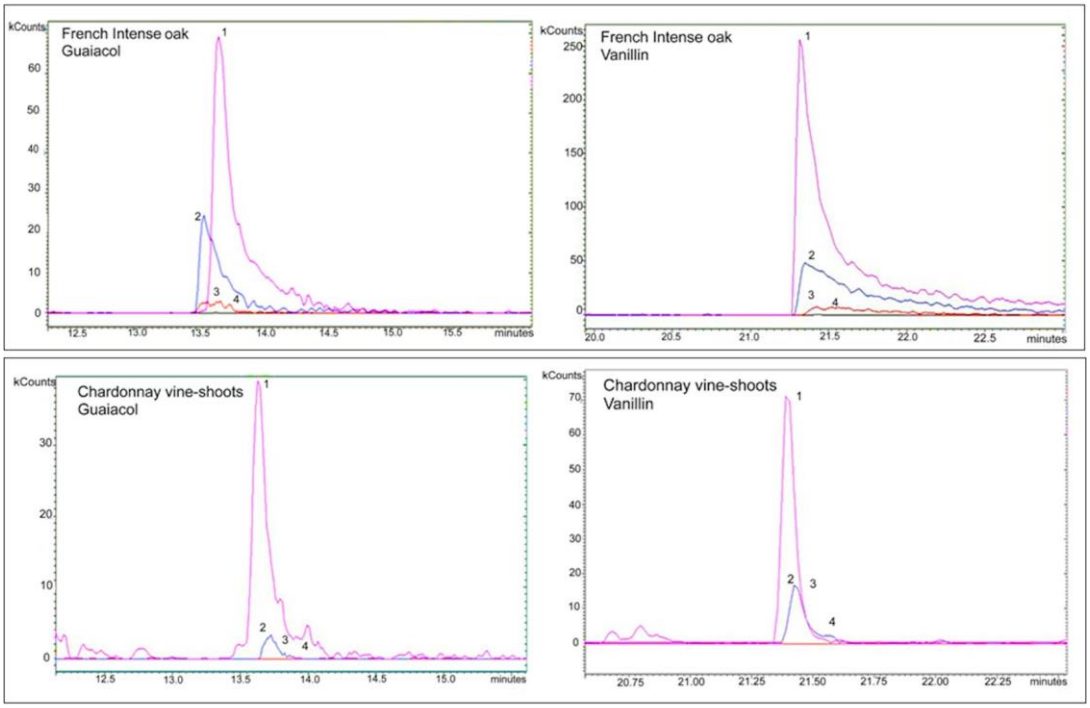

Figure 2. Extracted ion chromatograms from French Intense powder oak chips and Chardonnay powder vine-shoots for guaiacol and vanillin at 200, 180, 150 and 120 ºC (1, 2, 3 and 4, respectively).

proved that high toasting temperatures (heavy toasting, between 220 and 230 ºC) decreased the levels of furans and volatile phenols. The highest detected peaks corresponded to furfural, eluting at 5.8 min, and 5-methyl-2-furancarbox-

aldehyde, at 9.8 min, which are known to be responsible for the almond, toasty and toasted almond flavor and contribute to stabilize wines (15-17). Glycerin, which is known for its contribution to round organoleptic properties of wines (23), appears in the first part of the chromatogram, at 2.97 min. In oak wood, the concentration of glycerin decreases when the temperature increases; appearing the opposite effect on vine-shoots. Additionally, vanillin and guaiacol, known for their vanilla and smoky taste, respectively, also increased the intensity of their chromatographic peaks in vine-shoots and oak chips when the heating temperature was increased, as can be seen in **Figure 2**. On the other hand, two chromatographic peaks were identified as oak lactone in all varieties of oak wood (at 18.9 and 19.6 min). According to the literature, the *cis* isomer is the predominant form present in oak wood, eluting after the *trans* isomer, which is consistent with our results, as the second peak showed a higher intensity in all cases. The relative concentration of the *cis* isomer of whisky lactone increased by increasing the heating temperature, as shows **Figure 3.A**. Additionally, the content of this compound was considerably higher in American oak chips than in French varieties, as shown in **Figure 3.B**, which is also in agreement with previous results *(22,24)*.

The influence of the heating temperature was also globally evaluated by comparison of volatile profiles using multivariate statistical analysis. For this purpose, AMDIS generic files were aligned according to the protocol described in the experimental section. This step allowed obtaining a data set composed by the molecular features detected in the volatile fraction of the different varieties of vine-shoots and oak chips. Filters by frequency algorithms were applied to ensure representativeness of the data set by keeping the detected molecular features at least in a preset percentage of samples.

Due to variability between oak chips and vine-shoots, and also among samples within each class, a 50% frequency filter of all samples was selected, which led to a final data set formed by 65 molecular features. Comparison of volatile profiles was carried out by Principal Component Analysis (PCA). **Figure 4** shows the PCA scores plot, which allows identifying the heating temperature as

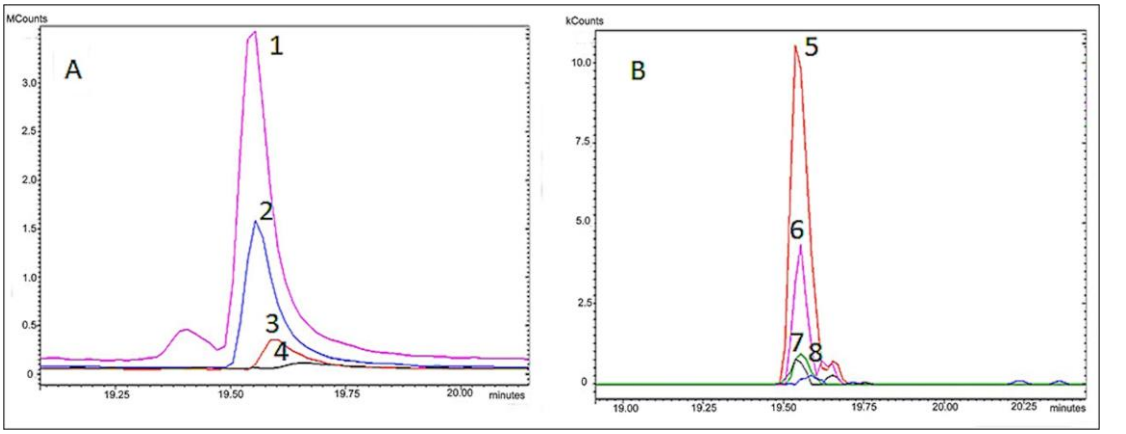

Figure 3. Extracted ion chromatograms from: (A) American Fresh oak powder monitoring characteristic ions for *cis* oak lactone at 200, 180, 150 and 120 ºC (1, 2, 3 and 4, respectively). (B) American Blend, American Fresh, French Intense, French Sweet and French Spice oak wood powder heated at 200 ºC (5, 6, 7 and 8, respectively).

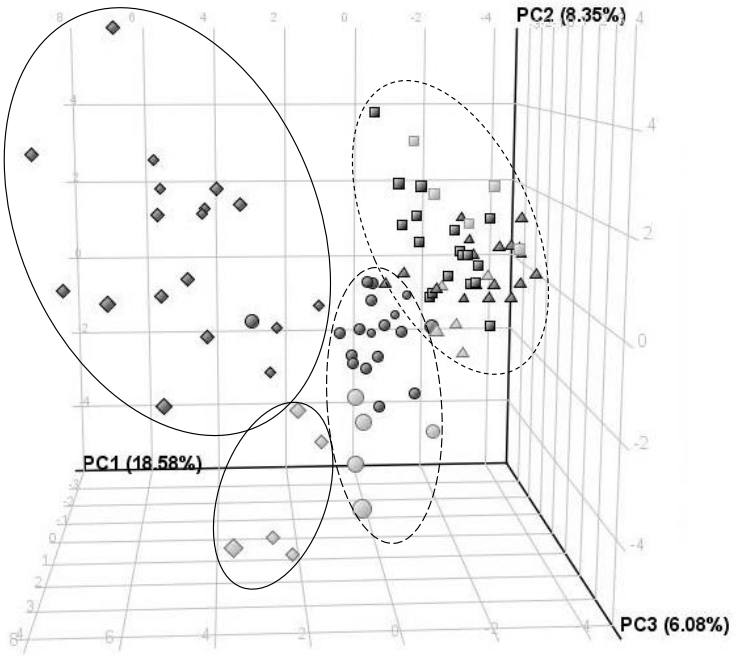

Figure 4. PCA scores plot to evaluate the influence of the heating temperature (squares: 120 ºC, triangles: 150 ºC, circles: 180 ºC, diamonds: 200 ºC) on the volatile fraction isolated from oak chips (grey) and vine-shoots (black).

the main factor explaining the observed variability. The lowest tested temperatures, 120 and 150 ºC, led to volatile profiles with a similar composition, while profiles for 180 and 200 ºC formed separated clusters. However, low temperatures in the autosampler did not lead to volatile profiles involving a high number of compounds. Complete discrimination between vine-shoots and oak chips samples was only found at the two highest temperatures. In fact, the number of molecular features detected in the samples decreased considerably with the increase of the heating temperature. Thus, the number of molecular features in the set of samples heated at 200 ºC was 286 (after application of a 50% frequency filter algorithm), while this number decreased up to 95 at 180 ºC and to 33 at the two lowest temperatures. Taking into account the profile of compounds formed at 200 ºC for oak chips and vine-shoots, this temperature was selected for further studies.

3.2. Qualitative comparison of the volatile fraction of vine-shoots and oak chips

Once the influence of the main variables involved in the heating process was assessed, the next step was the comparative analysis of samples pertaining to both materials, vine-shoots and oak chips. Qualitative comparison was carried out by Venn diagrams that allowed establishing the overlapping in composition between both types of samples.

The results are illustrated in **Figure 5** that shows the similarity between the volatile fractions from vine-shoots and oak chips after heating at high temperatures, since most of the molecular features were detected in both types of samples. As shown, 59 molecular features were detected in both types of wood at 180 ºC, however, only 7 and 29 features belonged exclusively to vine-shoots and oak chips, respectively, which allowed setting characteristic differences between both types of wood. On the other hand, at 200 ºC, all molecular features belonging to oak chips were detected in both types of wood, while only 40 molecular features were detected exclusively in vine-shoots;

therefore, despite the high similarity in the composition of both samples, differences allowing complete discrimination of both types of wood must be ascribed to differences in the intensity of the molecular features.

These results could open a door to the use of vine-shoots for wine ageing to obtain a wine flavor similar to that of oak, but with bouquet differences, the prevalence of which will be a function of the partition coefficient of the target compounds between the given wine and vine-shoots. Quantitation of these compounds in wine after ageing is necessary to know the magnitude of the transfer step, thus allowing handling of the flavor.

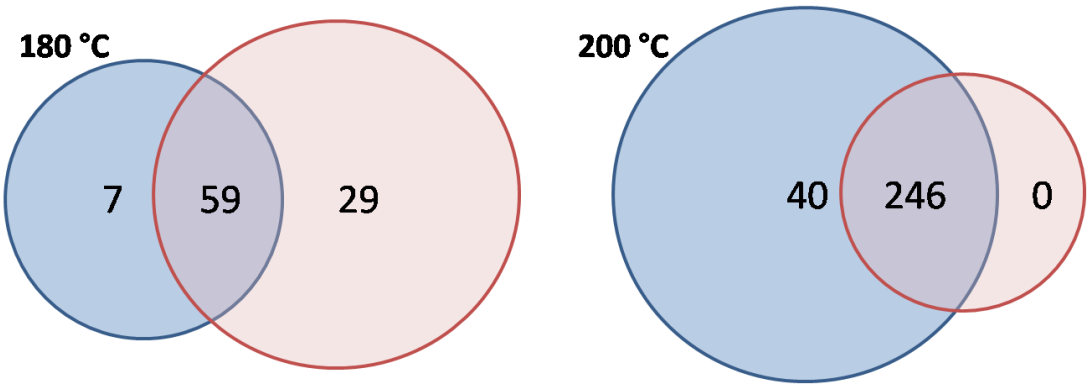

Figure 5. PCA scores plot to evaluate the influence of the heating temperature (squares: 120 ºC, triangles: 150 ºC, circles: 180 ºC, diamonds: 200 ºC) on the volatile fraction isolated from oak chips (grey) and vine-shoots (black).

3.3. Identification of volatile compounds in samples of vine-shoots and oak chips

Among the compounds identified in the different varieties of vine-shoots and oak chips (**Tables 2** and **3**) it is worth emphasizing the presence of several furans at the shortest retention times in the chromatograms from both materials. They encompass aldehydes such as furfural and 5-methy-2-furan-carboxaldehyde, which reported predominant signals with high intensity as compared to the rest of signals. These compounds participate in many chemical

reactions involved in wine aging (e.g. formation of furfuryl alcohol or wine adducts with (+)catechin), thus lowering their concentration as free species and making them difficult to reach their high detection thresholds (20–45 mg/L).

Some authors have described a synergistic effect between furans and the sensory incidence of oak lactone, particularly that of the *cis* isomer *(25)*, associated to toasted, wood and coconut notes. Oak lactone was detected in all studied oak chip varieties in growing concentrations as the heating temperature increased. The highest concentration of oak lactone in American varieties of oak chips in relative terms as compared to French varieties (**Figure 3.B**) confirms the results described in the literature *(26)*. The absence of whisky lactones in vine-shoots could be overcome by optimization of wine ageing with mixtures between vine-shoots and oak chips. Other furan derivatives such as alcohols, mainly 2-furanmethanol and 5-methyl-2-furylmethanol, and ester derivatives, mainly 2-furanmethanol acetate and 5-acetoxymethyl-2-furaldehyde, were only detected in the volatile profile associated to vine-shoots. Furan derivatives commonly detected in oak chips and vine-shoots were ketones such as 1-(2-furanyl)ethanone, 5-methyl-2-propionylfuran, 2-acetylfuran and 2-furan-methanol acetate. These compounds, which have been related to coffee-like, toasty caramel, honey and almond odors, were detected in both types of sample regardless of the heating temperature *(24,27)*. Differences were observed depending on the furan derivatives; thus, only furfural and 2-pentylfuran were present in all samples heated at 120 ºC, while American oak species allowed describing a more varied furan composition. **Supplementary Tables 1** and **2** list the compounds identified in the volatile fraction sampled at 120 ºC in the varieties of vine-shoots and oak chips thus demonstrating similarities and differences between them.

Since phenolic compounds are the final products of lignin thermo-degradation, the abundance of guaiacyl- and syringyl-type structures was expected. High heating temperature confers a varied phenolic composition to vine-shoots, mostly common with that in oak chips. These compounds are

responsible for smoky and vanilla aroma, especially ethyl vanillin, which is widely used in the aroma industry because it offers a warm vanilla note, three times stronger than vanillin *(28)*.

Ferulic acid was only detected in oak chips at low temperature (120 ºC) but its absence in the volatile fraction isolated from vine-shoots could be tentatively explained by thermal degradation. This compound was identified in previous studies as a precursor of other phenolic compounds formed by thermal degradation such as 4-vinylguaiacol (carnation odor descriptor), 4-ethylguaiacol (roasted wood), vanillin (vanilla), and guaiacol (smoke), among others, that are main products of pyrolysis *(29)*. This trend was confirmed by the presence of these compounds in vine-shoots and oak wood. By heating at 200 ºC the chromatographic peak of vanillin is one of the most intense in all samples. Other compounds such as methoxy-eugenol, syringaldehyde and vanillyl methyl ketone were detected in the volatile fraction from all samples, except from oak wood heated at 200 ºC, at which these compounds were degraded to the corresponding alcohol derivatives (*viz.* eugenol, dihydroeugenol and homovanillyl alcohol).

Some other interesting compounds detected were simple and ramified alcohols such as phenylethyl alcohol, known for its floral aroma and antimicrobial properties *(30)*, as well as 1,3-octanediol, 1,9-nonanediol, 2-hexyl-1-decanol or 3,4-dimethyl-1-penten-3-ol. Ethyl and methyl esters of aliphatic acids also constitute an important aromatic family in the aroma profile of wines, which were detected in practically all samples. Among them, ethyl and methyl palmitate have previously been detected in different types of wines, being related to fruity odours *(31)*. Terpenes such as copaene were detected in vine-shoots at low heating temperature, while phytol and isophytol were present in most of the samples regardless of their origin and heating temperature. Terpene derivatives are also important contributors to the aroma of wines because of their low perception threshold and their relationship with floral odor and antimicrobial activity *(32)*.

On the other hand, some compounds associated to undesirable characters (such as 6-nonenal, which is related with "sawdust" and "sappy" perception in some wines aged in new barrels) were also found *(24)*. This compound was detected in chromatograms of some vine-shoots corresponding exclusively to experiments carried out at high heating temperatures (180 and 200 ºC).

3.4. Confirmatory analysis of compounds with oenological interest

Some representative compounds (*viz*. vanillin, acetovanillone, syringol, eugenol, 4-vinylguaiacol, coniferaldehyde, guaiacol, 5-hydroxymethylfurfural, acetosyringone, furfural, *cis* and *trans* oak lactone and 4-ethylguaiacol) were selected because of their important sensory properties to confirm their identification. For this purpose, commercial standards were analyzed by using the same chromatographic method, which allowed confirming the presence of these compounds in the volatile fraction of oak chips and vine-shoots by retention times and mass spectra. **Table 4** shows the relative area, expressed as percentage, of these compounds in the volatile fraction sampled at 120, 150, 180 and 200 ˚C in two varieties of vine-shoots and oak wood. The relative areas were calculated in relation to the sum of the total area of the peaks corresponding to the selected compounds in each sample. The effect exerted by the heating temperature on the volatile fraction of oak chips and vine-shoots was especially noticeable for furfural. This compound was found at higher relative concentrations at intermediate heating temperatures (150, 180˚C) in all samples, while its relative concentration decreased at high temperature (200˚C), which agrees with the studies carried out by Chatonnet *et al*. (1999) *(22)*. The same trend was observed for vanillin, whose peak area increased with temperature, as **Figure 1** shows. Nevertheless, its relative abundance decreased due to generation of other compounds under these conditions. It is worth mentioning that threshold data had previously suggested that vanillin has a strong influence on wine aroma, while furfural and 5-methylfurfural possess minor impact due to their high detection thresholds *(33)*. The relative concentration of vanillin in the

analyzed samples strongly depended on the type of sample, being the highest levels found in the French varieties of oak wood, as previously described in the literature *(34)*, as well as in Pedro-Ximénez vine-shoot cultivar. On the other hand, oak lactones were always found at higher concentrations in American varieties of oak wood. The absence of oak lactones in vine-shoots is a key qualitative difference as compared to oak chips.

Coniferaldehyde was generally present at low heating temperatures and its concentration decreased when the temperature increased, which could be explained by thermal degradation since coniferaldehyde is the precursor in the synthesis of vanillin, among others. 5-Hydroxymethylfurfural was found at low temperatures in the case of French oak varieties, while its presence was detected at high temperature in the case of American varieties. Some other interesting compounds presented similar evolution patterns in oak chips and vine-shoots, being only found at medium and high heating temperatures. This is the case with syringol, 4-vinylguaiacol, guaiacol and eugenol, highly concentrated, in relative terms, in the volatile fraction of vine-shoots heated at 200 ˚C, as shows **Supplementary Figure 2**. These compounds were specially concentrated in the volatile fraction of Chardonnay and Syrah cultivars, while others, such as Baladí, Bobal, Montepila and Moscatel, reported low or undetectable levels. Vanillin presented higher relative concentrations in the volatile fraction of French varieties of oak wood, being the American varieties more similar to vine-shoots in this aspect. The opposite behavior was found in the case of furfural as vine-shoots exhibited higher concentrations than oak chips, except for the cases of the French Sweet and American Fresh oak varieties that showed levels similar to those in vine-shoots. Finally, 4-ethylguaiacol and coniferaldehyde were randomly present in some of the samples.

Table 4. Relative concentration of a panel of compounds sampled at 120, 150, 180 and 200 ˚C and expressed as the percentage of the sum of the areas of the selected compounds ("-" means the compound was not detected).

	Garnacha Tintorera vine-shoots				**Pedro-Ximénez vine-shoots**			
	120˚C	150˚C	180˚C	200˚C	120˚C	150˚C	180˚C	200˚C
Vanillin	-	-	0.24	1.15	10.25	0.89	0.75	2.53
Acetovanillone	-	-	-	0.21	-	-	-	0.16
4-Ethylguaiacol	-	-	-	-	-	-	-	-
Syringol	-	-	-	0.64	-	-	0.10	0.18
4-Vinylguaiacol	-	-	0.62	1.03	-	-	0.46	1.11
Coniferaldehyde	-	-	-	3.05	10.01	8.29	1.98	1.27
Guaiacol	-	-	0.17	1.18	-	-	-	0.86
trans-Oak lactone	-	-	-	-	-	-	-	-
cis-Oak lactone	-	-	-	-	-	-	-	-
5-Hydroxymethyl-furfural	-	-	-	-	76.57	1.12	-	-
Eugenol	-	-	0.07	1.38	-	-	-	1.00
Acetosyringone	-	-	-	0.03	-	-	-	-
Furfural	-	100	98.90	91.33	3.17	89.70	96.71	92.90
	French Sweet oak chips				**American Fresh oak chips**			
Vanillin	-	5.45	5.83	5.24	-	-	0.40	0.83
Acetovanillone	-	-	-	0.14	-	-	-	0.01
4-Ethylguaiacol	-	-	0.04	0.11	-	-	-	-
Syringol	-	-	0.28	0.56	-	-	-	-
4-Vinylguaiacol	-	-	0.32	0.72	-	-	0.50	0.76
Coniferaldehyde	5.25	-	-	-	92.66	3.92	-	-
Guaiacol	-	-	0.29	0.54	-	-	0.21	0.07
trans-Oak lactone	-	-	-	0.01	-	-	-	0.01
cis-Oak lactone	-	-	0.01	0.02	0.01	0.04	0.07	0.09
5-Hydroxymethyl-furfural	-	-	0.23	0.40	2.54	-	-	-
Eugenol	-	-	0.17	0.72	-	0.15	0.13	1.02
Acetosyringone	-	-	-	-	1.73	-	-	-
Furfural	94.75	94.55	92.83	91.55	3.06	95.89	98.69	97.21

Conclusions

The volatile profiles from different varieties of vine-shoots have been compared with those from different varieties of commercial oak chips, which are widely used in the oenological field to accelerate the ageing process of wines. A similar composition has been obtained in qualitative terms. The multivariate analysis also enabled identification of varieties of vine-shoots providing volatile

profiles with a more similar composition to those obtained from oak chips. These preliminary results could be considered for future tests intended to evaluate the utilization of vine-shoots for wine ageing either combined with oak chips or as single ageing agent to obtain flavors different to those provided by oak chips, a present trend in wines ageing processes *(35)*.

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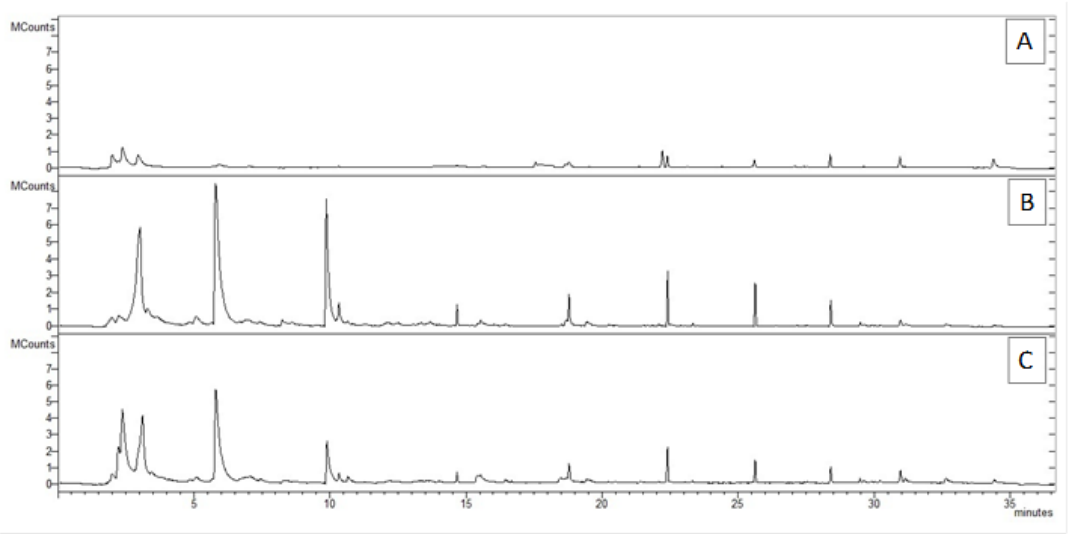
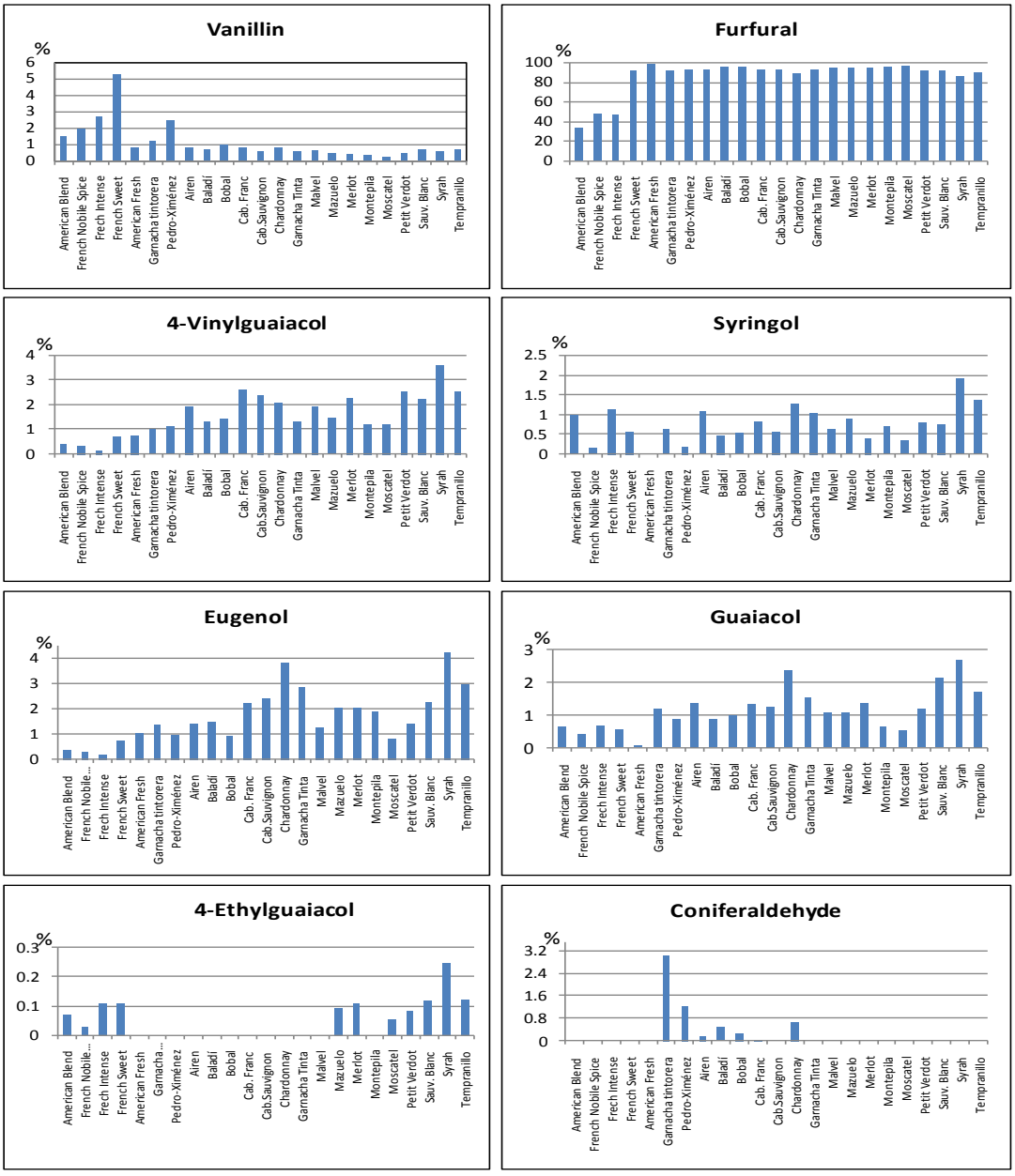
Supplementary material



Supplementary Figure 1. Chromatograms from Malbec vine-shoots after 6, 20 and 40 min heating time at 180 ºC (A, B and C, respectively).



Supplementary Figure 2. Relative concentration, expressed as percentage, of volatile compounds with oenological interest obtained at 200 ˚C as heating temperature.

Supplementary Table 1. Identification of compounds detected in the volatile fraction sampled at 120 ºC in the different varieties of vine shoots.

Vine shoots 120 ºC						
Compound	**Mass**	**RI (Ri)**	**Formula**	**Characteristic fragments**	**Red wine varieties**	**White wine varieties**
Difluorochloromethane	86	292 (258)	$CHClF_2$	51, 67, 85	✓	✓
Trichloromethane	118	642 (601)	$CHCl_3$	83, 50, 118	✓	
3-Methoxybutan-2-ol	104	677 (692)	$C_5H_{12}O_2$	59, 71, 89	✓	✓
3,4-Dimethyl-1-penten-3-ol	112	755 (742)	$C_7H_{12}O$	79, 43, 97		✓
Hexanal	100	784 (806)	$C_6H_{12}O$	56, 72, 82	✓	✓
1,3,5-Trioxacycloheptane	104	835 (806)	$C_4H_8O_3$	73, 61, 104	✓	
1,2-Butanediol	90	790 (824)	$C_4H_{10}O_2$	59, 89, 61	✓	✓
2-Hydroxypropionamide	89	872 (891)	$C_3H_7NO_2$	61, 55, 74	✓	
3-Pyridinol	95	915 (895)	C_5H_5NO	95, 68, 67		✓
4-Pyridinol	95	921 (895)	C_5H_5NO	95, 67, 55		✓
2,7-Dimethyl-3,5-octadiene	138	955 (903)	$C_{10}H_{18}$	95, 81, 67		✓
4,6-Dimethyl-2-heptanone	142	912 (924)	$C_9H_{18}O$	58, 69, 85		✓
Oxybisdichloromethane	182	980 (987)	$C_2H_2Cl_4O$	83, 85, 50		✓
1,1-Dimethoxyheptane	160	1055 (1004)	$C_9H_{20}O_2$	75, 55, 97	✓	
Cyclopentanecarboxylic acid	114	1036 (1017)	$C_6H_{10}O_2$	73, 86, 114		✓
2,3-Dimethylcyclohexanol	128	1022 (1030)	$C_8H_{16}O$	95, 81, 110	✓	
2-Pentylfuran	138	998 (1040)	$C_9H_{14}O$	81, 53, 138	✓	✓
Benzeneacetaldehyde	120	1102 (1081)	C_8H_8O	91, 65, 120		✓
Propanoic acid hexyl ester	158	1062 (1083)	$C_9H_{18}O_2$	57, 75, 84	✓	
3-Undecyne	152	1156 (1132)	$C_{11}H_{20}$	67, 81, 120		✓
5-Hydroxymethylfurfural	126	1185 (1163)	$C_6H_6O_3$	97, 126, 69		✓
2-Isopropyl-5-methylcyclohexanol	156	1202 (1164)	$C_{10}H_{20}O$	71, 81, 95		✓
6-Nonen-1-ol	142	1189 (1167)	$C_9H_{18}O$	67, 55, 82		✓
Decanal	156	1232 (1204)	$C_{10}H_{20}O$	57, 70, 82		✓
Copaene	204	1186 (1221)	$C_{15}H_{24}$	105, 119, 161	✓	
7-Methoxy-3,7-	186	1243	$C_{11}H_{22}O_2$	73, 55, 69		✓

dimethyloctanal		(1230)				
1-Formyl-4-hydroxymethyl-cyclohexane	142	1281 (1273)	$C_8H_{14}O_2$	81, 67, 95	✓	
Benzeneacetic acid, α-oxomethyl ester	164	1271 (1296)	$C_9H_8O_3$	105, 77, 51		✓
1-Undecanol	172	1388 (1357)	$C_{11}H_{24}O$	55, 69, 83		✓
Vanillin	152	1412 (1392)	$C_8H_8O_3$	152, 81, 123		✓
3,5-Dimethoxyacetophenone	180	1426 (1407)	$C_{10}H_{12}O_3$	165, 180, 137		✓
Vanillyl methyl ketone (Guaiacylacetone)	180	1530 (1538)	$C_{10}H_{12}O_3$	137, 180, 122		✓
Syringaldehyde	182	1602 (1581)	$C_9H_{10}O_4$	182, 111, 167		✓
Methoxyeugenol	194	1611 (1581)	$C_{11}H_{14}O_3$	194, 91, 119		✓
1-Hexadecyne	222	1633 (1609)	$C_{16}H_{30}$	81, 67, 55		✓
2,6-bis(1,1-Dimethylethyl)-2,5-cyclohexadiene-1,4-dione	220	1678 (1633)	$C_{14}H_{20}O_2$	177, 220, 135		✓
4-(3-Hydroxy-1-propenyl)-2-methoxyphenol (Coniferyl alcohol)	180	1622 (1653)	$C_{10}H_{12}O_3$	137, 124, 180	✓	
6-Methyl-2,4-di-tert-butyl-phenol	220	1660 (1668)	$C_{15}H_{24}O$	205, 220, 57	✓	
1-Heptadecyne	236	1688 (1709)	$C_{17}H_{32}$	81, 67, 55		✓
6,10,14-Trimethyl-2-pentadecanone	268	1791 (1754)	$C_{18}H_{36}O$	58, 71, 109		✓
9,12-Tetradecadien-1-ol, acetate	252	1814 (1795)	$C_{16}H_{28}O_2$	67, 81, 95	✓	✓
1-Octadecyne	250	1776 (1808)	$C_{18}H_{34}$	81, 67, 55		✓
2-Methyl-7-octadecyne	264	1869 (1863)	$C_{19}H_{36}$	81, 67, 95		✓
Hexadecanoic acid methyl ester (Methyl palmitate)	270	1891 (1878)	$C_{17}H_{34}O_2$	74, 87, 55	✓	✓
1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	278	1986 (1972)	$C_{16}H_{22}O_4$	149, 223, 104		✓
Hexadecanoic acid ethyl ester (Ethyl palmitate)	284	1993 (1978)	$C_{18}H_{36}O_2$	43, 73, 60	✓	✓
3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	296	2071 (2045)	$C_{20}H_{40}O$	81, 95, 123		✓
9,12-Octadecadien-1-ol	266	2096	$C_{18}H_{34}O$	67, 55, 81		✓

		(2069)				
9,12,15-Octadecatrien-1-ol	264	2044 (2077)	$C_{18}H_{32}O$	79, 67, 55		✓
Linolelaidic acid methyl ester	294	2045 (2093)	$C_{19}H_{34}O_2$	67, 55, 81	✓	✓

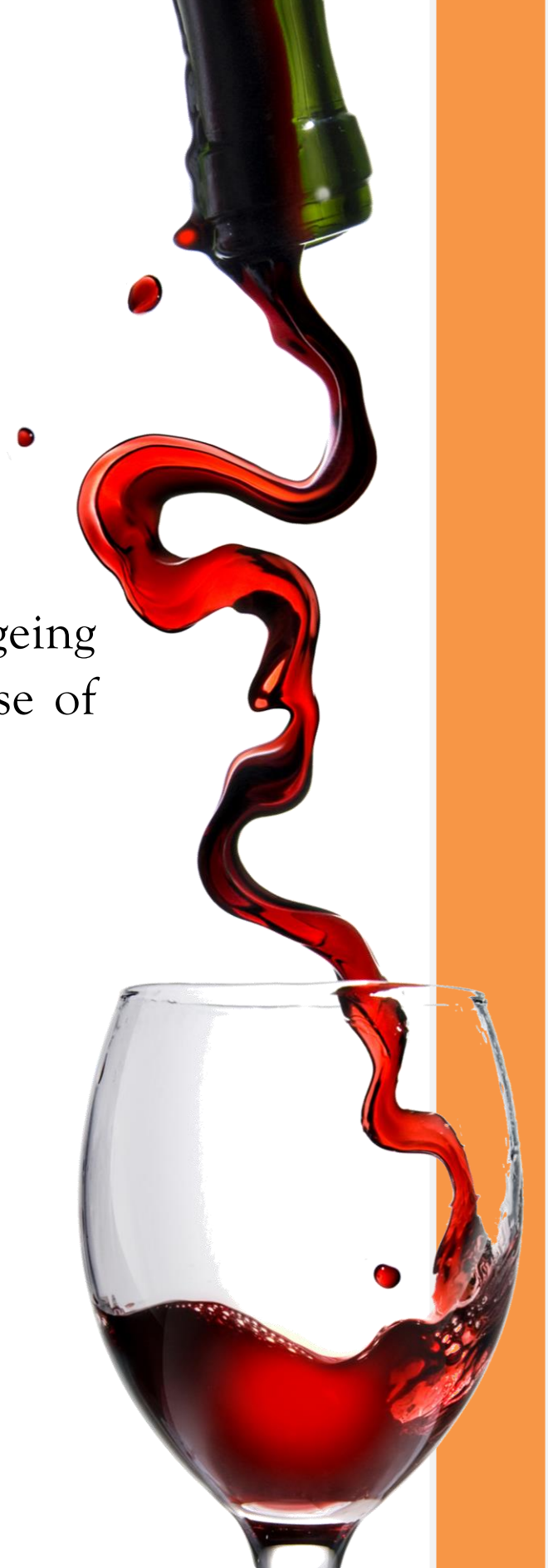
Supplementary Table 2. Identification of compounds detected in the volatile fraction sampled at 120 ºC in the different varieties of oak wood.

Oak wood 120 ºC						
Compound	**Mass**	**RI (Ri)**	**Formula**	**Characteristic fragments**	**French oak**	**American oak**
Propiolonitrile	51	580 (563)	C_3HN	51, 50, 52		✓
Trichloromethane	118	633 (601)	$CHCl_3$	83, 85, 50	✓	✓
Hexanal	100	785 (806)	$C_6H_{12}O$	56, 72, 82		✓
Furfural	96	836 (831)	$C_5H_4O_2$	96, 95, 67	✓	✓
2,5-Dimethyl-2-hexanol	130	823 (844)	$C_8H_{18}O$	59, 69, 97	✓	✓
4-Pyridinol	95	910 (895)	C_5H_5NO	95, 67, 55		✓
Methyl 2-furoate	126	932 (909)	$C_6H_6O_3$	95, 126, 68		✓
3-Methyl-6-hepten-1-ol	128	1016 (985)	$C_8H_{16}O$	55, 67, 95		✓
Octanal	128	1023 (1005)	$C_8H_{16}O$	56, 69, 84		✓
1-Ethynyl-1-cyclohexanol	124	1055 (1028)	$C_8H_{12}O$	81, 68, 95	✓	
2-Pentylfuran	138	1071 (1040)	$C_9H_{14}O$	81, 53, 138		✓
5-Hydrxoymethylfurfural	126	1182 (1163)	$C_6H_6O_3$	97, 126, 69		✓
1-Formyl-3,5,6-trimethyl-3-cyclohexene	152	1194 (1163)	$C_{10}H_{16}O$	121, 81, 67		✓
2,6-Dimethyl-2-octen-8-ol	156	1185 (1179)	$C_{10}H_{20}O$	69, 81, 55	✓	
3-Allyl-2,6,6-trimethylbicyclo[3.1.1] heptane	178	1150 (1187)	$C_{13}H_{22}$	83, 69, 55	✓	
1-Cyclohexyl-1-butanol	156	1209 (1241)	$C_{10}H_{20}O$	55, 95, 73		✓
5-Butyldihydro-4-methyl-2(3H)-furanone (*cis*-Oak lactone)	156	1226 (1245)	$C_9H_{16}O_2$	99, 71, 87	✓	✓
Vanillin	152	1369 (1392)	$C_8H_8O_3$	151, 109, 81		✓
3,4,5-Trimethoxyphenol	184	1499 (1468)	$C_9H_{12}O_4$	169, 184, 141		✓
2,6-Dimethyl-2-octen-8-ol butyrate	226	1487 (1501)	$C_{14}H_{26}O_2$	81, 69, 95	✓	
δ-Undecalactone	184	1526 (1503)	$C_{11}H_{20}O_2$	99, 71, 55	✓	

Vanillyl methyl ketone (Guaiacylacetone)	180	1506 (1538)	$C_{10}H_{12}O_3$	137, 180, 122	✓	
Dodecanoic acid	200	1562 (1570)	$C_{12}H_{24}O_2$	60, 73, 129	✓	
Methoxyeugenol	194	1566 (1581)	$C_{11}H_{14}O_3$	194, 91, 119	✓	
Syringaldehyde	182	1583 (1581)	$C_9H_{10}O_4$	182, 167, 139	✓	
Coniferaldehyde	178	1623 (1599)	$C_{10}H_{10}O_3$	178, 135, 107	✓	
Acetosyringone	196	1639 (1628)	$C_{10}H_{12}O_4$	181, 196, 153	✓	
Homovanillic acid	182	1644 (1659)	$C_9H_{10}O_4$	137, 182, 122	✓	
6-Methyl-2,4-di-tert-butyl-phenol	220	1654 (1668)	$C_{15}H_{24}O$	205, 220, 57	✓	
9,12-Tetradecadien-1-ol	210	1659 (1672)	$C_{14}H_{26}O$	68, 81, 55	✓	
6,10,14-Trimethyl-2-pentadecanone	268	1779 (1754)	$C_{18}H_{36}O$	58, 71, 109		✓
Ferulic acid	194	1772 (1767)	$C_{10}H_{10}O_4$	194, 179, 133		✓
14-Methyl-8-hexadecenal	252	1858 (1843)	$C_{17}H_{32}O$	55, 70, 83		✓
2-Methyl-7-octadecyne	264	1881 (1863)	$C_{19}H_{36}$	81, 67, 95		✓
Methyl palmitate	270	1861 (1878)	$C_{17}H_{34}O_2$	74, 87, 55	✓	✓
9-Hexadecenoic acid methyl ester	268	1897 (1886)	$C_{17}H_{32}O_2$	55, 69, 74		✓
3,7,11,15-Tetramethyl-1-hexadecen-3-ol (Isophytol)	296	1915 (1899)	$C_{20}H_{40}O$	71, 57, 82		✓
Ethyl palmitate	284	1990 (1978)	$C_{18}H_{36}O_2$	43, 73, 60	✓	✓
3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	296	2091 (2045)	$C_{20}H_{40}O_8$	81, 95, 123		✓
Methyl octadecadienoate	294	2122 (2093)	$C_{19}H_{34}O_2$	67, 81, 95		✓
Linoleic acid	280	2211 (2183)	$C_{18}H_{32}O_2$	67, 81, 55	✓	✓
1,2-Benzenedicarboxylic acid butyl cyclohexyl ester	304	2263 (2299)	$C_{18}H_{24}O_4$	149, 55, 223	✓	

SECTION II

Exploitation of vine–shoots.
Comparison of wine ageing by vine-shoots *versus* use of oak chips



En la Sección II de esta Memoria se recogen dos capítulos que proporcionan información sobre la posibilidad del aprovechamiento de los sarmientos de la vid para su uso en el proceso de envejecimiento del vino tinto.

Las dos publicaciones que se recogen como Capítulos 6 y 7 tienen una temática común: El estudio y comparación de vinos tintos envejecidos en ausencia y en presencia de virutas de roble o de sarmientos de vid, abarcando aspectos distintos que justifican su división. En el Capítulo 6 se muestran los resultados del estudio sensorial de los vinos por un panel de catadores expertos, estableciendo los aspectos sensoriales que experimentan modificaciones más o menos significativas con cada estrategia de envejecimiento. El uso de herramientas quimiométricas permitió traducir estos datos para el estudio en profundidad de los patrones de similitud de los vinos. El Capítulo 7 recoge el estudio comparativo de los perfiles obtenidos, tanto con los vinos envejecidos en presencia de virutas de roble y de sarmientos, como en ausencia de ellas, mediante análisis por GC–MS haciendo uso de herramientas quimiométricas. Finalmente, se llevó a cabo la identificación de los compuestos más representativos así como la comparación de vino envejecido con cada material en comparación con el vino envejecido sin su adición.

Section II in this PhD Book is devoted to two publications that provide information on the feasibility for exploitation of vine-shoots for ageing of red wine.

Chapters 6 and 7 have a common subject: the study and comparison of red wines that have been aged in the absence or presence of oak chips or vine-shoots, encompassing different aspects that justify division. Chapter 6 shows the results of a sensory study on the treated and untreated wines developed by a panel of experts, establishing the sensory aspects more significantly modified by each ageing strategy. The use of chemometric tools allows the translation of these data for in depth study of the similarity/dissimilarity patterns among wines. Chapter 7 contains a comparative study of the profiles obtained from wines aged in the presence/absence of oak chips or vine-shoots by GC–MS analysis taking advantage of chemometric tools. Finally, identification of the most representative compounds found in each fraction was carried out, as well as in wine aged with each material as compared to wine aged without them.

Chapter 6:

Comparative sensory profile of red wines aged with vine-shoots or oak chips

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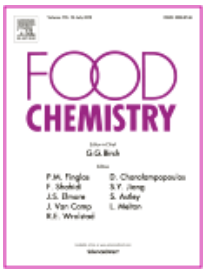

Comparative profile of red wines aged with vine-shoots or oak chips.

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Comparative sensory profile of red wines aged with vine-shoots or oak chips

M. Pilar Delgado de la Torre, Pedro Manuel Pérez-Juan, Eva Sánchez-Palomo, Miguel Ángel González-Viñas, Feliciano Priego-Capote and M. Dolores Luque de Castro**

Abstract

This study was devoted to the changes in the sensory characteristics of red wine caused by contact with either vine-shoots or oak chips. The changes undergone by the presence of each of the materials after ageing for 6 or 9 months were subjected to evaluation by a panel of experts, then compared among them and with a control wine. Wines aged in the presence of oak chips presented a similar sensory profile to control wines, with some tobacco, coffee and sweet spice notes. Vine-shoots gave sensory profiles with more perceptible prune-/raisins and liquorize attributes, more acid and astringent character and a less intense red fruit, tobacco and leather profile. This study provides a feasible alternative to traditional ageing methods. This is the first time that wines aged in the presence of vine-shoots are sensory evaluated by an expert judge panel.

1. Introduction

The sensory characteristics of wine depend on multiple aspects, among which the grape variety, the winemaking and ageing processes and the wine maturation are the most influential *(1)*. Wine aroma is modified along each of these steps. Traditionally, oak barrels have been used during wine ageing, since oak wood promotes changes in wine as its stabilization, increases wine complexity, and flavor changes by transference of compounds from lignin degradation *(2)*. Thermal treatment of oak wood has proven to be a key factor influencing the quantity of volatile phenols and furan compounds transferred to wines *(3-5)*; thus, higher toasting degrees are linked to wines with higher nutty aromas and earthy flavors in the mouth, while wines in contact with medium toasted chips are more smoky, woody, vegetative and spicy *(6)*. An alternative to barrel ageing is the addition of oak chips in different formats (e.g. segments, staves, and, more commonly, oak chips) to stainless steel wine containers *(7-10)*. The use of oak chips could have a positive effect on wine complexity and decrease the ageing time as compared with ageing in oak barrels *(8-13)*. The final profile of the aged wine depends on the characteristics of the oak wood in contact with wine (viz. origin, size, toasting process) but also by the dosage, period of contact with wine and possible oxygenation of the aged product *(14)*. A study by García-Carpintero *et al*. focused on the effect of oak chips added along different stages of wine ageing concluded that the oak character of wines was stronger when the chips were added during the malolactic fermentation *(15)*.

In general, research on wood maturation of wines is focused on compositional changes, with few examples in the literature devoted to the sensory character. Descriptive sensory analysis of wines provides an objective profile of wine flavor, taking into account the different perception threshold of some compounds responsible for desirable attributes, such as vanillin, acetovanillone and acetosyringone that, thanks to their low perception thresholds, have a strong influence on the organoleptical properties of wine because their vanilla and toffee flavoring contribution *(16)*. For this reason, descriptive analysis allows setting

sensory differences among wines, as demonstrated in the research by Campo *et al.*, who studied these differences among six wines from different grape cultivars *(17)*.

A number of new ageing alternatives using wood sources such acacia, cherry, chesnut or mulberry have been used for wine ageing *(18,19)*. In this context, the possibility of using vine-shoots arises as other alternative with the added value of taking benefit from an agricultural by-product. The phenolic profile and the volatile fraction of vine-shoots and oak chips have been compared in previous research using extracts obtained by superheated liquid extraction and liquid chromatography with diode array or time-of-flight mass detectors (LC–DAD, LC–TOF/MS), or gas chromatography and mass detector (GC–MS) for analysis *(16-18)*. As demonstrated, both materials have a very similar composition in compounds interesting from the oenological point of view as vanillin, which, although it has demonstrated to be slightly more concentrated in extracts from oak chips, it can be found at comparable levels depending on the vine-shoots cultivar used. Other example is 5-hydroxymethylfurfural, responsible for the toasty and caramel like flavor of wine, that, depending on the extraction conditions, is found in vine-shoots even at higher concentrations than in oak wood *(18,20,21)*. Based on the results of this research, the feasibility of vine-shoots as a potential alternative or complement to oak wood in wine ageing was considered a matter of study. Therefore, the aim of this study was to characterize the sensory profile of red wines aged in the presence of different varieties of oak chips or vine-shoots and compare them in terms of olfactory, gustatory and visual attributes. Two varieties of vine-shoots, two of oak chips (French and American oak chips both toasted and non toasted) were used for ageing wine up to 9 months. The subsequent sensory analysis provided an objective profile of wine aroma and flavor-by-mouth, and allowed evaluating the feasibility of vine-shoots to be used in the ageing process of wine with a double aim: to obtain wines with new organoleptic characteristics and valorize this viticulture residue.

2. Experimental

2.1. Vine-Shoots and Oak Chips

Vine-shoots from Vitis vinifera cultivars were sampled from the same plantation located at "Bodega y Viñedos Vallebravo" (Sierra de Segura, Jaén, Spain) in 2011, after leaf-fall, and by randomized selection of ten similar stocks of each cultivar. Two different vine cultivars were selected for this study, a red grape cultivar such as Merlot and a white grape cultivar such as Montepila. The selected vines were cultivated under the same agronomic conditions in terms of soil properties, climate, hydric regime, etc. Several 10 cm pieces of of vine-shoots from each cultivar were cut at the height of the first leaf bud. The vine-shoot samples were milled to obtain a homogeneous particle size (less than 0.42 mm diameter) and dried for 72 h at 30 ºC. To evaluate the incidence of toasting the vine-shoots on the sensory evaluation of aged wines, some experiments were planned by using toasted vine-shoots for 20 min in an oven at 180 ºC.

Four types of commercial oak chips representing different types of oak barrels used for wine ageing were kindly supplied by Laffort España S.A. (Guipúzcoa, Spain). The types of oak chips were two French varieties (Intense and Nobile Spice corresponding to toasted and non-toasted chips, respectively) and two American varieties (Blend and Fresh corresponding to toasted and non-toasted chips, respectively). As oak chips are commercialized as a dried product, they were milled and stored following the same protocol.

2.2. Wine Ageing

The wine was made in Sierra de Segura (Jaén) with grape cultivars of Syrah, Merlot and Mazuelo in equivalent quantities. The grapes were harvested at their optimal ripening stage (23±1 ºBrix) and good sanitary conditions. A cold prefermentative maceration was made, followed by alcoholic and malolactic fermentation at temperature below 22 ºC. Fermentation was made with the

indigenous yeasts and the total SO_2 concentration was under 60 mg/L. After this process, the obtained wine was used as control without addition of any oak chips or vine-shoots. Two bottles of 750 mL of control wine were stored at –20 ºC until sensory analysis. The scheme of the experimental plan is exposed in **Figure 1.**

Wines were aged with addition of either oak chips, toasted and non toasted vine-shoots after malolactic fermentation at a dose rate of 4 g/L. Wine ageing was performed in individual bottles of 750 mL (four bottles for each of the different wines subjected to ageing, from which two of each were subjected to 6 month-ageing, and the other two to 9 month-ageing). Thus, wines were aged under warehouse conditions in Pago Casa del Blanco (Manzanares, Ciudad Real) with a total number of 34 bottles subjected to ageing. The amount of chips used in this study was selected to avoid an excessive impact of the wood character in wines that could produce a negative effect on the taster *(11,22)*.

2.3. Standard Chemical Analyses of Wines

Total acidity, ethanol, pH, volatile acidity, total and free SO2 were determined according to the methods proposed by the O.I.V. (2012) *(23)*. Where all parameters met the expected standards for this type of wine and total SO2 was below 60 mg/L.

2.4. Sensory Descriptive Analysis

Wines were analyzed in duplicate by 15 expert trainers during eight sessions using a balanced, complete block design. Wines were stored at 12 ºC and 20 mL of each sample was presented at 15 °C for visual, olfactory and gustatory evaluation.

The expert trained judges were selected according to their sensitivity to perceive: astringency in standard solutions containing concentrations of pure compounds slightly above the threshold limit, basic tastes; differences in the Nez Du Vin, traditionally perceived in red wines such as cloves, violet, oak, black

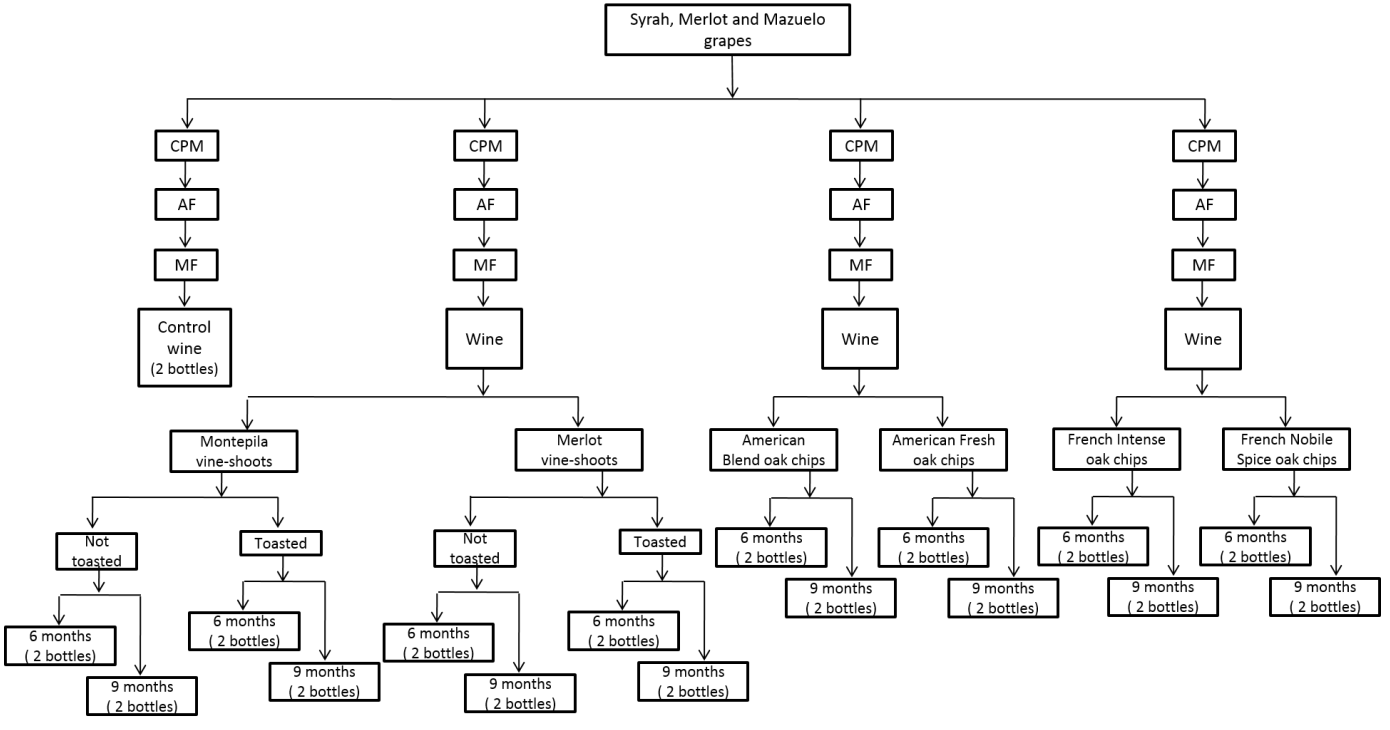


Figure 1. Diagram of the experimental plan followed in this study and identification of winemaking treatments. (CPM: cold prefermentative maceration; AF: alcoholic fermentation; MF: malolactic fermentation)

coffee, toast or vanilla.

2.4.1. Lexicon and Panel Training

Control and aged wines were evaluated by the trained judges with a total of 24 olfactory, 19 gustatory and 4 visual descriptors **(Supplementary Table 1**). The panellists received four wine samples in each session (with a total of eight sessions) and described individually their similarities and differences considering their visual profile, aroma, taste and mouth-feel properties. Then, the panellists debated the terms generated individually, and defined the terms describing the aroma and taste characteristics of the evaluated wines under the supervision of a panel leader. Finally, 4 visual, 9 olfactory an 11 flavor-by-mouth and mouth-feel attributes were considered among the best at describing the sensory characteristics of the wines and the main differences among them. The aim of the judges was two-fold: reaching a consensus about the meaning of each attribute and achieving a reliable intensity rating.

Physical-chemical standards were used to define attributes *(25)*. In addition, the panellists elaborated a sensory descriptive ballot for the wines where each attribute was rated according to a 0–10 unstructured scale. The left-hand end of the scale (0) was "attribute not perceptible" and the right-hand end (10) was "attribute strongly perceptible".

Previously, a final selection of the panellists was carried out after a training period as made by García-Carpintero *et al.* Thus, each judge evaluated six red wines with five replicates using the descriptive ballot *(25)*. Analysis of variance (ANOVA), where the sources of variation were the wine and the replications, was carried out for each judge and each attribute. The level of significance (*p*-value) of the F value, calculated by the ANOVA for the source of variation ´wine´ (pFwine), was used as the criterion to estimate the discriminating power or each judge, and the level of significance of the F value calculated for the source of variation ´replication´ (pFreplication) was used as

the criterion to estimate the reproducibility of each judge. Only individuals that showed an adequate discriminating power (pFwine≤0.30), reproducibility (pFreplication≥0.05) and consensus with the rest of the panel for at least 80% of the descriptors present in the ballot were selected to take part in the descriptive panel *(25)*.

2.4.2. Sensory Profile

Red wine samples (20 mL) were presented in standard wine-testing glasses according to the International Organization of Standarization, (UNE-EN ISO 8589, 2010) *(26)*, covered with a watch-glass to minimize the escape of volatile components, and coded with random three-digit numbers. The temperature of the wines was maintained at 15±1 ºC, the assessment took place in a standard sensory-analysis chamber according to the standard 87022 (UNE 87022:1992) *(27)*, equipped with separate booths and under white light. The wines were sniffed and tasted.

Data collection was made using the descriptive ballot generated by the panel. Each judge evaluated each wine with two repetitions. A different bottle of wine was used for each repetition, where the order of presentation of samples was balanced to avoid the first-order effect *(28)*.

2.5. Statistical Analysis

All statistical analyses were performed using the Statgraphics Centurion XV (v15.1.02 for Windows). The data set was evaluated by ANOVA to discriminate among the means of sensory data. Principal component analysis (PCA) was used as a tool for screening, extracting and compressing data as well as to find the dominant sensory descriptors of the target wines.

3. Results and Discussion

3.1. Sensory Profile of Aged Wines

After sensory analysis, the average scores for all attributes considered in the wines aged under different experimental conditions and the control wines were calculated. **Table 1** shows the mean scores for all the olfactory attributes according to the panel evaluation and the standard deviation associated to each score. The variability in the mean scores provided by the panellists, expressed in terms of relative standard deviation, ranged from 9.25 to 42.27% for control wines; while it ranged from 0 to 10.09% for wines aged with vine-shoots and from 0.26 to 13.98% for those aged with oak chips. These estimations allow deducing that the different olfactory attributes were consolidated with the ageing process, while control wines were characterized by a high variability of the olfactory profile found by the members of the panel. The differences in the profiles thus provided are shown in **Figure 2**. The scores for the control wine are also observed in each diagram to pinpoint the main changes occurring during wine ageing for 6 or 9 months. In the case of vine-shoots, two diagrams were plotted to show the influence of ageing by using the two selected cultivars of vine-shoots, Merlot and Montepila, from red grape and white grape varieties, respectively. Also, the olfactory profiles provided by ageing with toasted and non-toasted material are included. On the other hand, two diagrams were prepared for oak chips to compare French and American oak varieties. In the diagrams, the central point indicates low intensity for each descriptor increasing in the scale up to 10 at the end of the axes.

The control wine was predominantly marked by odor intensity and red fruit notes followed by spice and fresh perception and by low notes of other attributes such as tobacco, coffee, liquorize, leather and, particularly, prune/raisin odor. On the other hand, in all wine samples aged under the different conditions the odor intensity tends to remain at constant levels, similar

Table 1. Mean scores (SD in brackets) for attributes considered in the olfactory descriptive analysis of red wines by 15 judges (2 replicates). Relative standard deviation (RSD%) is also presented to show the variability of the panel for the different attributes according to the sample.

Sample	Intensity	Red fruit	Prune/raisin	Liquorize	Fresh	Spice	Leather	Tobacco	Coffee	RSD range (%)
Control wine	7.85 (0.75)	7.85 (0.75)	2.20 (0.93)	4.65 (0.80)	5.83 (0.74)	6.01 (0.74)	3.37 (0.92)	3.37 (0.92)	3.72 (0.98)	9.55-42.27
VS Merlot T 6M	7.94 (0.13)	5.19 (0.04)	3.50 (0.02)	4.94 (0.08)	4.63 (0.06)	4.97 (0.08)	3.09 (0.02)	3.73 (0.11)	3.79 (0.02)	0.53-2.95
VS Merlot T 9M	7.34 (0.24)	4.48 (0.16)	3.90 (0.01)	5.24 (0.02)	4.25 (0.12)	5.28 (0.12)	3.49 (0.00)	4.16 (0.08)	4.11 (0.04)	0-3.57
VS Merlot NT 6M	7.79 (0.13)	5.34 (0.06)	4.06 (0.08)	5.50 (0.31)	5.28 (0.18)	5.38 (0.23)	3.75 (0.32)	4.32 (0.20)	4.45 (0.22)	1.12-8.53
VS Merlot NT 9M	7.84 (0.18)	4.99 (0.03)	4.35 (0.02)	5.49 (0.42)	4.75 (0.08)	5.51 (0.02)	4.10 (0.38)	4.60 (0.22)	4.91 (0.10)	0.36-9.27
VS Montepila T 6M	7.67 (0.22)	5.52 (0.34)	4.30 (0.11)	5.46 (0.10)	5.13 (0.30)	5.34 (0.18)	3.58 (0.18)	3.95(0.14)	4.19 (0.09)	1.83-6.16
VS Montepila T 9M	7.53 (0.01)	4.46 (0.19)	4.67 (0.05)	5.77 (0.23)	4.36 (0.44)	5.13 (0.12)	3.68 (0.09)	4.41 (0.32)	4.61 (0.09)	0.13-10.09
VS Montepila NT 6M	8.21 (0.14)	7.21 (0.06)	3.70 (0.15)	4.23 (0.12)	5.46 (0.10)	4.06 (0.09)	3.80 (0.24)	4.08 (0.06)	3.87 (0.24)	0.83-6.32
VS Montepila NT 9M	8.08 (0.25)	6.84 (0.07)	4.02 (0.02)	4.43 (0.04)	5.00 (0.15)	4.62 (0.05)	4.34 (0.24)	4.22 (0.02)	4.20 (0.24)	0.47-5.71
Oak Am. Blend T 6M	7.98 (0.21)	6.62 (0.06)	3.19 (0.11)	4.98 (0.08)	4.30 (0.05)	5.26 (0.06)	3.80 (0.16)	4.28 (0.13)	4.10 (0.04)	0.91-4.21
Oak Am. Blend T 9M	8.07 (0.20)	6.36 (0.12)	3.69 (0.01)	5.10 (0.14)	3.92 (0.01)	5.68 (0.05)	4.13 (0.05)	4.65 (0.19)	4.40 (0.14)	0.26-4.09
Oak Fr. NSNT 6M	7.50 (0.25)	6.95 (0.28)	3.88 (0.08)	5.78 (0.15)	4.75 (0.11)	5.84 (0.19)	4.14 (0.18)	4.26 (0.14)	4.51 (0.39)	2.06-8.65
Oak Fr. NSNT 9M	7.49 (0.31)	6.58 (0.68)	3.80 (0.10)	5.91 (0.34)	4.63 (0.11)	6.04 (0.10)	4.31 (0.12)	4.47 (0.19)	4.37 (0.70)	2.38-16.02
Oak Fr. Intense T 6M	7.93 (0.08)	6.64 (0.20)	3.85 (0.05)	5.78 (0.15)	4.90 (0.02)	5.76 (0.04)	4.04 (0.07)	4.32 (0.10)	4.92 (0.40)	0.41-8.13
Oak Fr. Intense T 9M	8.05 (0.08)	6.34 (0.31)	4.26 (0.15)	5.91 (0.02)	4.40 (0.15)	6.23 (0.04)	4.65 (0.28)	4.72 (0.66)	5.02 (0.25)	0.34-13.98
Oak Am. Fresh NT 6M	7.61 (0.10)	6.78 (0.22)	4.27 (0.16)	5.95 (0.28)	4.81 (0.04)	5.20 (0.05)	4.88 (0.45)	4.73 (0.04)	4.43 (0.09)	0.83-9.22
Oak Am. Fresh NT 9M	7.39 (0.34)	6.41 (0.16)	4.43 (0.14)	6.19 (0.11)	4.55 (0.09)	5.37 (0.20)	4.08 (0.12)	4.82 (0.09)	4.57 (0.29)	1.78-6.35

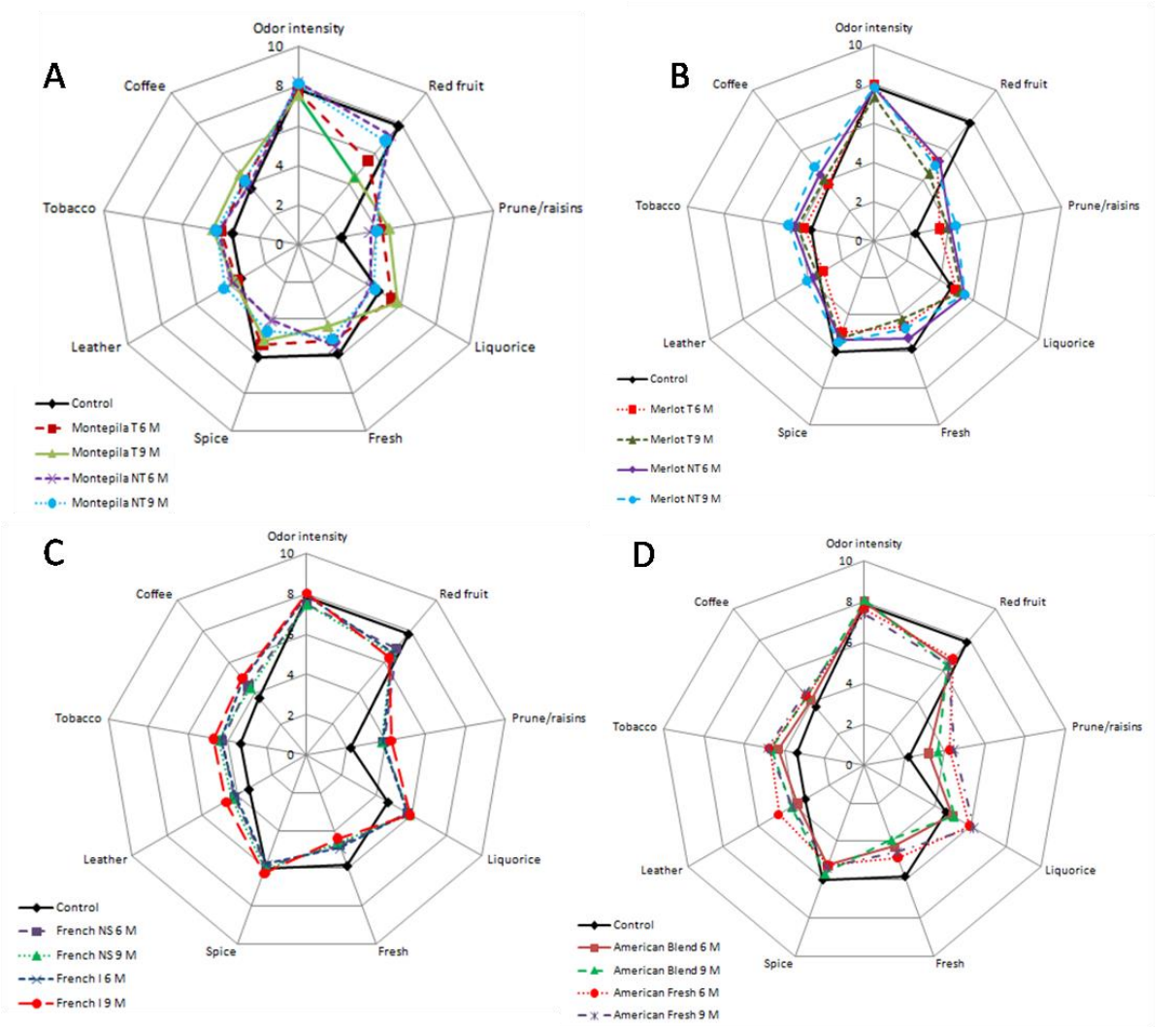


Figure 2. Olfactory attribute scores of the control wines *versus* wines aged in the presence of 4 g/L of: (A) Montepila vine-shoots, (B) Merlot vine-shoots, (C) French oak varieties and (D) American oak varieties. (T: toasted; NT: non toasted).

to that of control wine. The score for the red fruit attribute was significantly decreased under all ageing conditions as compared to the control wine, which is consistent with other previous studies found in the literature (6,15). However, this decreased effect was moderated in wines aged in contact with oak chips, especially with non-toasted chips as American Fresh and French Nobile Spice chips as American Fresh and French Nobile Spice chips, but also in those aged

with Montepila non-toasted vine-shoots, which reported the highest scores for this attribute.

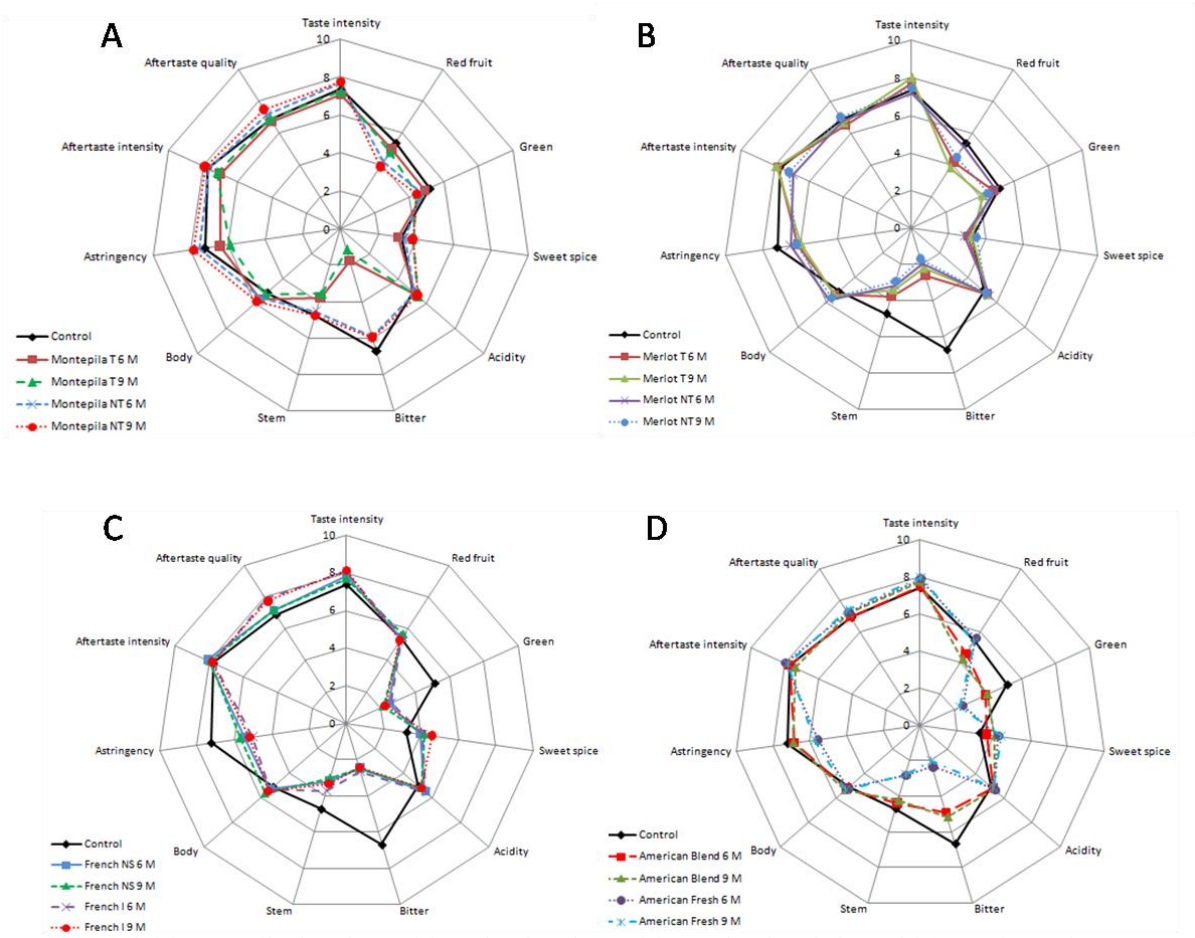
Some other attributes such as prune/raisin, liquorize, leather, tobacco and coffee were more perceptible in almost all aged wines than in the control wine. The prune/raisin notes were slightly more intense in wines aged in the presence of Montepila non toasted vine-shoots. The liquorize attribute was less intense in wines aged in contact with vine-shoots as compared to those in contact with oak chips, but in the particular case of ageing with toasted Montepila vine-shoots, this attribute was enhanced. The leather aroma was generally more acute in wines treated with oak chips, regardless the contact time, than in wines treated with vine-shoots, the lowest corresponding to toasted vine-shoots.

The tobacco and coffee aroma attributes increased in all cases, from low intensity perception in the control wine, to medium intensity in wines aged in the presence of oak chips or vine-shoots, but no statistical differences were found among treated wines. Regarding to the fresh attribute, a slight decrease in this characteristic was found in all treated wines, especially in those in contact with oak chips, where the decrease was more significant. Among wines treated with vine-shoots, those aged in contact with Montepila cultivar maintained this attribute up to score values, similar to the control wine. Concerning the intensity of the spice aroma, this attribute decreased for aged wines, except for those aged in the presence of French oak chips. The decrease was especially significant for wines aged with vine-shoots.

Regarding to the gustatory profile, the average scores for taste-intensity attributes and the standard deviation for all wines are listed in **Table 2**. Relative standard deviations of taste-intensity attributes ranged from 5.82 to 24.46% for control wines, from 0.23 to 13.30% for wines aged with vine-shoots and from 0.12 to 24.63% for wines aged with oak chips. Therefore, no variability differences were established between control wines and aged wines concerning gustatory attributes. **Figure 3** illustrates the main changes occurring in wines

aged with oak chips or vine-shoots. According to the panellists, several changes were found in the gustative profiles of the wines under study. The control wine displayed a profile characterized by some predominant notes, such as bitterness, astringency, aftertaste intensity, aftertaste quality and taste intensity, that were rated with values higher than 6.7 out of 10.

Figure 3. Gustatory attribute scores of the control wines *versus* wines aged in the presence of 4 g/L



of: (A) Montepila vine-shoots, (B) Merlot vine-shoots, (C) French oak varieties and (D) American oak varieties. (T: toasted; NT: non toasted).

Several changes were observed in the gustative attributes by comparing aged wines with control wines. The most perceptible changes were ascribed to the

bitter and stem attributes. Thus, bitterness, which is a negative attribute, was considerably reduced in all aged wines, except for wines aged with American Blend oak chips (toasted variety) and with Montepila non-toasted vine-shoots. The stem taste, also a negative attribute when in excess, was also decreased in aged wines, except in those aged with American Blend oak chips and Montepila non-toasted vine-shoots. Thus, the wines aged with both materials were characterized by a marked bitter taste with stem notes.

The perception of astringency and green attributes was also decreased in aged wines as compared to the control wine. The effect was more significant in wines aged with vine-shoots and, specially, in those aged in contact with American Fresh or French varieties of oak chips. A similar pattern was also followed by the sweet spice character that was slightly increased in wines aged with oak chips, especially with French varieties. The red fruit attribute also presented a particular trend during ageing, which is in agreement with the behavior found for the red fruit olfactory attribute, the perception of which was decreased with ageing, except from wines aged with French oak chips.

Concerning the rest of the attributes, it is worth emphasizing the acidity and body characters that were not practically altered by the ageing process. A similar trend was observed for the taste intensity, aftertaste intensity and aftertaste quality, with no statistically observed differences between control and aged wines.

The evaluated visual attributes did not show remarkable differences among the ageing experimental conditions since all them were slightly reduced in aged wines in comparison to the control wine. **Supplementary Table 2** shows the mean scores and standard deviations provided by all panellists for visual attributes, where relative standard deviations ranged from 6.84 to 12.56% for the control wine, from 0 to 5.92 for wines aged with vine-shoots and from 0.13 to 9.40% for wines aged with oak chips.

3.2. Ageing of Wines with Oak Chips

The resulting wines were compared among them on the basis of their ageing strategy by ANOVA with a 95% confidence level. For this purpose, the mean scores given by the panelists for the studied attributes (visual, gustatory and olfactory attributes) were used as dependent variable to establish significant differences as a function of different factors. Firstly, the control wine was compared to wines treated with oak chips for nine months to have a global view of the changes occurred in aged wines. In general, the use of oak chips altered a less number of mouth-feel attributes than aroma attributes, which is consistent with the results obtained by Cano-López *et al.* and García-Carpintero *et al. (25,29)*. The most significant differences were observed in olfactory features, where the characters corresponding to leather, prune/raisins and tobacco aromas showed to be statistically higher (p-values 0.049, 0.019 and 0.004, respectively) in wines aged with oak chips for 9 months, while fresh and red fruit attributes experienced a significant decrease (p-values 0.026 and 0.001, respectively) with ageing. For the gustative and visual levels, the only statistically significant variations corresponded to sweet spice and hue attributes, being the former taste higher in the case of aged wines (p-value 0.026), while the hue attribute resulted higher in the control wine (p-value 0.010).

To evaluate the influence of the ageing time, wines aged for 6 and 9 months in the presence of oak chips were compared. No statistically significant differences were found at the gustatory level; however, regarding to aroma notes, the red fruit aroma was significantly higher in the case of wines aged for 6 months, which is explained by a shorter contact time with oak chips. This effect was also found by Koussissi *et al.*, who found that the red fruit notes were more acute in no treated wines, those aged in barrels or those slightly treated with toasted oak chips than in wines treated with heavy toasted oak chips *(6)*. At the visual level, hue and opacity showed significant differences among samples aged

Table 2. Mean scores (SD in brackets) for attributes considered in the gustatory descriptive analysis of red wines by 15 judges (2 replicates). Relative standard deviation (RSD%) is also presented to show the variability of the panel for the different attributes according to the sample.

Sample	Intensity	Red fruit	Green	Sweet spice	Acidity	Bitter	Stem	Body	Astringency	Aftertaste intensity	Aftertaste quality	RSD range (%)
Control wine	7.38(0.63)	5.40 (0.75)	5.15 (0.73)	3.23 (0.79)	5.07 (0.44)	6.71 (0.57)	4.73 (0.82)	5.13 (0.31)	7.23 (0.59)	7.73 (0.45)	6.87 (0.58)	5.82-24.46
VS Merlot T 6M	7.72 (0.03)	4.18 (0.08)	4.79 (0.06)	3.01 (0.09)	5.36 (0.10)	2.62 (0.10)	3.81 (0.24)	5.39 (0.03)	6.12 (0.03)	7.81 (0.50)	6.53 (0.06)	0.39-6.40
VS Merlot T 9M	8.00 (0.02)	3.82 (0.23)	4.16 (0.35)	3.22 (0.03)	5.38 (0.05)	2.20 (0.17)	3.41 (0.09)	5.43 (0.14)	5.95 (0.10)	7.91 (0.03)	6.67 (0.14)	0.25-8.41
VS Merlot NT 6M	7.16 (0.06)	5.16 (0.13)	4.94 (0.12)	2.85 (0.15)	5.46 (0.19)	1.98 (0.26)	3.15 (0.12)	5.83 (0.27)	6.50 (0.18)	6.91 (0.02)	6.81 (0.09)	0.29-13.3
VS Merlot NT 9M	7.45 (0.13)	4.48 (0.02)	4.48 (0.06)	3.51 (0.03)	5.27 (0.16)	1.69 (0.20)	2.94 (0.12)	5.64 (0.06)	6.18 (0.01)	7.19 (0.03)	7.01 (0.05)	0.16-11.83
VS Montepila T 6M	7.08 (0.12)	5.00 (0.05)	4.88 (0.26)	3.08 (0.06)	5.23 (0.02)	1.74 (0.09)	3.76 (0.10)	5.66 (0.08)	6.44 (0.05)	7.00 (0.20)	6.73 (0.03)	0.38-5.33
VS Montepila T 9M	7.24 (0.12)	4.81 (0.17)	4.43 (0.38)	3.83 (0.06)	5.42 (0.17)	1.14 (0.01)	3.54 (0.10)	5.26 (0.12)	5.90 (0.04)	7.17 (0.27)	6.90 (0.08)	0.68-8.58
VS Montepila NT 6M	7.70 (0.09)	4.20 (0.03)	4.64 (0.11)	3.41 (0.31)	5.16 (0.08)	5.91 (0.14)	4.53 (0.12)	5.57 (0.12)	7.53 (0.09)	7.66 (0.12)	7.17 (0.08)	0.71-9.09
VS Montepila NT 9M	7.75 (0.02)	3.89 (0.06)	4.42 (0.01)	3.84 (0.15)	5.36 (0.26)	5.96 (0.10)	4.76 (0.06)	5.84 (0.09)	7.84 (0.19)	7.88 (0.09)	7.48 (0.04)	0.23-4.85
Oak Am. Blend T 6M	7.45 (0.15)	4.53 (0.01)	3.86 (0.25)	3.59 (0.16)	5.18 (0.13)	4.93 (0.42)	4.41 (0.02)	5.26 (0.12)	6.88 (0.09)	7.77 (0.17)	6.93 (0.27)	0.22-8.53
Oak Am. Blend T 9M	7.73 (0.35)	4.20 (0.00)	3.94 (0.48)	4.05 (0.11)	5.29 (0.12)	5.19 (0.55)	4.27 (0.26)	5.38 (0.14)	6.93 (0.33)	7.43 (0.16)	7.17 (0.37)	0-12.18
Oak Fr. NSNT 6M	7.80 (0.11)	5.41 (0.25)	2.48 (0.04)	3.96 (0.37)	5.51 (0.05)	2.49 (0.26)	3.21 (0.44)	5.35 (0.27)	5.43 (0.11)	8.08 (0.21)	7.12 (0.41)	0.91-13.71
Oak Fr. NSNT 9M	7.68 (0.05)	5.61 (0.21)	2.16 (0.17)	4.20 (0.18)	5.26 (0.17)	2.48 (0.16)	3.05 (0.32)	5.70 (0.41)	5.69 (0.04)	7.93 (0.07)	7.15 (0.35)	0.65-10.49
Oak Fr. Intense T 6M	8.03 (0.14)	5.44 (0.01)	2.61 (0.22)	4.08 (0.07)	5.60 (0.06)	2.66 (0.16)	3.74 (0.08)	5.37 (0.06)	5.03 (0.09)	7.75 (0.29)	7.84 (0.05)	0.18-8.43
Oak Fr. Intense T 9M	8.11 (0.25)	5.25 (0.26)	2.26 (0.28)	4.59 (0.64)	5.27 (0.52)	2.44 (0.16)	3.34 (0.49)	5.50 (0.11)	5.20 (0.15)	7.83 (0.40)	7.70 (0.25)	2.00-14.67
Oak Am. Fresh NT 6M	7.84 (0.18)	5.53 (0.15)	2.51 (0.14)	4.24 (0.03)	5.32 (0.06)	2.36 (0.03)	2.84 (0.24)	5.23 (0.24)	5.60 (0.64)	8.02 (0.32)	7.20 (0.08)	0.71-8.45
Oak Am. Fresh NT 9M	7.95 (0.03)	5.30 (0.16)	2.31 (0.42)	4.37 (0.16)	5.16 (0.16)	2.03 (0.50)	2.78 (0.15)	5.36 (0.42)	5.77 (0.87)	7.89 (0.14)	7.38 (0.13)	0.38-24.63

Note: VS: vine-shoots, T: toasted, NT: non toasted, 6M: 6 months, 9M: 9 months, Am. Blend: American Blend, Am. Fresh: American Fresh, Fr. Intense: French Intense, Fr. NS: French Nobile Spice, RSD: relative standard deviation

for 6 or 9 months, being more marked in the former samples, and corresponding the highest scores to the control wine.

The influence of the type of oak chips, American or French, was also studied by comparing wines aged with both varieties of oak. Few statistically significant differences were found between these wines since only the astringency was found different at the sensory level (*p*-value 0.046), which was clearly higher in wines treated with American oak chips. Apart from tannins, more than 26 sensory-active nonvolatile compounds were identified as key astringent components in red wine, among which several hydroxybenzoic acids, hydroxyl-cinnamic acids, flavon-3-ol glycosides and dihydroflavon-3-ol rhamnosides, and a structurally undefined polymeric fraction (>5 kDa) were found *(30,31)*. Also significant differences (*p*-value 0.077) were found in the spice aroma note that showed to be higher in wines aged with French oak chips. Regarding to visual attributes, no statistical differences were observed.

When wines treated with toasted and non toasted oak chips were compared, no statistically significant visual differences were reported by the judges. However, for gustative attributes, aftertaste intensity and stem showed to be statistically significant (*p*-value 0.029 and 0.012, respectively), being the intensity of the former higher in wines aged in the presence of non toasted oak chips and the stem notes higher in those aged in contact with toasted chips. At the olfactory level, the odor intensity was found to be highly-significant among samples at a 99.99% confidence level (*p*-value 0.0001), showing the highest levels wines aged in contact with toasted oak chips.

3.3. Ageing of Wines with Vine-Shoots

By analogy to oak chips, an ANOVA test at a 95% confidence level was used to evaluate wine ageing with vine-shoots. For this purpose, the mean scores given by the panellists for all considered attributes in the wines aged with vine-shoots were compared as a function of different factors. The comparison between the control wines and wines aged for 9 months in contact with vine-shoots

denoted a decrease of green taste in the latter (*p*-value 0.017) and an increase of acidity (*p*-value 0.027) respect to the control wine, as significant changes at the gustatory level. At the olfactory level, ageing in contact with vine-shoots had a significant effect on wine by increasing the intensity of the tobacco and prune/raisins descriptors (*p*-value 0.022 and 0.014, respectively). On the contrary, the fresh character and the hue attribute at the visual level experienced a decrease on treated wines (*p*-value 0.049 and 0.013), which was significantly perceived.

The influence of the ageing time with vine-shoots (6 or 9 months) on the wine attributes revealed no statistical differences in aroma descriptors. However, at the gustatory level, the green attribute generally linked to the presence of *trans*-3-hexen-1-ol and hexanol *(32),* was statistically less perceptible in wines treated for 9 months (*p*-value 0.004), while the sweet spice notes were clearer with longer contact time (*p*-value 0.0352). Regarding to visual changes, only the hue character had a significant change, being clearly less intense in wines aged for 9 months (*p*-value 0.003). The variability caused by the two vine-shoot cultivars (white and red grape cultivars Montepila and Merlot, respectively) was also analyzed, but no statistical differences were found.

The influence of wood toasting was also studied by comparing the wines aged in contact with toasted or non toasted vine-shoots. In general, the use of toasted vine-shoots had scant effects on aroma attributes, being the mouth-feel attributes the most affected. Thus, only the leather aroma showed an evident change (*p*-value 0.029), being this character more intense in wines treated with non toasted vine-shoots. On the contrary, the gustative descriptors body, aftertaste quality and astringency showed to be less perceptible in wines treated with non toasted vine-shoots (*p*-values 0.038, 0.044 and 0.043, respectively); effects explained by the strong influence of the toasting processes on the tannic composition of oak wood, decreasing its concentration during this process in all instances *(33,34)*. The quality and quantity of phenolic compounds in oak wood are closely related to toasting intensity and conditions *(34,35)*; since vine-shoots

have a similar composition and lignocellulosic structure *(20,21)*, a similar evolution during the toasting process could be expected.

3.4. Comparison of Wines Aged with Oak Chips or Vine-Shoots

When wines aged with oak chips or vine-shoots for 9 months were compared by PCA biplots, olfactory and gustative differences were found. **Figure 4** illustrates the PCA biplots obtained with the correlation matrix generated from the mean rating of each experimental condition across the sensory attributes. The two bidimensional PCAs, which correspond to olfactory and gustative attributes, explained 65.7% of the total variability. In the PCA corresponding to the olfactory attributes, wines aged with oak chips were separated from wines aged with vine-shoots along PC1, except for the wine aged with American Blend oak chips for 6 months, which was more similar to wines aged with oak chips. A similar situation was found in the gustative PCA biplot, but in this case all wines aged with American Blend oak chips (for 6 and 9 months) were grouped with wines aged with vine shoots. An additional discrimination source was observed in the olfactory PCA plot for wines aged with vine-shoots since separation along the olfactory PCA plot for wines aged with vine-shoots since separation along PC2 was observed depending on the raw material was toasted or not.

The biplot allows deducing the attributes responsible for the observed discrimination. Thus, in the case of the olfactory profile (**Figure 4**), red fruit, odor intensity and fresh attributes contributed to separate wines aged with vine shoots, while the resting attributes (leather, spice, coffee, tobacco, liquorize and prune) supported the separation of wines aged with oak chips. Similarly, gustative attributes such as body, astringency, stem and bitterness were responsible for separation of wines aged with vine-shoots, while acidity, red fruit, sweet spice, taste intensity and aftertaste quality and intensity emphasized the separation of wines aged with oak chips.

The discriminating significance of these attributes was statistically assessed by an ANOVA test. Thus, the main differences in the olfactory profile

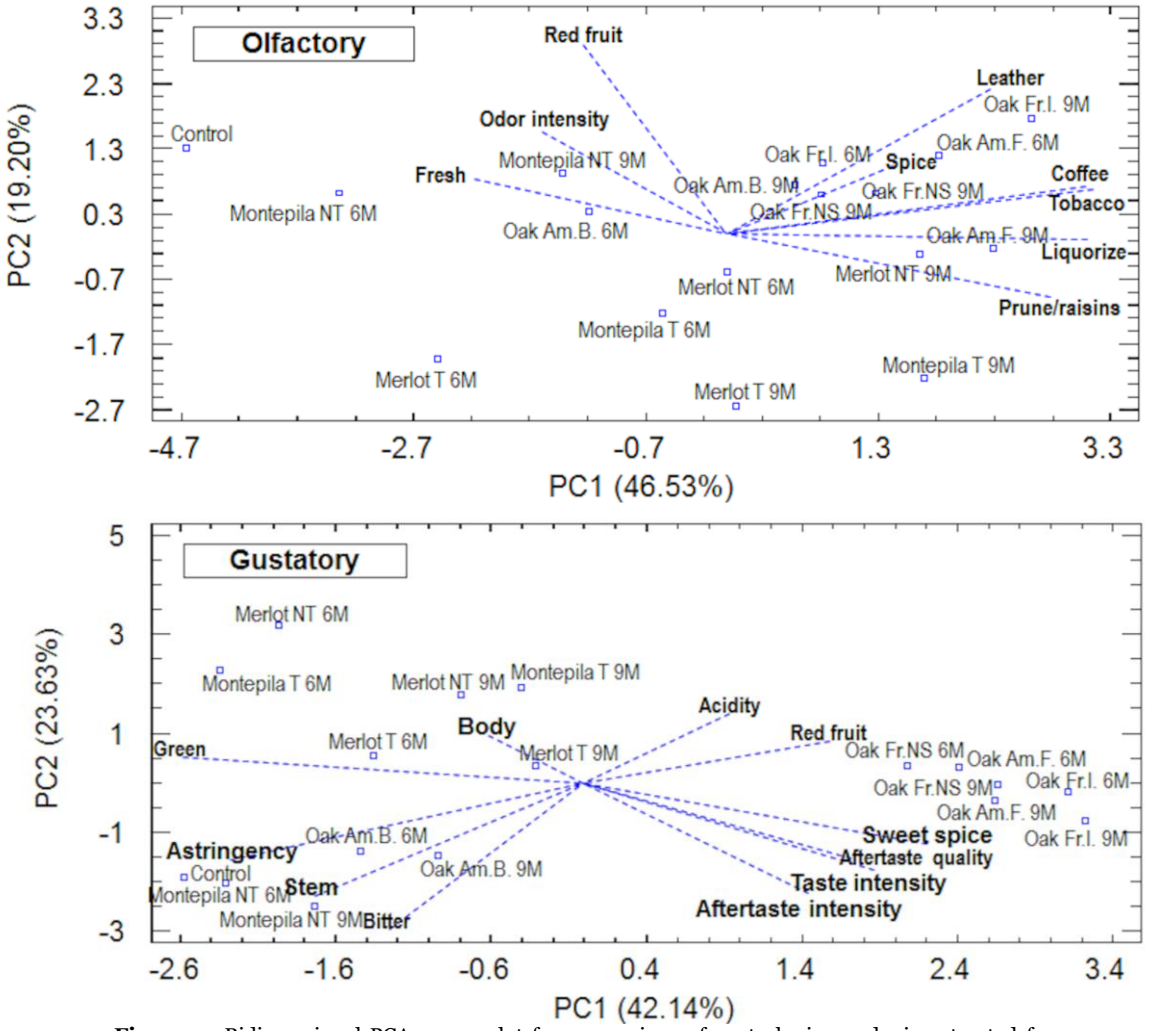


Figure 4. Bidimensional PCA scores plot for comparison of control wine and wines treated for 9 months with datasets corresponding to olfactory and gustatory attributes.

were found in the tobacco and spice characters (*p*-values 0.043 and 0.041, respectively) that were more pronounced in the case of wines treated with oak chips. Concerning to gustative attributes, the sweet spice character was more intense in wines treated with oak chips (*p*-value 0.009), while the acidity and green attributes were more perceptible in wines treated with vine-shoots (*p*-values 0.039 and 0.007, respectively).

Conlusions

Based on the results, it can be concluded that there are clear changes in wine by ageing in the presence of vine-shoots or oak chips, being the former more acid and astringent, and with more evident liquorize and prune/raisins attributes than control wines, while wines aged in the presence of oak chips were characterized by a more perceptible tobacco, coffee and sweet spice notes. Also, differences depended on the variety of ageing agent used and thermal treatment applied. This study shows, for the first time, that vine-shoots can be a cheap alternative to traditional ageing methods that should result in valorization of this raw material.

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Supplementary material

Supplementary Table 1. Standard references presented during the training periods for aroma, flavor-by-mouth and mouth-feel sensations.

Descriptor	Odour reference	Quantity
Aroma		
Red fruit	One piece fresh each of raspberry, blackberry and strawberry jam (Hero)	¶
Tobacco	Fresh tobacco for pipe	10 g†
Fresh	Herb extract (Firmenich)	20 mL†
Prune/raisin	Prune	One unit†
Liquorize	Liquorize stick	One unit (2cm X 1cm) †
Leather	Standard Sentosphère	§
Coffee	Coffee aroma, Vahiné	1 mL†
Spice	Black pepper (Amora)	Two small round grains
Flavour-by-mouth		
Red fruit	One piece fresh each of raspberry, blackberry and strawberry jam (Hero)	One piece¶
Sweet spice	Mixture of commercial clove, cinnamon and vanilla in equivalent proportion	Base wine sprinked with sweet spice mixture ††
Green	Herb extract (Firmenich)	20 mL ††
Stem	Macerated stems of red grape in hydroalcoholic solution 12% (v/v) 5g/L ethanol	10g/L in synthetic wine
Mouth-feel sensations		
Body	Full versus light-bodied wine	¶
Astringency	Tannic acid	1.2 g/L added to base wine ††
Acidity	Tartaric acid	§§
Bitterness	Caffeine	¶¶

Note: Descriptor, reference (chemical or natural) and employed quantities. †Contained in a 125-mL glass amber flask. (i) Solution Firmenich: concentration is expressed in relation to the concentration of the commercial solution. The solvent employed was mineral oil from Sigma (St. Louis, Pennsylvania, USA). (ii) Natural product: diluted with purified water (Milli-Q, Millipore, Massachusetts, USA). §Standard Sentosphère directly placed in a glass amber flask of 125 mL. ¶Added to 100 mL of red wine. ††Base wine: Cencibel Corcovo 2009. §§Synthetic wine (12% v/v ethanol; 5 g/L tartaric acid). ¶¶Full-bodied, high-alcohol wine: Finca La Estacada 12 months, 2008; light-bodied, low-alcohol wine: Lando tinto 2008.

Chapter 7:

Ageing red wines by oak chips or vine–shoots: a comparison of gas chromatography–mass spectrometry profiles

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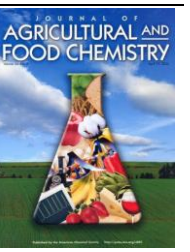

Ageing red wines by oak chips or vine–shoots: a comparison of gas chromatography–mass spectrometry

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Ageing red wines by oak chips or vine-shoots: a comparison of gas chromatography–mass spectrometry profiles

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*and M. D. Luque de Castro**

Abstract

Changes in the composition of red wine promoted by contact with either vine-shoots or oak chips have been studied by gas chromatography–mass spectrometry (GC–MS). The wine was aged in the presence of each of the materials for 3 or 9 months, and the chromatographic profiles of the different samples were compared among them and with control wine which was not in contact with any of the ageing agents. This strategy allowed obtaining a representative profile of compounds present in each sample. In general, at short ageing times both materials affected similarly the composition of wine by increasing the levels of compounds responsible for floral, sweet and smoky attributes with respect to control wines. After 9 months ageing, wines in the presence of oak chips were statistically more concentrated in compounds responsible for herbaceous notes, floral aroma, green, smoky and sweet taste than wines aged in the presence of vine-shoots and control wines. Finally, identification of the compounds representative of the differences was carried out; thus showing vine-shoots as a feasible alternative or complement to traditional

ageing methods. This is the first time that wines aged in the presence of vine-shoots are characterized.

1. Introduction

Oak barrels are traditionally used for ageing of wine and other beverages thanks to the properties this wood transfer to wine. Among others, wood contact enhances the organoleptic properties of wine and actively contributes to improve the complexity, stability and astringency of wine, giving a characteristic bouquet to aged wines. This change occurs partly by transference of lignin degradation compounds, such as low-molecular mass alcohols, aldehydes, ketones, acids and furanic compounds formed during thermal treatment of oak wood. This treatment has proven to be a key factor on the quantity of compounds transferred to wines *(1–3)*; thus, wines with higher nutty aromas and earthy flavors are linked to high toasting degrees, while smoky, woody, vegetative and spicy notes are traditionally related to wines aged in contact with medium toasted wood *(4,5)*. Indeed, it has been proved that high toasting temperatures (heavy toasting, between 220 and 230 ºC) decrease the levels of furans and volatile phenols *(6)*, while medium toasting temperatures provide an increase in the level of compounds generated by pyrolysis of polysaccharides (furanic aldehydes) or Maillard reaction (furanones and pyranones), and those formed by degradation of lignin (phenolic aldehydes and volatile phenols) *(7)*. The use of oak chips during the ageing process, either combined with oak barrels to maximize the transfer of interesting compounds and reduce the ageing time, or added in stainless steel tanks to simulate the barrel ageing process is a common practice at present *(8–11)*. The wood chips added to the wine are residues from staves used to construct barrels. Chips are dried similarly to staves, milled to the desired particle size and roasted in a rotary drum similarly to coffee or cocoa *(12)*. There is also a current trend towards the use of other different wood sources (*e.g.* such as acacia, chestnut, cherry or mulberry) for the ageing process *(13,14);* or to

involve oak species different to the traditional American oak (*Quercus Alba*) and French varieties (*Q. Robur* and *Q. Petraea*); for example Spanish oak varieties (*Q. Pyrenaica*, and *Q. Faginea*) *(15),* and other European oaks (Hungarian, Russian, German and Polish) *(16)*.

Vine-shoots are a very abundant residue in wine-producing countries, such as Spain, France or Italy. These residues, of few or null utility and traditionally burned or cast upon the ground to rot, have a high structural similarity to oak wood, with a 68% holocellulose content and about 20% of lignin (dry weight). The phenolic profile of vine-shoots and oak chips has been studied using extracts obtained by superheated liquids and analyzed by liquid chromatography with diode array or time-of-flight mass detectors (LC–DAD, LC–TOF/MS) *(17,18)*. Moreover, the volatile fraction of extracts from raw vine-shoots and oak chips has been characterized and compared using gas chromatography with mass detection with or without a headspace device (HS–GC–MS, GC–MS) *(7,19)*. In view of these results, and given the similar composition in both families of compounds present in vine-shoots and oak wood from the oenological point of view, the feasibility of vine-shoots as a potential alternative or complement to oak wood for wine ageing is considered a matter of study. Therefore, the aim of the present study was to characterize the differences existing in the composition of red wines aged in the presence of oak chips or vine-shoots. Two varieties of vine-shoots and four varieties of oak chips (French and American oak chips) were used for ageing wine for periods of 3 and 9 months. GC–MS analysis of the different wines enabled to identify the compounds that presented significant differences in the concentration profile of wines. This is the first time that the profile of wines aged in the presence of vine-shoots or oak chips under the same conditions is compared.

2. Material and methods

2.1. Vine-Shoots and Oak Chips

Vine-shoots from *Vitis vinifera* cultivars were sampled from the same plantation located at "Bodega y Viñedos Vallebravo" (Sierra de Segura, Jaén, Spain) in 2011, after leaf-fall, and by randomized selection of ten similar stocks of each cultivar. Two different vine cultivars were selected for this study, a red grape cultivar (Merlot) and a white one (Montepila), that were chosen due to the high similarity to oak chips in terms of phenolic and volatile composition *(7, 17-19)*. The selected vines were cultivated under the same agronomic conditions in terms of soil properties, climate, hydric regime, etc. Several pieces of 10 cm of vine-shoots from each cultivar were cut at the height of the first leaf bud. The vine-shoot samples were milled to obtain a homogeneous particle size (less than 0.42 mm diameter) and dried for 72 h at 30 ºC. To evaluate the incidence of toasted vine-shoots on aged wines, some experiments were planned by toasting them for 20 min in an oven at 180 ºC.

Four types of commercial oak chips representative of different types of oak barrels used for wine ageing were kindly supplied by Laffort España S.A. (Guipúzcoa, Spain). The types of oak chips were two French varieties (Intense and Nobile Spice corresponding to toasted and non-toasted chips, respectively) and two American varieties (Blend and Fresh corresponding to toasted and non-toasted chips, respectively). As oak chips are commercialized as a dried product, they were milled and stored following the same protocol as for vine-shoots.

2.2. Wine Ageing

The wine was made in Sierra de Segura (Jaén) with grape cultivars of Syrah, Merlot and Mazuelo in equivalent quantities. The grapes were harvested at their optimal ripening stage (23±1 ºBrix) and good sanitary conditions. A cold prefermentative maceration was made, followed by alcoholic and malolactic

fermentation at temperature below 22 ºC. Fermentation was made with the indigenous yeasts and the total SO2 concentration was under 60 mg/L. After this process, the obtained wine was used as control without addition of any oak chips or vine-shoots. Two bottles of 750 mL of the control wine were stored at –20 ºC until sensory analysis. Two bottles of the original control wine were also aged for 3 and 9 months to study the influence of the addition of oak chips or vine-shoots on the ageing process. The scheme of the experimental plan is exposed in **Figure 1**. Wines were aged with addition, after malolactic fermentation, of either oak chips or vine-shoots at a dose rate of 4 g/L under warehouse conditions in Pago Casa del Blanco (Manzanares, Ciudad Real). Wine ageing was performed in individual bottles of 750 mL (six bottles for each experiment) involving oak chips or vine-shoots, toasted or non-toasted, for 3 and 9 months). The total number of bottles used in this study was 54 (6 bottles for the control wine, 16 for wine ageing with vine-shoots and 32 for wine ageing with oak chips). The amount of chips used in this study was selected according to recommendations provided by the manufacturer.

2.3. Standard Chemical Analyses of Wines

Total acidity, ethanol, pH, volatile acidity, total and free SO2 were determined according to the methods proposed by the O.I.V. (International Organization of vine and wine) (2012). All the parameters met the expected standards for this type of wine, and total SO2 was below 60 mg/L.

2.4. Apparatus and Instruments

Vine shoots and oak chips were grinded and sieved by using a 40-mesh sieve (particle size less than 0.42 mm diameter). Toasting of vine shoots was performed for 20 min in an oven at 180 ºC.

Individual separation of compounds was carried out by a Varian CP 3800 gas chromatograph (Walnut Creek, CA, USA) equipped with a programmable

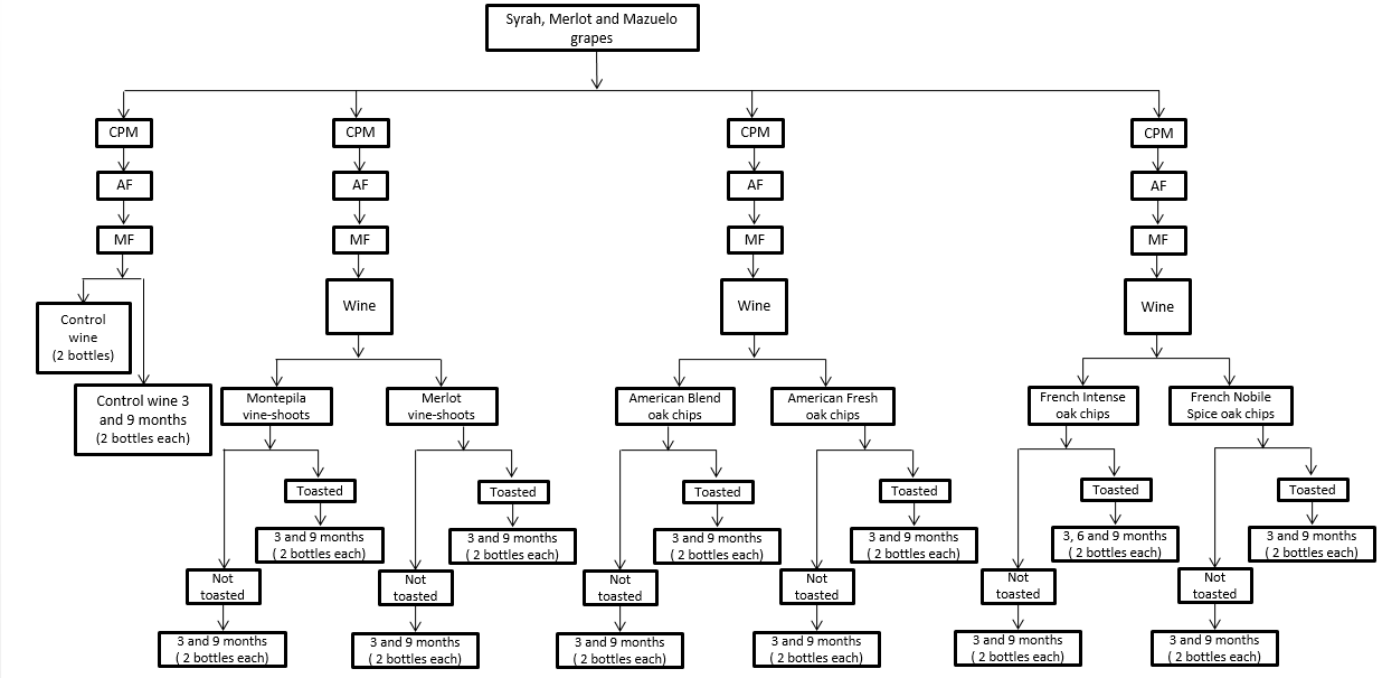

Figure 1. Diagram of the experimental plan followed in this study and identification of winemaking treatments. (CPM: cold prefermentative maceration; AF: alcoholic fermentation; MF: malolactic fermentation)

temperature injector and coupled to a Saturn 2200 ion-trap mass spectrometer (Sunny valley, TX, USA). A Factor Four VF-5ms fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness, Varian) completed the experimental approach.

2.5. Reagents

Anhydrous sodium sulfate and LC-grade dichloromethane (supplied by Scharlab, Barcelona, Spain) were used for liquid–liquid extraction. 1-Octanol (internal standard) and a standard mixture of *n*-alkanes (C_{10}–C_{40}) for the performance test of GC systems were purchased from Sigma–Aldrich (St. Louis, USA).

2.6. Sample Preparation

After confirmation by GC–MS analysis that 1-octanol was not present in the studied wines, this was used as internal standard at 25 μg/mL in 2-mL aliquots of each wine Aditionally 0.4 mL of dichloromethane was added for liquid-liquid extraction. After 10 min stirring in a vortex, the extracts were subjected to 8 min centrifugation at 850 × *g*. Finally, the organic fraction was isolated and water traces were removed by addition of 0.2 g of anhydrous sodium sulfate. The resulting analytical sample was filtered using a 0.20 μm pore-size filter before chromatographic analysis.

2.7. GC–MS Analysis

One μL of the analytical sample was injected into the chromatograph in the splitless mode. The injector temperature was fixed at 270 ºC. The oven temperature program was as follows: initial temperature 40 ºC (held for 3 min), increased at 7 ºC/min to 230 ºC (held for 3 min), and, finally, increased at 6 ºC/min to 250 ºC (held for 10 min). The total analysis time was 47 min, and 5 min extra time was necessary for re-establishing and equilibrating the initial conditions. The ion-trap mass spectrometer was operated in the electron impact

ionization (EI) fixed mode, for which the instrumental parameters were set as follows: filament emission current 70 μA; transfer-line, ion trap and manifold temperatures were kept at 280, 200 and 60 ºC, respectively. The recording window was set between 40 *m/z* and 350 *m/z* and the data were acquired using total ion current (TIC) scan mode. Digital selected ion monitoring (SIM) was applied as data treatment to remove the chromatographic background. Additionally, chromatograms were smoothed by application of a Savitzky-Golay filter (5 points).

2.8. Data Treatment

All statistical analyses were performed using the Mass Profiler Professional (MPP) software package (Version 12.0, Agilent Technologies, Santa Clara, CA, USA). MassHunter GC/MS translator (version B.07.00, Agilent Technologies, Santa Clara, CA, USA) was used to transform .sms data files into generic .d files that were exported to the Unknown Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) to process all data obtained by GC–MS. Treatment of raw data files started by deconvolution of potential molecular features (MFs) with the suited algorithm included in the software. For this purpose, the deconvolution algorithm considered all ions exceeding 4000 counts for the absolute area parameter. Additionally, the accuracy error and the window size factor were set at 50 ppm and 100 units, respectively. After extraction of MFs, data files in compound exchange format (.cef files) were created for each sample and exported into the MPP software package for further processing.

In the next step, the data were processed by alignment of the potential MFs according to their retention time and *m/z* value using a tolerance window of 0.3 min and an accuracy error of 15 ppm. In the last step, the MFs resulting after data pretreatment were exported (.cef file) for recursive analysis. For this purpose, the Quantitative Analysis software (version 7.0, Agilent Technologies, Santa Clara, CA, USA) was used to reintegrate all potential compounds found in all analyzed samples. The resulting table was exported in coma separated value format (.csv file) and reprocessed with the Mass Profiler Professional (MPP)

software package, where the 105 resulting molecular entities were realigned and statistical analyses were performed. Data pretreatment was based on baselining to the mean of all samples for removal of background noise and normalizing by logarithmic transformation to reduce relatively large differences among the respective MF abundances. The extraction algorithm confirmed the efficiency of this filtering step. This correction was applied to all the tested samples. The data set was evaluated by ANOVA to discriminate among the means of acquired data.

As **Figure 2** shows, the chromatographic profile obtained for an aged wine sample concentrates most of the signals on the first part of the chromatogram; that is, small retention indexes, and characteristic of low molecular mass compounds, which is supported on the absence of a derivatization step. The NIST Mass Spectral Search Program 2008 v.2.0 (NIST, Washington, DC, USA) was used for the spectral search (Mainlib and Replib libraries). Tentative identification was supported on correlation between experimental mass and database spectra above 700 in the normal search mode. A series of n-alkanes (C_{10}–C_{40}) was analyzed to establish the experimental retention

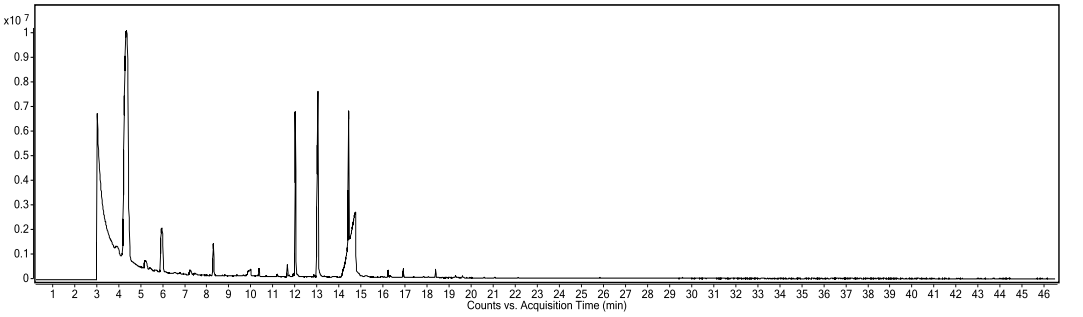

Figure 2. Chromatogram from a wine aged for six months in the presence of not toasted vine-shoots from Merlot variety.

index (RI) for each tentative compound, calculated on the basis of retention times, which were compared with experimental or theoretical values included in the NIST database (Ri), with the same or an equivalent chromatographic column. The cut-off limit for *I* difference was set at 100 units for theoretical references, while 30 units was the value used for experimental references.

3. Results and Discussion

3.1. Short-Term Changes Ocurring in Wines Aged for Three Months

Traditional wine ageing is an expensive and time-consuming process owing to the long duration of traditional methods. For this reason, the addition of oak chips to barrels arose as a technique to accelerate the ageing process and making it more convenient and economical than the traditional ageing. In this study, two types of materials, oak chips and vine-shoots, were added to wine to evaluate their effect on the final composition of aged wines. The influence of the added material on the composition of wine was assessed by ANOVA at a 95% confidence level with Bonferroni Holm FWER (Familywise Error Rate) correction by comparing wines aged with each material and control wine. The comparison was carried out at the two sampling stages as an attempt to identify the main changes occurring at 3 and 9 months.

At the first ageing stage corresponding to three months, changes affecting to 11 and 13 compounds were observed in wines aged with oak chips and vine-shoots, respectively. None of these compounds resulted statistically significant in comparison between wines aged for 3 months without addition of any material and control wine. Therefore, these compositional changes should be attributed to the ageing process using oak chips and/or vine-shoots. These compounds are listed in **Table 1** according to their chemical taxonomy. Among them, it is worth emphasizing the significant presence of ethyl esters of fatty acids in wines aged with oak chips or vine-shoots. These compounds, produced during the alcoholic fermentation, play a positive role in the generation of quality aroma in wines with fruity and floral notes *(21,22)*. Examples of this behavior are acetic acid methoxy ethyl ester, benzoic acid 4-ethoxyethyl ester and butanedioic acid monoethyl ester, which were found to be statistically lower in control wines than in wines aged in the presence of oak chips or vine-shoots (p-value <0.001 in all cases). **Supplementary Figure 1** shows the extracted ion chromatograms (EICs) provided by analysis of control wine, wine aged for 3 months without any

material and wines aged in presence of oak chips and vine-shoots for the same period of time. The same behavior was also found for a phenolic compound such as 5-hydroxyvanillin (vanilla aroma) and two branched fatty alcohols such as 3,7-dimethyl-1-octanol (tetrahydrogeraniol, known for its fresh odor and floral taste) and 4-methyl-1-heptanol, that is a branched chain primary alcohol found in wine and produced during fermentation (*p*-value <0.001 in all cases). EIC chromatograms for these three compounds are illustrated in **Supplementary Figure 2**.

The opposite situation was found in the case of pentanedioic acid diethyl ester, which was statistically present at higher level in control wine than in aged wines (*p*-value <0.001). Similarly, homosyringic acid (*p*-value 0.022), a product of wood hydrothermal degradation that contributes to wine aroma with woody and sweet notes, was also found at lower level in wines aged for 3 months with oak chips and vine-shoots as compared to control wine *(17)*.

When wines aged in the presence of oak chips and vine-shoots were compared among them, most of the significant compounds were found at statistically higher level in wines aged in the presence of oak chips. Examples of these compounds are syringol, a product of lignin degradation highly appreciated from the oenological point of view thanks to the smoky flavoring it transfers to wine (*p*-value 0.007); tyrosol, a phenolic compound also present in olive oil and beer and endowed with several health-enhancing properties, such as free radical scavenging, antioxidant, anticarcinogenic, antimicrobial and cardio-preventive *(23)* (*p*-value <0.001); 3-penten-1-ol, a linear alcohol to which green attributes of wines have been assigned (*p*-value 0.012); butanoic acid ethyl ester, that contributes to wine aroma, with sweet, fruity odor and hints of banana and pineapple *(24,25)* (*p*-value <0.001); 2-decanynoic acid, which has been previously identified as part of the volatile fraction of honey *(26)* (*p*-value 0.001); 2,6-dimethoxybenzoquinone, a product of lignin oxidation (*p*-value 0.032); 2,3,4-trimethylpentane, that according to the Yeast Metabolome Data Base (YMDB) (www.ymdb.ca), is a branched-chain alkane found in wine and produced

Table 1. Identification of representative compounds detected in the volatile fraction of wines aged for 3 months.

Compound	Characteristic fragments	Formula	Rt	RI (Ri)	Wines aged for 3 months (*p*-value)		
					Control *vs.* oak chips	Control *vs.* vine-shoots	Oak chips *vs.* vine-shoots
2,3,4-Trimethylpentane	71, 55, 43	C_8H_{18}	3.77	623 (624)	-	-	*
2,3-Octanedione	71, 55, 43	$C_8H_{14}O_2$	4.32	588 (512)	*	***	-
3,3-Dimethyl-2-pentanol	70, 55, 43	$C_7H_{16}O$	4.34	654 (795)	-	-	***
Butanoic acid, ethyl ester	88, 71, 60	$C_6H_{12}O_2$	5.77	730 (785)	-	***	-
3-Methyl-hexanoic acid	87, 60, 43	$C_7H_{14}O_2$	6.19	762 (753)	-	-	*
3-Penten-1-ol	68, 56, 55	$C_5H_{10}O$	6.91	792 (769)	**	-	*
β-Methyl-γ-butyro-lactone	56, 42, 41	$C_5H_8O_2$	8.38	871 (886)	***	***	***
Acetic acid, methoxy ethyl ester	88, 70, 45	$C_5H_{10}O_3$	8.66	885 (836)	***	***	-
Pentanoic acid (Valeric acid)	73, 60, 41	$C_5H_{10}O_2$	9.96	955 (938)	-	-	***
4-Methyl-1-heptanol	69, 55, 41	$C_8H_{18}O$	11.61	1044 (995)	***	***	-
3,7-Dimethyl-1-octanol (Tetrahydrogenariol)	70, 55, 41	$C_{10}H_{22}O$	11.69	1048 (1130)	***	***	-
Butanedioic acid, monoethyl ester	128, 101, 73	$C_6H_{10}O_4$	14.30	1168 (1079)	***	***	-
Butanedioic acid, diethyl ester	129, 101, 55	$C_8H_{14}O_4$	14.84	1217 (1151)	-	***	***
Pentanedioic acid, diethyl ester	143, 114, 87	$C_9H_{16}O_4$	16.41	1300 (1282)	***	***	-
Syringol	154, 139, 111	$C_8H_{10}O_3$	17.62	1386 (1279)	-	-	**
Tyrosol	107, 77, 51	$C_8H_{10}O_2$	19.24	1452 (1356)	***	***	***
2-Decanynoic acid	67, 55, 43	$C_{10}H_{16}O_2$	20.76	1470 (1389)	-	-	**
Benzoic acid, 4-ethoxyethyl ester	194, 149, 121	$C_{11}H_{14}O_3$	21.04	1537 (1549)	***	***	-
2,6-Dimethoxybenzoquinone	168, 69, 53	$C_8H_8O_4$	21.57	1577 (1485)	-	-	*
3,4-Dihydroxy-5-methoxybenzaldehyde (5-Hydroxyvanillin)	168, 125, 97	$C_8H_8O_4$	26.54	1615 (1612)	***	***	-
Homosyringic acid	212, 167, 123	$C_{10}H_{12}O_5$	26.62	1920 (1848)	-	**	-

*Note: *(0.05-0.01); **(0.01-0.001); ***(0.001-0)*

by vinification yeasts (*p*-value 0.046). Other two compounds with a similar behaviour were pentanoic acid (*p*-value <0.001) and 3,3-dimethyl-2-pentanol (*p*-value <0.001). Pentanoic acid together with hexanoic acid are known for their cheese, fatty and rancid contribution to wine flavor; however, according to Jiang *et al.* these fatty acids impart mild and pleasant aroma to wine at concentrations from 4 to 10 mg/L; exerting a negative impact only at levels higher than 20 mg/L *(27)*.

3.2. Differences in Wines Aged with Oak Chips and Vine-Shoots for Nine Months

The same strategy was applied to wines aged for nine months in the presence of either oak chips or vine-shoots, which were compared among them and with control wine by statistical analysis. **Table 2** lists the identified compounds that resulted statistically significant in this comparison. In the particular case of wines aged with oak chips, 7 compounds were statistically significant with a 95% confidence level. The most significant differences were related to three phenolic compounds such as guaiacol, homosyringic acid and 2-hydroxybenzoic acid butyl ester as well as two short chain fatty acid ethyl esters such as butanoic acid 3-hydroxyethyl ester (isobutyl salicylate) and acetic acid methoxy ethyl ester, a short chain alcohol as 3-penten-1-ol and, finally, a lactone derivative such as β-methyl-γ-butyro-lactone. Some of these compounds are well-known descriptors of wine aroma such as the linear alcohol 3-penten-1-ol that has been assigned to green taste of red wines (*p*-value 0.028). Guaiacol and homosyringic acid are interesting compounds from the oenological point of view derived from lignin degradation, which are representative of smoke and sweet characters *(17-19)* (*p*-value <0.001 in both cases), and isobutyl salicylate, that is known for its floral aroma (*p*-value <0.001). On the other hand, β-methyl-γ-butyro-lactone has been reported to be present in wines, contributing with an aromatic, faint sweet character to aroma properties. Nevertheless, Ribéreau-Gayon *et al.* have pointed out the presence of high levels of this lactone in red wines (about 1 mg/L) that they did not consider an important flavor component

due to its high detection threshold *(25)*. In all cases, the significant compounds were characterized by the same trend, the concentration of them was significantly higher in wines aged in the presence of oak chips as compared to control wines.

When control wines were compared to wines aged in contact to vine-shoots, 11 compounds were found at statistically different concentrations in control wine and wine aged with added vine-shoots as **Table 2** shows. Among them, it is worth mentioning compounds of interest such as tetrahydrogeraniol, appreciated by its sweet, rosy odour and bitter taste (*p*-value 0) *(28)*. 4-methyl-1-heptanol that, as previously commented, is a primary alcohol produced during fermentation (*p*-value 0), hexanoic acid (*p*-value 0.006) and β-methyl-γ-butyro-lactone (*p*-value 0). Other interesting compounds that were also marked by a significant increase in wines treated with vine-shoots as compared to control wine where butanoic acid ethyl ester with sweet, fruity odor (*p*-value 0.007) *(24,25)* butanedioic acid diethyl ester and butanedioic acid monoethyl ester which, according to the Yeast Metabolome Data Base (YMDB), is one of the major volatiles found in wine and is produced during wine fermentation by the reaction of a single ethanol unit with succinic acid (*p*-values 0.004 and 0, respectively).

Finally, wines aged in the presence of oak chips or vine-shoots were compared among them. A total number of 21 compounds statistically significant were identified, as **Table 2** shows. Most of the significant compounds were more concentrated in wines aged with oak chips than in those aged with vine-shoots. As can be seen, most of them are highly appreciated in the wine industry. Among them, 4,5-dimethyl-1-hexene has been recognized by Pérez-Olivero *et al.* as a minoritary compound of wine, but appreciated by its contribution to wine flavor with floral aroma (*p*-value 0.008).29 Other aroma compounds were 1-hexanol, which is generally linked to the green taste of wines (*p*-value 0.003)30 and esters such as propanoic acid 2-[(tetrahydro-2H-pyran-2-yl)oxy]ethyl ester and pentanedioic acid diethyl ester (*p*-values 0.023 and 0.005 respectively). Phenolic compounds such as syringol (smoky taste) and alcohols such as 1,8-nonadien-1-ol

Table 2. Identification of representative compounds detected in the volatile fraction of wines aged for 9 months.

Compound	Characteristic fragments	Formula	Rt	RI (Ri)	Wines aged for 9 months (*p*-value)		
					Control *vs.* oak chips	Control *vs.* vine-shoots	Oak chips *vs.* vine-shoots
Ethylenediamine	60, 56, 54	$C_2H_8N_2$	3.23	594 (611)	-	-	**
3,3-Dimethyl-2-pentanol	70, 55, 43	$C_7H_{16}O$	4.34	654 (795)	-	-	*
2,3-Dimethyl-1-butanol	84, 71, 43	$C_6H_{14}O$	4.38	655 (732)	-	-	**
4,5-Dimethyl-1-hexene	71, 55, 43	C8H16	4.50	662 (747)	-	-	**
2-Methyl-1-pentanol	71, 55, 43	C6H14O	4.96	687 (796)	-	-	***
Butanoic acid, ethyl ester	88, 71, 60	$C_6H_{12}O_2$	5.77	730 (785)	-	**	-
2-Methylbutanoic acid	87, 74, 57	$C_5H_{10}O_2$	6.88	789 (811)	-	-	**
3-Penten-1-ol	68, 56, 55	$C_5H_{10}O$	6.91	792 (769)	*	-	-
1-Hexanol	69, 56, 43	$C_6H_{14}O$	7.15	805 (841)	-	-	**
Butanoic acid, 3-hydroxyethyl ester	117, 71, 43	$C_6H_{12}O_3$	8.91	898 (947)	*	-	***
β-Methyl-γ-butyro-lactone	56, 42, 41	$C_5H_8O_2$	8.38	871 (886)	***	***	**
Acetic acid, methoxy ethyl ester	88, 70, 45	$C_5H_{10}O_3$	8.66	885 (836)	**	-	***
Hexanoic acid	87, 73, 60	$C_6H_{12}O_2$	10.01	958 (973)	-	**	**
1,8-Nonadien-3-ol	84, 57, 49	$C_9H_{16}O$	11.46	1035 (1058)	-	-	*
4-Methyl-1-heptanol	69, 55, 41	$C_8H_{18}O$	11.61	1044 (995)	-	***	***
3,7-Dimethyl-1-octanol (Tetrahydrogenariol)	70, 55, 41	$C_{10}H_{22}O$	11.69	1048 (1130)	-	***	***
Guaiacol	124, 109, 81	$C_7H_8O_2$	12.40	1085 (1090)	***	***	-
Butanedioic acid, monoethyl ester	128, 101, 73	$C_6H_{10}O_4$	14.30	1168 (1079)	-	***	***
Butanedioic acid, diethyl ester	129, 101, 55	$C_8H_{14}O_4$	14.84	1217 (1151)	-	***	**
2-(Hexyloxy)heptane	85, 57, 43	$C_{13}H_{28}O$	15.85	1272 (1325)	-	-	**
Pentanedioic acid, diethyl ester	143, 114, 87	$C_9H_{16}O_4$	16.41	1300 (1282)	-	-	**
Syringol	154, 139, 111	$C_8H_{10}O_3$	17.62	1386 (1279)	-	-	***
Propanoic acid, 2-[(tetrahydro-2H-pyran-2-yl)oxy], ethyl ester	101, 85, 41	$C_{10}H_{18}O_4$	18.49	1412 (1346)	-	-	*
Tyrosol	107, 77, 51	$C_8H_{10}O_2$	19.24	1452 (1356)	-	*	**
2-Hydroxybenzoic acid, butyl ester (Isobutyl salicylate)	138, 120, 92	$C_{11}H_{14}O_3$	23.41	1676 (1579)	***	***	-
Homosyringic acid	212, 167, 123	$C_{10}H_{12}O_5$	26.62	1920 (1848)	***	***	-

*Note: *(0.05-0.01); **(0.01-0.001); ***(0.001-0)*

(fruity), 2-methyl-1-pentanol, 3,3-dimethyl-2-pentanol (herbaceous) and 2,3-dimethyl-1-butanol (herbaceous scent) (*p*-values 0, 0.046, 0 and 0.014 and 0.001, respectively) were significantly at higher concentration in wine aged with oak chips. Other compounds such as 2-methylbutanoic acid, ethylenediamine and 2-(hexyloxy)heptane (*p*-values 0.023, 0 and 0.007, respectively) were also in the list of significant compounds.

Only four identified compounds were found to be significantively more concentrated in the case of wines aged in the presence of vine-shoots, namely: 4-methyl-1-heptanol, tetrahydrogeraniol, butanedioic acid diethyl ester and butanedioic acid monoethyl ester (*p*-value <0.001 in all cases). Interestingly, guaiacol, a very appreciated compound on the oenological field (smoke, sweet character) *(17-19)*, was less concentrated in control wines when they were compared with treated wines (*p*-value <0.001 in all cases). However, no statistical differences were found when treated wines were compared among them, which means that both materials were suitable to transfer similar quantities of guaiacol to wine.

3.3. Influence of the Toasting Degree on Wine Ageing

Wood toasting is a key treatment applied to oak wood during barrel manufacture. During this process, oak wood suffers degradation at some extent, liberating compounds that are dissolved into wine during wine ageing, thus contributing to wine sensory characteristics. Toasted and no toasted chips by one hand, and toasted and no toasted vine-shoots by the other were compared by applying the ANOVA strategy (95% confidence level, unpaired *t*-test and Bonferroni Holm FWER *p*-value correction).

In the case of oak chips, only five compounds resulted statistically significant, as shows **Table 3**. In all cases, the representative compounds were found to be more concentrated in wines aged in the presence of toasted oak chips, a logical behavior since thermal treatment enhances the release of multitude of compounds. The most representative of these compounds are 3-penten-1-ol and

syringol at confidence level of 99% in both cases (*p*-value <0.001 in both cases); desaspidinol, a phenolic compound produced by ligning fractionation and used as a flavoring agent and food additive, was also found to be significantly higher in the case of toasted chips (*p*-value 0.003) *(31)*. Finally, other identified compounds that showed to be relevant were butanoic acid ethyl ester and tyrosol, with *p*-values of 0.038 and 0.003, respectively.

The analysis of wines treated with no toasted or toasted vine-shoots led to identify five significant compounds, most of which can be linked to floral descriptors such as 4,5-dimethyl-1-hexene, 1-hexanol and tetrahydrogeraniol (*p*-values 0.016, 0.020 and 0.050, respectively). Only tetrahydrogeraniol was found to be statistically higher in wines treated with toasted vine-shoots, being 4,5-dimethyl-1-hexene and 1-hexanol at higher concentrations in the case of wines aged in the presence of no toasted vine-shoots.

According to the YMDB, 2,3,4-trimethylpentane is a branched-chain alkane found in wine and produced by vinification yeasts. This compound was found to be statistically higher in the case of wines aged in the presence of non toasted vine-shoots (*p*-value 0.031). The opposite situation was found in the case of β-methyl-γ-butyro-lactone (aromatic, sweet character), that was found significantly higher in the case of wines aged with toasted vine-shoots (*p*-value 0.009).

Conclusions

Based on the above results, it can be concluded that there are clear changes in the composition of wine by ageing in the presence of vine-shoots or oak chips, although these changes were essentially quantitative since wines aged with both materials were qualitatively quite similar. In general, at short ageing times both materials showed to affect the composition of wine in the same extent, with subtle differences, increasing the levels of compounds responsible for floral, sweet and smoky attributes with respect to control wines. However, at nine months ageing an increase in the number of representative compounds was

Table 3. Identification of representative compounds detected in the volatile fraction of wines aged in the presence of not toasted or toasted oak chips and vine-shoots.

Compound	Characteristic fragments	Formula	Rt	RI (Ri)	*p*-Value	
					Not toasted *vs.* toasted oak chips	Not toasted *vs.* toasted vine-shoots
Butanoic acid ethyl ester	88, 71, 60	$C_6H_{12}O_2$	5.77	730 (785)	*	-
3-Penten-1-ol	68, 56, 55	$C_5H_{10}O$	6.91	792 (769)	***	-
Syringol	154, 139, 111	$C_8H_{10}O_3$	17.62	1386 (1279)	***	-
Tyrosol	107, 77, 51	$C_8H_{10}O_2$	19.24	1452 (1356)	**	-
Desaspidinol	210, 195, 167	$C_{11}H_{14}O_4$	24.9	1796 (1858)	**	-
2,3,4-Trimethylpentane	71, 55, 43	C_8H_{18}	3.77	623 (624)	-	*
4,5-Dimethyl-1-hexene	71, 55, 43	C8H16	4.50	662 (747)	-	*
1-Hexanol	69, 56, 43	$C_6H_{14}O$	7.15	805 (841)	-	*
β-Methyl-γ-butyro-lactone	56, 42, 41	$C_5H_8O_2$	8.38	871 (886)	-	**
3,7-Dimethyl-1-octanol (Tetrahydrogenariol)	70, 55, 41	$C_{10}H_{22}O$	11.69	1048 (1130)	-	*

*Note: *(0.05-0.01); **(0.01-0.001); ***(0.001-0)*

observed when treated wines were compared among them, being wines aged in the presence of oak chips statistically more concentrated in compounds responsible for herbaceous notes, floral aroma, green, smoky and sweet taste than wines aged in the presence of vine-shoots. Ageing of wine with vine-shoots can be considered an alternative or a complement to the use of oak chips, with multiple possibilities that depend on the variety of vine-shoot and the thermal treatment applied. This study shows, for the first time, that vine-shoots can be a cheap alternative to traditional ageing methods that would constitute a valorization of this abundant raw material.

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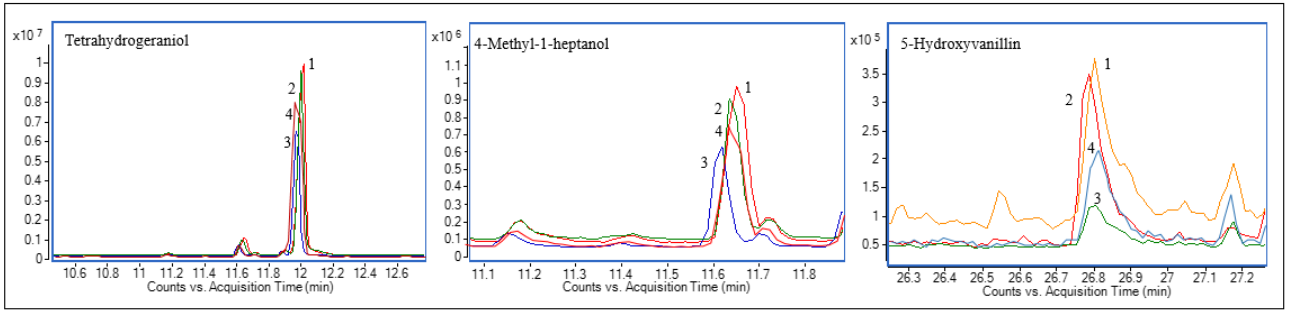
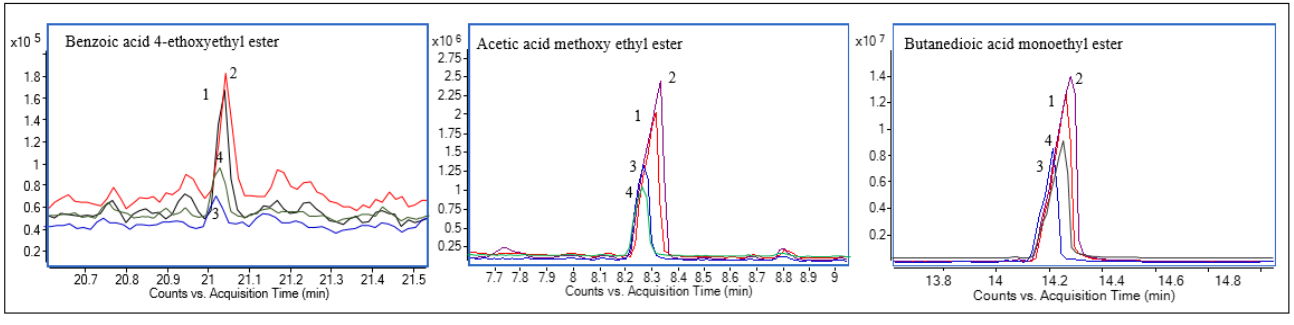
Supplementary Material



Supplementary Figure 1. Extracted ion chromatograms (EICs) obtained by GC–MS analysis of wines aged for 3 months in the presence of; vine-shoots (1), oak chips (2), control wine (3) and control wine at 3 months (4) for tetrahydrogeraniol, 4-methyl-1-heptanol and 5-hydroxyvanillin.



Supplementary Figure 2. Extracted ion chromatograms (EICs) obtained by GC–MS analysis of wines aged for 3 months in the presence of; vine-shoots (1), oak chips (2), control wine (3) and control wine at 3 months (4) for benzoic acid 4-ethoxyethyl ester, acetic acid methoxy ethyl ester and butanedioic acid monoethyl ester.

SECTION III

Characterization of vinification lees and quantitation of target families of compounds

Las lías de vinificación son un residuo muy abundante de la industria vinícola que durante el proceso de envejecimiento del vino retienen algunos compuestos presentes en el mismo, por lo que son susceptibles de ser una fuente de compuestos de interés nutracéutico. La Sección III de esta Memoria se dedica al estudio de este residuo.

El Capítulo 8 abarca el estudio de la fracción polar y de polaridad media de extractos de lías secas, obtenidas de 11 bodegas españolas, para lo que se desarrolló un método de análisis no orientado mediante el uso de un cromatógrafo de líquidos acoplado a un espectrómetro de masas de alta resolución–tiempo de vuelo (LC—QTOF), que permitió la comparación de los perfiles obtenidos y la identificación de una amplia variedad de compuestos. En el Capítulo 9 se recoge una investigación que se planteó como la continuación de la anterior, en la que usando la misma metodología se realizó el análisis e identificación de los compuestos más representativos presentes en lías líquidas, o "vino embebido", y se compararon con los obtenidos de extractos de lías secas.

El Capítulo 10 se centra en el desarrollo de un método cromatográfico para, haciendo uso de la versatilidad del triple cuadrupolo, llevar a cabo el análisis de extractos de lías secas y de vinos embebidos mediante tres modos de adquisición diferentes —ion producto, ion precursor y pérdida de masa neutra— para el análisis confirmatorio de antocianinas, antocianidinas y proantocianidinas sin necesidad del uso de patrones analíticos. Posteriormente, se abordó el análisis cuantitativo mediante la modalidad monitorización de reacciones seleccionadas (SRM, del inglés "Selected Reaction Monitoring")

Vinification lees are a very abundant residue of the wine industry that retain some of the compounds present in wine during the wine ageing process; therefore, this residue is susceptible of being a source of interesting nutraceuticals. Section III of this PhD Book is devoted to the study of wine lees.

Chapter 8 encompasses the study of the polar and mid-polar fraction of extracts from dry lees, obtained from 11 Spanish wineries, by untargeted analysis using a liquid chromatograph coupled to a high resolution mass spectrometer quadrupole–time of flight (LC–QTOF), which allowed comparison of the obtained profiles and identification of a wide variety of compounds. Chapter 9 shows the research planned as a continuation of the previous one. In that, the analysis and identification of the most representative compounds in liquid lees or "imbibed wine" was performed using the same methodology as in Chapter 8. The obtained profiles were further compared with those provided by extracts from dry lees.

Chapter 10 is devoted to the development of a chromatographic method, taking advantage of the versatility of triple quadrupole equipment, to carry out the analysis of extracts of dried lees and imbibed wines by three different adquisition modes —product ion, precursor ion and neutral loss— scanning for confirmatory analysis of anthocyanins, anthocyanidins and proanthocyanidins without the use of analytical standards. Then, quantitative analysis of these compounds was addressed by the selected reaction monitoring (SRM) mode.

Chapter 8:

Tentative identification of polar and mid-polar compounds in extracts from wine lees by liquid chromatography–tandem mass spectrometry in high–resolution mode

WILEY

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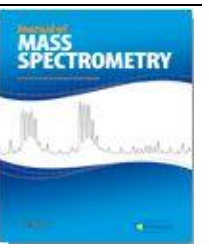

Tentative identification of polar and mid-polar compounds in extracts from wine lees by liquid chromatography–tandem mass spectrometry in high–resolution mode

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Abstract

Sustainable agriculture has a pending goal in the valorization of agrofood residues. Wine lees are an abundant residue in the oenological industry. This residue has so far been used to obtain tartaric acid or pigments, but not for being qualitatively characterized as a source of polar and mid-polar compounds such as flavonoids, phenols and essential amino acids. Lees extracts from eleven Spanish wineries have been analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) in high resolution mode. The high-resolution power of LC–MS/MS has led to the tentative identification of the most representative compounds present in wine lees, comprising primary amino acids, anthocyans, flavanols, flavonols, flavones and non-flavonoid phenolic compounds, among others. Attending to the profile and content of polar and mid-polar compounds in wine lees, this study underlines the potential of wine lees as an exploitable source to isolate interesting compounds.

1. Introduction

Wine lees, also known as dregs, are defined by the 337/79 EEC regulation either as the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, or as the residue obtained following wine filtration or centrifugation. Lees are mainly composed by microorganisms (particularly yeasts) and, in a less proportion, by tartaric acid, phenolic compounds and inorganic matter *(1,2)*. Wine lees are so far the less studied by-product from the winery industry, where most attention has been set on the study of wine itself, and also on that of grape skins and seeds. The scant literature on wine lees composition has reported the phenolic content of this residue *(3,4)*; thus, Pérez *et al.* optimized a method for the extraction of phenolic compounds from wine lees and determined the antioxidant activity of the extracts *(3)*. The volatile profile of lees is also of interest, as some studies have reported that wine lees retain part of the volatile compounds that oak wood transfers to wine during ageing, preventing wine from having an excessive, unpleasant woody flavor, and diminishing the impact of undesirable compounds such as toxins, pesticides, antifoaming and volatile compounds as 4-ethylphenol and 4-ethylguaiacol, which have been described as responsible for the associated horsy, medicinal and spicy aroma of wines *(5–7)*. Pigments in wine lees have also been studied since the intense color of red wine lees indicates their anthocyanin and anthocyanidin content *(8,9)*.

Apart from this, most published articles on wine lees are focused on the positive effect on the overall bouquet of wine aged over lees *(10,11)*. Despite the varied studies on wine lees composition, as far as the authors know, there is not published research on the global profiling of this residue.

On the other hand, the use of microwave assisted extraction (MAE) has been deeply investigated and applied in analytical chemistry during the last decades to accelerate sample digestion and chemical reactions. MAE has become a viable alternative to conventional methodologies such as maceration or Soxhlet

due to the substantial improvements this energy produces on extraction, such as shortening of extraction time and lowering extractant consumption, among others *(12–14)*. There are numerous studies comparing the extraction efficiency of MAE for isolation of natural products. In 2010 Taamalli *et al.* evaluated the leaching efficiency of MAE and other assisted extraction techniques (*viz.* accelerated solvent extraction —ASE—, supercritical fluid extraction —SFE—, and conventional solid–liquid extraction) to recover phenolic compounds from Tunisian olive leaves *(15)*. The evaluation was supported on the analysis of the extracts by LC–ESI–TOF/MS and LC–ESI–IT–MS *(15)*. The profiles of the extracts showed a larger number of phenolic compounds in the extracts obtained using MAE, followed by those of the conventional method. In general, MAE and conventional methods were the best choice for extracting the majority of polar compounds. The current trend is to optimize protocols involving organic solvents compatible with human consumption such as ethanol or acetone, which, in addition, can be easily removed from the extracts. Also wine lees and MAE have been used by Pérez *et al.* to obtain ethanolic phenols extracts *(3)*, and Delgado de la Torre *et al.* to obtain 60:40 (v/v) ethanol–water extracts of anthocyanins, anthocyanidins and proanthocyanidins *(9)*.

The research reported here was aimed at obtaining a global profile of polar and mid-polar compounds in wine lees as unexploited raw material. With this aim, the extracts obtained by ethanol–water mixtures and MAE were the analytical samples subjected to liquid chromatography separation with tandem mass spectrometry detection using a quadrupole time-of-flight (LC–QTOF MS/MS) hybrid detector to take benefit from high resolution measurements.

2. Experimental

2.1. Samples

Wine lees, as the semisolid residue decanted after alcoholic fermentation of grape juice, were provided by eleven different Spanish wineries, namely: Cune, Muga, Ramírez de la Piscina, Viña Hermosa and López de Heredia from La Rioja; Real Sitio de Ventosilla and Torres de Anguix from Burgos; Selección de Torres from Valladolid, Valdeviña from Soria, La Unión from Córdoba and Fernández de Manzanos from Navarra. Each sample was homogenized by agitation at room temperature for 15 min, centrifuged at 855 g for 5 min and the liquid phase discarded. The solid phase was dried at 40 ºC for 48 h in an oven, milled in a mortar, sieved to < 0.5 mm particle size and stored at 4 ºC until use.

2.2. Reagents

Acetonitrile and formic acid (MS grade) were from Scharlab (Barcelona, Spain). Deionized water (18 mΩ•cm) was obtained by a Milli-Q water purification system from Millipore (Billerica, MA, USA). Folin–Ciocalteu (F–C) reagent, sodium carbonate.

2.3. Apparatus and Instruments

A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for MAE.

Shaking and centrifugation of wine lees and extracts were carried out by an MS2 minishaker (IKA, Germany) and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

Analysis of the extracts was performed by an Agilent 1200 Series LC system interfaced to an 6540 Agilent UHD Accurate-Mass LC–QTOF MS/MS detector (Palo Alto, CA), equipped with an Agilent Technology dual electrospray ion source (Dual ESI) operating in the negative and positive ionization modes.

2.4. Sample Preparation

6 g of dried lees was placed into the extraction vessel of the microwave-assisted digestor with 50 mL of 60:40 (v/v) ethanol–water adjusted at pH 4 with formic acid. The vessel was positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 140 W irradiation power for 10 min, after which the solid residue was removed by centrifugation and the extract filtered by a 20 μm pore size filter and 1:2 diluted with 60:40 (v/v) ethanol–water adjusted at pH 4 with formic acid.

2.5. LC–QTOF MS/MS Analysis

Chromatographic separation was performed using a C18 Inertsil ODS-2 column (250 mm×4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), kept at 25 °C. Mobile phases in this case were water (phase A) and acetonitrile (phase B), both LC–MS/MS grade and with 0.1% formic acid. The LC pump was programmed at a flow rate of 0.9 mL/min, and the following elution gradient was carried out: held from 0–2 min 4% phase B, 2–7 min 10% phase B, 7–60 min 100% phase B and held up to min 70. A post run of 4 min was included to equilibrate the column. The injection volume was 10 μL, and the injector needle was rinsed five times with 70% methanol. Furthermore, the needle seat back was flushed for 12 s at a flow rate of 4 mL/min with 70% methanol to clean it. The operating conditions of the mass spectrometer were as follows: gas temperature, 325 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3500 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; and focusing voltage, 175 V. Data acquisition (2.5 Hz) in both the centroid and profile modes was governed via the Agilent MassHunter Workstation software.

The instrument was operated in MS-high accuracy mode and in auto MS/MS mode. The mass range and detection window were set at *m/z* 100–3000 and 100 ppm, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in positive ionization mode, while ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.0007 (formate adduct of HP-921) were used in negative ionization mode. The collision energy was set at 22.5 V for the whole run.

2.6. Data Processing

The MassHunter Workstation software package (B.05.00 Qualitative Analysis and B.06.00 Profinder, Agilent Technologies, Santa Clara, CA, USA) was used to process all data obtained by LC–QTOF in the MS/MS mode. The recursive feature extraction algorithm in the software MassHunter Profinder was used to extract and align potential molecular features in all injections. This algorithm initially deconvolutes chromatograms and aligns features across the selected sample files in terms of mass and retention time; then, it uses the mass and retention time of each feature for recursive targeted feature extraction. This two-step procedure reduces the number of both false negatives and false positives in feature extraction. The target parameters for feature extraction included a threshold of 1500 counts and a maximum charge state of 2. In addition, the isotopic distribution for a valid feature had to be defined by two or more ions — with a peak spacing tolerance of 0.0025 *m/z*, plus 10.0 ppm. Adduct formation in the positive (+Na) and negative ionization mode (+HCOO) as well as protonated (+H) and deprotonated ions (–H) were also used to identify features of the same molecule. Features were aligned by using a tolerance window of 0.30 min and a mass accuracy of 10 ppm for retention times and *m/z* values across all data files, respectively. The minimum absolute height required for feature extraction was

set at 3000 counts, which was also used for 100% of samples in the recursive step. The files from this treatment were created in comma separated value files (.csv) for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies, Santa Clara, CA) for further processing. Compound identification was performed using the METLIN Metabolite and MS/MS (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp/), MetFrag (http://msbi.ipb-halle.de/MetFrag/), and PlantCyc (http://plantcyc.org/) databases. The allowed negative precursor ions for identification were formate adducts and deprotonated ions. Dehydratation neutral losses were also allowed.

3. Results and discussion

3.1. Microwave Assisted Extraction of Representative Compounds from Wine Lees

In the present study, the extraction protocol optimized by Delgado de la Torre *et al.* for anthocyanins profiling in wine lees was used *(9)*. **Figure 1** shows the base peak chromatogram (BPC) provided by the extract of wine lees provided by Muga winery. As can be seen, the abundance of chromatographic peaks at the beginning and the intermediate zone of the chromatogram (elution time window from 3 to 40 min) is indicative of the presence of mainly polar and mid-polar compounds in the extracts. Additionally, the observed chromatographic profile revealed a great heterogeneity in the composition of the extracts. Characterization of representative families of these compounds was carried out by tandem mass spectrometry in high-accuracy mode. Identification and confirmatory detection was supported on mass accuracy of the precursor ion and representative products ions, the structure of which was tentatively elucidated, and also on isotopic distribution of signals detected in full-scan mode. **Table 1** lists the tentatively identified compounds organized by families (information

about theoretical and experimental *m/z* values as well as characteristic fragment ions are included).

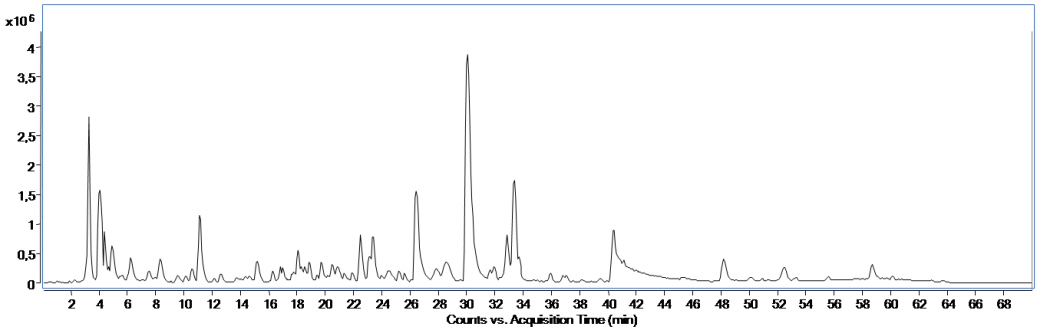

Figure 1. Base peak chromatogram obtained by LC–MS/MS analysis in negative ionization mode of an extract from wine lees provided by Muga winery.

Table 1. Compounds in extracts from wine lees identified by LC–QTOF MS/MS analysis.

Compound	*m/z*	RT (min)	Formula	Species	Error (ppm)	Mass	Fragments
Primary amino acids							
L-Arginine	175.1193	2.35	$C_6H_{14}N_4O_2$	$[M+H]^+$	-1.71	174.112	130.0936 121.0374 116.0710
L-Proline	116.0709	3.21	$C_5H_9NO_2$	$[M+H]^+$	1.72	115.0633	116.0711
3-Hydroxy-L-tyrosine	196.0618	3.27	$C_9H_{11}NO_4$	$[M-H]^-$	1.53	197.0688	152.0731 139.0391 111.0097
L-Valine	118.0865	3.47	$C_5H_{11}NO_2$	$[M+H]^+$	-1.69	117.0793	118.0865
β-Homoproline	130.0863	3.92	$C_6H_{11}NO_2$	$[M+H]^+$	0	129.0793	128.0340
L-Asparagine	133.0602	5.24	$C_4H_8N_2O_3$	$[M+H]^+$	4.50	132.0531	116.0353
L-Tyrosine	182.0794	5.30	$C_9H_{11}NO_3$	$[M+H]^+$	9.13	181.0739	165.0531 136.0740 121.0509
Aminocaproic acid	132.1019	7.21	$C_6H_{13}NO_2$	$[M+H]^+$	0	131.0946	132.0821 115.0046
L-Tryptophan	205.0974	15.17	$C_{11}H_{12}N_2O_2$	$[M+H]^+$	0.97	204.0904	146.0587 132.0738 118.0616
L-Phenylalanine	166.0860	17.01	$C_9H_{11}NO_2$	$[M+H]^+$	1.80	165.0793	120.0787 103.0529
Nitrotyrosine	227.0663	27.36	$C_9H_{10}N_2O_5$	$[M+H]^+$	0.44	226.0590	181.0606

							168.0286 133.0520
L-Tyrosine octadecyl ester	432.3442	40.56	$C_{27}H_{47}NO_3$	$[M-H]^-$	6.48	433.3556	432.3429 431.3372
Flavonoids							
Kaempferol 3-(2',3-dyacetylrhamnoside)-7'-rhamnoside	661.1820	3.69	$C_{31}H_{34}O_{16}$	$[M-H]^-$	-6.95	662.1847	601.1626 175.0260 131.0372
3,4,5-Trimethoxyflavone	335.0890	7.97	$C_{18}H_{16}O_5$	$[M+Na]^+$	0	312.0998	298.0187 280.0701 252.0822
Epigallocatechin 3-glucuronide	481.0975	14.40	$C_{21}H_{22}O_{13}$	$[M-H]^-$	2.70	482.106	301.0338 257.0463 175.0017
Gallocatechin	305.0673	14.46	$C_{15}H_{14}O_7$	$[M-H]^-$	-1.99	306.074	167.0333 137.0235 125.0237
4'-Methyl-epigallocatechin-7-glucuronide	495.1140	16.17	$C_{22}H_{24}O_{13}$	$[M-H]^-$	0.80	496.1217	301.0338 257.0463 175.0017
Pelargonidin 3-(6-*p*-coumarylglucoside)	578.1424	16.25	$C_{30}H_{27}O_{12}$	$[M-H]^-$	1.04	579.1497	289.0687 407.0733 125.0229
Rutin	609.1488	16.63	$C_{27}H_{30}O_{16}$	$[M-H]^-$	-4.43	610.1534	300.0277 271.0245 255.0311
Procyanidin B2	577.1357	17.47	$C_{30}H_{26}O_{12}$	$[M-H]^-$	-1.03	578.1419	425.0932 407.0767 289.0731
Catechin	291.0856	17.49	$C_{15}H_{14}O_6$	$[M+H]^+$	2.40	290.079	147.0442 139.0385 123.0410
Malvidin 3-galactoside	492.1252	18.16	$C_{23}H_{25}O_{12}$	$[M-H]^-$	4.26	493.1346	329.0645 313.0353 299.0128
Peonidin-3-O-glucoside	463.1232	20.96	$C_{22}H_{23}O_{11}$	$[M+H]^+$	2.63	462.1162	301.0723
Epicatechin	291.0859	21.13	$C_{15}H_{14}O_6$	$[M+H]^+$	-1.37	290.079	147.0437 139.0387 123.0443
Cinnamtannin A1	865.1964	21.72	$C_{45}H_{38}O_{18}$	$[M-H]^-$	2.42	866.0241	739.1640 575.1171 287.0582
Quercetin 4'-glucuronide	477.0679	23.37	$C_{21}H_{18}O_{13}$	$[M-H]^-$	-0.83	478.0747	301.0370 202.9375 151.0197
Malvidin 3-(6-*p*-coumarylglucoside)	639.1706	24.59	$C_{32}H_{30}O_{14}$	$[M+H]^+$	0	638.1636	477.1248 331.0786 229.1407
Quercetin-3-O-glucoside	463.0891	24.83	$C_{21}H_{20}O_{12}$	$[M-H]^-$	-1.72	464.0955	301.0219 300.0139 271.0129
Myricetin	317.0337	26.62	$C_{15}H_{10}O_8$	$[M-H]^-$	0.63	318.0376	179.0020 151.0070 137.0273

Peonidin 3-(6"-*p*-coumarylglucoside)	609.1600	27.13	$C_{31}H_{28}O_{13}$	$[M+H]^+$	0.49	608.1530	447.1067 433.2554 301.0707
Quercetin	301.0370	29.99	$C_{15}H_{10}O_7$	$[M-H]^-$	-5.31	302.0427	178.9996 151.0048 121.0293
Isorhamnetin	315.0517	35.94	$C_{16}H_{12}O_7$	$[M-H]^-$	2.22	316.0583	315.0512 300.0279 151.0036
Kaempferol	285.0401	36.86	$C_{15}H_{10}O_6$	$[M-H]^-$	1.40	286.0477	257.0464 239.0363 151.0015
Non-flavonoid phenolic compounds							
4-(2-Hydroxy-3-isopropyl-aminopropyl) benzoic acid	276.1182	4.23	$C_{13}H_{19}NO_4$	$[M+Na]^+$	8.69	253.1314	213.0826 147.0735 110.0575
Gallic acid	169.0142	11.40	$C_7H_6O_5$	$[M-H]^-$	0	170.0215	125.0281 107.0159
2-Methoxyphenol	106.0716	13.17	$C_{12}H_{18}O_3$	$[M+2H]^{2+}$	-9.33	210.1256	106.0669 103.0539
Ferulic acid	193.0508	15.31	$C_{10}H_{10}O_4$	$[M-H]^-$	-1.03	194.0579	178.0276 134.0352
3,4-Dihydroxyphenyl-acetic acid	167.0352	16.54	$C_8H_8O_4$	$[M-H]^-$	-1.48	168.0423	123.0591 108.0179
Protocatechuic acid	153.0198	16.70	$C_7H_6O_4$	$[M-H]^-$	3.26	154.0266	108.0218 109.0297
Caffeic acid	179.0351	18.66	$C_9H_8O_4$	$[M-H]^-$	-0.55	180.0425	135.0429 117.0320 107.0477
p-Hydroxybenzoic acid	137.0244	18.69	$C_7H_6O_3$	$[M-H]^-$	0	138.0317	136.0149 108.0198
Protocatechualdehyde	137.0238	19.46	$C_7H_6O_3$	$[M-H]^-$	4.38	138.0317	136.0153 108.0201
p-Coumaric acid	163.04	19.75	$C_9H_8O_3$	$[M-H]^-$	0.61	164.0473	135.0554 119.0496
Sinapic acid	223.0623	21.52	$C_{11}H_{12}O_5$	$[M-H]^-$	-4.93	224.0685	209.0450 193.0365 164.0473
Cinnamic acid	147.0451	23.05	$C_9H_8O_2$	$[M-H]^-$	0.68	148.0521	132.1676 103.0554
Piceid	389.1278	25.02	$C_{20}H_{22}O_8$	$[M-H]^-$	-9.25	390.1315	227.0741 185.0569 159.0807
*trans-R*esveratrol	227.0715	25.75	$C_{14}H_{12}O_3$	$[M-H]^-$	-0.44	228.0786	185.0681 143.0536
Vanillin	151.0387	26.62	$C_8H_8O_3$	$[M-H]^-$	9.26	152.0473	136.1382 108.0218
2-Phenylacetaldehyde	119.05	26.81	C_8H_8O	$[M-H]^-$	1.67	120.0575	103.0418
Syringic acid	197.0454	31.65	$C_9H_{10}O_5$	$[M-H]^-$	0.50	198.0528	182.0203 166.9970

							123.0071
Others							
4-Acetamido-butanoate	144.0673	3.19	$C_6H_{11}NO_3$	$[M-H]^-$	-4.85	145.0739	128.0291 102.0552
Galactobioside	683.2250	3.23	$C_{24}H_{44}O_{22}$	$[M-H]^-$	0.14	684.2324	179.0563 161.0475 101.0247
Galactaric acid	209.0305	3.42	$C_6H_{10}O_8$	$[M-H]^-$	-0.95	210.0376	191.0976 133.0129 115.0031
2-Carboxy-2,3-dihydro-5,6-dihydroxyindole	194.0400	3.62	$C_9H_9NO_4$	$[M-H]^-$	3.04	195.0532	129.0190 113.0258 101.0238
Digalacturonic acid	369.0682	3.66	$C_{12}H_{18}O_{13}$	$[M-H]^-$	1.89	370.0747	193.0357 131.0348 113.0249
Lactobionic acid	357.1043	3.83	$C_{12}H_{22}O_{12}$	$[M-H]^-$	-1.40	358.1111	131.0431 114.0257 101.0229
6-Methyl-D-Galactopyranuronosyl (1-4) D-galactopyranuroic acid	559.1143	3.88	$C_{19}H_{28}O_{19}$	$[M-H]^-$	1.63	560.1225	365.0694 193.0318 175.0211
D-Galactopyranuronosyl -(1-4)D-Galactopyranuronosyl (1-4)D-galacturonic acid	545.0987	4.03	$C_{18}H_{26}O_{19}$	$[M-H]^-$	1.65	546.1068	369.0672 193.0328 175.0229
Shikimic acid	173.0462	4.20	$C_7H_{10}O_5$	$[M-H]^-$	-4.04	174.0528	137.0236 111.0439
Malic acid	133.0145	4.47	$C_4H_6O_5$	$[M-H]^-$	-2.25	134.0213	115.0043 114.9987
Tartaric acid	149.0096	4.57	$C_4H_6O_6$	$[M-H]^-$	-2.68	150.0164	143.7544 132.6965 105.0172
Trehalose-6-phosphate	421.0751	5.26	$C_{12}H_{23}O_{14}P$	$[M-H]^-$	0.47	422.0825	259.0174 171.0101
B-D-Xylopyranosyl (1-5)-L-arabinofuranosyl (1-5)L-arabinose	413.1280	5.39	$C_{15}H_{26}O_{13}$	$[M-H]^-$	5.08	414.1373	257.1127 213.1221 155.0092
2,3-Dihydroxy-3-methylbutanoate	135.0543	6.41	$C_5H_{10}O_4$	$[M+H]^+$	7.77	134.0579	119.0429 107.0455
Citric acid	191.0204	9.21	$C_6H_8O_7$	$[M-H]^-$	-3.66	192.027	129.0239 111.0130
Pantothenic acid	218.1035	14.25	$C_9\ H_{17}NO_5$	$[M-H]^-$	-0.45	219.1109	146.0820 116.0732
Glutathione	306.0783	14.37	$C_{10}H_{17}N_3O_6S$	$[M-H]^-$	-5.88	307.0838	272.0865 244.0236 128.0370
Cytosine	110.0635	16.83	$C_4H_5N_3O$	$[M-H]^-$	-4.30	111.0433	109.0281 108.0195

Benzyl O-(arabinofuranosyl-(1-6)glucoside)	401.1444	18.12	$C_{18}H_{26}O_{10}$	$[M-H]^-$	2.24	402.1526	269.0984
							113.0255
							101.0261
Hydroxycitric acid	207.0143	30.73	$C_6H_8O_8$	$[M-H]^-$	1.44	208.0219	143.0485
							135.0487

3.2. Primary Amino Acids

The presence of primary amino acids in grapes and wine lees has been extensively reported in the literature *(16,17)*, since these compounds are contributors to the nutritional value of wine, but also a significant source of yeast nutrition. Microbial and enzymatic conversion of amino acids produces key aroma compounds in foods such as cheese, wine, honey and other fermented foodstuffs *(18)*, while decarboxylation of amino acids by bacteria leads to the formation of non desirable biogenic amines *(17,18)*, such as tyramine, phenyl-amine and phenylethylamine, formed from tyrosine, phenylalanine and L-arginine. Eight primary amino acids and five derivatives were tentatively identified in the extracts in this research. One of the most important primary amino acids found was L-tyrosine. The extracted ion chromatogram (EIC) of this compound by monitoring the *m/z* value at 182.0794 in the positive mode revealed its presence at 5.30 min (**Figure 2.A**), which was clearly identified by MS/MS fragmentation of the precursor ion [M+H]+ that provided three representative fragments: one at *m/z* 165.0531 assigned to the structure shown in **Figure 2.B**, formed by the cleavage of the ammonia unit (17 Da); the second characteristic fragment was assigned to *m/z* 136.0740, produced by the consecutive loss of a water moiety and a carbonyl group, producing the [M+H–H2O–CO]+ ion. Subsequent fragmentation of this fragment produced the third characteristic fragment corresponding to *m/z* 121.0509, which is the result of the cleavage of the ammonia unit (17 Da) *(19–21)*. This fragmentation pattern was similar for most amino acids, which made easier their tentative identification.

Other important primary amino acid identified was L-phenylalanine. **Figure 3.A** shows the EIC for this compound by monitoring the *m/z* ratio at

166.086 in the positive mode, corresponding to the peak eluted at 17.0 min; the MS/MS fragmentation of this [M+H]+ precursor ion, led to two fragments, as shown in **Figure 3.B**; at *m/z* 120.0787 by cleavage of the COOH unit (45 Da) from the original L-phenylalanine structure that corresponds to the structure of the phenylethylamine molecule, and the second product ion at *m/z* 103.0529 by subsequent loss of the ammonia unit (17 Da) *(19)*.

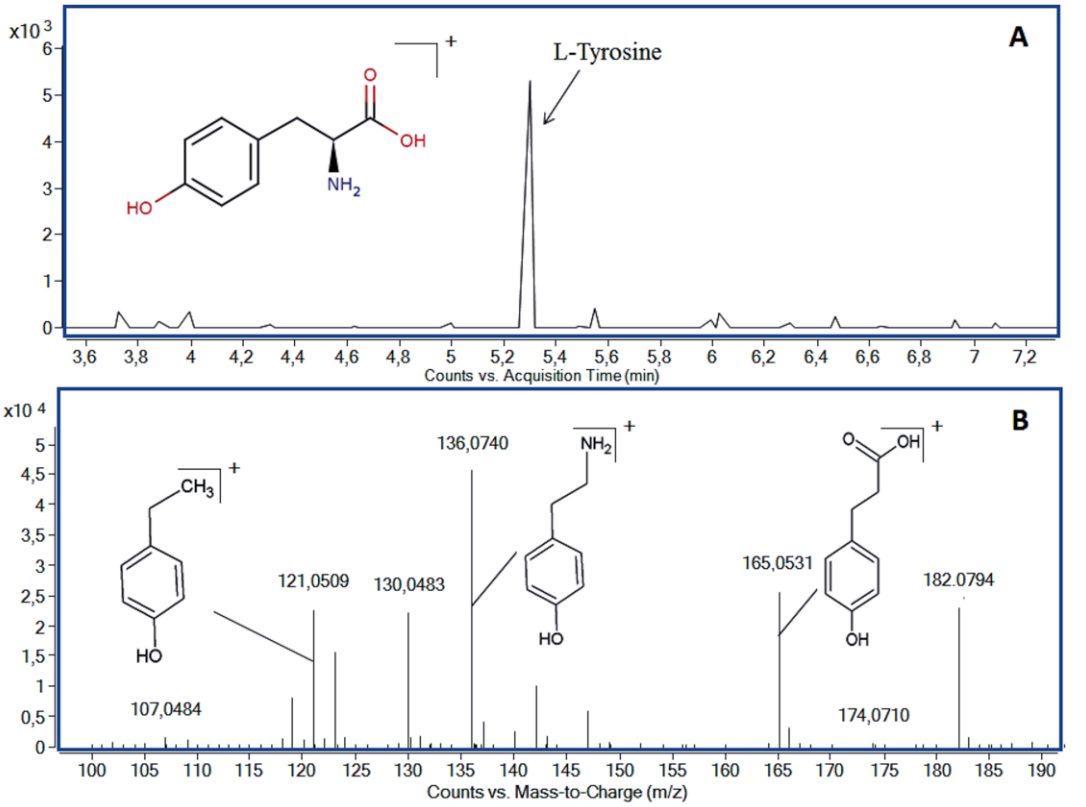

Figure 2. (A) Extracted ion chromatogram obtained by LC–MS/MS analysis in positive ionization mode for L-tyrosine. (B) MS/MS spectrum for L-tyrosine.

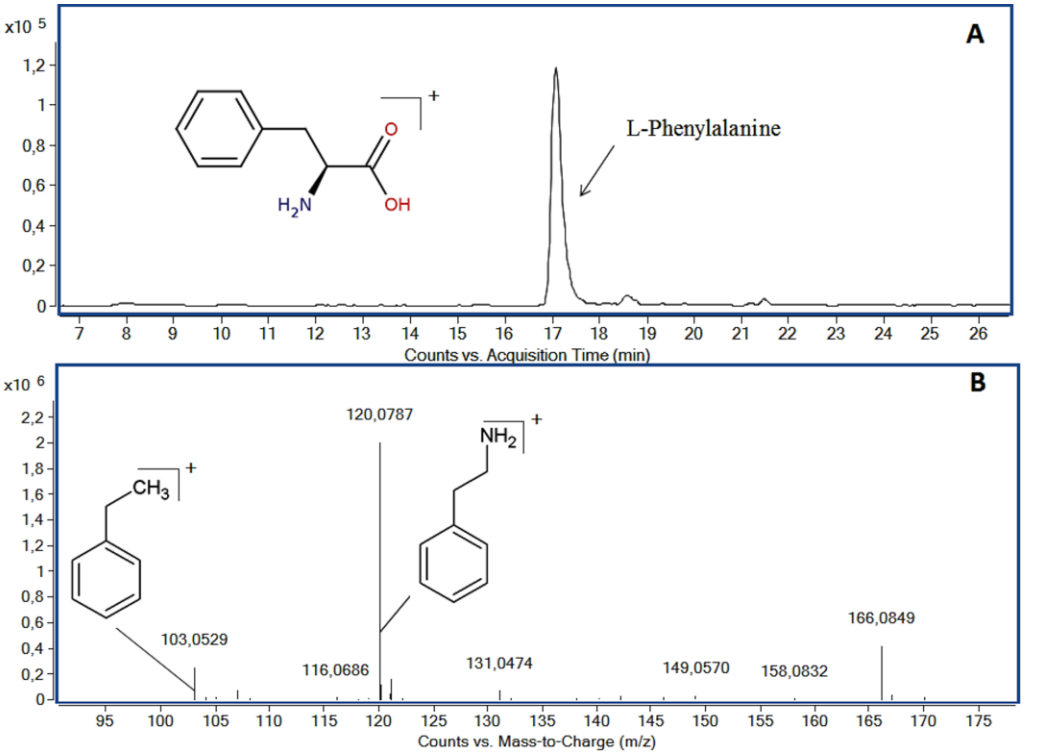

Figure 3. (A) Extracted ion chromatogram obtained by LC–MS/MS analysis in positive ionization mode for L-phenylalanine. (B) MS/MS spectrum for L-phenylalanine.

3.3. Flavonoids

It is well documented the abundance of flavonoids in wine and in grapes in general. In fact, these compounds, together with the rest of phenolic families, are responsible for the so called "French paradox", linked to a moderate consumption of wine, given the nutraceutical properties attributed to the intake of these compounds. Protection by wine flavonoids against LDL oxidation and atherosclerosis *(22)*, as well as their anticarcinogenic and radical scavenging activity have been proposed *(23–24)*. Flavonoids are a large family of more than 4000 secondary plant metabolites, comprising anthocyans, flavanols, flavonols, flavanonols and flavones *(25)*. Among them, flavonols were the main compounds

identified in wine lees, including quercetin, myricetin, kaempferol and isoharmmetin, which are the most common flavonols identified in grapes *(25)*. Quercetin was detected by elution at 30 min, as **Figure 4.A** shows by monitoring the *m/z* ratio at 301.0370. The MS/MS spectrum of this precursor ion [M−H]− provided representative product ions at *m/z* 121.0293, 151.0048 and 178.9996 (**Figure 4.B**), where the fragment at *m/z* 151.0048 is produced by Retro Diels-Alder (RDA) reaction of the A ring, being a common product ion for different flavonols, flavanones and flavones. Fragment at *m/z* 178.9996 was produced by retrocyclation of the original structure of quercetin, as proposed by Dubber *et al. (26)*. Additionally, MS/MS fragmentation of quercetin also led to two product ions at *m/z* 273.0417 and *m/z* 255.0313, which correspond to the fragments [M−H−CO]− and [M−H−CO−H2O]−, respectively. Other representative fragment for quercetin used for its identification is the ion found at *m/z* 107.0137 obtained by decarboxylation after RDA reaction [M−H−CO−CO2]−.

As most flavonoids, myricetin shows a fragmentation pattern where the precursor ion [M−H]− at *m/z* 317.0337 produces representative product ions at *m/z* 137.0273, 151.0070 and 179.0020, being the product ion at *m/z* 137.0273 the only distinctive fragment from quercetin (**Figure 5**). Isorhamnetin was also identified in the negative mode, exhibiting a [M−H]− precursor ion at *m/z* 315.0517 and representative fragments at *m/z* 300.0279 and 151.0036, which is in agreement with the results obtained by Justensen *et al.* in 2000, who explained the fragment at *m/z* 300.0279 by the loss of CH3• from the deprotonated molecular ion *(27)*. In the case of kaempferol, the [M−H]− precursor ion eluted at 36.86 min with a *m/z* of 285.0401, exhibiting representative product ions at *m/z* 257.0464, 239.0363 and 151.0015 produced by subsequent loss of a CO moiety (28 Da), a water molecule (18 Da), and RDA reaction of the A ring, following the same fragmentation pattern as myricetin.

A glucoside derivative of quercetin (namely, quercetin 3-O-glucoside) was also identified. This glucoside conjugate gave a [M−H]− precursor ion at *m/z* 463.0891 and, by loss of the glucose moiety (162 Da), the corresponding aglycone

at *m/z* 301.0219. Additionally, **Figure 4.C** shows the fragments derived from the aglycone fragmentation.

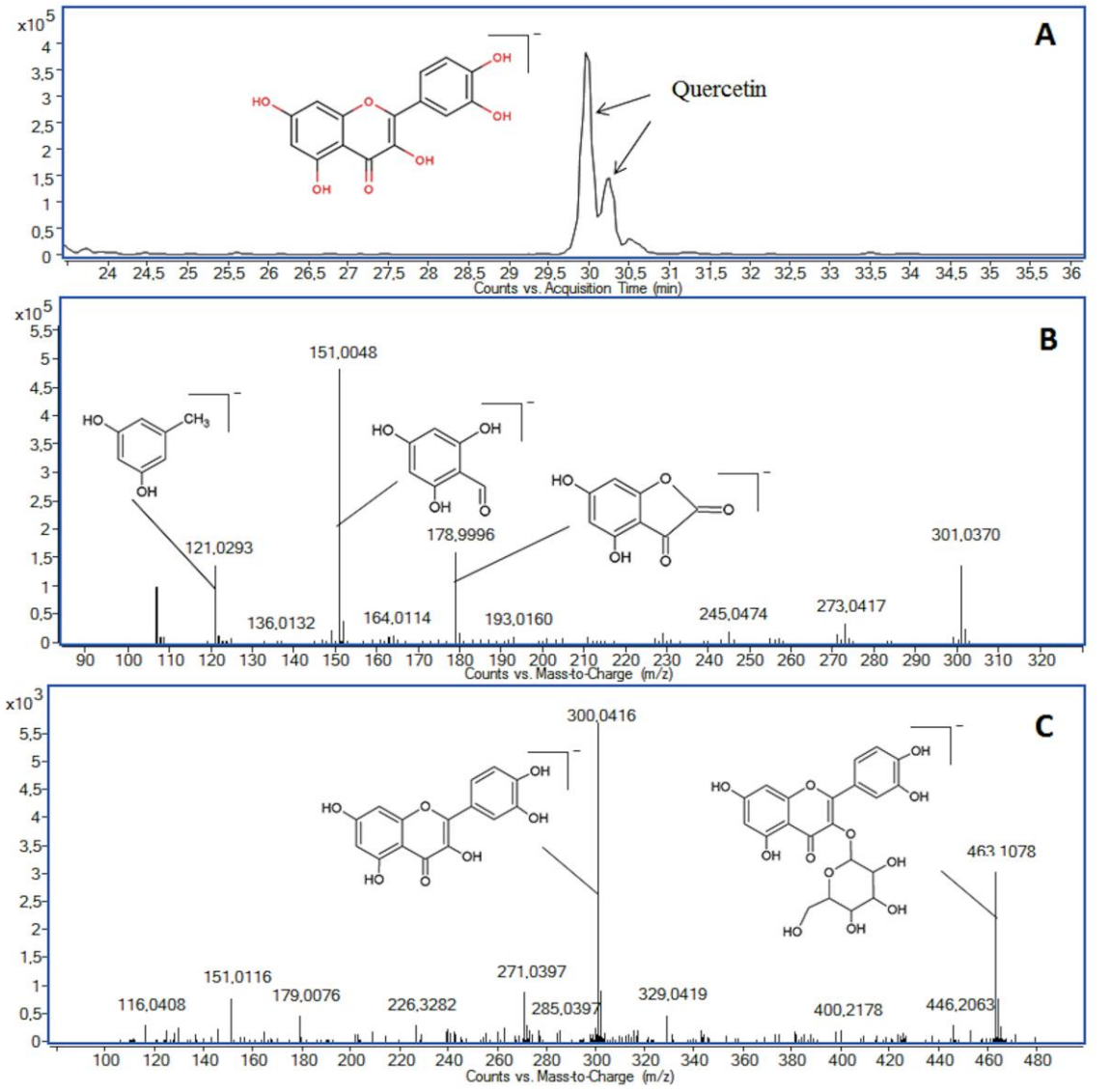

Figure 4. (A) Extracted ion chromatogram obtained by LC–MS/MS analysis in negative ionization mode for quercetin. (B) MS/MS spectrum for quercetin.

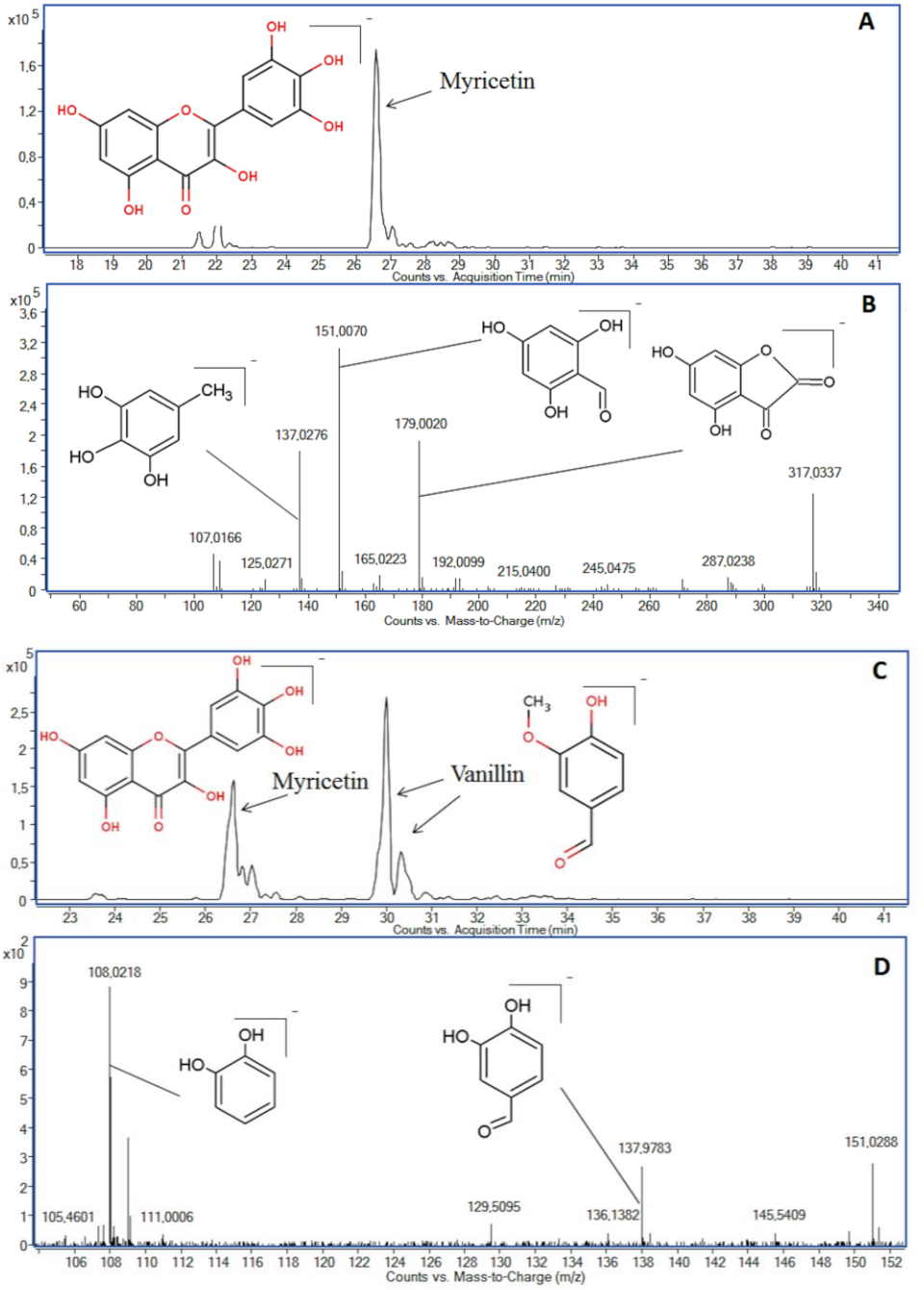

Figure 5. (A) Extracted ion chromatogram obtained by LC–MS/MS analysis in negative ionization mode for myricetin. (B) MS/MS spectrum for myricetin. (C) BPC chromatogram for vanillin. (D) MS/MS spectrum for vanillin.

Among the identified flavanols, it is worth mentioning catechin, epicatechin, gallocatechin, procyanidin B2 and cinnamtanin A1. Catechin and epicatechin, were eluted at 17.5 and 21.1 min, respectively, with [M+H]+ precursor ions at *m/z* 291.0856 and 291.0859, respectively, where representative fragments were found at *m/z* 147.0442, 139.0385 and 123.0410 in the case of catechin, and *m/z* 147.0437, 139.0387 and 123.0443 for epicatechin; with the fragment at 147 as the most abundant in the case of epicatechin. The fragments at 147 are explained by heterocyclic ring fission (HRF) and loss of a water molecule; the fragment at 139 is produced by RDA reaction of the original molecule; finally, the fragments at 123 can be explained either by direct benzofuran forming fission (BFF) or by loss of a water molecule following BFF, as described by Li *et al. (28)*.

Gallocatechin was also identified by elution at 14.5 min with [M–H]– *m/z* 305.0673 as precursor ion. The characteristic fragments found were those at *m/z* 167.0333, 137.0235 and 125.0237 that, following a similar fragmentation pattern to catechin and epicatechin, could be explained by BFF, RDA and HRF reactions, respectively *(28)*. Some interesting flavones have also been identified in extracts from wine lees. Thus, 3,4,5-trimethoxyflavone eluted at 8.0 min as [M+Na]+ precursor ion (*m/z* 335.0890), where the most abundant fragments were at *m/z* 298.0187, 280.0701 and 252.0822, consistent with the results obtained by Huck *et al*. and explained by the loss of a CH_3 molecule from the original structure (15 Da), the loss of a water molecule from the fragment at 298 (18 Da), and the loss of a carbonyl group from the fragment at 280, respectively (28 Da) *(29)*.

Procyanidin B2 was identified as the precursor ion [M–H]– at *m/z* 577.1357, with representative fragments at *m/z* 407.0767; 289.0731 and 425.0932. The detection of these fragments is consistent with the fragmentation pathway proposed by Delgado de la Torre *et al*., where fragments at *m/z* 271.0 and 289.0 were explained by a quinone methide (QM) cleavage of the interflavan bond, and those at *m/z* 407.0 and 425.1 would correspond to an RDA reaction *(9)*. One other procyanidin derivative identified in the extracts was cinnamtannin

A1 (procyanidin C1), eluted at 21.7 min, with [M–H]– as precursor ion at *m/z* 865.1964. Its fragmentation led to characteristic product ions at *m/z* 739.1640, 575.1171 and 287.0582, where the fragments at *m/z* 575.1171 and 287.0582 correspond to the dimer and monomer of epicatechin, respectively, and the fragment at *m/z* 739.1640 corresponds to the loss of a $C_6H_6O_2$ and a hydroxyl unit.

Finally, some anthocyanins were also found, as is the case of peonidin 3-O-glucoside, peonidin 3-(6-p-coumarylglucoside) and malvidin 3-(6-p-coumarylglucoside). Peonidin 3-O-glucoside was found eluting at 20.9 min as the adduct [M+H]+ corresponding to *m/z* 463.1232, where the loss of the glucoside moiety (–162 Da) led to the fragment found at *m/z* 301.0723, corresponding to the aglycone peonidin. A similar fragmentation pattern was observed in the case of peonidin 3-(6-p-coumarylglucoside), which was detected as the [M+H]+ cation at *m/z* 609.1600 eluting at 27.1 min. In this case, the fragment corresponding to the aglycone peonidin was also detected at *m/z* 301.0707, and the fragment corresponding to the loss of the coumaroyl moiety (146 Da) at *m/z* 447.1067. This fragmentation pattern is also found in the case of malvidin 3-(6-p-coumarylglucoside), where the consecutive loss of the coumaroyl and glucoside moieties explains the main fragments found at *m/z* 477.1248 and 331.0786, respectively. The last fragment corresponds to the aglycone malvidin. This glucoside derivative was found to elute at 24.6 min showing a [M+H]+ precursor ion at *m/z* 639.1706.

3.4. Non Flavonoids

A wide range of the non-flavonoid phenols was identified, including mainly phenolic aldehydes, stilbenes, and phenolic acids, which can be classified into hydroxycinnamic and hydroxybenzoic acids depending on their structure. This family of compounds is in general highly appreciated since most of them contribute actively to the final bouquet of wines. Some of these compounds with interest from the oenologial point of view were tentatively identified in extracts from wine lees. In the case of phenolic aldehydes, it is worth emphasizing the

case of vanillin, highly appreciated for the vanilla scent it transfers to wine and making it one of the most popular wine descriptors. As shown in **Figure 5.C**, this compound was identified by monitoring the *m/z* ratio at 151.0387 in the negative mode, corresponding to the peak eluted at 29.8 min, [M–H]–; the MS/MS fragmentation of this precursor ion produced two characteristic fragments, one at *m/z* 137.1382 by loss of a CH_3 (15 Da) group from the vanillin original structure and corresponding to the structure of protocatechualdehyde, as shown in **Figure 5.D**, and one other fragment at *m/z* 108.0218 produced by consecutive loss of a CO unit (28 Da). Protocatechualdehyde was also found eluting at 19.5 min, exhibiting a [M–H]– precursor ion at *m/z* 137.0238, where the only product ion obtained by MS/MS fragmentation was found at *m/z* 108.0201 and, as in the case of vanillin, it is explained by the loss of a CO unit (28 Da).

The stilbenes piceid and *trans*-resveratrol were found in wine lees. *Trans*-resveratrol was detected to elute at 25.7 min, as can be seen in **Figure 6.A**, with a precursor ion [M–H]– at *m/z* 227.0715, and exhibiting representative fragments at *m/z* 185.0681, 143.0536, 159.0858 and 119.0470, which are results consistent with those obtained by Careri *et al.* in 2004 and Stella *et al.* in 2008. According to the fragmentation pattern discussed by Stella *et al.*, the fragment at *m/z* 185.0681 is attributable to the loss of a C_2H_2O moiety with H rearrangement, and corresponds to the structure in **Figure 6.B**. Fragment ions at *m/z* 183.0764, 159.0868 and 143.0536 correspond to losses of H_2, CO and C_2H_2O, respectively, which agree with the proposed structures *(30,31)*.

A key group of phenolic acids is that composed by hydroxycinnamic acids, a class of aromatic compounds that are implicated in the flavor of red wine. One example of them is sinapic acid, a hydroxycinnamic acid detected by the precursor ion at *m/z* 223.0623, which produces significant product ions at *m/z* 209.0450 by loss of a CH_3 group (15 Da), and at *m/z* 109.0223 by consecutive loss of a second CH_3 group. Other characteristic fragment was found at *m/z* 164.0413, caused by loss of a CH_3 group and a carboxylic group, leading to the

structure shown in **Figure 7.A**. Caffeic acid was also identified at 18.7 min by monitoring the *m/z* ratio at 179.0351, which corresponds to the [M–H]– precursor ion of this compound, and verified by the presence of two representative fragments at *m/z* 135.0429 and 117.0320 caused by cleavage of the carboxylic group (44 Da) and a second loss of a hydroxyl unit (17 Da), respectively, as can be seen in **Figure 7.B**.

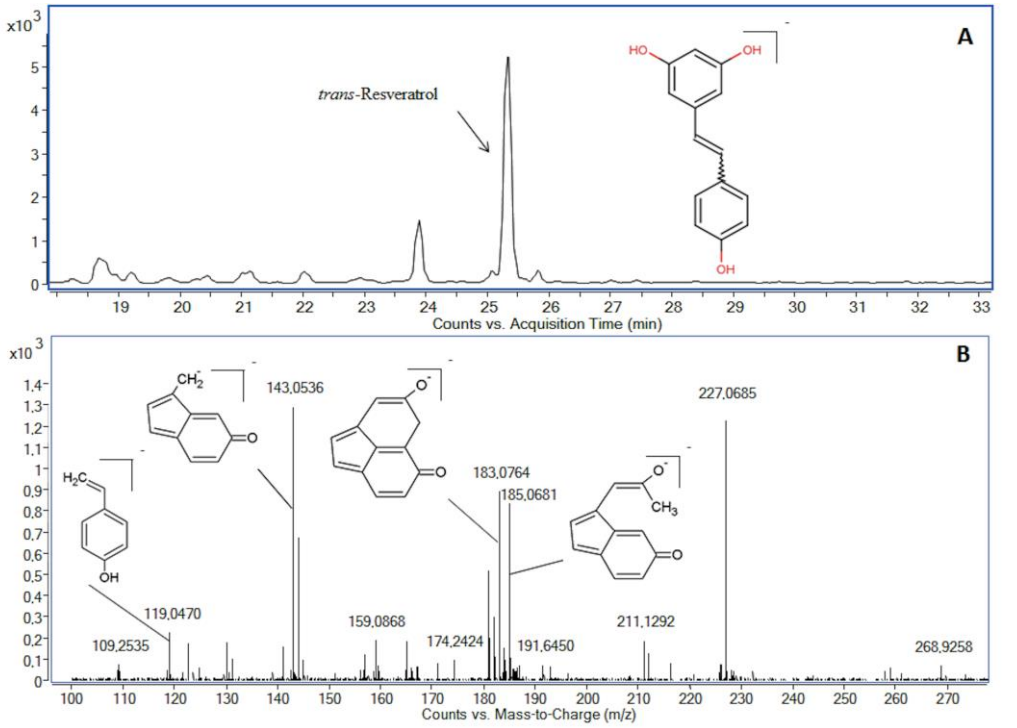

Figure 6. (A) Extracted ion chromatogram obtained by LC–MS/MS analysis in negative ionization mode for *trans*-resveratrol. (B) MS/MS spectrum for *trans*-resveratrol.

One other subgroup of phenolic acids are hydroxybenzoic acids, among which syringic, gallic, protocatechuic and hydroxybenzoic acids were identified in the extracts from wine lees. Syringic acid was found at 31.6 min by a [M–H]– precursor ion of *m/z* 197.0454, with representative fragments at *m/z* 182.0203 by loss of a CH_3 group, at *m/z* 166.9970 following the loss of a second CH_3 group and at *m/z* 123.0071 produced by the subsequent loss of the carboxylic group. A similar fragmentation pattern is that of gallic acid, with the [M–H]– precursor

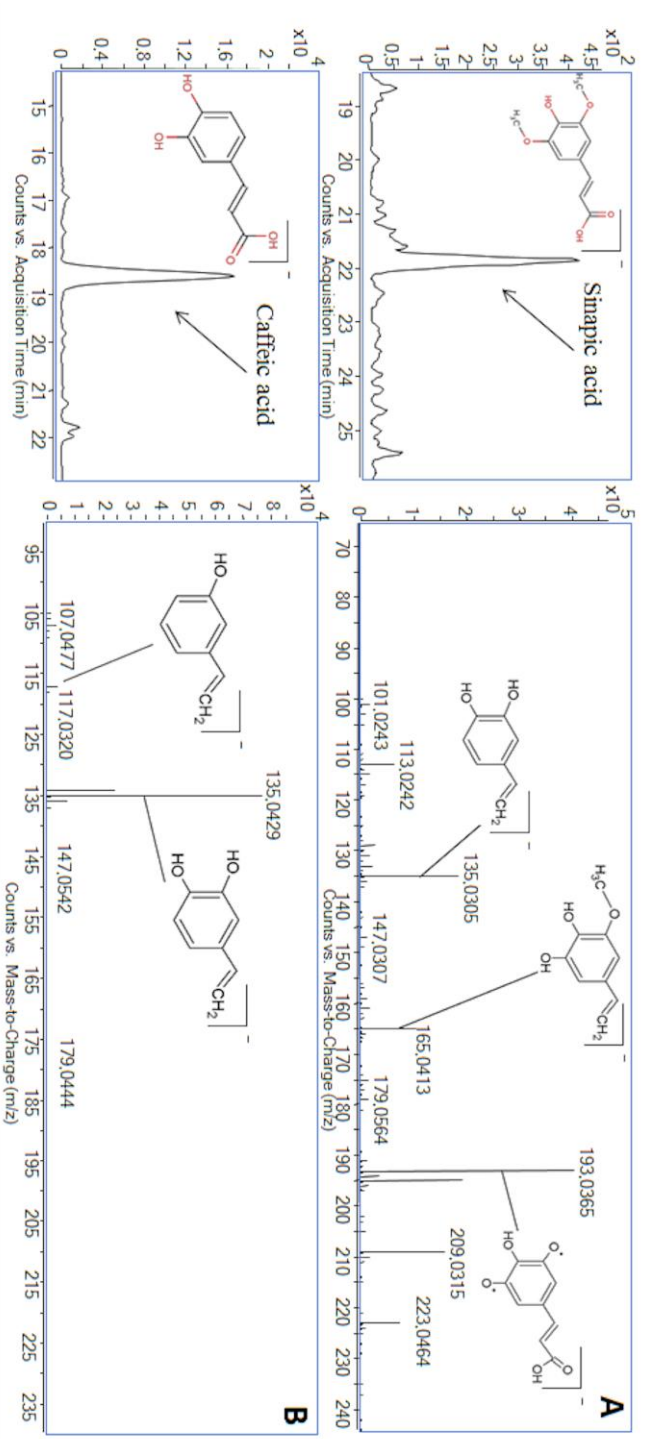

Figure 7. Extracted ion chromatogram obtained by LC–MS/MS analysis in negative ionization mode and MS/MS spectrum of sinapic acid (A), and caffeic acid (B).

ion found at *m/z* 169.0142 and characteristic fragments at *m/z* 125.0281 and *m/z* 107.0159 by loss of the carboxylic and hydroxyl groups, respectively.

3.5. Other Compounds in Wine Lees

Apart from the two families of compounds discussed above, a wide range of compounds has also been identified, some of them of high importance in the oenological field. This is the case of organic acids such as malic, tartaric and citric acids, where the first two are the major acids found in grape that play a key role in the acidity of wines. Citric acid is a minor component in grapes, but generally added during the winemaking process to improve the sensory characteristics of wine by increasing acidity to complement a specific flavor or prevent ferric hazes *(32)*.

Malic acid was identified by eluting at 4.5 min, in the [M−H]− form at *m/z* 133.0165, and presenting a characteristic fragment at *m/z* 115.0071 produced by the loss of a hydroxyl group (17 Da). Similarly, tartaric acid eluted at 4.6 min, with [M−H]− precursor ion at *m/z* 149.0086, presented characteristic fragments at *m/z* 130.9981, 105.0172 and 103.0139, produced by the consecutive losses of a hydroxyl (17 Da) and a carboxyl groups, respectively. Citric acid eluted at 9.2 min with a characteristic [M−H]− precursor ion at *m/z* 191.0240 and with identificative fragments at 129.0239 and 111.0130, which can be explained by the conversion of citric acid into itaconic acid by loss of a carboxyl group, followed by the loss of a hydroxyl group and subsequent dehydration of the molecule to form the ion with *m/z* 111.0130.

It is worth noting the presence of shikimic acid, a precursor in the synthesis of phenolic and aromatic compounds *(33)*. It was found eluting at 4.2 min by monitoring the *m/z* ratio at 173.0462, which corresponds to the [M−H]− precursor ion. Representative fragments were found at *m/z* 137.0236 and 111.0439, produced by the loss of two hydroxyl groups in the case of *m/z* 137.0236, and by the loss of a third hydroxyl group and a carboxylic group in the case of *m/z* 111.0439.

One other interesting identified compounds was phantotenic acid (vitamin B_5) that has an important role as a growth factor of yeasts. This acid was identified eluting at 14.2 min by monitoring the *m/z* ratio at 218.0135 as [M–H]– precursor ion. The MS/MS fragmentation of this compound yielded two representative fragments: at *m/z* 146.0820, explained by the loss of a CH_3CH_2COO– moiety, and at *m/z* 116.0732 by the subsequent loss of a CH_2OH unit.

Finally, some sugar derivatives were also detected, as the galactaric acid, which eluted at 3.4 min and was identified exhibiting a [M–H]– precursor ion at *m/z* 209.0305. Its fragmentation produced representative fragments as that found at *m/z* 191.0976, explained by the loss of a water molecule (18 Da), or that detected at *m/z* 133.0129 and ascribed to the loss of a CH_2OHCOO– unit from the original structure. The third fragment at *m/z* 115.0031 is explained by the subsequent loss of a hydroxyl group.

Conclusions

Extracts of dried wine lees have been examined by LC–QTOF MS/MS analysis to obtain a global profile of polar and mid-polar compounds. Attending to the wide variety of compounds identified in wine lees, this study underlines the potential of wine lees as an exploitable source to isolate interesting compounds, which would valorize this residue. This study complements previous studies on wine lees and other oenological by-products.

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Chapter 9:

Characterization and comparison of wine lees by liquid chromatography–mass spectrometry in high-resolution mode

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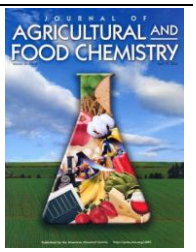

Characterization and comparison of wine lees by liquid chromatography−mass spectrometry in high-resolution mode

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Characterization and comparison of wine lees by liquid chromatography–mass spectrometry in high-resolution mode

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Abstract

Wine lees from eleven different wineries pertaining to two denominations of origin in Spain (La Rioja and Ribera del Duero) have been characterized in this research by LC–MS/MS in high resolution mode. For this purpose, the wine lees were separated into the liquid phase (imbibed wine from lees) and the solid residue, which was dried and subjected to solid–liquid extraction assisted by microwaves. Both fractions were separately analyzed and each fraction from the eleven wineries compared in order to find similarity in their patterns. The statistical analysis enabled to find both differences and common aspects in the composition of imbibed wine from lees and dried lees from all wineries. MS/MS tentative identification of representative compounds in each fraction revealed the varied composition of wine lees with special emphasis on flavonoids such as quercetin, myricetin and malvidin-3-galactoside, identified in extracts of dried lees, or other compounds such as kaempferol 3-(2',3'-dyacetylrhamnoside)-7"-rhamnoside, aminocaproic acid and citric acid, exclusively identified in imbibed wine from lees. The adsorbent capacity of the solid residue justified the high concentration of phenolic compounds in the extracts from solid lees. The differences found in the composition of the two phases support the separated exploitation of them.

1. Introduction

The oenological industry produces different kind of residues with a high content in biodegradable compounds. Spain was the second larger wine producer in 2013, according to the International Organisation of vine and wine (OIV), with 50.6 million hL of wine, which also produced as residues or wastes around 3.29×10^5 tons of stems, 9.2×10^5 tons of grape pomace, 3.94×10^5 tons of wine lees and about 28 million tons of wastewaters. Research on the exploitation of these residues/wastes has stated the possibilities of obtaining lactic acid, bio-surfactants, ethanol and other compounds from marcs, vine shoots and wine lees *(1–4)*. Grape skins and grape seeds are also rich in phenolic compounds, generally endowed with antioxidant properties; while imbibed wine from lees (the liquid residue obtained after centrifugation of wine lees) contain a high quantity of tartaric acid, which can be extracted and commercialized *(5–7)*. Moreover, a recent business has emerged in this industry, since lipid antioxidants or dietary supplements are being isolated from grape seed and grape skin extracts for application in foods *(8–10)*. Extracts from wine production are also used in cosmetics as active ingredients, as in the case of hair and skin products, and in the pharmaceutical industry *(11,12)*. The range of interesting compounds obtained from the residues of winemaking has a positive economic impact, since they contribute to the valorization of these residues. However, a long way is still ahead, and the volume of residues generated and not reused is particularly high. In this sense, wine lees are one of the most underexploited residues in the oenological industry.

Wine lees, also known as dregs, are defined by the EEC regulation no. 337/79 as the residue formed at the bottom of recipients containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following filtration or centrifugation of this product. Wine lees are obtained in different decanting steps during the vinification process, and can be found as semi-solid residue constituted by liquid and solid lees. Eventually,

this semisolid residue is centrifuged and dehydrated prior to storage, which is known as wine lees cake, whereas the liquid phase is usually discarded together with the wastewater that, depending on the manufacturer, is processed for alcohol and tartaric salts isolation or treated as an organic residue. Traditionally, aging over lees was a process almost exclusively applied to some white and sparkling wines; however, at present, ageing over lees is a common practice in the red wine production since it gives to wine interesting organoleptic properties such as reduction of astringency and an increased stability of the color. This practice has also a positive influence on the mouth feel and body of wines, as well as on their aroma *(13-14)*.

Lees are mainly composed by microorganisms, particularly yeasts produced during alcoholic fermentation and, in a less proportion, by phenolic compounds, bacteria, remnants from plant cells, inorganic matter and tartaric salts, which are deposited during alcoholic fermentation *(15,16)*. Nevertheless, the composition of wine lees is subjected to a strong variability and depends on the winery procedures. Once the alcoholic fermentation has ended, autolysis of wine yeast starts, which is characterized by degradation of cell walls and releasing of some enzymes and mannoproteins, amino acids and precursors of volatile compounds into wine as well as some phenolic and sulphur compounds that are adsorbed on wine lees *(17,18)*. Chassagne *et al.* established that the adsorption phenomenon is directly linked to the degree of autolysis, ethanol concentration, pH of wine and temperature *(19)*; while Zea *et al.* found differences in the concentration of volatile compounds in cells and musts when different yeast strains were used *(20–22)*.

Up to date, wine lees have been mainly exploited as a source of tartaric acid and calcium tartrate *(15)*; however, some attempts have also been made to use this raw material for feeding cattle, then discarded in view of the extremely poor nutritional value of wine lees *(23,24)*. Additionally, their use as a biosorbent for removal undesirable compounds from wine has also been proposed *(25,26)*, since wine lees have been reported to retain part of the volatile compounds that oak wood transfers to wine, preventing wine from having an excessive, thus

unpleasant woody flavor, as well as diminishing the impact of undesirable compounds such as toxins, pesticides, antifoaming and volatile compounds as 4-ethylphenol and 4-ethylguaiacol, which have been described as responsible for the associated horsy, medicinal and spicy aroma of wines *(25–27)*. From the compositional point of view, some published research has focused on the pigment content of red wine lees, since their intense red coloration suggests a high anthocyanin and anthocyanidin content *(28,29)*. Some other studies have focused on the production of phenolic extracts from wine lees, and the study of their phenolic content and their antioxidant activity *(30,31)*.

The aim of this research was to study and compare the composition profile of wine lees by independent analysis of imbibed wine from lees obtained by centrifugation, and solid dried lees. For this purpose, ethanol–water extracts of solid lees were obtained by microwave assisted extraction (MAE) as previously did Pérez *et al.* and Delgado de la Torre *et al. (29,30)*. Both fractions were subjected to liquid chromatography separation with tandem mass spectrometry detection using a quadrupole time-of-flight (QTOF) hybrid detector. Finally, both fractions were characterized and compared with the aim of studying the potential of these fractions to be recycled together or independently, depending on their composition. Multivariate analysis is a key tool to treat the large data sets provided by the QTOF detector, and to understand the similar/dissimilar patterns among a range of samples from different wineries pertaining to the most characteristic denomination of origin wines in Spain.

2. Material and Methods

2.1. Reagents

Wine lees as semisolid residue were provided in 2010 by eleven Spanish wineries from regions of Spain where different varieties of red grapes are used. The wineries correspond to the two most popular denominations of origin of red wines in Spain, namely, Ribera del Duero and Rioja (see **Table 1**). In all cases,

wine lees were in contact with wine for three months during alcoholic fermentation, after which wine lees were isolated and used for this study. The sample from each winery was homogenized by agitation at room temperature for 15 min and centrifuged at 855 *g* for 5 min. The solid phase was dried at 40 °C for 48 h in an oven, milled in a mortar, sieved to a 0.5 mm particle size and stored at −20 °C until use. The liquid phase was directly stored at −20 °C until use.

Table 1. Samples provided by wineries from two of the most characteristic denominations of origin in Spain.

Code	Grape	Location	Denomination of origin
R1	Tempranillo	Azagra (Navarra)	Rioja
R2	Tempranillo	Haro (La Rioja)	Rioja
R3	Tempranillo	Haro (La Rioja)	Rioja
R4	Tempranillo	San Vicente de la Sonsierra (La Rioja)	Rioja
R5	Tempranillo (Ecological)	Aldeanueva de Ebro (La Rioja)	Rioja
R6	Tempranillo, Garnacha	Gimileo (La Rioja)	Rioja
R7	Mazuelo	Haro (La Rioja)	Rioja
RD1	92% Tempranillo, 3% Cabernet, 5% Merlot	Gumiel de Mercado (Burgos)	Ribera del Duero
RD2	Tempranillo	Langa del Duero (Soria)	Ribera del Duero
RD3	Tempranillo	Fompedraza (Valladolid)	Ribera del Duero
RD4	Tempranillo	Anguix (Burgos)	Ribera del Duero

2.2. Apparatus and Instruments

A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for MAE.

Shaking and centrifugation of wine lees and extracts were carried out by an MS2 minishaker (IKA, Germany) and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

Analysis of the studied compounds was performed in an Agilent 1200 Series LC system interfaced to an Agilent UHD Accurate-Mass QTOF MS/MS detector (Palo Alto, CA), equipped with an Agilent Technology dual electrospray ion source (Dual ESI).

2.3. Sample Preparation

Six g of dried lees was placed into the extraction vessel of the microwave-assisted digestor with 50 mL of 60:40 (v/v) ethanol–water mixture adjusted to pH 4 with formic acid. The vessel was positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 140 W irradiation power for 10 min, after which the solid residue was removed by centrifugation and the liquid fraction filtered by a 20 μm pore size filter and 1:2 diluted with 60:40 (v/v) ethanol–water adjusted to pH 4 with formic acid.

2.4. LC–QTOF MS/MS Analysis

Chromatographic separation was performed using a C18 Inertsil ODS-2 column (250 mm×4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), kept at 25 °C. Mobile phases in this case were water (phase A) and ACN (phase B), both LC–MS/MS grade and with 0.1% formic acid as ionization agent. The LC pump was programmed at a flow rate of 0.9 mL/min, and the following elution gradient was carried out: held from 0–2 min 4% phase B, 2–7 min 10% phase B, 7–60 min 100% phase B and held up to min 70. A post run of 4 min was included to equilibrate the column. The injection volume was 10 μL, and the injector needle was rinsed five times with 70% methanol. Furthermore, the needle seat back was flushed for 12 s at a flow rate of 4 mL/min with 70% methanol to clean it. The operating conditions of the mass spectrometer were as follows: gas temperature, 325 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3500 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; and focusing voltage, 175 V. MS and MS/MS data were collected in both polarities using the centroid and the profile modes at a rate of 2 spectrum

per second in the extended dynamic range mode (2 GHz). Accurate mass spectra in auto MS/MS mode were acquired in MS *m/z* range 100–3000 and MS/MS *m/z* range 100–3000. The auto MS/MS mode was configured with 2 maximum precursors per cycle and an exclusion window of 0.25 min after two consecutive selections of the same precursor. The collision energy selected was 22.5 eV. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by the use of signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in positive ionization mode, while ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.0007 (formate adduct of HP-921) were used in negative ionization mode.

2.5. Data treatment

The MassHunter Workstation software package (B.05.00) and MassHunter Profinder (B.06.00) from Agilent Technologies was used to process all data obtained by LC–QTOF in the auto MS/MS mode. The recursive feature extraction algorithm was used to extract and align potential molecular features in all the injections set. This algorithm initially deconvolutes the chromatograms and aligns the molecular features across the selected raw data files in terms of *m/z* value and retention time; then, it uses the mass and retention time of each feature for recursive targeted feature extraction in the raw data files. This two-step procedure reduces the number of both false negatives and false positives in the process of feature extraction. The target parameters for feature extraction included a threshold of 1500 counts and a maximum charge state of 2. In addition, the isotopic distribution for a valid feature had to be defined by two or more ions —with a peak spacing tolerance of 0.0025 *m/z*, plus 10.0 ppm. Adduct formation in the positive (+H, +Na) and negative ionization mode (–H, +HCOO) was also used to identify features of the same molecule. Features were aligned by using a tolerance window of 0.30 min and a mass accuracy of 10 ppm for retention times and *m/z* values, respectively, across all data files. The minimum absolute height required for feature extraction was set at 3000 counts, which was

also used for 100% of samples in the recursive step. Thus, ions with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as molecular features (MFs) or entities characterized by RT, intensity in the apex of the chromatographic peak, and accurate mass.

The resulting data files after this treatment were created as comma separated value files (.csv) for each sample and exported into the Mass Profiler Professional (MPP) software package (version 2.0, Agilent Technologies) for further processing. Compound identification was performed using the METLIN Metabolite and MS/MS (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp/), MetFrag (http://msbi.ipb-halle.de/MetFrag/) and PlantCyc (http://plantcyc.org/) databases.

3. Results and Discussion

3.1. Data Pretreatment

Wine lees are a complex matrix containing mainly polar and mid-polar compounds, as revealed by the LC–MS/MS elution profiles obtained in the positive and negative ionization modes. The elution profiles represented by the base peak chromatograms (BPCs) show the predominance of polar compounds in imbibed wines from lees in both ionization modes, where the peaks were found almost exclusively in the elution time window from 1 to 28 min, while the extracts from the solid residues provided peaks characterized by the presence of less polar compounds, as most of the peaks were associated to compounds eluted at longer times (elution time window from 1 to 60 min), which support the adsorbent role of this residue during wine ageing.

With the aim of ensuring the representativeness of the study, the data set obtained after alignment of molecular features was filtered to select a subdata set formed by the molecular features in all the samples of liquid and solid lees. This means that the data sets should contain molecular features present in all the samples analyzed for each class. This data mining operation reduced the data sets up to 90 and 68 molecular entities for imbibed wine from lees and extracts, respectively, in the positive ionization mode, and 34 and 67 molecular entities in the negative ionization mode. This pretreatment ended with mean-centering of the data set prior to statistical analysis. As the alignment diagram in **Figure 1** shows, the positive ionization mode reported a data set formed by molecular

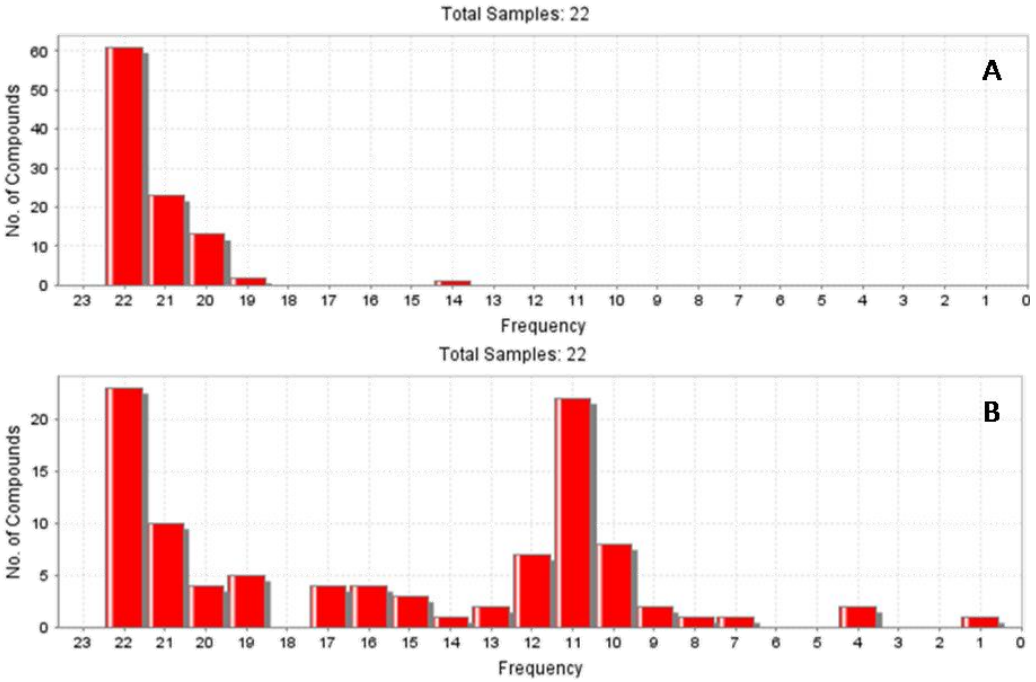


Figure 1. Alignment diagram corresponding to positive (A) and negative ionization modes (B).

features detected in most samples. On the other hand, the profile of molecular features in negative ionization mode allowed deducing higher variability than in positive mode since a representative proportion of molecular entities were

detected in 50% of the samples. This high proportion of entities detected in half of the samples should be assigned to features detected exclusively in liquid or solid dried lees; therefore, to differences in their composition.

3.2. Multivariate Analysis

The next step was the comparative analysis of samples pertaining to both classes of samples, imbibed wine from lees and extracts of dried lees. Qualitative comparison was carried out by application of Venn diagrams that allow establishing the overlapping in compositional terms between both types of samples. The diagrams in **Figure 2** show a high similarity between the molecular entities found in imbibed wine from lees and in the extracts of solid dried lees in positive ionization mode, since 61 features were common to the two types of samples, while 7 and 29 features were exclusively detected in the extracts and imbibed wine from lees, respectively. Concerning the negative ionization mode (**Figure 2.B**), the Venn diagram agreed with the alignment diagram (**Figure 1.B**) about the observed variability in the composition of extracts and imbibed wine from lees. Thus, only 23 entities were common to the two types of samples, while 44 entities were exclusively detected in extracts from the solid residue. The different behavior in positive and negative ionization could be explained by the influence of the chemical structure on the ionization efficiency. Therefore, it is evident that data sets from the two ionization modes should include molecular features corresponding tentatively to different compounds, which are preferentially ionized in one mode.

Comparison of the compositional profiles of both fractions was made in terms of no supervised analysis using principal component analysis (PCA) to evaluate the distribution of the samples according to the variability sources. **Figure 3** shows the PCA scores plot obtained for imbibed wine from lees and extracts of dried lees after data pre-treatment. As can be seen, both ionization modes led to similar results since a clear discrimination was observed between the samples corresponding to imbibed wine from lees and extracts from dried solid wine lees. In negative ionization mode, PC1/PC2/PC3 explained 54.1% of

the total variability, while in positive mode the variability explained by the three first components increased up to 61.7%. In both cases, discrimination between the two types of samples was ascribed to PC1. Therefore, it is evident that the composition of both separated materials was quite different. Apart from this discrimination, no trends were observed according to the origin of the wineries such as designation of origin or localization of wineries.

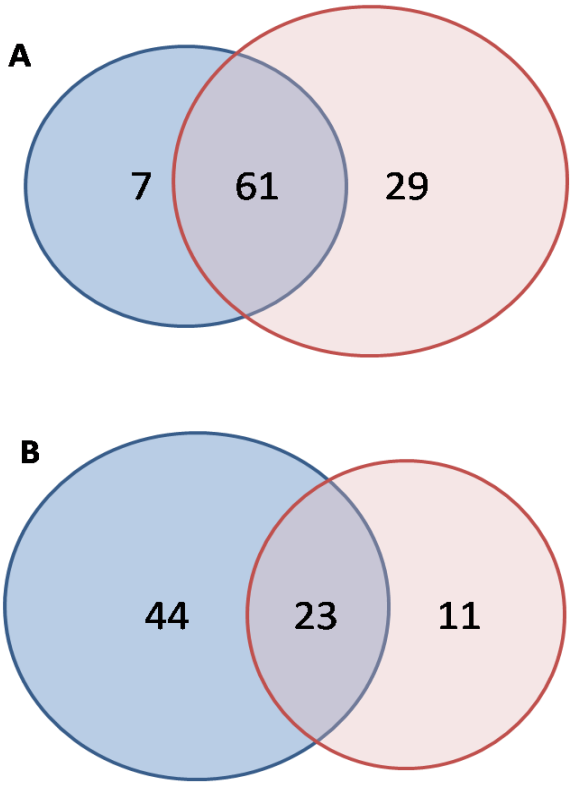
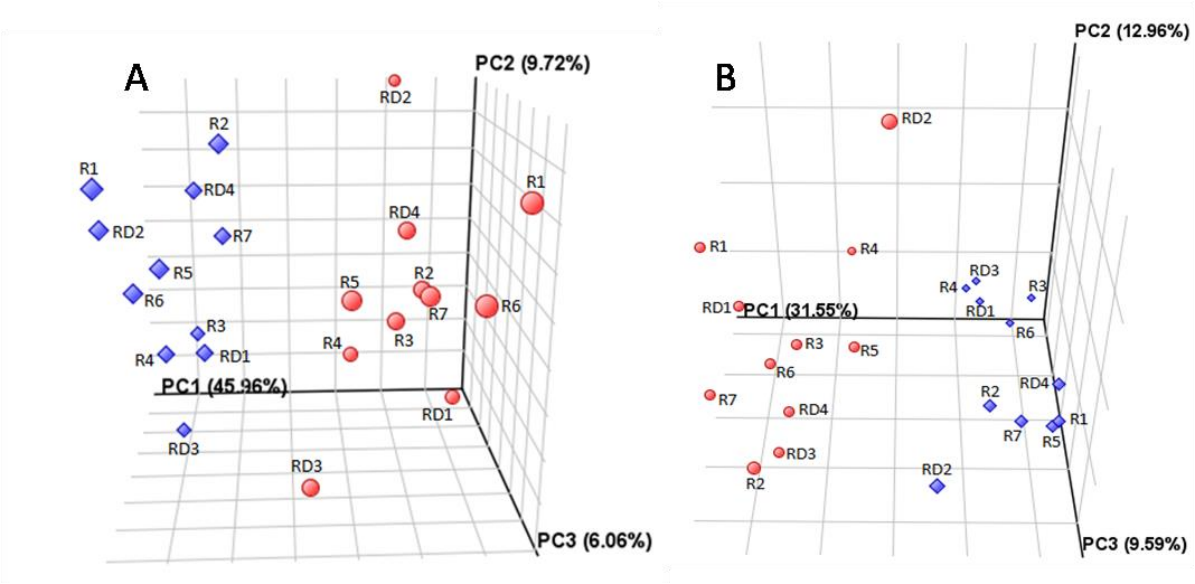


Figure 2. Venn diagrams obtained by qualitative comparison of the molecular entities for imbibed wine from lees (right side) and extracts of dried lees (left side) after application of a 100% frequency filter in the positive (A) and negative ionization modes (B).



Note: R1-R7: Rioja; RD1-RD4: Ribera del Duero denomination of origin.

Figure 3. PCA scores plot obtained for imbibed wine from lees (diamonds) and extracts of dried lees (circles) using the data set formed after data pretreatment for the positive (A) and the negative (B) ionization modes.

3.3. Identification of Compounds in Positive Ionization Mode

Characterization of representative families of compounds in the samples was carried out by tandem mass spectrometry in high resolution mode. Tentative identification of compounds was supported on mass accuracy measurement of the precursor ion and representative product ions. The isotopic distribution of signals detected in full-scan mode was also taken into account. The combination of these data allowed tentatively elucidating the structure of the compounds listed in **Table 2**, which are organized by families showing information about theoretical and experimental *m/z* values as well as characteristic fragment ions used to support identification.

Concerning the positive ionization mode, it is worth emphasizing the presence of compounds identified exclusively in imbibed wine from lees, those

only identified in extracts of dried lees and common to both fractions. Few amino acids were identified in wine lees. Thus, only one primary amino acid was detected exclusively in imbibed wine from lees in this polarity; aminocaproic acid, an analogue of the amino acid lysine. Other two compounds exclusively identified in imbibed wine from lees were 8-oxo-guanosine monophosfate and ureidoglycolate. The former results by degradation of 8-oxo guanosine triphosphate, produced in nucleic acids during cellular metabolic processes and responsible for eliminating oxidized nucleotides in plants, thus preventing replicational and translational errors by misincorporation of 8-oxo guanosine triphosphate into DNA and RNA, which would cause mutation and phenotypic suppression *(32,33)*. On the other hand, ureidoglycolate is involved in purine metabolism, and also in metabolic pathways and microbial metabolism in diverse environments. Particularly, it is present in Saccharomyces cerevisiae, where it is produced by degradation of allantoin with the concomitant production of urea *(34)*.

Concerning to compounds found exclusively in extracts from dried lees, only two of them were tentatively identified as phenolic compounds, namely: 3,4,5-trimethoxyflavone and malvidin 3-(6-*p*-coumarylglucoside). The former elutes at 8.0 min with *m/z* ratio at 335.0890 as precursor ion, which corresponds to the [M+Na]+ adduct. The most abundant fragments were at *m/z* 298.0187, 280.0701 and 252.0822, which is consistent with the results obtained by Huck *et al.* and explained by the loss of a CH3 group from the original structure (−15 Da), the subsequent loss of a molecule of water (−18 Da), and the loss of a carbonyl group (−28 Da) *(35)*. Malvidin 3-(6-*p*-coumarylglucoside) was found at 24.6 min, with [M+H]+ as precursor ion at *m/z* 639.1706. The consecutive loss of the coumaroyl and glucoside moieties explains the characteristic fragments found at *m/z* 477.1248 and 331.0786, respectively, where the fragment at 331.0786 corresponds to the malvidin aglycone.

Table 2. Compounds exclusively found in imbibed wine from lees, extracts from dried lees and common compounds identified by LC–QqTOF MS/MS analysis.

Compound	*m/z*	RT (min)	Formula	Species	Error (ppm)	Mass	Fragments
Primary amino acids							
3-Hydroxy-L-tyrosine[C]	196.0618	3.27	$C_9H_{11}NO_4$	$[M-H]^-$	1.5	197.0688	152.0731 135.0391 119.0042
L-Valine[C]	118.0857	6.80	$C_5H_{11}NO_2$	$[M+H]^+$	3.1	117.0793	–
Aminocaproic acid[A]	132.1019	7.21	$C_6H_{13}NO_2$	$[M+H]^+$	0	131.0946	115.0046
L-Tyrosine octadecyl ester[B]	432.3442	40.56	$C_{27}H_{47}NO_3$	$[M-H]^-$	6.5	433.3556	–
Phenolic compounds							
Kaempferol 3-(2',3'-dyacetylrhamnoside)-7'-rhamnoside[A]	661.1820	3.69	$C_{31}H_{34}O_{16}$	$[M-H]^-$	–6.9	662.1847	601.1626 175.0260 131.0372
3,4,5-Trimethoxyflavone[B]	335.089	7.97	$C_{18}H_{16}O_5$	$[M+Na]^+$	0	312.0998	298.0187 280.0701 252.0822
Gallic acid[B]	169.0142	11.40	$C_7H_6O_5$	$[M-H]^-$	0	170.0215	125.0281 107.0159
Pelargonidin 3-(6-*p*-coumaryl)glucoside[C]	578.1424	16.25	$C_{30}H_{27}O_{12}$	$[M-H]^-$	1.1	579.1497	289.0687 407.0733 125.0229
Procyanidin B2[C]	577.1357	17.47	$C_{30}H_{26}O_{12}$	$[M-H]^-$	–1.0	578.1419	425.0932 407.0767 289.0731
Epicatechin[C]	291.0859	21.13	$C_{15}H_{14}O_6$	$[M+H]^+$	-1.4	290.0790	147.0437 139.0387 123.0443
Malvidin 3-galactoside[B]	492.1252	18.16	$C_{23}H_{25}O_{12}$	$[M-H]^-$	4.3	493.1346	329.0645 313.0353 299.0128
Peonidin 3-O-glucoside[C]	463.1232	20.96	$C_{22}H_{23}O_{11}$	$[M+H]^+$	2.6	463.1240	301.0723
Catechin[C]	291.0856	17.49	$C_{15}H_{14}O_6$	$[M+H]^+$	2.4	290.0790	147.0442 139.0385 123.0410
Quercetin 3-glucuronide[B]	477.0638	23.24	$C_{21}H_{18}O_{13}$	$[M-H]^-$	2.4	478.0747	300.9973 151.0065 178.9983
Malvidin 3-(6-*p*-coumarylglucoside)[B]	639.1706	24.59	$C_{32}H_{30}O_{14}$	$[M+H]^+$	0	638.1636	477.1248 331.0786 229.1407
Myricetin[B]	317.0301	26.62	$C_{15}H_{10}O_8$	$[M-H]^-$	0.6	318.0376	179.0020 151.0070 137.0276
Quercetin[B]	301.0354	29.81	$C_{15}H_{10}O_7$	$[M-H]^-$	5.2	302.0427	178.9996

							151.0049
							121.0293
Others							
Glicerophosphocholine[B]	258.1104	3.37	$C_8H_{20}NO_6P$	$[M+H]^+$	−1.2	257.1028	184.0723
							124.9994
							104.1067
Hexanethiol[C]	119.0977	3.51	$C_6H_{14}S$	$[M+H]^+$	−7.4	118.0816	107.9774
2-Carboxy-2,3-dihydro-5,6-dihydroxyindole[A]	194.0400	3.62	$C_9H_9NO_4$	$[M-H]^-$	3.0	195.0532	129.0190
							113.0258
							101.0238
Tartaric acid[C]	149.0096	4.57	$C_4H_6O_6$	$[M-H]^-$	−2.7	150.0164	132.6965
							105.0172
2,3-Dihydroxy-3-methylbutanoate[C]	135.0543	6.41	$C_5H_{10}O_4$	$[M+H]^+$	7.8	134.0579	119.0429
							107.0455
Norfuraneol[C]	115.0392	7.73	$C_5H_6O_3$	$[M+H]^+$	−3.5	114.0317	–
Hypoxantyne[C]	137.0444	8.48	$C_5H_4N_4O$	$[M+H]^+$	9.2	136.0385	119.0348
							110.0346
6-Imino-5-oxociclohexa-1,3-dienecarboxylate[B]	152.0331	9.63	$C_7H_5NO_3$	$[M+H]^+$	7.2	151.0269	106.0607
							118.0605
							135.0048
Citric acid[A]	191.0204	9.21	$C_6H_8O_7$	$[M-H]^-$	−3.7	192.027	129.0239
							111.0130
8-Oxo-guanosine-monophosphate[A]	380.0413	11.22	$C_{10}H_{12}N_5O_9P$	$[M+H]^+$	5.6	379.0529	145.9649
							161.0066
							133.0065
1,2-Benzoquinone[A]	109.0285	13.47	$C_6H_4O_2$	$[M+H]^+$	−0.9	108.0211	–
Kasugamycin[A]	362.1589	13.70	$C_{14}H_{25}N_3O_9$	$[M+H-H_2O]^+$	−7.2	379.1591	230.1133
							201.1223
							131.1144
S-Adenosyl-methioninamine[A]	354.1451	14.23	$C_{14}H_{23}N_6O_3S$	$[M-H]^-$	8.2	355.1552	255.0878
							128.0394
Glutathione[B]	306.0783	14.37	$C_{10}H_{17}N_3O_6S$	$[M-H]^-$	−5.9	307.0838	272.0865
							244.0236
							128.0370
5-Nitro-furancalboxaldehyde[B]	244.0540	15.26	$C_7H_9N_3O_4$	$[M+FA-H]^-$	−8.6	199.0593	124.0146
2-Formamido-N1-5-phospho-D-ribosyl-acetamidine[C]	314.0768	15.32	$C_8H_{16}N_3O_8P$	$[M+H]^+$	−6.4	313.0675	136.0600
							153.0146
							107.9478
Ureidoglicolate[A]	135.0436	16.75	$C_3H_6N_2O_4$	$[M+H]^+$	−2.7	134.0362	107.0524
							118.9161
							104.0001

Cytosine[B]	110.0635	16.83	$C_4H_5N_3O$	$[M-H]^-$	−4.3	111.0433	–
Phenolic steroid[A]	257.1896	18.77	$C_{18}H_{24}O$	$[M+H]^+$	1.5	256.1724	179.9834 144.1028 129.0588
Isoquinoline n-oxide[A]	146.0597	19.05	C_9H_7NO	$[M+H]^+$	2.1	145.0528	128.0465 118.0670
Benzamide[B]	120.0433	23.15	C_7H_7NO	$[M-H]^-$	−6.6	121.0528	104.0268
5'-Deoxy-5-fluorocytidine[B]	244.0758	25.18	$C_9H_{12}FN_3O_4$	$[M-H]^-$	−7.8	245.0812	201.0561 159.0452
3-Hydroxy-6-nonanoyl CoA[B]	906.2267	31.67	$C_{30}H_{52}N_7O_{17}P_3S$	$[M-H]^-$	1.4	907.2653	811.2179 359.0944

Note: [A]compounds found exclusively in imbibed wine from lees, [B]compounds found exclusively in extracts, [C]representative compounds common to both fractions.

Valine was the only primary amino acid found in all the samples analyzed in this polarity. It was found at 6.8 min elution time when the *m/z* ratio at 118.0857 was monitored as [M+H+] precursor ion. Two phenolic compounds were tentatively detected in imbibed wine from lees and extracts from the solid residue: two flavanol isomers, catechin and epicatechin, naturally found in diverse plants such as cocoa, tea, apples and berries, among others. Catechin and epicatechin elute at 17.5 and 21.1 min, respectively, with [M+H]+ precursor ions at *m/z* 291.0856. Representative fragments supporting the tentative identification of both isomers were detected at *m/z* 147.0442, 139.0385 and 123.0410. According to Li *et al.*, the fragment at 147.0442 is explained by heterocyclic ring fission and loss of a water molecule; the fragment at 139.0385 is produced by retro Diels-Alder (RDA) reaction of the original molecule and, that at 123.0410 can be explained either by direct benzofuran forming fission (BFF) or by loss of a water molecule following BFF *(36)*.

Other compounds tentatively identified in the positive mode in both types of samples were hypoxantyne, norfuraneol, 2-formamido-N1-5-phospho-D-ribosyl-acetamidine, hexanethiol and 2,3-dihydroxy-3-methylbutanoate. The presence of all them was verified by using the Plantcyc database (www.plantcyc.org). Hypoxanthine is a naturally occurring purine derivative and

a reaction intermediate in the metabolism of adenosine and in the formation of nucleic acids. It elutes at 8.5 min with a [M+H]+ precursor ion at 137.0444. The MS/MS fragmentation of this precursor ion produces two characteristic fragments, one at *m/z* 119.0348 by the loss of a molecule of water (–18 Da) from the original structure and another fragment at 110.0346 by the loss of a CO unit (–28 Da). Norfuraneol is known for being one of the caramel descriptors in wine, together with furaneol and homofuraneol, as supported by Sarrazin *et al. (37)*. This compound was tentatively identified eluting at 7.7 min by monitoring the *m/z* ratio at 115.0392 as [M+H]+ precursor ion, with two representative product ions at *m/z* 113.0244 and 110.9751.

2,3-Dihydroxy-3-methylbutanoate was identified by a [M+H]+ precursor ion at *m/z* 135.0543, and with representative fragments at *m/z* 119.0429 produced by the loss of an oxygen unit (–16 Da) and 107.0455 produced by the loss of a CO unit from the original structure (–28 Da). According to the Yeast Metabolome Database (www.ymdb.ca), 2,3-dihydroxy-3-methylbutanoate is present in *Saccharomyces cerevisiae*, one of the yeast more widely used for winemaking, baking and also brewing. According to Morata *et al.*, the use of different strains of *Saccharomyces cerevisiae* can have a strong influence on the content of wine in different compounds such as vitisin A and B, pyruvate and acetaldehyde, among others *(38)*.

3.4. Identification of Compounds in Negative Ionization Mode

As **Table 2** shows, only one amino acid derivative and one phenolic compound were tentatively identified as exclusively present in imbibed wine from lees in the negative ionization mode: namely 3-hydroxy-L-tyrosine and kaempferol 3-(2',3'-dyacetylrhamnoside)-7''-rhamnoside. On the other hand, compounds exclusively detected in imbibed wine from lees were citric acid, S-adenosylmethioninamine and 2-carboxy-2,3-dihydro-5,6-dihydroxyindole. Citric acid is commonly added during the winemaking process to improve the sensory characteristics of wine by increasing acidity to complement a specific flavor or prevent ferric hazes *(39)*. This acid was found at 9.2 min with a $[M-H]^-$

precursor ion at *m/z* 191.0240. The MS/MS spectrum associated to this compound presented characteristic fragments at 129.0239 and 111.0130, interpreted by the conversion of citric acid into itaconic acid by decarboxylation following the loss of a molecule of water to form the ion at *m/z* 111.0130. On the other hand, S-adenosylmethioninamine and 2-carboxy-2,3-dihydro-5,6-dihydroxyindole are related to the methionine and tyrosine metabolic pathways, respectively. The former is a precursor of spermine and spermidine, which are biogenic amines undesirable in wine *(40,41)*.

Regarding to compounds exclusively detected in the extracts of wine lees, one other derivative of tyrosine is octadecyl ester, found at 40.6 min with a representative [M–H]– precursor ion at *m/z* 432.3442.

It should be highlighted the abundance of phenolic compounds tentatively identified in the extracts of dried lees in negative ionization, which is justified by the adsorbent power of wine lees during ageing. As shown in **Table 2**, most of them are flavonoids, widely spread in wine and grapes, such as quercetin, myricetin and conjugates such as malvidin-3-galactoside.

Quercetin was identified at 29.8 min with a characteristic $[M–H]^-$ precursor ion at *m/z* 301.0354, and with MS/MS fragments at 178.9996, 151.0049 and 121.0293 (**Figure 4.A**). The fragment at *m/z* 151.0049 is produced by RDA reaction of the A ring and is a common product ion from flavonols, flavanones and flavones. The fragment at *m/z* 121.0297 is produced by retrocyclation of the original structure of quercetin, with the mechanism proposed by Dubber *et al. (42)*. Other representative fragment for quercetin used for its identification is the ion found at *m/z* 107.0137, product of the cleavage of a CO_2 unit after RDA reaction takes place $[M–H–CO–CO_2]^-$. Quercetin was detected in free form, but also as a glucuronide derivative at 23.2 min showing a $[M–H]^-$ precursor ion at *m/z* 477.0638, as shown in **Figure 4.B**, with a representative ion at *m/z* 300.9973 produced by the loss of the glucuronide moiety to form the corresponding aglycone.

Following a similar pattern, **Figure 4.C** shows the MS/MS fragmentation pattern of malvidin 3-galactoside, eluted at 18.2 min with precursor ion $[M–H]^-$ at *m/z* 492.1252. The spectrum was marked by the presence of three representative product ions at *m/z* 329.0645, 313.0353 and 299.0128, where the ion at 329.0645 corresponds to the malvidin aglycone and the ion at 313.0353 is produced by the complete loss of the galactoside moiety (–179 Da).

Gallic acid was also tentatively detected in lees. This non-flavonoid phenolic compound is a final product in the hydrolysis of ellagitannins, which contribute to the astringent character of wines. Gallic acid elutes at 11.4 min as $[M–H]^-$ precursor ion at *m/z* 169.0196. Its fragmentation produces representative product ions at *m/z* 107.0159 and 125.0281, as shown in **Figure 4.E**, where the ion at *m/z* 125.0281 is explained by decarboxylation, and the ion at 107.0159 is produced by the subsequent loss of a OH unit (–17 Da).

Additionally, two phenolic compounds were tentatively found in the two samples from all wineries, procyanidin B2 and pelargonidin 3-(6-p-coumaroylglucoside). Procyanidin B2 reported a $[M–H]^-$ precursor ion at *m/z* 577.1357, with representative fragments at *m/z* 407.0767, 289.0731 and 425.0932. This fragmentation pattern is consistent with the scheme proposed by Delgado de la Torre *et al.,* where fragments at *m/z* 271.0 and 289.0 were explained by a quinone methide (QM) cleavage of the interflavan bond, while those at *m/z* 407.0 and 425.1 would correspond to the RDA reaction *(29)*.

Apart from these phenols, tartaric acid was also found in the two fractions of wine lees; a logical finding since this is one of the most concentrated organic acids in grapes and it plays a fundamental role in wine acidity. Tartaric acid was detected at 4.6 min, with a $[M–H]^-$ precursor ion at *m/z* 149.0086, and representative fragments at *m/z* 130.9981 and 105.0172. The fragment at *m/z* 130.9981 is explained by the loss of an OH group and the fragment at 105.0172 is obtained by the loss of a CO unit.

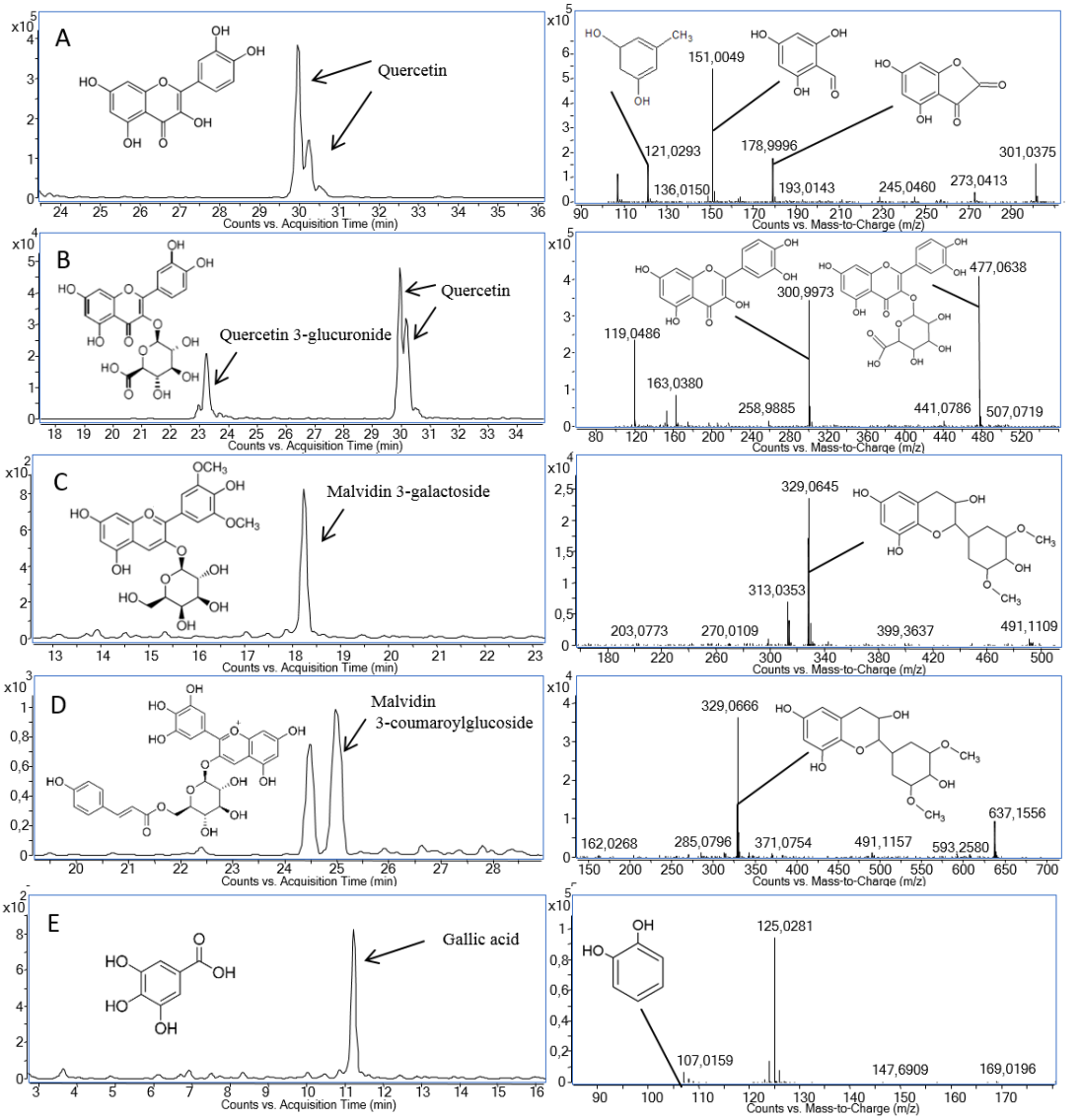


Figure 4. Extracted ion chromatograms for quercetin (A), quercetin 3-glucuronide (B), malvidin 3-galactoside (C), malvidin 3-(6-*p*-coumarylglucoside) (D), and gallic acid (E), with MS/MS spectra obtained by LC–MS/MS analysis in negative ionization mode.

Attending to these results, it can be concluded that both fractions imbibed wine from lees and extracts from dried lees can be considered an exploitable source for various interesting compounds, where the former proved to be rich in primary amino acids and phenolic compounds, especially flavonoids,

which can be ascribed to its adsorbent characteristics. On the other hand, a similar number of other uncommon compounds has been identified in both fractions, thus selection of a fraction for its exploitation would depend on the target product, since, for instance, citric acid was only found in imbibed wine from lees. Based on the number of compounds present in both fractions and those existing only in solid lees, this residue is proposed as the most adequate sample for exploitation, since the absence of water facilitates transport and storage. This study complements previous studies on wine lees and other oenological by-products.

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Chapter 10:

Anthocyanidins, proanthocyanidins and anthocyanins profiling in wine lees by solid-phase extraction–liquid chromatography coupled to electrospray ionization tandem mass spectrometry with data–dependent methods

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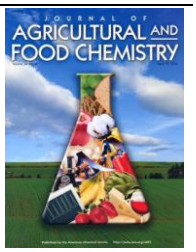

Anthocyanidins, proanthocyanidins and anthocyanins profiling in wine lees by solid-phase extraction–liquid chromatography coupled to electrospray ionization tandem mass spectrometry with data-dependent methods

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Abstract

A method has been developed to study the content of anthocyanidins, proanthocyanidicins and anthocyanins in wine lees, an abundant by-product from wineries. Detection/quantitation of the target compounds was carried out by a hyphenated system consisting of a solid-phase extraction workstation (Prospekt-2 unit) on-line coupled to a liquid chromatograph–triple quadrupole tandem mass spectrometer (LC–MS/MS), where standards were used for identification/quantitation of both anthocyanidins and proanthocyanidins. Owing to the lack of anthocyanins standards, advantages from the use of data-dependent methods were taken for their identification and confirmatory analysis. The combination of the scanning methods (*viz.* product-ion, precursor-ion and neutral-loss scanning), allowed identifying five different anthocyanins present in wine residues. The results thus obtained have been validated by complementary analysis of the extracts using LC–TOF/MS in high resolution mode. Quantitation of the monitored compounds was supported on selected reaction monitoring (SRM) and calibration curves run with standards of anthocyanidins and pro-anthocyanidins.

1. Introduction

A general trend in the last decade was the development of new nutraceuticals and supplemented foods based on extracts or compounds obtained from diverse natural sources as fruits or vegetables. Anthocyanins are a representative example of this trend as an onset nutraceutical industry is presently interested in this family of compounds because of their antioxidant properties and beneficial effects reported in the literature. These compounds have been featured with anti-carcinogenic *(1)* and anti-inflammatory activity *(2)*, with a significant role in prevention of cardiovascular diseases and diabetes *(3)*. This role is essentially based on their chemoprotective, vasoprotective *(4)* and free radical scavenging effects *(5)*, among others, most of them directly related to the flavilium ion with electron deficiency *(6)*. Apart from that, anthocyanins apparently are a suited option as food colorants because they are non-toxic, water soluble, and easily obtained from natural sources. All these benefits have promoted the development of studies on characterization of these compounds with the aim of replacing artificial colorants, the negative side-effects of which —such as attention deficit hyperactivity disorder in children *(7,8)* and carcinogenic activity, as is the case with Red Sudan colorants *(9)*— have been reported.

Most studies dealing with the analysis of these natural pigments have been focused on their extraction from non-valuable agricultural raw materials, which would involve their valorization. Present research on raw materials from the winemaking industry is mainly targeted at quantitation of anthocyanins and anthocyanidins from grapes (skin and pomace) *(5)*; but also research on anthocyanins encompasses wine itself *(10)*, where the anthocyanin content has demonstrated to be representative of each wine, thus allowing discrimination of adulterated wines *(11)*.

On the contrary, wine lees —the solid residue remaining at the bottom of reservoirs after wine fermentation— have received less attention. Despite their variable composition, wine lees are mainly composed by microorganisms

(particularly yeasts) and, in a less proportion, by tartaric acid and inorganic matter. In red wine production, the intense red color of wine lees suggests that this agricultural waste could have an added value as source of colorants. In fact, most of the research on wine lees is focused on adsorption of anthocyanins by yeasts, being of special interest the studies by Mazauric *et al.* in 2006 *(12)*, and Morata *et al.* in 2003 *(13),* who simulated aging of a model wine over lees to prove that about one third of the total content of free anthocyanins in wine was removed by lees after one week.

Methods for determination of anthocyanins are mainly based on liquid chromatography (LC) with detection by molecular absorption (usually a diode array detector —DAD), where identification is primarily based on UV–Vis spectra or retention times by comparison with standards *(14)*. However, DAD detection is unable to discriminate between co-eluted compounds, which is specially critical here taking into account the isomeric character of anthocyanins. In this sense, it is worth mentioning the research by Pati *et al.* in 2009, who developed a selective method for screening of anthocyanins and dimmers in crude grape extracts with the aid of LC–DAD–MS/MS *(15)*.

Additionally, quantitation of these compounds is a difficult task owing to the absence of commercially available standards for most of them. Two different strategies based on mass spectrometry (MS) can be used to overcome this problem. One is the utilization of a high resolution mass spectrometer such as the time-of-flight (TOF) detector, which supports identification by mass accuracy detection of precursor and product ions. Papoušková *et al. (16)* used a UPLC–QqTOF MS/MS configuration to study the anthocyanin profile of 68 certified red wines and stated this analytical arrangement as a conclusive tool for anthocyanin profiling. The second strategy takes benefits from the triple quadrupole mass detector to confirm the presence of known compounds by application of data-dependent methods. Among these methods, product-ion scanning enables acquisition of MS2 spectra, which provide with information on product ions formed by activation of a selected precursor ion. Other approaches are precursor-ion scanning and neutral-loss scanning, which are complementary

tools to screen the presence of specific compounds in complex matrices *(17)*. Precursor-ion scanning is useful to detect all precursor ions that release known product ions, while neutral-loss scanning allows detection of compounds with a given *m/z* difference between precursor ions and product ions (neutral loss), which should be representative of a characteristic fragmentation. These approaches, used for screening of anthocyanins in grapes and red wine varieties, have allowed identification of compounds such as monoglucoside and diglucoside derivatives as well as other conjugates *(17,19,20)*. In this research, a commercial automated solid-phase extraction (SPE) system coupled on-line to LC–MS/MS was selected to develop a method for detection and quantitation of anthocyanins, anthocyanidins and proanthocyanidins in wine lees and in the extracts from dried lees. To set the concentration range of the target compounds in this vinification residue, samples from eight winemakers located in different points in Spain were used. Application of data-dependent methods enabled to confirm the presence of anthocyanins in the target samples. Finally, validation of identifications was supported on complementary analysis by LC–TOF/MS.

2. Materials and Methods

2.1. Samples

Wine lees obtained as semisolid residue decanted after alcoholic fermentation of grape juice were provided by eight different wineries, where common Spanish grape varieties were used, namely: Cune (Tempranillo), López de Heredia (Mazuelo, Tempranillo, Graciano, Garnacha), La Unión (Syrah, Tempranillo), Ramírez de la Piscina (Tempranillo), Selección de Torres (Tempranillo), Torres de Anguix (Tempranillo), Real Sitio de Ventosilla (Tempranillo, Cabernet Franc, Merlot) and Viña Hermosa (Tempranillo, Garnacha). Each sample was homogenized by agitation at room temperature for 15 min and centrifuged at 855×*g* for 5 min to isolate the liquid fraction. The "dried solid phase" was the solid resulting from centrifugation and drying at 30ºC

for 48 h in an oven, milled in a mortar, sieved to a 0.5 mm particle size and stored at 4 ºC until use. Pools of the liquid and solid phases from the different varieties of wine lees were prepared by mixing aliquots of them. These two pools were used for optimization and validation of the method.

2.2. Standards and Solvents

Cyanidin chloride (Cy), delphinidin chloride (Dp), peonidin chloride (Pn), petunidin chloride (Pt), malvidin chloride (Mv), procyanidin B1 (PB1), procyanidin B2 (PB2) and procyanidin A2 (PA2) were from Extrasynthese, (Barcelona, Spain). A standard solution of each anthocyanidin was prepared in ethanol at 1000 μg/mL (pH=3).

Methanol, acetonitrile and formic acid (MS grade) were from Scharlab (Barcelona, Spain). Deionized water (18 mΩ•cm) was obtained by a Milli-Q water purification system from Millipore (Billerica, MA, USA). All chemicals were LC-grade and used without further purification.

2.3. Apparatus and Instruments

A Microdigest 301 digestor of maximum power 200 W (Prolabo, Paris, France) furnished with a microprocessor programmer (Prolabo) to control the microwave unit was used for microwave-assisted extraction.

Shaking and centrifugation of wine lees and extracts were carried out by an MS2 minishaker (IKA, Germany) and a Mixtasel (Selecta, Barcelona, Spain) centrifuge, respectively.

The on-line SPE–LC–MS/MS system was configured by an automated Prospekt-2 SPE workstation from Spark Holland (Emmen, the Netherlands) coupled to an Agilent 1200 Series LC system from Agilent Technologies (Palo Alto, USA) 6460 Triple Quad LC–MS detector equipped with a Jet Stream Technology electrospray ion source, also from Agilent. The SPE workstation comprises a unit for SPE cartridge exchange, an automatic cartridge exchanger, and a high pressure syringe dispenser for SPE solvent delivery, as shown in

Figure 1. The automated system was also coupled to a Midas autosampler furnished with a 200 µL sample loop. Peek tubing of 0.25 mm i.d. from VICI (Houston, TX, USA) was used for all connections between valves. The SPE step was fully automated via the Sparklink v.2.10 software.

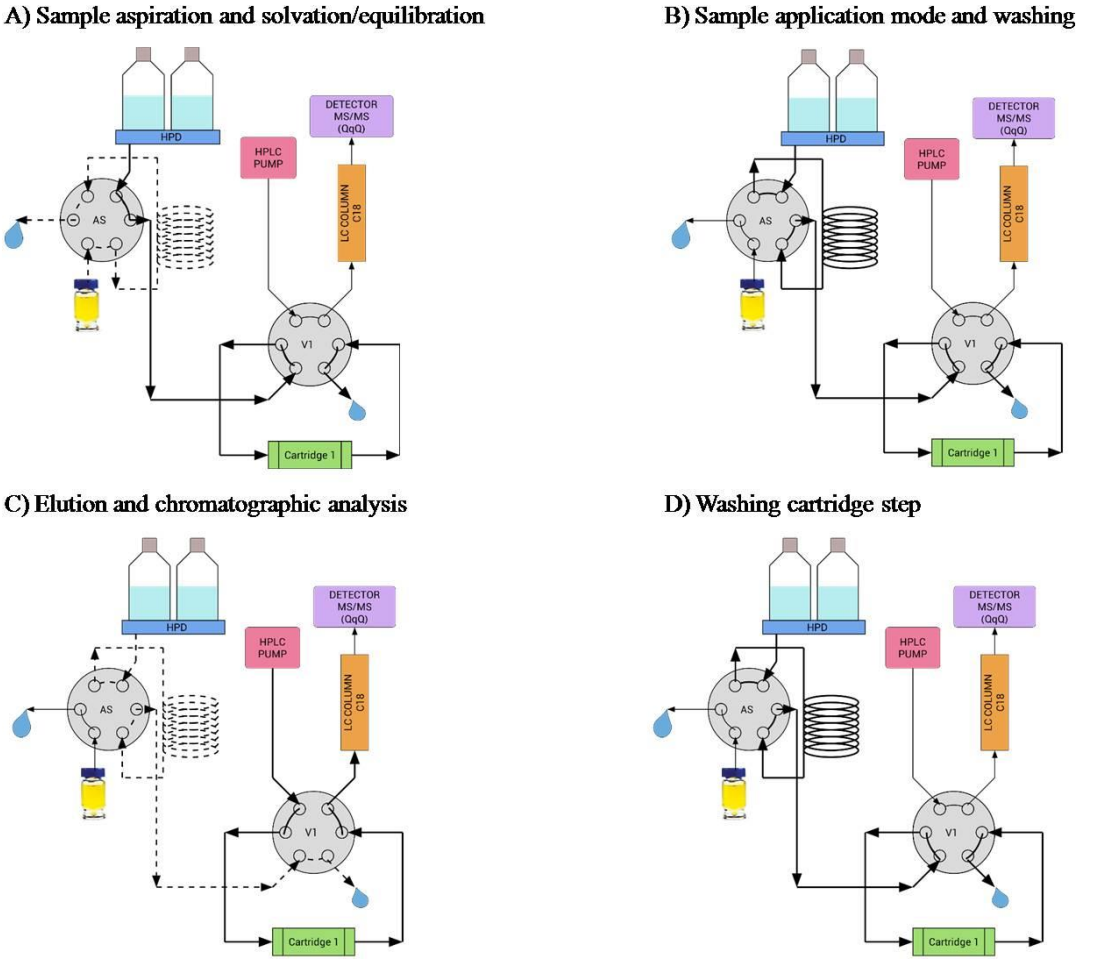

Figure 1. Scheme of the experimental approach based on SPE–LC–MS/MS. Automatic cartridge exchange (ACE), electrospray ionization module (ESI), high pressure dispenser (HPD) and triple quadrupole mass detector (QqQ).

HySpere C8 EC cartridges (endcapped silica based octyl phase, particle size 10 µm, 10×2mm i.d.) from Spark Holland were used in the SPE step, selected

after testing other types of cartridges such as CN (silica based cyanopropyl phase, particle size 7 μm), C2 (silica based ethyl phase, particle size 7 μm), C18 HD (end-capped silica based phase with a high density of octadecyl chains, particle size 7 μm), Resin GP (polymeric polydivinylbenzene phase, particle size 5–15 μm) and Resin SH (strong hydrophobic modified polystyrene–divinylbenzene phase, particle size 20–50 μm). All cartridges were 10×2 mm i.d.

Confirmatory analysis of the target compounds was performed by an Agilent 1200 Series LC system interfaced to an Agilent UHD Accurate-Mass LC–TOF/MS detector (Palo Alto, CA), equipped with an Agilent Technology dual electrospray ion source (dual ESI) operating in the negative and positive ionization modes.

2.4. Sample Preparation

The liquid phase of wine lees was filtered using a 20 μm pore size filter and 1:2 diluted with acidified milli-Q water (pH = 4) before analysis to avoid obstruction problems in the automated SPE system.

Concerning the solid residue of dried lees, 6.25 g was placed into the extraction vessel of the microwave-assisted digestor with 50 mL of 60:40 (v/v) ethanol–water adjusted at pH 4 with HCl. The vessel was positioned at the suited zone for irradiation with focused microwaves. The auxiliary energy was applied at 140 W irradiation power for 10 min, after which the solid residue was removed by centrifugation and the liquid fraction filtered by a 20 μm pore size filter and 1:2 diluted with acidified milli-Q water.

The analysis started by solvation of the selected SPE cartridges by 4 mL of MetOH at 5 mL/min and equilibration with 2 mL of milli-Q water acidified to pH 4 at 5 mL/min, and 1 mL of acidified milli-Q water at 2 mL/min. The cartridges were then loaded with the analytical samples (200 μL) by propelling 2 mL of acidified milli-Q water at 2 mL/min, and washed with 0.5 mL of acidified milli-Q water at 1.7 mL/min. Then, the analytes were eluted by the chromatographic mobile phase for 7.5 min according to the LC gradient program detailed

below, and finally chromatographically separated in the analytical column prior to MS detection. Rinsing of the cartridges with 4 mL of MetOH at 5 mL/min and 4 mL of milli-Q water at 5 mL/min allowed their repeated use. The sequence of automated SPE operations is listed in **Table 1**.

Table 1. Sequence of operations involved in the on-line SPE method.

Automated analytical protocol	Flow rate (mL/min)	Volume (mL)	Solvent	Comment
New cartridge				C8 EC
Start autosampler				Load sample (200 µL)
Solvation	5.0	4.0	Methanol	
Equilibration 1	5.0	2.0	Deionized water (pH 4)	
Equilibration 2	2.0	1.0	Deionized water (pH 4)	
Sample application	2.0	2.0	Deionized water (pH 4)	Injected sample (200 µL)
Wash cartridge	1.7	0.5	Deionized water (pH 4)	
Switch off valve				
Elution			LC mobile phase	7.5 min
Wash cartridge 1	5.0	4.0	MeOH	
Wash cartridge 2	5.0	4.0	Deionized water	

Separation of the analytes was performed by a Mediterranea Sea C18 analytical column (3 µm, 15×0.46 cm) from Teknokroma (Barcelona, Spain) using a flow rate of 0.8 mL/min. The gradient program —established by a mobile phase A (deionized water) and mobile phase B (methanol) both containing 0.5% formic acid (pH=3)— was as follows: 0–1 min, 16% eluent B; 1–6 min, 45% eluent B, held from 6–9 min; 9–16 min, 66.4% eluent B; 16–17 min, 100% eluent B and held up to min 20. The analytical column, thermostated at 20 ºC, was equilibrated by a post-run time of 8.5 min between analyses. Complete automation of the SPE and LC–MS/MS systems was accomplished by programming appropriate analysis sequences.

Detection by the triple quad mass spectrometer used high-purity nitrogen (99.999%) as collision gas. The data were processed using the Agilent

MassHunter Workstation Software (version B.05.00) for qualitative and quantitative analysis. Determination of the analytes was performed by ESI–MS/MS in selected reaction monitoring (SRM) mode. Flow and temperature of the drying gas and sheath gas (nitrogen) were 10 L/min and 325 °C, respectively. The nebulizer pressure was 40 psi and the capillary voltage was 2000 V for the positive ionization mode and 4000 V for the negative mode. The dwell time was set at 70 ms.

For LC–TOF/MS confirmatory analysis, chromatographic separation was performed using an Inertsil ODS-2 column (250 mm×4.6 mm i.d., 5 μm particle, Análisis Vínicos, Tomelloso, Ciudad Real, Spain), kept at a temperature of 25 °C. The mobile phases in this case were water (phase A) and acetonitrile (phase B), both LC–MS/MS grade, and with 0.1% formic acid as ionization agent. The LC pump was programmed at a flow rate of 0.9 mL/min, and the following elution gradient was carried out: held from 0–2 min 4% eluent B, 2–7 min 10% eluent B, 7–60 min 100% eluent B and held up to min 70. A post run of 4 min was included to equilibrate the column. The injection volume was 10 μL, and the injector needle was five times rinsed between samples with 70% methanol. Furthermore, the needle seat back was flushed for 12 s at a flow rate of 4 mL/min with 70% methanol to clean it. The operating conditions of the mass spectrometer were as follows: gas temperature, 325 °C; drying gas, nitrogen at 10 L/min; nebulizer pressure, 40 psi; sheath gas temperature, 380 °C; sheath gas flow, nitrogen at 10 L/min; capillary voltage, 3500 V; skimmer, 65 V; octopole radiofrequency voltage, 750 V; and focusing voltage, 175 V. Data acquisition (2.5 Hz) in both the centroid and the profile modes was governed via the Agilent MassHunter Workstation software. The instrument was operated in auto MS/MS mode. The mass range and detection window were set at *m/z* 100–3000 and 100 ppm, respectively. The instrument was calibrated and tuned according to procedures recommended by the manufacturer. To ensure the desired mass accuracy of recorded ions, continuous internal calibration was performed during analyses by using signals at *m/z* 121.0509 (protonated purine) and *m/z* 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921] in

positive ionization mode, while ions with *m/z* 119.0362 (proton abstracted purine) and *m/z* 966.000725 (formate adduct of HP-921) were used in negative ionization mode.

2.5. Data Processing

MassHunter Workstation software (version B.05.00) Qualitative Analysis (Agilent Technologies, Santa Clara, CA) was used for processing all data obtained by LC–TOF/MS. The feature extraction algorithm took into account all ions exceeding 5000 counts with a charge state equal to or above one, and a feature had to be composed of two or more ions to be valid (*e.g.* two ions in the isotope cluster). Peaks with identical elution profiles and related *m/z* values (representing different adducts or isotopes of the same compound) were extracted as molecular features (MFs) or entities characterized by retention time (RT), intensity in apex of chromatographic peak, and accurate mass.

3. Results and Discussion

3.1. Optimization of the LC–MS/MS Method

Based on the ESI–MS/MS ionization and fragmentation patterns for the target compounds, the negative ESI mode was selected for determination of proanthocyanidins, whilst the positive mode was selected for anthocyanidins. Both groups of compounds were enough separated in the chromatograms to require only one analysis per sample by fast polarity switching during the chromatographic run. The effect of the ionization agent on the ESI–MS/MS sensitivity and peak shape was studied by testing variable concentrations of formic and acetic acids taking into account that the stability of the target compounds requires acid pHs. Formic acid improved the sensitivity as compared to acetic acid, with 0.5% (v/v) as optimum concentration. The preferred precursor ions for proanthocyanidins were the $[M–H^-]$ forms, while anthocyanidins were mainly ionized as $[M^+]$ entities. The SRM method was developed for

compounds with available standards. Three SRM transitions were selected for each compound to confirm its presence in the target samples.

Table 2. Optimization of the MS/MS step for qualitative and quantitative determination of anthocyanidins and proanthocyanidins.

Analyte	Precursor ion (*m/z*)	Voltage MS1 (eV)	Product ion (*m/z*)	CE (eV)	Quantitation SRM transition	Confirmation SRM transitions	RT (min)
Proanthocyanidin B1	577.1	160	289.1	30	577.1 →289.1	577.1→407.1; 124.9	6.1
Proanthocyanidin B2	577.1	160	289.1	30	577.1 →289.1	577.1→407.1; 124.9	6.9
Proanthocyanidin A2	575.0	195	285.0	30	575.0 →285.0	575.0→539.2; 449.0	10.1
Delphinidin	303.1	175	229.0	35	303.1 →229.0	303.1→137.1; 257.0	10.7
Cyanidin	287.1	185	137.1	35	287.1 →137.1	287.1→213.0; 109.1	12.6
Petunidin	317.1	190	302.0	25	317.1 →302.0	317.1→217.0; 203.1	13.2
Peonidin	301.0	155	286.0	25	301.0 →286.0	301.0→258.0; 201.1	14.5
Malvidin	331.1	185	315.0	35	331.1 →315.0	331.1→287.1; 242.0	15.2

Note: CE: collision energy; RT: retention time.

Table 2 lists the optimum values for each anthocyanidin or proanthocyanidin. Activation of proanthocyanidins was characterized by the fragmentation pattern associated to quinone methide (QM) cleavage of the interflavan bond, which led to different fragments depending on the structure of the given proanthocyanidin. As shows **Supplementary Figure 1**, proantho-cyanidin A is characterized by a main fragment of *m/z* 285.0 and a secondary fragment at *m/z* 290.0 as a result of QM cleavage. On the other hand, B-type proanthocyanidins led to *m/z* 289.0 and 271.0 fragments at as a result of QM, as shown in **Supplementary Figure 2**. *(18)* Concerning anthocyanidins, malvidin, peonidin and petunidin shared the same fragmentation pattern by cleavage of a methyl group in position R1 (**Supplementary Figure 3**). On the other hand, cyanidin and delphinidin were fragmented by different mechanisms. Optimum quantitation transition of cyanidin can be explained by retro Diels–Alder reaction as described by Barnes *et al. (21)*, where fragments at *m/z* 137.1, 121.0 and 109.1 are produced. Fragmentation of delphinidin can be explained by the loss of a molecule of water and two carbonyl groups $[M\text{-}H_2O\text{-}2CO]^+$, as reported by Montoro *et al.(22)*

3.2. Data-Dependent Methods and High Resolution Mass Spectrometry

Data-dependent methods were used to scan derivatives of the target metabolites by LC–MS/MS. For this purpose, the versatility of the triple quad mass analyzer allowed application of three different scanning methods: precursor ion, neutral loss and product ion. Tentative identifications carried out by data-dependent methods were confirmed by LC–TOF/MS in high resolution mode by using the same chromatographic method. The sample used in all analyses was a pool of liquid lees. **Table 3** summarizes the main results obtained by combination of these methodologies. For precursor ion scanning, the precursor ions found for the three proanthocyanidins and the six anthocyanidins were simultaneously monitored in the third quadrupole (Q3) by scanning precursor ions corresponding to derivative metabolites formed by conjugation, such as glucosides, in the first quadrupole (Q1). Scanning of the precursor ion for delphinidin (*m/z* 303.1) allowed detecting two ions at *m/z* 465.0 and 611.0 at 8.3 and 14.5 min elution time, which fit to the molecular cations of delphinidin-3-glucoside and delphinidin-3-rutinoside, respectively. Neutral-loss scanning by tuning Q1 and Q3 to monitor common neutral losses of 162 Da and 146 Da, corresponding to the loss of glucose and rhamnose as substituents, confirmed the molecular cations of delphinidin-3-glucoside (loss of 162 Da) and delphinidin-3-rutinoside (loss of 146 Da). Thirdly, MS/MS product-ion scanning of delphinidin-3-glucoside and delphinidin-3-rutinoside led to the preferential formation of the aglycon cation (*m/z* 303.1, delphinidin) and a fragment at *m/z* 465.0 that corresponded to the cleavage of the rhamnose substituent, which is indicative of the fragmentation of the glycosidic bond between glucose and rhamnose.17 Identification of these conjugated derivatives was confirmed by high resolution mass spectrometry by extracting ions at *m/z* values, as shown in **Table 3**. Mono-isotopic masses were searched in the raw data files obtained by the analysis of the samples. Search parameters were mass accuracy 4 ppm, minimum peaks height of 2500 counts on the profile and centroid spectra and a peak spacing tolerance of 0.0025 *m/z* plus 7 ppm.

Table 3. Anthocyanins identification using data-dependent methods and confirmation by LC–TOF/MS.

Compound	Precursor ion[a] (*m/z*)	Product ion[b] (*m/z*)	Neutral loss[c] (Da)	LC–TOF/MS			
				Experimental *m/z*	Ion	Exact mass	Error (ppm)
Peonidin-3-glucoside	463.2	301.0	162	463.1223	[M+]	463.1235	2.63
Peonidin-3,5-diglucoside	625.2	301.0	162 × 2	625.1765	[M+]	625.1763	-0.29
Petunidin-3-glucoside	479.4	317.1	162	479.1193	[M+]	479.1184	-1.87
Delphinidin-3-glucoside	465.0	303.1	162	465.1020	[M+]	465.1028	1.51
Delphinidin-3-rutinoside	611.0	303.1	146, 162	611.1598	[M+]	611.1607	3.18

[a]*Detected by precursor-ion scanning.* [b]*Detected by product-ion scanning.* [c]*Detected by neutral-loss scanning*

The same strategy was used for other compounds. Thus, glucoside derivatives of peonidin (*m/z* 301.0) were also detected as the scanning of its precursor ion led to detection of two ions at *m/z* 625.2 and 463.2, which should correspond to the cationic adducts of peonidin-3,5-diglucoside and peonidin-3-glucoside found at min 7.5 and 9.6, respectively. Confirmation based on neutral loss scanning (162 Da) and the product ion scanning enabled to find the fragment ascribed to aglycone peonidin (*m/z* 301.0), as shows **Figure 2**. By analogy, high-resolution mass spectrometry confirmed identifications proved by data-dependent methods.

Finally, the scanning of the precursor ion of petunidin (*m/z* 317.1) allowed detecting the molecular cation of petunidin-3-glucoside (*m/z* 479.4) at min 8.9, which was also supported on neutral loss scanning of a glucose moiety. Therefore, the product ion scanning detected the aglycone cation of petunidin (*m/z* 317.1).

After identification and confirmatory analysis of conjugated derivatives in extracts from wine lees, optimization of the method for detection of these compounds by the SRM method was carried out. Voltage of the first quadrupole and collision energy applied to activate precursor ions were optimized resulting in the final method in **Table 4**.

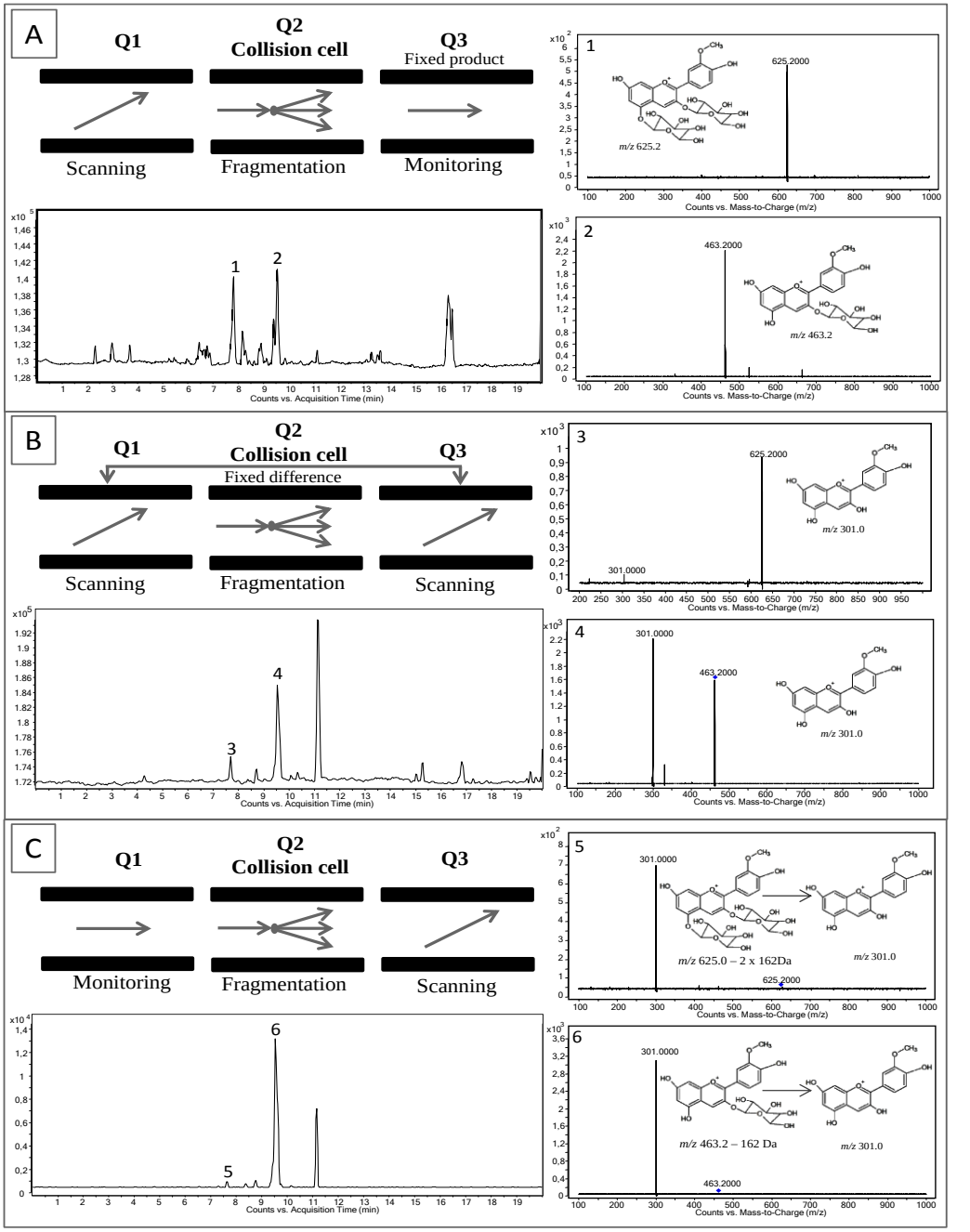

Figure 2. Data-dependent methods used for qualitative analysis. A) Product ion scanning for detection of peonidin-3,5-diglucoside and peonidin-3-glucoside; B) neutral loss scanning by monitoring the cleavage of the glucose moiety; and C) precursor ion scanning for detection of peonidin-3,5-diglucoside and peonidin-3-glucoside.

Table 4. Optimization of the MS/MS step for qualitative and quantitative determination of anthocyanins.

Analyte	Precursor ion (*m/z*)	Voltage MS1 (eV)	Product ion (*m/z*)	CE (eV)	Quantitation transition	RT (min)
Peonidin-3-glucoside	463.2	120	301.0	15	463.2 →301.0	**7.5**
Delphinidin-3-glucoside	465.0	120	303.1	25	465.0→303.1	**8.3**
Petunidin-3-glucoside	479.4	120	317.1	15	479.4→317.1	**8.9**
Peonidin-3,5-diglucoside	625.2	120	301.0	25	625.2→301.0	**9.6**
Delphinidin-3-rutinoside	611.0	100	303.1	25	611.0→303.1	**14.5**

Note: CE: collision energy; RT: retention time.

3.3. Optimization of On-line SPE–LC–MS/MS

A generic protocol was used for optimization of the sorbent, where the tested SPE cartridges were automatically solvated by 4 mL of MeOH at 4 mL/min, conditioned with 2 mL of deionized water at 4 mL/min, and equilibrated first with 2 mL of MeOH and secondly with 2 mL of deionized water at 4 mL/min. The cartridges were then loaded with 100 μL of the pool of liquid wine lees by propelling 2 mL of deionized water at 4 mL/min. The washing step was eliminated to assure 100% retention. Then, the analytes were eluted from the SPE cartridges by the mobile phase after switching off valve 1 during the LC gradient program. Finally, the eluated components were chromatographically separated by the analytical column prior to MS detection,

The capacity of the sorbents for analyte retention (expressed as average of relative percentage of peak areas for all compounds) decreased in the sequence: C8 EC (100) > C18 HD (80) > Resin SH (70) > Resin GP (65) > C2 (15) > CN (5). The best results were obtained by HySphere C8 EC cartridges, which were adopted for further testing as they provided the best overall recovery in these preliminary tests.

Response surfaces were developed to optimize the volume and flow rate for the loading solvent and washing solutions, being the best recoveries found at volumes of 0.5 mL for both and flow rates of 2.0 and 1.7 mL/min, respectively.

The effect of the type of elution solvent was also tested, obtaining the best results with acidified milli-Q water (pH 4) after checking mixtures with different percentages of acidified organic phase (i.e. 0, 10, 20, 30 and 40% MetOH). The retained analytes were eluted by pumping the LC mobile phase through the SPE cartridge to the analytical column for 7.5 min. Shorter times did not allow quantitative elution of the analytes from the cartridge, while longer elution times led to peak broadening. The efficiency of the SPE step was calculated by setting two C8 cartridges on-line for sequential passage of the sample through them. As shows **Supplementary Figure 4**, a modified configuration was used by implementation of a second valve (valve 2) in the system. Thus, if breakthrough occurs, the second cartridge should retain the fraction of compounds no retained in the first cartridge. Independent analysis of the eluted fraction from each cartridge should allow estimating the SPE retention efficiency, which was assessed by triplicate analysis of liquid wine lees and extracts of dried wine lees spiked at two concentrations (*i.e.* 1.0 and 1.5 ng/mL). The extraction efficiency was calculated as the ratio of analyte response in cartridge 1 and the sum of the response provided by both cartridges. Attending to these estimations, the extraction efficiency was above 93% in the extracts from solid lees, while in liquid lees this parameter was above 97% in all types of lees. With these premises, no breakthrough was observed for any target analyte at any concentration, despite the differences in polarity between conjugated and non-conjugated compounds.

3.4. Validation of the Method

Calibration graphs (n=7) made with both spiked liquid wine lees and extracts from solid lees exhibited excellent linearity for proanthocyanidins and anthocyanidins, with regression coefficients higher than 0.98 over the concentration range of each target analyte (**Table 5**). Three analytical replicates (n=3) of spiked samples for each concentration level within the linear dynamic range were injected. **Figure 3.A** shows the SRM chromatograms obtained by monitoring selected ions of the target analytes in liquid wine lees spiked with

standards (1 μg/mL) subjected to the above-described on-line SPE–LC–MS/MS procedure. **Figure 3.B** shows the SRM chromatograms obtained by monitoring selected ions of the target analytes in liquid wine lees from La Unión wineries.

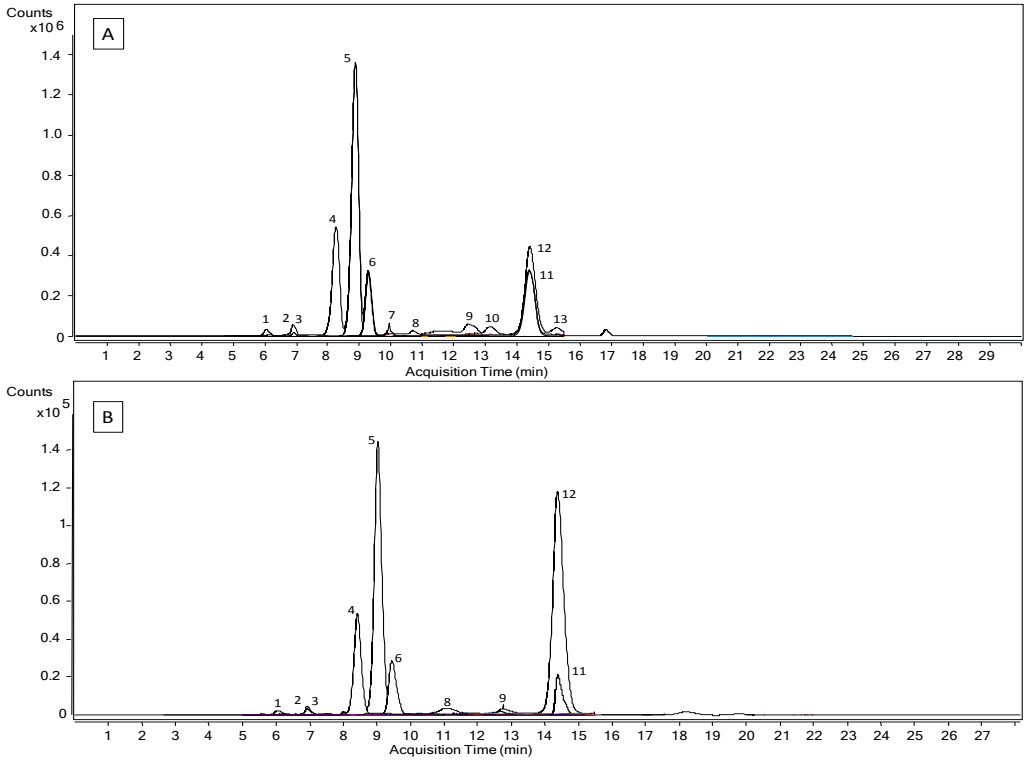

Figure 3. Selected reaction monitoring chromatogram obtained from A) liquid wine lees from Viña Hermosa wineries spiked with standards (1 μg/mL each), and B) extract of the solid residue from wine lees from La Unión wineries.

Note: 1, proanthocyanidin B1; 2, proanthocyanidin B2; 3, peonidin-3-glucoside; 4, delphinidin-3-glucoside; 5, petunidin-3-glucoside; 6, peonidin 3,5-diglucoside; 7, proanthocyanidin A2; 8, delphinidin; 9, cyanidin; 10, petunidin; 11, delphinidin-3-rutinoside; 12, peonidin; 13, malvidin.

The lower limit of detection (LLOD) and that of quantitation (LLOQ) were calculated by injecting dilution series of each analyte in both types of samples to obtain the concentrations which provided signals three and ten times above the background noise, respectively. The LLODs ranged from 15 to 45

pg/mL for liquid wine lees and from 15 to 78 pg/mL for a pool of extracts of dried wine lees.

The sensitivity was thus better for liquid lees, which could be ascribed to components of the solid matrix present in the extracts. **Table 5** summarizes the characteristics of the method. Matrix effects could justify the differences in calibration models for certain compounds. However, this effect was not matrix-dependent as it is random. This is an important aspect and explains the utilization of different calibration models.

Table 5. Analytical features of the method.

Analyte	Sample	Calibration equation	Linear range (ng/mL)[a]	R^2	Limit of detection		Limit of quantitation	
					(pg/mL)	On column (pg)	(pg/mL)	On column (pg)
Proanthocyanidin B1	L	y = 112318x + 5460,80	0.10–500	0.9804	30	3.0	100	10.0
	E	y = 117165x + 11500	0.10–800	0.9809	30	3.0	100	10.0
Proanthocyanidin B2	L	y = 11067x - 8684,50	0.08–600	0.9963	24	2.4	80	8.0
	E	y = 21862x + 9463,9	0.05–900	0.9951	15	1.5	50	5.0
Proanthocyanidin A2	L	y = 58250x + 503,32	0.08–900	0.9934	24	2.4	80	8.0
	E	y = 25397x - 637,68	0.08–750	0.9883	24	2.4	80	8.0
Cyanidin	L	y = 226620x - 3704,20	0.05–900	0.9936	15	1.5	50	5.0
	E	y = 221780x - 14067	0.1–900	0.9984	30	3.0	100	10.0
Peonidin	L	y = 950387x - 18233	0.05–800	0.9953	15	1.5	50	5.0
	E	y = 1E+06x - 73010	0.10–800	0.9979	30	3.0	300	30.0
Petunidin	L	y = 438493x - 4223,80	0.10–600	0.9909	30	3.0	100	10.0
	E	y = 356935x - 38870	0.20–900	0.9931	60	6.0	200	20.0
Malvidin	L	y = 196102x + 2370,40	0.15-750	0.9899	45	4.5	150	15.0
	E	y = 370529x - 3383,7	0.20–750	0.9992	60	6.0	200	20.0
Delphinidin	L	y = 138385x - 5007,70	0.05–900	0.9999	15	1.5	50	5.0
	E	y = 192184x - 785,22	0.26-750	0.9998	78	7.8	260	26.0

L: Liquid wine lees; E: Extracts of wine lees;R^2: Coefficient of regression; [a]The concentration in the extracts correspond to 6.50 g of dried lees quantitatively extracted with 50 mL of extractant —60:40 (v/v) ethanol water (see under "sample preparation").

The precision of the method was estimated by intra-day and inter-days studies. Both parameters were evaluated by analysis of pools of liquid lees and extracts spiked at three concentrations (0.5, 1.0 and 1.5 ng/mL) for seven consecutive days with three replicates per day. As shows **Table 6**, intra-day and inter-day variability, expressed as RSD, ranged from 0.1% to 10.9% and from 0.4% to 10.1%, respectively, depending on the target analyte.

Table 6. Precision study by estimation of intra-day and inter-days variability expressed as relative standard deviation.

Analyte	Liquid lees 1.5 (ng/mL)		Liquid lees 1.0 (ng/mL)		Liquid lees 0.5 (ng/mL)		Extract 1.5 (ng/mL)		Extract 1.0 (ng/mL)		Extract 0.5 (ng/mL)	
	Intra-day	Inter-days	Intra-day	Inter-days	Intra-day	Inter-days	Intra-day	Inter-days	Intra-day	Inter-days	Intra-day	Inter-days
Proanthocyanidin B1	2.6	3.7	7.5	7.5	3.2	7.4	2.0	6.8	5.6	6.2	1.9	6.9
Proanthocyanidin B2	2.5	9.8	8.9	9.9	8.0	9.9	3.6	7.4	3.7	8.8	3.6	9.9
Proanthocyanidin A2	2.0	9.3	2.6	7.4	4.0	6.4	5.5	6.9	0.1	8.9	0.4	7.1
Delphinidin	1.2	5.2	0.9	1.9	0.5	8.3	7.7	9.6	6.9	9.7	3.4	8.3
Cyanidin	1.0	1.3	0.1	0.4	0.4	9.5	8.4	9.9	0.7	10.1	1.5	7.4
Petunidin	1.1	2.4	1.4	2.4	0.1	2.5	3.2	6.7	1.9	5.3	2.3	8.5
Peonidin	0.2	1.5	1.6	0.9	0.1	4.7	5.9	7.4	0.3	4.8	0.4	8.6
Malvidin	3.8	4.9	6.8	7.2	8.4	8.6	4.7	5.7	2.1	6.6	1.7	8.6

3.4. Analysis of Anthocyanins, Anthocyanidins and Proanthocyanidins Content in Lees

The proposed on-line SPE–LC–MS/MS method was used to determine the anthocyanins, anthocyanidins and proanthocyanidins profile in liquid wine lees and extracts of dried wine lees. **Table 7** shows the quantitation data obtained after interpolation of peak areas within their calibration curves for all analyzed samples. Concerning analytes with no commercial standards, it is worth mentioning that they were quantified by the aglycon form, which is the usual approach since it is supossed that the glucoside and the aglycon forms have similar biological effects.

In general terms, the concentrations of the target compounds in the extracts of dried lees were higher than in liquid lees, especially for glucoside derivatives. B-type proanthocyanidins were always found at higher concentrations than those of A-type, with levels between 2.0 and 31.5 ng/g in dried lees extracts and between 0.3 and 23.5 ng/mL in liquid lees. Exceptional levels were

found in the case of liquid lees from López de Heredia, Ramírez de la Piscina and Viña Hermosa wineries, in which the concentration of proanthocyanidin B2 reached 17.5, 8.5 and 23.5 ng/mL, respectively.

Concerning anthocyanidins, the concentrations found varied between 1.06 and 77.5 ng/g in the extracts of dried lees, and between 0.09 and 7.5 ng/mL in liquid lees. The highest concentrations of petunidin and malvidin were found in extracts from Selección de Torres dried lees: 71.3 and 77.5 ng/g, respectively. The detection of anthocyanidins in wine lees had previously been described by Mazauric *et al.* and Salmon *et al.*, who stated that yeast lees can modify the color of wines either by weak and reversible interactions between anthocyanins and yeast walls or by a periplasmic β-glucosidase enzyme *(12,22)*.

Variability of the results was characteristic in the case of anthocyanins. Thus, high levels of anthocyanins were found in samples from specific wineries such as Cune, Selección de Torres, Torres de Anguix, Real Sitio de Ventosilla and Viña Hermosa. It should be emphasized the high concentration of peonidin-3-glucoside, petunidin-3-glucoside, delphinidin-3-glucoside and delphinidin-3-rutinoside (191.7, 3400.5, 4000.2 and 1697.4 ng/g, respectively) detected in extracts isolated from Selección de Torres as compared to other wineries. One other interesting result was ascribed to lees from Real Sitio de Ventosilla, in which concentrations of petunidin-3-glucoside and delphidinin conjugates were high both in liquid lees and extracts from dried lees. The lowest concentrations of anthocyanins were found in lees from La Unión and Ramírez de la Piscina wineries.

The application of quantitative methods enabled to compare the target analytes content of wine lees from different wineries and also the content of liquid lees and extracts isolated from dried lees. Attending to these results, it is evident the influence of the winery on the colorant content, especially that of anthocyanidins. This could be explained by the differences of wine ageing protocols adopted by wineries, also influenced by the region and cultivar. This study also proved the capability of data-dependent methods combined with high

Table 7. Concentration of anthocyans detected in liquid lees and extracts from solid lees expressed as ng/mL and ng/g.

Analyte	Sample[a]	Cune[b]	López de Heredia[b]	La Unión[b]	Ramírez de la Piscina[b]	Selección de Torres[b]	Torres de Anguix[b]	Real Sitio de Ventosilla[b]	Viña Hermosa[b]
Pro B1	A	18.4	n.d.	14.4	11.2	25.1	16.9	17.2	4.5
	B	nd	1.9	0.3	3.3	0.3	n.d.	0.3	3.7
Pro B2	A	28.3	n.d.	24.9	2.0	31.5	19.5	14.6	4.8
	B	2.3	17.5	3.1	8.5	3.9	2.0	5.4	23.5
Pro A2	A	1.4	3.7	1.5	4.2	1.9	1.2	0.6	1.1
	B	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Cyanidin	A	1.5	1.5	1.1	1.7	6.0	3.0	2.3	1.1
	B	0.1	0.1	0.1	0.1	0.5	0.5	0.3	0.1
Peonidin	A	n.d.	n.d.	n.d.	n.d.	3.6	2.8	n.d.	n.d.
	B	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Petunidin	A	8.0	10.1	2.7	4.9	71.3	20.9	16.5	1.9
	B	n.d.	0.4	n.d.	0.3	0.4	n.d.	3.7	1.1
Malvidin	A	7.7	n.d.	n.d.	1.9	77.5	25.7	21.6	n.d.
	B	0.2	0.3	n.d.	0.7	2.1	0.2	4.5	1.0
Delphinidin	A	30.2	n.d.	n.d.	4.6	27.9	37.7	24.0	4.9
	B	0.1	2.4	0.09	0.2	0.2	0.1	7.5	4.6
Peonidin-3-glucoside[c]	A	39.9	n.d.	n.d.	13.3	192	100	36.2	6.1
	B	0.6	3.6	0.9	0.3	0.2	0.3	3.5	0.5
Peonidin-3,5-diglucoside[c]	A	11.4	n.d.	n.d.	4.8	27.9	40.3	9.3	n.d.
	B	n.d.	0.06	0.05	0.09	0.06	n.d	0.2	0.1
Petunidin-3-glucoside[c]	A	421	2.1	2.2	112	3401	717	424	80.3
	B	0.9	33.4	0.6	9.8	9.9	0.9	113	85.5
Delphinidin-3-glucoside[c]	A	426	n.d.	n.d.	65.0	4000	538	342	71.7
	B	0.4	31.8	0.3	1.2	1.7	0.8	103	61.9
Delphinidin-3-rutinoside[c]	A	552	n.d.	n.d.	148	1697	690	992	39.6
	B	0.2	4.3	0.6	5.5	3.3	0.3	30.2	8.4

Note: [a]A: Extract of dry lees, B: Liquid wine lees, [b] n.d.: Not detected, [c] Concentration estimated in terms of the calibration curve of the corresponding anthocyanidin.

resolution MS to detect and confirm the identity of unknown compounds without use of standards.

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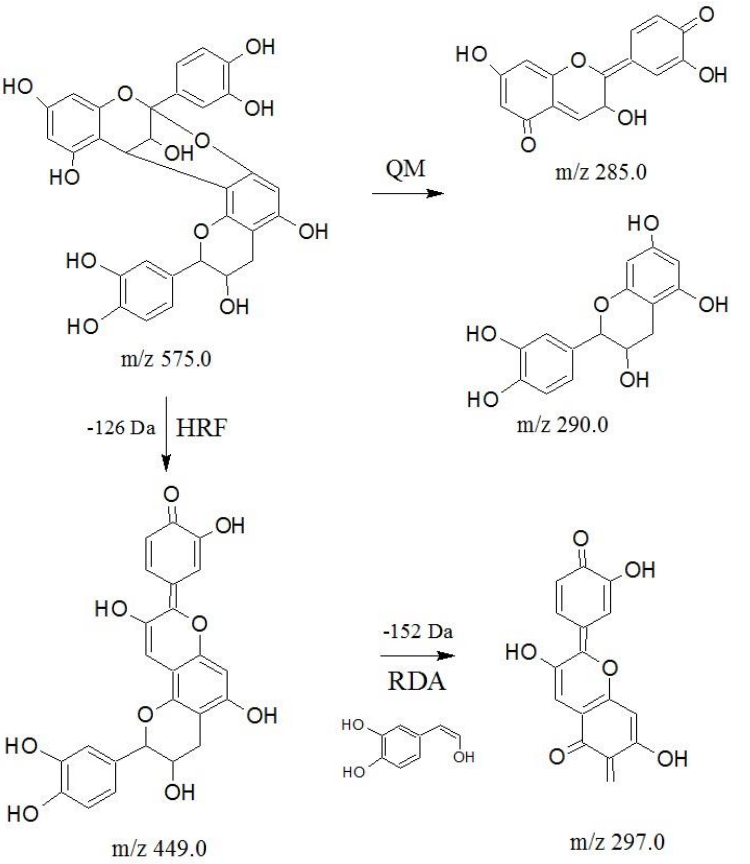
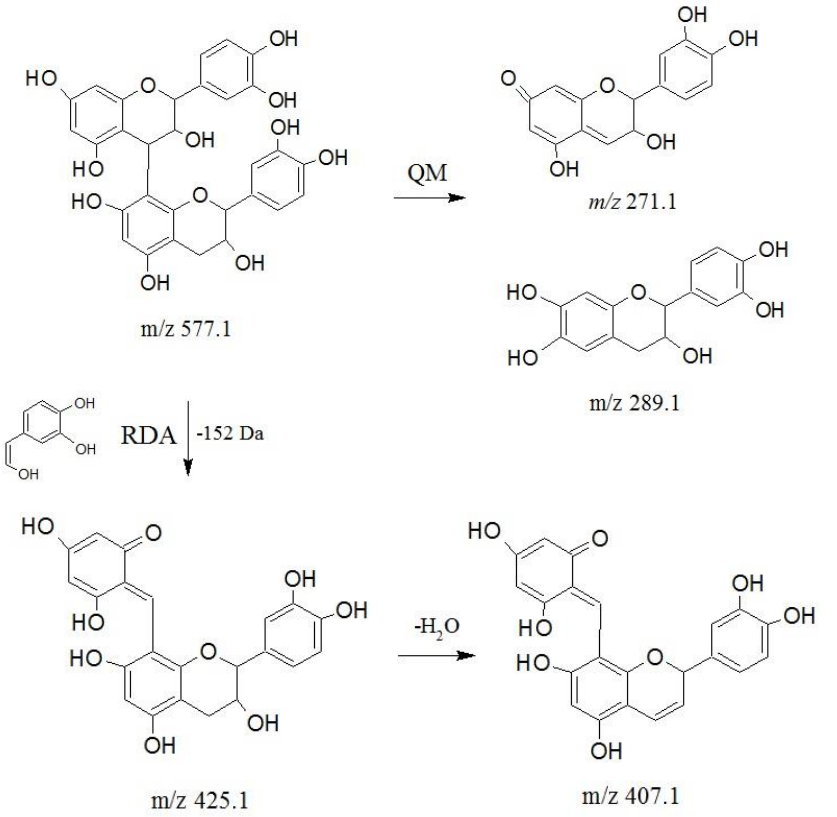
Supplementary material

Supplementary Figure 1. Fragmentation pathway of proanthocyanidin A2



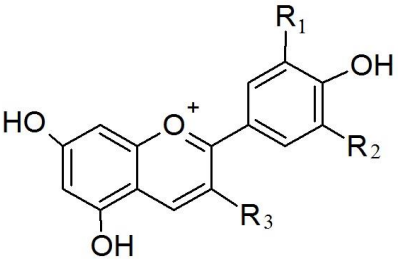
Note. QM: quinone methide cleavage of the interflavan bond; HRF: Heterocyclic ring fission; RDA: Retro Diels-Alder reaction.

Supplementary Figure 2. Fragmentation pathway of proanthocyanidin B



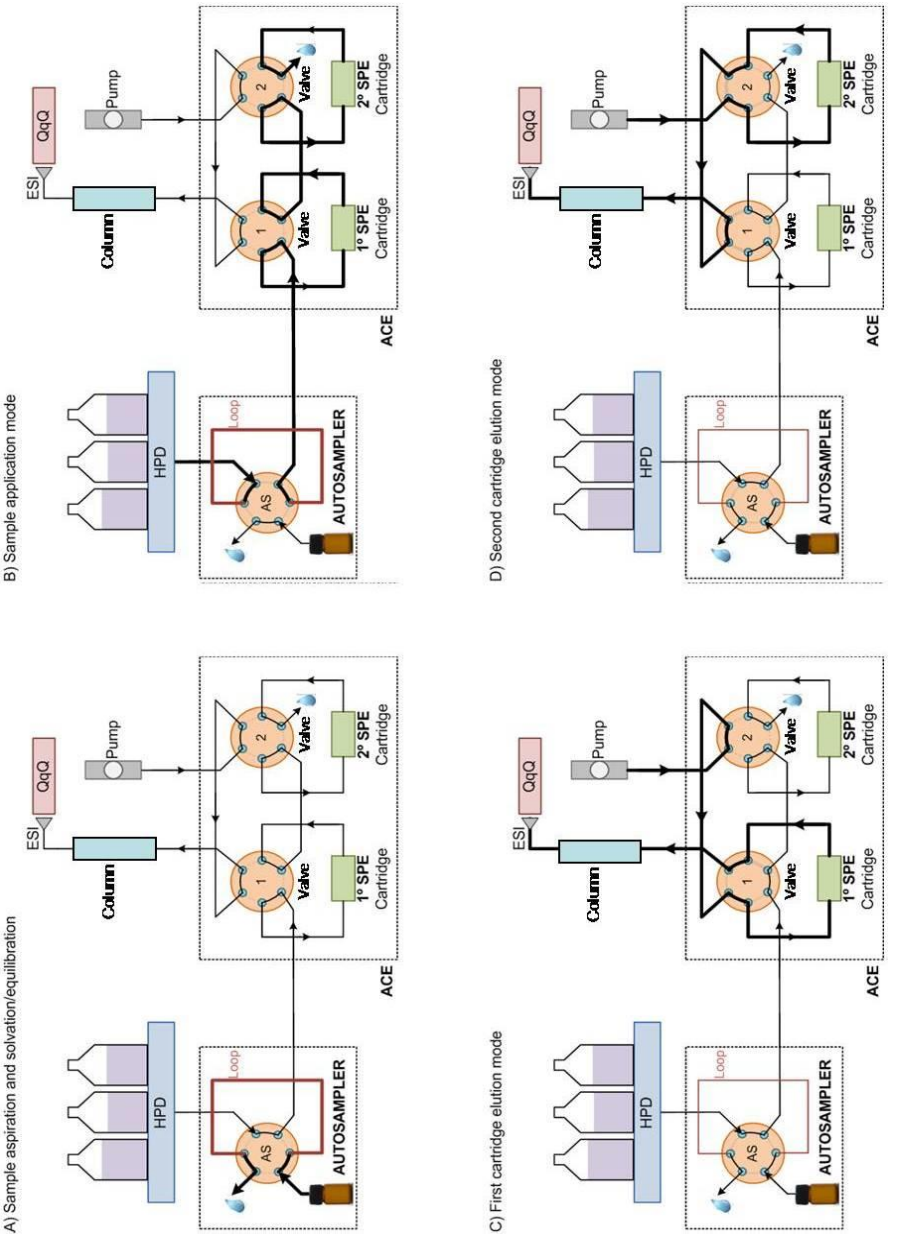
Note. QM: quinone methide cleavage of the interflavan bond; RDA: Retro Diels-Alder reaction.

Supplementary Figure 3. Basic structure of anthocyanidins



Aglycone	R_1	R_2	R_3
Delphinidin	OH	OH	OH
Cyanidin	OH	H	OH
Petunidin	OCH_3	OH	OH
Peonidin	OCH_3	H	OH
Malvidin	OCH_3	OCH_3	OH

Supplementary Figure 4. Scheme of the experimental approach based on SPE–LC–MS/MS. Automatic cartridge exchange (ACE), electrospray ionization module (ESI), high pressure dispenser (HPD) and triple quadrupole mass detector (QqQ).



DISCUSIÓN DE LOS RESULTADOS

La normativa actual de la Universidad de Córdoba para la presentación de una Memoria de Tesis Doctoral, en la modalidad en la que se incluyen los artículos (publicados o próximos a su publicación) como tales, requiere una discusión conjunta de los resultados de la investigación realizada, cuya estructura depende de su homogeneidad. En la presente Memoria la investigación ha tenido como denominador común el estudio de materiales de desecho de la vid y de la industria vitivinícola para su posible explotación, ya sea mediante la obtención de extractos ricos en compuestos de interés o para su uso en el envejecimiento de vinos (en este caso como alternativa o complemento de las virutas de roble). El objetivo, por tanto es único: La caracterización de residuos que actualmente tienen escaso o nulo valor mediante su explotación, tanto desde el punto de vista nutracéutico como del organoléptico.

La Memoria en la que se recoge la investigación realizada se ha dividido en tres secciones en función tanto del objetivo como del tipo de muestra utilizado, de modo que las Secciones I y II tienen en común un material de desecho agrícola poco estudiado y menos aprovechado: Los sarmientos de la vid. Por otra parte, la Sección III está dedicada a un desecho de la industria vinícola que también ha recibido escasa atención tanto en investigación como en el aprovechamiento de sus variados componentes: Las lías de vinificación.

El contenido de las Secciones I y II abarca dos aspectos diferenciados y gradacionales de toda investigación, ya que la primera abarca el estudio exhaustivo de la materia prima: Estudio de los métodos de extracción (lixiviación, más propiamente dicha), identificación de los componentes presentes en los sarmientos en función del cultivar y comparación de este material con virutas de roble con vistas a una sustitución de éstas por sarmientos o al uso conjunto de ambos en el envejecimiento del vino. Dado el escaso o nulo coste de los sarmientos, tan abundantes en la agricultura de la vid, este uso podría proporcionar una interesante fuente de ingresos. Una comparación del perfil de volátiles de ambos

materiales también constituye materia de esta sección para conocer cómo puede contribuir cada uno al aroma del vino envejecido en su presencia.

El conocimiento exhaustivo y comparativo de las características químicas de los dos tipos de materiales condujo a la siguiente etapa, también comparativa, del uso de ambos (tradicional y potencial) en el envejecimiento del vino, tal como se recoge en la Sección II: La comparación del comportamiento de ambos materiales en esa etapa, realizada en las mismas condiciones para los dos, con el mismo tipo de vino tinto y durante tiempos iguales. El estudio sensorial mediante un panel de expertos y el tratamiento informatizado de los informes proporcionados por los panelistas, junto con un estudio más objetivo realizado mediante cromatografía de gases con detección por espectrometría de masas completa esta información que se pone al servicio de las industrias del sector interesadas en innovación en envejecimiento de vinos.

La Sección III abarca la investigación realizada sobre las lías de vinificación para proporcionar una información de su composición que permita decidir qué componentes son los que pueden y deben explotarse. Por tanto, la primera etapa de la investigación fue la identificación de los componentes, polares y de polaridad media; seguida de otra de comparación de los componentes de las lías sólidas y el vino embebido en ellas en función de la bodega de procedencia. Finalmente, la identificación y cuantificación de los componentes de mayor interés, colorantes tales como proantocianidinas, antocianidinas y antocianinas, establecen una vía sólida del uso de las lías para la obtención de colorantes naturales, tan demandados en la actualidad.

Teniendo en cuenta la relativa independencia de los diferentes bloques de la investigación realizada, se ha planteado la discusión de los resultados obtenidos por secciones.

Sección I. Caracterización y comparación de sarmientos de la vid y virutas de roble

La Sección I recoge el desarrollo de diferentes estrategias de extracción y análisis orientadas a la posible explotación de los sarmientos de la vid como una alternativa al uso de madera de roble, utilizada actualmente para la elaboración de barricas y de virutas empleadas en el proceso de envejecimiento del vino. La gran similitud que ambos materiales tienen en su estructura y composición (muy rica en compuestos fenólicos, alcoholes de bajo peso molecular, aldehídos, cetonas y ácidos, así como en otros tipos de compuestos entre los que destacan furanos, lactonas, polisacáridos, ácidos grasos, taninos y pigmentos; todos ellos desempeñando un papel clave en la mejora y estabilización de las características organolépticas del vino) permite prever éxito en la aplicación de los sarmientos para este uso.

Esta sección engloba 5 capítulos en los que la investigación que se recoge tiene un doble objetivo: Conocer de forma exhaustiva los estudios realizados hasta la fecha sobre las diversas técnicas de extracción asistidas por las diferentes formas de energía de las que la investigación puede servirse, así como hacer uso de estas energías para obtener los extractos en los que estudiar las características tanto de la fracción polar y de polaridad media como de la fracción volátil de los sarmientos de la vid y compararlas con las de las virutas de roble comerciales.

Capítulo 1

El Capítulo 1 es a su vez uno de los que conforman un libro multiautor en el que se explora el estado actual y se discuten en profundidad tanto las características como las ventajas e inconvenientes de las técnicas más utilizadas en la actualidad para la extracción de productos naturales, con especial énfasis en la lixiviación con líquidos sobrecalentados (SHLE) que fue comparada con la extracción asistida por microondas y ultrasonidos. En el capítulo también se

discute la aplicabilidad de la SHLE frente a la extracción asistida por energías auxiliares dependiendo de la familia de compuestos que se pretende extraer, y se comparan con técnicas de extracción convencionales, como la maceración, la Soxhlet o el reflujo. La redacción de este capítulo de libro ha permitido la comparación crítica de las metodologías que se han empleado en las investigaciones publicadas en los últimos años, teniendo en cuenta factores como el tiempo necesario para que se complete la extracción, el volumen de extractante, la responsabilidad respecto al uso de extractantes no tóxicos, la eficiencia de la extracción y su coste atendiendo tanto al material de partida como a los compuestos que se pretende extraer. La redacción de este capítulo de libro ha proporcionado a la doctoranda una puesta al día con relación a lo publicado sobre la materia en cuestión, con lo que ha adquirido una base sólida en la que soportar la investigación a desarrollar, así como formación para la interpretación crítica de la investigación publicada por otros autores.

La experiencia adquirida en el estudio bibliográfico que dio lugar al Capítulo 1 proporcionó la base para la investigación que se recoge en el Capítulo 2.

Capítulo 2

La comparación de distintas técnicas de extracción asistidas por diferentes formas de energía para la obtención de extractos ricos en compuestos fenólicos —importantes en enología— a partir de distintas variedades de sarmientos de la vid es la investigación que se recoge en este capítulo. El punto de partida para esta investigación fue la gran similitud de los sarmientos de la vid con la madera de roble que se utiliza comúnmente en el proceso de envejecimiento del vino para mejorar sus características sensoriales. La composición de los sarmientos de la vid se caracteriza por tres grandes fracciones: Celulosa, hemicelulosa y lignina, donde esta última se encuentra en torno al 20% del peso seco. Esta fracción libera al hidrolizarse multitud de compuestos fenólicos proporcionando así al vino características organolépticas que son muy apreciadas. La fracción celulósica también es fuente de compuestos muy interesantes desde el

punto de vista organoléptico, como los furanos y sus derivados, que se forman por degradación de azúcares mediante el tratamiento térmico de la madera y que contribuyen al flavor del vino aportando notas tostadas, acarameladas y a almendra.

Con el objetivo de obtener extractos ricos en estos compuestos para comparar el perfil que pueden proporcionar las distintas variedades de sarmientos, se utilizaron y compararon tres métodos para mejorar/acelerar la extracción mediante la asistencia de ultrasonidos, microondas o con líquidos sobrecalentados. Estas metodologías se optimizaron utilizando estrategias multivariantes y se eligieron las condiciones más adecuadas de cada tipo de extracción determinando el contenido fenólico total en los extractos con el método de Folin-Ciocalteu y la concentración de 5-hidroximetilfurfural mediante separación/-detección por cromatografía de líquidos/detector de diodos en fila (LC–DAD). De este modo se demostró que la extracción realizada con líquidos sobrecalentados a 180 ºC y durante 60 minutos proporciona extractos más ricos en compuestos fenólicos y mayores concentraciones de 5-hidroximetilfurfural que los otros métodos de extracción. Este derivado furánico es clave desde el punto de vista enológico, ya que proporciona al vino notas tostadas y acarameladas, que son muy deseables desde el punto de vista organoléptico; sin embargo, se trata de un compuesto que puede presentar toxicidad por lo que su concentración en alimentos está regulada —en el caso de los extractos obtenidos su concentración se encontró bastante por debajo del límite máximo permitido en vinos.

La separación–detección individual de los compuestos fenólicos de interés mediante LC–DAD requirió 73 min. Los compuestos de interés se identificaron y cuantificaron por comparación de sus tiempos de retención y sus espectros en la zona ultravioleta con los correspondientes de patrones con los que se realizaron las rectas de calibrado. Además, se confirmó la identificación mediante análisis por LC y espectrometría de masas de tiempo de vuelo (LC–QTOF).

La cuantificación individual de los compuestos de interés en cada variedad de sarmientos permitió evaluar las diferencias en concentración fenólica entre variedades, tanto total como individual. Las variedades con un mayor contenido fenólico total fueron Chardonnay y Montepila, mientras que Pedro Ximénez y Baladí fueron las de menor contenido.

La comparación de la capacidad antioxidante de los extractos mediante el método ORAC dio resultados consistentes con los obtenidos mediante el método de Folin-Ciocalteu y la determinación individual del contenido fenólico de los extractos, ya que la variedad Chardonnay, que fue la de mayor contenido fenólico, resultó ser también la variedad con mayor capacidad antioxidante, lo que verifica la solidez de los resultados obtenidos.

El amplio abanico de concentraciones relativas de compuestos fenólicos en función de la variedad de sarmientos abre también un amplio abanico de matices que pueden transferirse al vino si se usan para el envejecimiento, además de las características saludables atribuidas a estos compuestos.

Capítulo 3

La investigación que conforma este capítulo pretendió ampliar la información obtenida hasta el momento sobre las posibilidades del uso de sarmientos de la vid en el envejecimiento del vino que se recoge en el Capítulo 2. Con este fin, se prepararon extractos de cinco tipos de virutas comerciales de roble, tanto francés como americano, y se compararon con los extractos obtenidos de los sarmientos de la vid. La comparación abarcó tanto su composición cuantitativa como el estudio estadístico mediante herramientas quimiométricas para tener una visión general de las características de similaridad/disimilaridad de los extractos de ambos materiales.

Para la extracción y análisis cuantitativo de los extractos de roble se aplicaron los métodos optimizados en el Capítulo 2, en los que se utilizaron líquidos sobrecalentados y separación–cuantificación por comparación de los

tiempos de retención con los proporcionados por los patrones mediante LC–DAD y verificación por LC–QTOF.

Se encontraron concentraciones de 5-hidroximetilfurfural muy similares a las ya encontradas en sarmientos de vid, siempre inferiores al límite máximo establecido. Del mismo modo, se encontraron concentraciones muy similares a las obtenidas en el caso de los sarmientos de vid para muchos otros compuestos de interés enológico, como el ácido gálico, producto final de la hidrólisis de elagintaninos y que es responsable del carácter astringente de los vinos; o el pirogalol, formado por descarboxilación del ácido gálico y que se encontró en todos los extractos en concentraciones similares, lo que demuestra que ambos compuestos están relacionados a través de una ruta bioquímica. Algunos compuestos como la vainillina o el sinapaldehído se encontraron ligeramente más concentrados en los extractos de madera de roble, pero de forma general se puede concluir que los extractos de madera de roble tienen una composición muy similar a la de los sarmientos, por lo que no se puede discriminar atendiendo a este aspecto.

La utilización de la espectrometría de masas de alta resolución permitió un segundo enfoque en el análisis de la fracción polar y de polaridad media de los extractos de ambos materiales. Además de verificar la identificación de compuestos, hizo posible el desarrollo de métodos de análisis comparativo para evaluar la variabilidad de esta fracción. Para ello, utilizando LC–QTOF y análisis no orientado, se analizaron los extractos obtenidos de las 18 variedades de sarmientos de la vid y las 5 variedades de virutas de roble comerciales.

En el estudio entre variedades de sarmientos se observó una discriminación significativa dependiente de la variedad de sarmiento, que se evaluó comparando el número de entidades moleculares (potenciales compuestos) en función de la variedad. Se utilizó el filtrado por frecuencia como pretratamiento de datos para eliminar las entidades moleculares no representativas, lo que redujo el número de estas entidades considerablemente. Asimismo, se llevó a cabo un análisis estadístico no supervisado de componentes principales (PCA), que explicó

el 89 y el 87% de la variabilidad para los modos negativo y positivo, respectivamente. El análisis de varianza para un nivel de confianza de un 95% redujo las entidades moleculares significativas a 47 y 27 para los modos de ionización positivo y negativo, respectivamente; mientras que un intervalo de confianza del 98.5% redujo estas entidades a 10 y 11, respectivamente, y a 8 entidades en el caso de un 99.0% de nivel de confianza. Las representaciones mediante PCA de estos sets de datos proporcionaron información acerca de las variedades que tienen una composición similar. Para un nivel de confianza del 98.5%, aparte del conjunto principal, se obtuvieron otros dos conjuntos, uno formado por las variedades Pedro-Ximénez, Montepila, Merlot, Garnacha Tintorera y Bobal, y otro formado por las variedades Moscatel, Chardonnay y Syrah.

La identificación de estas entidades moleculares que poseen una contribución representativa dio resultados interesantes, ya que se identificaron compuestos como 5-hidroximetilfurfural, pirogalol, acetovainillona, siringaldehído y alcohol coniferílico, entre otros.

Para la comparación de los extractos de sarmientos de la vid con los de roble se aplicó un filtro por frecuencia para reducir la dimensionalidad de los datos. Este filtrado es crucial puesto que trabajando con las entidades que están presentes en todas las muestras, extractos de roble o de sarmientos, se asegura la representatividad de cada tipo de muestra. De esta forma el número de entidades moleculares se redujo a 69 y 37 para los modos de ionización positivo y negativo, respectivamente. Como era de esperar, la aplicación de PCA mostró dos grupos bien diferenciados, ya que ambos extractos provienen de dos tipos de muestras distintos, por lo que se empleó otro filtro menos restrictivo para estimar el nivel de similitud entre ambos tipos de muestras. Aplicando un filtro por frecuencia del 40% las entidades moleculares fueron 647 en ambos modos de ionización. El PCA de este set de datos mostró una serie extractos de variedades de sarmientos que poseían mayor similitud con los extractos de roble. Las componentes principales que explicaron más del 90% de la variabilidad correspondieron a Garnacha Tinta, Malbec, Cabernet Sauvignon, Sauvignon Blanc, Petit Verdot, Pedro-Ximénez, Chardonnay, Garnacha Tintorera, Merlot y Montepila.

Este estudio constituye un sólido soporte para la caracterización de los sarmientos de la vid como una potencial alternativa al uso de virutas de roble con fines enológicos, ya sea como sustitutos de éstas o para su uso simultáneo con ellas.

Capítulo 4

Para profundizar en el estudio del posible uso de los sarmientos con fines enológicos, se consideró conveniente estudiar y comparar el perfil de los compuestos obtenidos mediante GC–IT/MS en los extractos obtenidos a partir de sarmientos y de virutas comerciales de roble, dada la enorme influencia de este perfil de la madera de roble en el aroma y el sabor del vino durante y tras el proceso de envejecimiento. Con este propósito se aplicó el método optimizado en el Capítulo 2 para la extracción mediante líquidos sobrecalentados. Los perfiles cromatográficos obtenidos se compararon mediante herramientas quimiométricas. Estos perfiles, si bien fueron relativamente similares entre sí, revelaron diferencias entre los tipos de madera y entre las variedades de sarmientos. El perfil fenólico fue muy similar y en él se identificaron compuestos tan importantes desde el punto de vista enológico como la vainillina, la acetovanillona o la propiovanillona, con ligeras diferencias en cuanto a su concentración, que generalmente es más baja en el caso de los sarmientos, aunque ciertas variedades como Chardonnay presentan niveles comparables a los de los extractos de roble. Este es el caso también del etil vainillato, un producto derivado de la vainillina que confiere al vino características de olor a quemado y ahumado; por tanto, un exceso de etil vainillato puede tener un efecto negativo en el bouquet del vino. Este compuesto se identificó en la mayoría de las muestras, independientemente de su origen, siendo su concentración algo más elevada en las muestras de roble.

Entre los compuestos que se encontraron en mayores concentraciones en los sarmientos que en las virutas de roble estuvieron el eugenol, que proporciona un sabor afrutado al vino, y compuestos de tipo siringil como el siringol, siringaldehído y ácido siríngico. Los extractos de roble mostraron mayores

concentraciones de alcohol homovainíllico y coniferaldehído, que proporcionan aromas dulce y a madera, respectivamente.

Algunos compuestos se identificaron sólo en ciertas variedades, independientemente del tipo de muestra. Éste es el caso de la acetosiringona y el ácido homosiríngico, que también se asocian a aromas dulces y a madera, mientras que el metoxieugenol se asocia a sabores picantes y a quemado. En cuanto a los compuestos furánicos, cuyo aroma se ha descrito como a caramelo o azúcar quemada, no se encuentra una variabilidad significativa entre extractos, lo que se debe probablemente a pérdidas en la etapa de preparación de muestra. Sin embargo, en la investigación recogida en los anteriores capítulos se ha demostrado que los compuestos furánicos están en cantidades más significativas en los extractos de sarmientos que en los de virutas de roble. Los niveles de 5-hidroximetilfurfural en los extractos de ambos tipos de muestras fueron similares, mientras que el resto de derivados furánicos estaban más concentrados en los extractos de sarmientos.

La primera etapa para la comparación de los extractos de sarmientos de vid con los de virutas de roble fue aplicar un filtro por frecuencia para reducir la dimensionalidad de los datos. Se aplicaron estos filtros desde dos puntos de vista; considerando todas las muestras como un único grupo y como grupos de muestras por separado: Sarmientos y virutas de roble. La aplicación de los distintos filtros por frecuencia puso de manifiesto la gran variabilidad que existe, no sólo entre ambos grupos de muestras, sino también entre las distintas variedades de sarmientos y de roble. Atendiendo a esta variabilidad, se seleccionaron dos sets de datos; aquellos formados por las entidades moleculares presentes en al menos el 70% de todas las muestras y los formados por las entidades moleculares que se detectaron en al menos el 90% de las muestras pertenecientes a sarmientos o a virutas de roble. El porcentaje considerado en el primer filtro es menos restrictivo, ya que se aplicó al set de muestras completo que, obviamente, presenta una mayor variabilidad. De esta forma el número de entidades moleculares se redujo de 80 y 275 a 42 y 30, en muestras de sarmientos y virutas de roble, respectivamente.

Se utilizó PCA para evaluar la similitud entre los extractos de sarmientos de vid y de virutas de roble en los que, si bien la representación tridimensional de las componentes principales no reveló una completa discriminación entre ambos tipos de extractos, la representación bidimensional sí reveló cuáles son las variedades de sarmiento cuya composición es más similar a las de virutas de roble. Merlot, Bobal, Pedro-Ximénez, Mazuelo y Baladí proporcionaron extractos más similares a los de las virutas de roble americano, en tanto que los extractos de las variedades Cabernet Sauvignon, Sauvignon Blanc y Chardonnay resultaron ser más similares a las variedades de roble francés.

Este estudio, al igual que el recogido en el anterior capítulo, pone de manifiesto la posibilidad de jugar con la variedad de la vid para conseguir, en este caso un bouquet del vino envejecido similar o con los matices diferenciadores que se deseen jugando con la variedad o con la mezcla de variedades de sarmientos.

Capítulo 5

La investigación recogida en este capítulo pretendió ampliar la información discutida en el Capítulo 4 centrándose en el perfil de volátiles de los sarmientos de vid y de las virutas de roble. Para ello se consideró conveniente estudiar y comparar el perfil de volátiles de ambos materiales producido durante el proceso de tostado de la madera, dada la enorme importancia que tiene esta etapa en el proceso de fabricación de barricas de roble para su uso en el envejecimiento de vinos y otras bebidas alcohólicas. Con este propósito se optimizó un método sencillo para el tostado, en un dispositivo de espacio de cabeza, de las 5 variedades de virutas de roble y las 18 variedades de sarmientos de vid y se analizó el contenido del espacio de cabeza mediante GC–IT/MS. Esta estrategia permitió obtener un perfil de compuestos volátiles representativo de cada muestra.

La optimización del tostado de muestras contempló tanto el tiempo de tostado como la temperatura, siendo finalmente 20 min y 200 ºC las condiciones para la máxima extracción de compuestos.

La comparación de los perfiles de volátiles obtenidos a distintas temperaturas de tostado mediante PCA permitió establecer la temperatura de tostado como el principal factor de variabilidad, proporcionando las temperaturas de tostado más bajas (120 y 150 ºC) un perfil más similar entre sí, mientras que a las temperaturas más altas (180 y 200 ºC) aparecen como clusters separados, que a su vez permiten una completa discriminación entre sarmientos y madera de roble.

La identificación de compuestos de interés en los perfiles de volátiles de ambos tipos de muestras puso de manifiesto una composición muy similar en ambos casos. El aumento de la temperatura proporcionó perfiles ricos en compuestos generados por pirólisis de polisacáridos (aldehídos furánicos) o por la reacción de Maillard (furanonas y piranonas), así como de compuestos generados por degradación de la lignina (aldehídos fenólicos y fenoles volátiles).

Entre los compuestos que se identificaron en las distintas variedades de virutas de roble y en las de sarmientos hay que destacar algunos muy interesantes desde el punto de vista enológico, como furfural y 5-metil-2-furancarboxaldehído, que participan en numerosas reacciones relacionadas con el envejecimiento del vino; o las lactonas del roble, que se identificaron sólo en las virutas de roble y en concentraciones crecientes a medida que aumentó la temperatura de tostado y de forma más intensa en los robles americanos que en los franceses. Otros derivados de compuestos furánicos, como el 1-(2-furanil)etanona o el 2-furanmetanol-acetato, que se han relacionado con aromas a café, tostado, miel o caramelo, se identificaron en todas las muestras, independientemente de la temperatura de tostado.

Como era de esperar teniendo en cuenta que los compuestos fenólicos son el producto final de la degradación térmica de la lignina, se detectó gran cantidad de compuestos de estructura tipo guayacil y siringil, siendo la gran mayoría comunes en sarmientos de vid y en virutas de roble. El ácido ferúlico fue el único que sólo se detectó en virutas de roble a baja temperatura (120 ºC), aunque su ausencia en el resto de muestras puede explicarse por degradación térmica, ya

que este compuesto ha sido descrito en otras investigaciones como precursor de compuestos fenólicos (4-vinilguayacol, 4-etilguayacol, vainillina y guayacol, entre otros); lo que se confirma por la presencia de estos compuestos tanto en sarmientos de vid como en virutas de roble cuando se tuestan a 200 ºC.

Finalmente, y mediante el empleo de patrones de los compuestos más significativos desde el punto de vista sensorial, se llevó a cabo el análisis confirmatorio de la identificación, tanto por tiempo de retención como por espectro de masas, lo que, además, permitió calcular la concentración relativa de estos compuestos en las distintas muestras.

Por tanto, se demuestra en este estudio que, en términos cualitativos, tanto las virutas de roble comerciales, que se usan tradicionalmente en el proceso de envejecimiento del vino, como los sarmientos de vid tienen un perfil volátil muy similar. Asimismo, el análisis multivariante permitió identificar las variedades de sarmiento que tienen un perfil más similar al de las virutas de roble, por lo que estos resultados preliminares pueden considerarse en futuros ensayos de uso de los sarmientos de vid en el proceso de envejecimiento del vino, ya sean solos o combinados con virutas de roble.

La comparación de los resultados obtenidos en los Capítulos 4 y 5 pone de manifiesto la gran similitud en cuanto a compuestos interesantes desde el punto de vista enológico que tienen los sarmientos de la vid y las virutas de roble, siendo su presencia de forma general algo más elevada en el caso de la virutas de roble. Si bien en el Capítulo 5 el interés está focalizado en el estudio de la fracción volátil de ambos materiales, son muchos los compuestos comunes tanto en extractos como en el espacio de cabeza obtenido por calentamiento directo de ambos materiales, este es el caso de compuestos tan interesantes desde el punto de vista enológico como la vainillina, la etilvainillina y la acetovanillona, conocidos por las notas avainilladas que transmiten al vino. O compuestos del tipo siringil como son el siringol, siringaldehído o acetosiringona, que fueron igualmente detectados tanto en los extractos de ambos materiales como en el espacio de cabeza de estos, y que son responsables de sabores dulces y aromas a madera. Igualmente es llamativa la

abundancia de compuestos de tipo furánico en ambos casos, que dotan a los vinos con sabores a madera y almendra tostada. Así como los extractos preparados a partir de ambos materiales destacan por ser más ricos en etil esteres de ácidos grasos, los cuales juegan un papel positivo en el aroma del vino contribuyendo con notas afrutadas y florales, como es el caso del ácido 2-butenedioico dietil ester o el ácido hexanoico etil ester.

Por tanto, se plantea la preparación o no de extractos de estos materiales para su aprovechamiento dependiendo de la finalidad, tal y como podría ser el caso del uso directo de virutas en el envejecimiento de vinos y que constituye el cuerpo principal de la Sección II de esta tesis doctoral.

Sección II. Explotación de los sarmientos de vid. Comparación de vinos envejecidos en presencia de sarmientos de vid *versus* virutas de roble

La investigación recogida en la sección anterior se basó en la hipótesis de que el uso de sarmientos de vid para el envejecimiento de vino tinto puede producir caldos con características similares a las de los obtenidos con virutas de roble. La investigación que se recoge en esta Sección II abarca vías para la confirmación de la hipótesis: Una subjetiva apoyada en la experiencia de un panel de 15 expertos para evaluar las características organolépticas que los sarmientos de la vid pueden otorgar al vino y sus diferencias respecto a las virutas de roble. La opinión de los expertos sobre el perfil sensorial de los vinos, expresada en cuestionarios bien diseñados, permitió un estudio estadístico sólido del conjunto de las respuestas incluidas en cada uno de ellos. La otra vía estuvo basada en elementos objetivos como los que proporcionó el perfil de volátiles de estos vinos mediante GC–IT/MS y el subsiguiente estudio quimiométrico. Estos estudios constituyen los Capítulos 6 y 7, respectivamente.

Capítulo 6

En este capítulo se recoge la evaluación sensorial por un panel de expertos de un vino tinto que había sido envejecido durante 6 y 9 meses en presencia de virutas de roble francés y americano, y de dos variedades de sarmientos de la vid (Merlot y Montepila), tostados y no tostados; todos ellos se compararon con un vino control que no estuvo en contacto con ningún material que modificara el envejecimiento natural. De este modo se pretendió verificar la hipótesis de partida acerca del potencial uso de los sarmientos de vid para el envejecimiento del vino. Este estudio es el primero de sus características, llevado a cabo con una evaluación del perfil sensorial de vinos envejecidos en presencia de sarmientos de vid.

El vino envejecido en presencia de virutas de roble presentó un perfil sensorial muy similar al del vino control, con algunas notas a tabaco, cuero, ciruelas/pasas, café y especias dulces. Los sarmientos de vid proporcionaron perfiles con notas más pronunciadas a pasas/ciruelas, en el caso de Montepila no tostado, y regaliz en el caso de Montepila tostado, mostrando también un carácter más ácido y astringente y con un perfil a frutos rojos, tabaco y cuero menos pronunciado que en los vinos envejecidos con virutas de roble. Se observó un incremento en aromas a tabaco y a café en todos los vinos tratados con respecto a los control, obteniéndose intensidades medias de percepción en todos los casos. Por el contrario, el carácter fresco se vio disminuido respecto al vino de control en todos los casos, siendo más evidente esta disminución en el caso de las virutas de roble.

En cuanto al perfil gustativo, el vino control estuvo marcado por su amargor, astringencia, intensidad y calidad del dejo e intensidad de sabor. Ciertos atributos negativos se vieron disminuidos en los vinos tratados, como el amargor en el caso de robles tostados americanos y sarmientos del tipo Montepila no tostados. También disminuyó la percepción de la astringencia, especias dulces y gusto a verde, especialmente en vinos tratados con virutas de roble. El resto de atributos no presentaron una variación significativa en los vinos tratados, excepto

los envejecidos con sarmientos de la variedad Merlot que mostraron un menor gusto a frutos rojos que el resto de vinos tratados y mucho menor que el vino de control, siendo, en general, esta variedad de sarmientos la que produjo una variación más discreta de todos los atributos sensoriales.

Finalmente, la comparación estadística de las valoraciones emitidas por el panel de cata mediante ANOVA permitió discriminar las diferencias más significativas entre los caldos. Se comparó el vino control con los vinos tratados durante 9 meses para tener una visión global de los cambios producidos. En general, el uso de virutas de roble tuvo mayor efecto en los atributos olfativos, en los que los caracteres correspondientes a cuero, pasas/ciruelas y tabaco fueron estadísticamente más intensos en los vinos tratados, al contrario que los aromas a fresco y frutos rojos. En los vinos envejecidos en presencia de sarmientos de vid fue significativa la reducción en la percepción del gusto a verde y el incremento de la acidez con respecto al vino control. En los atributos olfativos se encontraron diferencias evidentes en la intensidad del aroma a tabaco y pasas/ciruelas, que aumentó con respecto al vino control, al contrario que el aroma a fresco, que disminuyó especialmente en los vinos tratados con la variedad Merlot.

El tiempo de envejecimiento no ejerció influencia significativa en los vinos tratados con virutas de roble; sin embargo, los vinos tratados con sarmientos de vid experimentaron una disminución estadísticamente significativa en la percepción del gusto a verde cuando el envejecimiento se prolongó 9 meses, mientras que las notas a especias fueron más significativas a este tiempo de envejecimiento más largo. La influencia de la etapa de tostado en los materiales para el envejecimiento ejerció cierta influencia en el cuerpo del vino, la calidad del dejo y la astringencia; influencia que fue menos perceptible en vinos tratados con sarmientos no tostados, mientras que la intensidad del aroma fue mayor cuando se utilizaron virutas de roble tostadas.

Finalmente, la comparación de los vinos mediante PCA bidimensional permitió discernir qué variedades estaban más próximas entre sí de acuerdo con

sus características sensoriales, así como deducir qué atributos son responsables de las diferencias.

Capítulo 7

En este capítulo se continúa el estudio de los vinos envejecidos en presencia de virutas de roble o de sarmientos de la vid desde un punto de vista más analítico, basado en el estudio del perfil de volátiles de estos vinos obtenido mediante GC–IT/MS y el subsiguiente estudio quimiométrico e identificación de los compuestos más representativos.

La evaluación por separado de los cambios ocurridos a corto y a largo plazo en los vinos tratados mostró diferencias significativas en cuanto a la concentración de compuestos interesantes. Así, los vinos tratados durante 3 y 9 meses en presencia de virutas de roble o de sarmientos de vid poseían compuestos comunes muy interesantes desde el punto de vista organoléptico que, de forma general, se encontraron más concentrados en los vinos que estuvieron en contacto con virutas de roble, especialmente en el caso de vinos tratados durante 9 meses. Son ejemplos de este comportamiento el 4,5-dimetil-1-hexeno (aroma floral), el 1-hexanol y 3-penten-1-ol (gusto a verde), el siringol (sabor ahumado) y el 1,8-nonadien-1-ol (frutal).

Es llamativo que la existencia de multitud de compuestos más concentrados en los vinos tratados que en los vinos de control no causara diferencias significativas entre vinos tratados en espacios de tiempo cortos, de lo que se deduce que tanto las virutas de roble como los sarmientos de vid los transfieren al vino en cantidades similares a corto plazo. Ejemplos de este comportamiento son la 5-hidroxivainillina (aroma a vainilla), el isobutil salicilato (aroma floral), el guayacol (sabor ahumado), el tetrahidrogeraniol (aromas florales) y otros como los ácidos pentanedioico dietil éster, el butanedioico monoetil éster, o el homosiríngico. En el caso de vinos envejecidos durante 9 meses, este comportamiento sólo se observó en el guayacol, el isobutil salicilato y el ácido homosiríngico.

El estudio comparativo de los datos proporcionados por un perfil de cata y de los obtenidos mediante análisis GC–IT/MS arroja datos interesantes, la disminución del gusto a verde con respecto al vino de control justifica las diferencias significativas encontradas en el caso de 3-penten-1-ol, 1-hexanol, ácido butanoico 3-hidroxietil ester y tetrahidrogeraniol, este último responsable del gusto a verde y a notas florales, así como el 2,3-dimetil-1-butanol, responsable del gusto herbáceo y que también puede contribuir de formas generales al atributo verde. Del mismo modo, el aumento de la astringencia en vinos tratados con madera de roble puede ser justificada por la presencia de compuestos clave en la manifestación de este atributo, como son el ácido hidroxibenzóico e hidroxicinámico entre otros compuestos. Tanto guayacol como siringol mostraron diferencias estadísticamente significativas cuando se compararon los vinos tratados frente a los vinos control, sin embargo los catadores no apreciaron el sabor tostado o ahumado, característico de estos compuestos como un rasgo significativo de los vinos catados, pudiendo esto atribuirse al umbral de percepción de estos. Sin embargo, el gusto a especias que también suele ser asociado al siringol sí que fue detectado como rasgo significativo por los catadores, experimentando una reducción significativa en los casos de vinos tratados con respecto a los vinos control tal y como se comprueba en el análisis mediante GC–IT/MS. Del mismo modo, el aumento del gusto a regaliz y a café experimentado en todos los vinos tratados puede ser explicado por la elevada cantidad de compuestos furánicos que se han detectado en los trabajos que dieron lugar a los capítulos 4 y 5 de esta tesis doctoral, y que entre otros, suelen ser responsables de notas tostadas, acarameladas, regaliz, café, pan tostado y serrín.

Por lo tanto, se puede concluir que los sarmientos de vid constituyen una posible y novedosa materia para el envejecimiento del vino, pudiéndose obtener vinos con distintas cualidades mediante la manipulación de la duración del proceso y la variedad de los sarmientos utilizados.

Sección III. Caracterización de las lías de vinificación y cuantificación de familias de compuestos de interés

Esta última sección de la Tesis se dedica al estudio de la composición de lías de vinificación de vino tinto para su posible valorización. La sección se compone de 3 capítulos en los que la investigación que se recoge tuvo un doble objetivo: Evaluar el potencial de las lías de vinificación como fuente de compuestos de interés dada su riqueza en antioxidantes, como fenoles (entre los que destacan flavonoides como las antocianinas y sus derivados, y no flavonoides, como los ácidos gálico, ferúlico y caféico) y en aminoácidos esenciales y sus derivados, entre otros compuestos de interés, así como desarrollar un método cromatográfico para la detección y cuantificación de colorantes en lías sin necesidad de usar patrones analíticos. Con este doble objetivo se estudiaron las fracciones polar y de polaridad media mediante análisis no orientado LC–QTOF y se llevó a cabo la identificación de compuestos representativos mediante bases de datos MS/MS de calidad como Metlin, Massbank, Plantcyc y Metfrag. Se desarrolló un método de análisis orientado ("Data Dependent Methods") para la cuantificación de antocianinas, antocianidinas y proantocianidinas, que finalmente se verificó mediante análisis LC–QTOF, tal como se recoge en los Capítulos 8, 9 y 10.

Capítulo 8

Este capítulo recoge la investigación sobre la evaluación de las lías de vinificación como posible materia prima para la extracción de compuestos de interés, ya que tradicionalmente este residuo se ha utilizado como fuente de ácido tartárico o de colorantes, pero nunca se había caracterizado cualitativamente. Por tanto, se realizó la extracción de lías de vinificación de vino tinto procedentes de 11 bodegas españolas con ayuda de un digestor de microondas focalizadas. Los extractos se analizaron mediante LC—QTOF con deteción MS/MS en modo de alta resolución.

Hay que destacar que para obtener una buena exactitud en la medida de la relación *m/z*, necesaria para la identificación del máximo número de compuestos representativos, característico de un análisis global, se necesita un equipo de masas de alta resolución, esta instrumentación permite la obtención de espectros MS/MS de los iones precursores detectados y proporciona la información necesaria para la identificación de cada compuesto.

La alta capacidad de resolución del análisis mediante MS/MS permitió la identificación de una amplia variedad de compuestos representativos, pertenecientes a diferentes familias y entre los que figuran aminoácidos primarios, antocianos, flavanoles, flavonoles, flavonas y compuestos fenólicos no flavonoides, entre otros; lo que demostró que el protocolo basado en LC–MS/MS es una buena estrategia para el análisis de este tipo de muestra.

Se identificaron 8 aminoácidos esenciales y 4 derivados de aminoácidos (e.g. L-arginina, L-prolina, L-valina, L-asparraguina, L-tirosina, 3-hidroxi-L-tirosina, y ácido aminocaproico). También se identificaron multitud de flavonoides, entre los que destacan flavonoles como quercetina, miricetina, kaemferol y isoharmmetina, que se encuentran comúnmente en uvas; derivados glucósidos como la quercetina-3-*O*-glucósido; flavanoles, como la catequina, epicatequina, galocatequina o la procianidina B2 y antocianinas como la peonidina-3-*O*-glucósido, peonidina 3-(6-*p*-cumarilglucósido) y la malvidina 3-(6-*p*-cumarilglucósido). Entre los compuestos no flavonoides identificados se encontraron principalmente aldehídos fenólicos (vainillina, aldehído protocatéquico), estilbenos (resveratrol-3-*O*-glucósido y *trans*-resveratrol) y ácidos fenólicos, como los hidroxicinámicos (ácido sinápico, caféico e hidroxicinámico) y ácidos hidroxibenzóicos, como el ácido siríngico, gálico, protocatequico e hidroxibenzóico.

Otros compuestos de interés identificados fueron ácidos orgánicos, como el málico, el tartárico, el cítrico, el shikímico, el pantoténico y el galactárico.

Este estudio de identificación ha contribuido a caracterizar este residuo tan poco estudiado con fines de explotación y valorización.

Capítulo 9

La investigación recogida en este capítulo se plantea como la continuación natural del estudio iniciado en el capítulo anterior; por tanto, utilizando la misma metodología se realizó el análisis e identificación de compuestos representativos presentes en la fracción líquida de las lías de vinificación o "vino embebido" y se comparó con la información obtenida en el caso de extractos de lías secas mediante aplicación de estrategias de análisis multivariante. La hipótesis de partida fue la posibilidad de explotación por separado de ambas fracciones, dado que actualmente la fracción líquida se viene desechando junto con las aguas residuales durante el proceso de elaboración del vino y, por tanto, tienen escaso o nulo valor económico. Para el estudio se utilizaron lías de vinificación pertenecientes a 11 bodegas españolas y correspondientes a vinos tintos de dos denominaciones de origen muy valoradas en España: Ribera del Duero y Rioja.

La utilización de la espectrometría de masas de alta resolución en modo no orientado permitió el análisis de la fracción polar y de polaridad media de ambos materiales e hizo posible el desarrollo de métodos de análisis comparativo.

La comparación de los perfiles de elución de ambas fracciones mostró que los vinos embebidos poseen mayor cantidad de compuestos polares, mientras que en los extractos de lías sólidas predominan compuestos menos polares, con perfiles de elución a tiempos más largos, siendo también más abundantes, lo que se explica por el carácter adsorbente de este residuo.

Dada la gran abundancia de datos que proporciona el análisis no orientado, es de vital importancia el diseño adecuado para su tratamiento. Son fundamentales un correcto alineamiento y filtrado de datos para asegurar la representatividad. La comparación cualitativa de ambos tipos de muestra se llevó a cabo mediante aplicación de diagramas de Venn, que pusieron de manifiesto una gran similitud entre ambas fracciones, encontrándose 61 entidades moleculares comunes en el modo de ionización positivo y 23 en el negativo. Las

entidades moleculares exclusivamente detectadas en vinos embebidos y en extractos de lías fueron 7 y 29, respectivamente, en el modo de ionización positivo, mientras que en el negativo fueron 44 y 11, respectivamente. La diferencia en número de entidades moleculares entre ambas polaridades se explica por la influencia que la estructura química de estos compuestos tiene en la eficiencia de la ionización.

La evaluación de las muestras mediante PCA confirmó la suposición de la distinta composición de ambas fracciones, ya que ambos tipos de muestra aparecieron bien diferenciados en las dos polaridades, siendo la variabilidad explicada por componentes principales de un 54.1% y de un 61.7% en los modos positivo y negativo, respectivamente. El origen de cada una de las muestras no mostró tener influencia.

Del mismo modo que en el Capítulo 8, en el presente capítulo se identificaron 40 entidades moleculares representativas, entre las que se detectaron 4 aminoácidos esenciales, dos de ellos comunes a ambas fracciones (3-hidroxi-L-tyrosina y L-valina), uno propio de los vinos embebidos (ácido aminocaproico) y uno exclusivo de los extractos de lías secas (L-tirosina octadecil éster). Entre los compuestos fenólicos encontrados destaca la abundancia de flavonoides, muy comunes en el vino y en las uvas, como la 3,4,5-trimetoxiflavona, la malvidina 3-(6-*p*-cumarilglucósido), la quercetina, la miricetina y conjugados como la malvidina-3-galactósido, que fueron identificados exclusivamente en extractos de lías sólidas. En el vino embebido se encontraron compuestos tan interesantes desde el punto de vista nutracéutico como la catequina y la epicatequina, entre otros; mientras el ácido gálico la procianidina B2 y la pelargonidina 3-(6-*p*-cumarilglucósido) fueron comunes a ambas fracciones. Otros compuestos identificados diferenciadores del vino embebido fueron la 8-oxo-guanosina monofosfato, el ureidoglicolato, la benzoquinona, el ácido cítrico o la S-adenosilmetioninamina. Se concluye, por tanto, que ambas fracciones pueden ser consideradas fuentes de obtención de compuestos de interés, siendo las lías de vinificación especialmente ricas en compuestos fenólicos, concretamente flavonoides, lo que está ligado al carácter adsorbente de esta fracción.

Capítulo 10

La investigación recogida en este capítulo se orientó al desarrollo y aplicación de métodos de adquisición de datos ("data-dependent methods") basados en la versatilidad del equipo analítico —un espectrómetro de masas de triple cuadrupolo— utilizado para la identificación de antocianinas, antocianidinas y proantocianidinas en extractos de lías de vinificación y vinos embebidos en lías pertenecientes a ocho variedades. Se utilizaron tres modos de adquisición: el barrido de ion precursor y de ion producto, y el barrido de pérdida de masa neutra, que son de gran utilidad en análisis confirmatorio cuando no existen patrones comerciales. Posteriormente se llevó a cabo el análisis mediante SRM para la cuantificación de los principales compuestos que constituyen el perfil de antocianos de las muestras y, en el caso de compuestos para los que no había patrón analítico disponible, se utilizó el de la correspondiente aglicona.

La plataforma instrumental utilizada estuvo constituida por una estación de SPE conectada en serie con un cromatógrafo de líquidos y un espectrómetro de masas de triple cuadrupolo (SPE–LC–MS/MS), con la que se realizó una optimización exhaustiva de cada etapa: Primero de la fragmentación y medida, con la ayuda de patrones analíticos, donde se utilizó el modo de ionización negativo para la identificación de proantocianidinas y el positivo para el caso de las antocianidinas; después se optimizó la separación cromatográfica. La posterior optimización de la etapa de preparación de la muestra se realizó utilizando distintos tipos de sorbentes con diferente tamaño de partícula y sometidos a diferentes acondicionamientos, estudiando la posible dilución de la muestra previa a su paso por el sorbente y la etapa de retención, la disolución de lavado y la de elución consideran-do su naturaleza, volumen y caudal a través del sorbente. Este estudio, previo al de estabilidad, finalizó con la validación del método —utilizando muestras reales de vino embebido y extractos de lías de vinificación fortificadas y no fortificadas— y con la evaluación de su sensibilidad y precisión a 3 niveles de concentración. Los límites de detección y cuantificación obtenidos estuvieron

entre 15 y 45 pg/mL para vinos embebidos y entre 15 y 78 pg/mL para extractos de lías. La variación en el día y entre días, expresada como desviación estándar relativa, varió desde 0.1% a 10.9% y de 0.4% a 10.1%, respectivamente, para vinos embebidos y extractos de lías, dependiendo del analito estudiado.

Finalmente, la confirmación de la identificación realizada mediante los métodos de adquisición descritos anteriormente se llevó a cabo con LC–MS/MS en modo de alta resolución utilizando un analizador de tiempo de vuelo y el mismo método cromatográfico.

En términos generales, las concentraciones de analitos en los extractos de lías secas fueron mayores que en los vinos embebidos, especialmente para los derivados glucósido. La proantocianidina B se encontró siempre en mayores concentraciones que la de tipo A, a niveles entre 2.0 y 31.5 ng/g en extractos de lías secas y entre 0.3 y 23.5 ng/mL en vinos embebidos. Los niveles detectados de antocianidinas oscilaron entre 1.16 y 77.5 ng/g en los extractos de lías y entre 0.1 y 7.5 ng/mL en los vinos embebidos.

La aplicación de métodos cuantitativos permitió la comparación de la concentración de los analitos de interés en muestras de lías secas y de vino embebido en ellas procedentes de varias bodegas españolas. La capacidad de los métodos dependientes de datos combinados con MS de alta resolución para la confirmación de la identidad de compuestos desconocidos sin necesidad de patrones analíticos comerciales fue clave en este caso para el éxito de la investigación.

DISCUSSION OF THE RESULTS

The current regulation of the University of Córdoba on proposing doctoral dissertations dictates that the articles resulting from the doctoral work, whether published or in the way for publication, should be included in the Thesis report as such, and that the report should include a section presenting a joint discussion of the results, the structure of which depends on the homogeneity of the developed research. In the present PhD Book, the research has as a common aspect the study of byproducts, residues or wastes from both vineyards and the subsequent winery industry for a potential exploitation as integral as possible. These raw materials have been used either to obtain extracts rich in valuable compounds or to age wines. Therefore, the objective was unique: to characterize residues that presently have a scant or nil value by exploitation from two points of view, nutraceutical and organoleptic.

The research in the PhD Book has been divided into three sections as a function of both the objective and the type of sample, in such a way that Sections I and II have in common an agricultural waste scantly studied and less exploited so far: vine-shoots. On the other hand, Section III is devoted to a waste of the winery industry that has also received limited attention: vinification lees.

The content of Sections I and II encompasses two different and gradational aspects of any investigation. Thus, the first section is devoted to the exhaustive study of the raw material: study of the extraction (more properly expressed as leaching or lixiviation) methods, identification of the compounds present in the vine-shoots as a function of the given cultivars and comparison of this material with oak chips with the final aim of either substituting the latter by vine-shoots or using both together in wine ageing. Taking into account the scant of nil costs of vine-shoots, so abundant in vineyards, this use could provide an interesting economical incoming. A comparison of the volatile profiles from both materials also constitutes a subject of this research to know how each of them can contribute to wine aged in its presence.

An exhaustive and comparative study of the chemical characteristics of both types of materials led to the next step, also comparative, on the traditional and potential use of both for wine ageing, as shown in Section II: comparison of the behavior of both materials in wine ageing, carried out under the same conditions for both, on the same type of red wine and for the same ageing time. The sensory study by an expert panel and the chemometric treatment of the reports provided by the experts, together with the more objective study developed by gas chromatography with mass spectrometry detection, completed the obtained information that has been put at the service of the winery industries interested in innovation on wine ageing.

Section III is devoted to the research on vinification lees to provide information on their composition that allowed deciding what of their components can and must be exploited. Therefore, the first step of this research was to identify lees polar and mid-polar components; followed by the step for comparison of the components of solid lees with those of the wine imbibed in them, as a function of the winery. Finally, identification and quantitation of the most interesting components —colorants such as proanthocyanidins, anthocyanidins and antho-cyanins— established a solid basis to obtain natural colorants, very demanded at present.

Taking into account the relative independence of the sections, the discussion of the obtained results is divided into the given sections.

Section I. Characterization and comparison vine-shoots and oak chips

Section I is devoted to the development of different extraction strategies focused on a potential exploitation of vine-shoots as an alternative to the use of oak wood, presently the most used wood for barrel fabrication and for obtainment of chips both used for wine ageing. The similar composition and

structure of oak and vine-shoots (both rich in aromatic compounds such as phenols, low-molecular weight alcohols, aldehydes, ketones and acids, as well as other types of compounds among which furans, lactones polysaccharides, fatty acids, tannins and pigments as the most important) make foreseeable a successful use in application of vine-shoots for ageing.

This section is composed by 5 chapters, in which the research has a dual objective: to know in an exhaustive way the studies developed so far on the different extraction techniques assisted by the different types of energy used in analytical research, and to use them for the study of the characteristics of the polar, mid-polar and volatile fractions of vine-shoots in comparison with those of commercial oak chips.

Chapter 1

Chapter 1 is also one chapter of a multiauthor book. In it, the characteristics, and the advantages and disadvantages of the techniques more used at present for the extraction of natural products with special emphasis on superheated liquid extraction (SHLE) that was compared with ultrasound and microwave-assisted extraction. The applicability of SHLE was also compared with that of extraction assisted by these energies depending on the families of target compounds to be extracted are discussed in the chapter, together with a comparison with conventional extraction techniques such as maceration, Soxhlet or reflux. Critical comparison of the methods published in the last years, concerning factors such as the time required for complete extraction, extractant volume, the use of toxic solvents, the extraction efficiency and costs attending to both the raw material and the compounds to be extracted, has been the key for updating the knowledge of the PhD student on the matter.

The experience acquired in the bibliographic study in Chapter 1 provided to the PhD student the basis for the research in Chapter 2.

Chapter 2

Comparison of extraction techniques assisted by different forms of energy to obtain extracts rich in phenol compounds —of key importance in oenology— from different vine-shoots varieties is the matter of Chapter 2. The starting point of this research was the similitude between vine-shoots and oak wood, the material usually employed in wine ageing to improve its sensory properties. Vine-shoots are characterized by three main fractions: cellulose, hemicellulose and lignin, of which the last constitutes 20% of dry-weight. Upon hydrolysis, this fraction liberates a huge variety of phenol compounds that confers to wine very appreciated organoleptic characteristics. Also, the cellulosic fraction is a source of compounds of interest from the organoleptic point of view (*e.g.* furans and derivatives, formed by sugars degradation under thermal treatment of wood and being contributors of wine flavor with toasted, caramelized and almond notes).

With the objective of obtaining extracts rich in these compounds to compare the profile provided by the different vine-shoots varieties, three methods were compared to improve/accelerate extraction by assistance of ultrasound, microwaves or superheated liquids. These methods were optimized using multivariate strategies, and the most suited values of the influential variables characteristic of each type of extraction were selected to obtain the extracts, the total phenols content of which was determined by the Folin–Ciocalteu method, while the individual concentration of 5-hydroxymethylfurfural was obtained by separation–detection using liquid chromatography–diode array detection (LC–DAD). In this way, it was demonstrated that extraction by liquids superheated at 180 ºC for 60 min provided the extracts more rich in phenols and with higher concentrations of 5-hydroxymethylfurfural than those obtained by the other methods. This furanic derivative is a key compound from the enological point of view, as it endows wine with the very desirable toasted and caramelized profiles; nevertheless, this compound can be toxic above a certain concentration level, so that its concentration in food is regulated. In the obtained extracts its concentration was quite lower than the maximum limit allowed in wines.

The individual separation–detection of the phenol compounds in extracts by LC–DAD took 73 min. The target compounds were identified and quantified by comparison of their retention times and their spectra in the ultraviolet zone with the corresponding standards used to run the calibration curve of each phenol. In addition, identification was confirmed by LC–QTOF analysis.

Individual quantitation of the target compounds allowed evaluation of the differences among varieties in phenols concentrations, both total and individual. Varieties with higher phenols content were Chardonnay and Montepila; while Pedro Ximénez and Baladí were those with the lowest contents.

Comparison of the antioxidant capacity of the extracts by the ORAC method provided results in agreement with both those obtained by the Folin-Ciocalteu method and the individual phenols content of the extracts: as the Chardonnay variety, with the highest phenols content, also provided the highest antioxidant capacity, thus demonstrating the robustness of the results.

The wide fan of relative concentrations of phenol compounds depending of the vine-shoots variety opens an also wide fan of profiles that can be transferred to wine if vine-shoots are used for ageing (in addition to the healthy characteristics attributed to phenols).

Chapter 3

The research in this chapter was aimed at improving the information in Chapter 2 on the possibilities of the use of vine-shoots for wine ageing. With this aim, extracts from five types of commercial oak chips both French and American were prepared and compared with those from vine-shoots. Comparison encompas-sed both quantitative composition and statistical study by using chemometric tools to obtain an overview of the similarity/dissimilarity of the profiles of the extracts from both types of materials.

The methods in Chapter 2 were used for extraction and quantitative analysis of oak extracts obtained by superheated liquids and LC–DAD, verified by LC–QTOF. The found concentrations of 5-hydroxymethylfurfural were similar to those in vine-shoots, always lower than the maximum allowed level. Also other compounds of oenological interest were found at concentrations similar to those in vine-shoots (*e.g*. gallic acid, final product in the elagitannins hydrolysis and responsible for the astringency of wines; pyrogallol, formed by decarboxylation of gallic acid and obtained at similar concentrations in all extracts, thus demonstrating the relationship of both compounds by a biochemical pathway). Compounds such as vanillin o synapaldehyde were slightly more concentrated in the extracts from oak wood, but, in general, a very similar composition of extracts from oak wood and vine-shoots made impossible discrimination between them.

The use of high resolution MS allowed a different approach in the analysis of the polar and mid-polar fractions of the extracts from both raw materials. In addition to assess identification of the target compounds, development of comparative analysis methods to evaluate the variability of this fraction was possible. LC–QTOF for untargeted analysis was used in this case, and extracts from 18 vine-shoots varieties and 5 commercial oak chips varieties were analyzed.

In the study among vine-shoots varieties a significant discrimination dependent on the vine-shoots variety was detected, then evaluated by comparison of the number of molecular features (potential compounds) as a function of the variety. A filter by frequency was applied as data pretreatment to remove non-representative molecular features, drastically decreasing the number of these entities. A no supervised statistical analysis by principal components (PCA) explained 89 and 87% of the variability for the negative and positive ionization modes, respectively. The variance analysis with a confidence level of 95% decreased the significant molecular features to 47 and 27 for the positive and negative ionization modes, respectively; while a confidence level of 98.5% decreased these entities to 10 and 11, respectively, and to 8 entities when the confidence level was 99.0%. The PCA plots from these data sets provided

information on the varieties with similar composition. For a confidence level of 98.5%, apart from the main discrimination block, there were two other blocks, one of them formed by varieties Pedro-Ximénez, Montepila, Merlot, Garnacha Tintorera and Bobal, and the other formed by the varieties Moscatel, Chardonnay and Syrah.

Identification of the molecular entities with significant contribution provided interesting results, as compounds such as 5-hydroxymethylfurfural, pyrogallol, acetovanillone, syringaldehyde and coniferyl alcohol, among others, were identified.

A filter by frequency was applied to reduce dimensionality for comparison between extracts from vine-shoots and oak. This filter is the key to ensure comparability of the data, as working with entities present in all the samples, extracts from oak and vine-shoots, ensured representativeness of each type of sample. In this way, the number of molecular features was decreased to 69 and 37 for the ionization modes positive and negative, respectively. As foreseeable, PCA analysis showed two well differentiated groups, as the extracts come from two different types of raw samples; therefore, a less restrictive filter was used to estimate the level of similitude between both types of samples. By application of a 40% filter by frequency, the number of molecular entities was 647 for both ionization modes. The PCA of this data set showed a series of extracts from vine-shoots varieties with higher similitude to oak extracts. The principal components that explained more than 90% of the variability corresponded to Garnacha Tinta, Malbec, Cabernet Sauvignon, Sauvignon Blanc, Petit Verdot, Pedro-Ximénez, Chardonnay, Garnacha Tintorera, Merlot and Montepila.

This study constitutes a solid support for valorization of vine-shoots as a potential alternative to the traditional use of oak chips with oenological purposes, either as substitutes of oak chips or for use simultaneous with them.

Chapter 4

To go in-depth into the potential use of vine-shoots with oenological purposes it was considered convenient to study and compare the compounds detected by GC–IT/MS in the extracts obtained from vine-shoots and commercial oak chips, taking into account the enormous influence of oak wood on the aroma and taste of wine during and after the ageing process. With this purpose, the method for extraction of this fraction based on the use of superheated liquids and optimized in Chapter 2 was applied, and the extracts thus obtained were analyzed by GC–IT/MS. The profiles from the different extracts were compared using chemometric tools.

Despite the obtained profiles were relatively similar among them, differences among the types of wood and among the vine-shoots varieties were found. The phenolic profiles were very similar and key compounds from the oenological point of view that were identified in them included vanillin, aceto-vanillone, propiovanillone, with slight differences in concentration, usually lower in vine-shoots than in oak wood, despite some vine-shoots varieties such as Chardonnay showed levels comparable to those in oak. This is the case with ethyl vanillate, a derivative product from vanillin that endows wine with burned and smoked smell; therefore, an excess of this compound can exert a negative effect on wine bouquet. Ethyl vanillate was identified in the majority of the samples, independendly of their origin, with a concentration slightly higher in the extracts from oak wood.

Among the compounds more concentrated in vine-shoots than in oak chips were eugenol that provides to wine a fruity taste, and syringil compounds such as syringol, syringaldehyde and syringic acid. Oak extracts showed higher concentrations in homovanillyl alcohol and coniferaldehyde that provide wine with sweet and wood aromas, respectively.

Some compounds were identified only in certain varieties, independent of the raw materials. Such is the case with acetosyringon and homosyringic acid, which are associated to sweet and wood aromas; while methoxyeugenol are

associated to spice and burnt tastes. Concerning to furanic compounds, the aroma of which has been described as caramelized or toasted sugar, did not have a significant variability among extracts; probably owing to losses during the sample preparation step. Nevertheless, it has been shown in the research in previous chapters that furanic compounds are in more significant quantities in vine-shoots extracts than in those from oak chips. While the levels of 5-hydroxymethylfurfural were similar in all extracts, the rest of furanic derivatives were more concentrated in vine-shoots extracts.

The first step for comparison of the extracts from vine-shoots with those from oak chips was to apply a filter by frequency to reduce the dimensionality of the data. Two filters by frequency were applied from two points of view: by considering all the samples as a unique group and as two separate groups, vine-shoots and oak chips. Application of the two types of filters by frequency showed the enormous variability both between the two groups of samples and among the different varieties of vine-shoots and those of oak chips. Two data sets were selected taking into account variability: those formed by molecular entities present in at least 70% of all the samples, and those formed by molecular entities detected in at least 90% of the samples belonging to either vine-shoots or oak chips. The percentage in the former filter is less restrictive than that in the latter as the former was applied to the overall data set that, obviously, is affected by a wider variability. In this way, the number of molecular entities decreased from 80 and 275 to 47 and 30 for vine-shoots and oak chips, respectively.

PCA was applied to evaluate the similitude between the extracts from vine-shoots and from oak chips. While the tridimensional PCA plot did not revealed complete discrimination between both types of extracts, the dimensional PCA plot revealed the vine-shoots varieties more similar to oak chips. Merlot, Bobal, Pedro-Ximénez, Mazuelo and Baladí provided the extracts more similar to those from American oak chips; while extracts from Cabernet Sauvignon, Sauvignon Blanc and Chardonnay varieties were more similar to the French oak varieties.

Similarly to the study in the previous chapter, the present research shows the possibility to select the vine-shoot variety to achieve, in this case, a bouquet of aged wine similar to, or with the desired differentiating nuances, by using a given variety of a mixture of them.

Chapter 5

The research in this chapter was addressed at expanding the information in Chapter 4 on the volatile profile of both vine-shoots and oak chips. It was considered convenient to study and compare the volatile profile of both materials during the toasting step, taking into account the importance of this step in the fabrication of oak barrels to be used for ageing both wines and spirits. With this aim, a simple method for toasting the raw materials (18 varieties of vine-shoots and 5 of oak chips) in a head-space device was optimized; then, the content of the headspace was analyzed by GC–IT/MS. This strategy allowed obtaining the representative profile of volatile compounds in each sample.

The optimization step encompassed the toasting time and temperature, being 20 min and 200 ºC the optimum conditions.

Comparison of the volatile profiles at different toasting temperatures by PCA allowed establishing the toasting temperature as the main variability factor, showing that the lowest toasting temperatures (120 and 150 ºC) provided a more similar profile; while at the highest temperatures (180 and 200 ºC) the profiles appeared as separate clusters that allowed a complete discrimination between vine-shoots and oak wood.

Identification of interesting compounds in the volatile profiles of both types of samples showed a very similar composition in both cases. The increase in temperature provided profiles rich in compounds generated by pyrolysis of polysaccharides (furanic aldehydes) or by the Maillard reaction (furanones and pyranones), as well as compounds generated by degradation of lignin (phenolic aldehydes and volatile phenols).

Among the compounds of interest from the oenological point of view identified in the different varieties of oak wood and vine-shoots are furfural and 5-methyl-2-furancarboxyaldehyde that participate in a number of reactions related to wine ageing, or the oak lactones, which were identified only in oak chips at growing concentrations as the toasting temperature increased, and were more intense in American oak than in French oak. Other derivatives from furanic compounds —as 1-(2-furanyl)ethanone or 2-furanmethanolacetate—, which have been related to coffee, toasted, honey or caramel aromas, were identified in all samples, independently of the toasting temperature.

As foreseeable taking into account that phenol compounds are the final products of the thermal degradation of lignin, a great amount of compounds with guaiacyl and syringyl structures were detected, the majority being common to vine-shoots and oak chips. Only ferulic acid was detected at low temperatures (120 ºC) in oak chips, despite its absence in the resting samples can be explained by thermal degradation, as this compound has been described in other investigations as a precursor of phenol compounds (4-vinylguaiacol, 4-ethylguaiacol, vanillin and guaiacol, among others); then confirmed by their presence both in vine-shoots and oak chips when toasted at 200 ºC.

Finally, and by using standards of the most significant compounds from the sensory point of view, the analysis confirmatory of identification was developed. It involved the retention times and mass spectra and, in addition, allowed calculation of the relative concentration of these compounds in the different samples.

This study showed the similarity, in qualitative terms, of the volatile profiles of commercial oak chips (traditionally used in wine ageing) and vine-shoots. Also, multivariate analyses allowed identification of the vine-shoots varieties with volatile profiles more similar to those of oak chips; therefore, these preliminary results can be taken into account in future assays on the use of vine-shoots in wine ageing, either alone or combined with oak chips.

The general comparison of the results in Chapter 4 and 5 highlights the high similarity of vine-shoots and oak chips in interesting compounds from the oenological point of view, being their presence generally higher in oak chips. Although Chapter 5 is focused on the study of the volatile fraction of both materials, many identified compounds are also found in extracts. This is the case of vanillin, ethylvanillin and acetovanillone, known for the vanilla notes that all of them transfer to wine. Syringyl type compounds, such as siringol, syringaldehyde or acetosyringone, detected in extracts and the headspace of both materials, are responsible for the sweet taste and woody aroma. It is also worth emphasizing the abundance of furanic compounds in both cases, which contribute mainly with woody and toasted almond notes. On the other hand, the extracts are rich in ethyl esters of fatty acids, which play a key role in wine aroma, with fruity and floral notes, as is the case with 2-butenedioic acid diethyl ester or hexanoic acid ethyl ester.

Therefore, either preparation of vine-shoots extracts to be exploited for wine ageing, or the direct use of this material in a way similar to that of oak chips are proposed and their study developed, thus constituting the main body of Section II of this Doctoral Thesis.

Section II. Exploitation of vine-shoots. Comparison of wines aged in the presence of vine-shoots *vs* oak chips

The research in the previous section was based on the hypothesis that the use of vine-shoots for ageing red wines can produce wines with characteristics similar to those aged in the presence of oak chips. The research in Section II encompassed two ways to confirm the hypothesis: one of them is subjective and supported on the experience of a panel of 15 experts to evaluate the organoleptic characteristics with which vine-shoots can endow wine and their differences as compared with oak chips. The experts' opinion on the sensory profile of wines,

expressed as well-designed reports, allowed a solid statistical study of the responses in each report. The other way was based on objective aspects, as those provided by the volatile profiles of the target wine by GC–MS/IT and the subsequent chemometric study. These studies constitute Chapters 6 and 7, respectively.

Chapter 6

The sensory evaluation by an experts panel of a red wine aged for 6 and 9 months in the presence of either toasted and non toasted French or American oak chips or two varieties of vine-shoots (Merlot and Montepila); all them compared with a control wine, which was not in contact with any type of material that modified its natural ageing. In this way, the starting hypothesis about the potential use of vine-shoots for wine ageing was intended to be verified. This has been the first time that evaluation by an experts panel of the sensory profile of wines aged in the presence of vine-shoots has been carried out.

The wine aged in the presence of oak chips showed a profile very similar to that of the control wine, with some tobacco, leather, prune/raisins, coffee and sweet spice attributes. Vine-shoots provided profiles with more pronounced prune/raisins notes in the case of non toasted vine-shoots, and liquorize notes in toasted vine-shoots, with also a more acid and astringent character and a red fruits, tobacco and leather profile less pronounced than that of wines aged with oak chips, except for those aged with Merlot vine-shoots that showed less intense red fruit taste than both the control wine and the rest of treated wines, being this variety of vine-shoots characterized by a change on sensory characteristics smaller with respect to control wine. An increase in tobacco and coffee aromas was observed in all treated wines as compared with the control wine. On the contrary, the fresh character decreased as compared with the control wine, being more evident the decrease in the case of vine-shoots, especially in dealing with the Merlot variety.

The gustative profile of the control wine was defined by bitterness, astringency, aftertaste intensity, aftertaste quality and taste intensity. Certain negative attributes were decreased in treated wines, as bitterness when treated with toasted American oak and no toasted Montepila vine-shoots. Also astringency, sweet spices and green perception decreased, particularly in wine aged in the presence of oak chips. The resting attributes did not change significantly in all wines.

Finally, statistical comparison of the opinions from the expert panel by ANOVA allowed discrimination among the most significant differences among wines. The control wine was compared with wines treated for 9 months to obtain an overall information of the produced changes. In general, the use of oak chips had more effects on olfactory aspects, among which those corresponding to leather, prune/raisins and tobacco were statistically more important in treated wines, contrarily to fresh and red fruit aromas. The decrease in green perception and increase of acidity in wines aged in the presence of vine-shoots with respect to wine control was significant; on the contrary, olfactory attributes such as tobacco and prune/raisins aromas increased regarding wines control.

The ageing time did not exert significant influence on wines treated with oak chips; on the contrary, wines treated with vine-shoots underwent a statistically significant decrease on the green perception when the ageing time was 9 months, while spicy notes were more significant at this ageing times. The influence of the toasting step on the materials for ageing had certain influence on the wine body, quality of aftertaste and astringency intensity; influence that was less perceptible in wines treated with non toasted vine-shoots and increased with the use of toasted oak.

Finally, comparison of the obtained wines by dimensional PCA allowed to discriminate the varieties closer among them according to their sensory characteristics, as well as to establish the attributes responsible for the differences.

Chapter 7

This chapter constitutes a new step in the study of wines aged in the presence of oak chips or vine-shoots from a more analytical point of view, based on the volatile profile of the target wines by GC–MS/IT and the subsequent chemometric study and identification of the representative compounds.

The separate evaluation of the changes at short and long times occurred in the treated wines demonstrated the more significant differences concerning the compounds of oenological interest. Thus, wines treated for 3 and 9 months in the presence of oak chips or vine-shoots shared compounds of interest from the organoleptic point of view that, in general, were more concentrated in wines in contact with oak chips, particularly in wines treated for 9 months. Examples of this behavior were 4,5-dimethyl-1-hexene (floral aroma), 1-hexanol y 3-penten-1-ol (green taste), syringol (smoked taste) and 1,8-nonadien-1-ol (fruity taste).

It is worth emphasizing the existence of a multitude of compounds that were more concentrated in treated wines than in control wines but did not cause significant differences among wines aged for short times, that can be explained because both types of materials for ageing transfer to wines similar amounts of these compounds in short times. Examples of this behavior are 5-hydroxyvanillin (vanilla aroma), isobutyl salicylate (floral aroma), guaiacol (smoked sabor), tetrahydrogeraniol (floral aroma), and others such as acids pentanedioic diethyl ester, butanedioic monoethyl ester or homosyiringic. In wines aged for 9 months this behavior was only observed for guaiacol, isobuthyl salicylate and homosyringic acid.

The comparative study of the data provided by the sensory analysis and those from GC–IT/MS analysis yields interesting conclusions. The decrease of the green taste in treated wines with respect to control wine justifies the statistically significant differences found for 3-penten-1-ol, 1-hexanol, butanoic acid 3-hydroxyethyl ester and tetrahydrogeraniol, being the latter responsible, not only for the green taste but also for floral notes; in addition, 2,3-dimethyl-1-butanol, is responsible for the herbaceous taste and can also contribute to the

green attribute perception. The increase in the astringency perception of wines aged in the presence of oak chips can be justified by the presence of some key compounds in the expression of this attribute, as hydroxybenzoic and hydroxycinnamic acids, among others. Guaiacol and syringol showed to be statistically significant when treated wines were compared to the control wine; however, at the sensory level, the smoky and toasted attributes were reported not to be relevant, a behavior that can be ascribed to the perception threshold of these compounds. However, syringol is also commonly associated to spice taste of wines, an attribute that was detected as significant by the panel of experts. The spice character showed to be decreased in treated wines with respect to control wines, behavior supported on the significant decrease of syringol on treated wines. Similarly, the increase of the liquorize and coffee taste reported in all treated wines with respect to the control wine can be explained by the high number of furanic compounds that has been detected in both materials in the research that constitutes chapters 4 and 5 of this Doctoral Thesis. Among others, these compounds are usually responsible for the toasted, caramel like, liquorize, coffee, toasted bread and sawdust notes.

Thus, it could be conclude that vine-shoots can be considered a potential new material for wine ageing, which allows obtaining wines with different qualities by manipulation of both duration of the step and vine-shoots used.

Section III. Characterization of vinification lees and quantitation of interesting families of compounds

This last section of the developed research was devoted to studying the vinification lees from red wine with a view on their possible valorization. Thus, Section III is composed by 3 chapters in which the research had a dual objective: evaluate the potential of vinification lees as a source of interesting compounds

taking into account their richness in antioxidants (among which the most noticeable are flavonoids as anthocyanins and derivatives, and non flavonoids as gallic, ferulic and caffeic acids), in essential amino acids and derivatives, among other compounds of interest. The second objective was focused on the development of a chromatographic method for the detection and quantification of pigments on wine lees without the need for analytical standards.

Thus, polar and mid-polar fractions of wine lees were studied by no supervised analysis (LC–QTOF), and identification of representative compounds was developed with the help of MS/MS data bases as Metlin, Massbank, Plantcyc and Metfrag. Also, a method for targeted analysis based on "Data Dependent Methods" was developed for quantitation of anthocyanins, anthocyanindins and proanthocyanidins; then validated by LC–QTOF analysis, as presented in Chapters 8 to 10.

Chapter 8

This chapter involves the research on evaluation of vinification lees, which have traditionally been used as a source of either tartaric acid or pigments, but never lees had been qualitatively characterized. The research started with the extraction, with the help of a focalized microwave digestor, of vinification lees from 11 Spanish wineries. The obtained extracts were analyzed by LC–QTOF with detection in the MS/MS mode.

It deserves to be emphasized that accuracy in the measurement of the *m/z* relationship is mandatory for identification of a number as high as possible of representative compounds, which is characteristic of global analysis and requires high resolution mass equipment. This instrumentation permits obtainment of MS/MS spectra of the detected precursor ions and provides the necessary information for unequivocal identification of each compound.

The high capability for resolution of MS/MS analysis allowed identification of a wide variety of representative compounds belonging to an also

wide variety of families, among which essential amino acids, anthocyans, flavonoids, flavonols, flavones and non flavonoid phenolic compounds are the most important; thus demonstrating that the analysis protocol based on LC–MS/MS is an excellent strategy for this type of sample.

Eight essential amino acids and 4 derivatives (*e.g*. L-arginine, L-proline, L-valine, L-asparagine, L-tyrosine, 3-hydroxy-L-tyrosine, and aminocaproic acid) were identified together with a multitude of flavonoids, including flavonols such as quercetin, myricetin, kaemferol and isoharmmetin; glucoside derivatives such as quercetin-3-*O*-glucoside; flavanols such as la catechin, epicatechin, gallocatechin or procyanidin B2, and anthocyanins such as peonidin-3-*O*-glucoside, peonidin 3-(6-*p*-cumaroylglucoside) and malvidin 3-(6-*p*-cumaroylglucoside). Among identified no flavonoid compounds are mainly no phenolic aldehydes (vanillin, protocatechuic aldehyde, stilbens (resveratrol-3-*O*-glucoside and *trans*-resveratrol), and phenolic acids such as hydrxoycinnamic acids (sinapic, caffeic and hydroxycinamic acids) and hydroxybenzoic acids as syringic, gallic, protocatechuic and hydroxybenzoic acids.

Among other compounds of interest that were identified are organic acids such as malic, tartaric, citric, shikimic, pantothenic and galactaric acids.

This identification study has contributed to the knowledge of this scantly studied residue with exploitation and valorization purposes.

Chapter 9

The research in this chapter was planned as a natural continuation of the study in the previous chapter; therefore, representative compounds present in the liquid fraction of vinification lees (or imbibed wine) were identified and compared with those in dry lees by applying multivariate analysis strategies. The starting hypothesis was the possibility of separated exploitation of both fractions, taking into account that, at present, the liquid fraction is wasted together with residual water during the vifinication process; therefore, the present economic

value of imbibed wine is nil. Vinification lees from 11 Spanish wineries of red wines from two of the most important denominations of origin in Spain (Ribera del Duero and Rioja) were used.

High resolution mass spectrometry in untargeted mode allowed analysis of the polar and mid-polar fractions in both materials. In addition, development of methods for comparative analysis was possible.

Comparison of the elution profiles of both fractions showed that imbibed wine possesses higher amounts of polar compounds, while less polar compounds predominate in solid lees, with elution profiles at longer times, explained by the adsorptive character of this residue.

Taking into account the abundance of data provided by untargeted analysis, the design of an appropriate treatment of these data is crucial. Correct data alignment and filtering are the basis to ensure representativeness of the research. Qualitative comparison of both types of samples was carried out by Venn diagrams, which showed a great similitude between both fractions, and found 61 common molecular features in the positive ionization mode and 23 in the negative mode. The molecular features exclusively found in imbibed wines and extracts from lees were 7 and 29, respectively, in the positive ionization mode; while in the negative mode were 44 and 11, respectively. This difference in the number of molecular features between both polarities can be explained by the influence of the chemical structure of the target compounds on the ionization efficiency.

Evaluation of the data by PCA confirmed the different composition of the fractions, as both types of samples appeared well differentiated in both polarities, being the variability explained by the principal components of 54.1% and 61.7% in the positive and negative ionization modes, respectively. The origin of the different samples did not be influential.

As in Chapter 8, also in the present chapter 40 representative molecular entities were identified, including 4 essential amino acids, two of them common to both fractions (3-hydroxy-L-tyrosine and L-valine), one of them exclusive of

imbibed wine (aminocaproic acid) and other exclusive of extracts from dry lees (L-tyrosine octadecyl ester). Among the phenolic compounds found it is remarkable the abundance of flavonoids, very common in wine and in grapes, such as 3,4,5-trimetoxiflavone, malvidin 3-(6-*p*-cumaroylglucoside), quercetin, myricetin and conjugates such as malvidin-3-galactoside, only identified in extracts from solid lees. Compounds of high interest from the nutraceutical point of view such as catechin and epicatechin, among other, were found in imbibed wine; while gallic acid, procyanidin B2 and pelargonidin 3-(6-*p*-cumaroylglucoside) were common to both fractions. Others among the identified compounds that differentiate imbibed wine from dry lees extracts were 8-oxo-guanosin monophosphate, ureidoglycolate, benzoquinone, citric acid or S-adenosyl-methioninamine.

It can be concluded that both fractions are sources of compounds of interest, being vinification lees particularly rich in phenolic compounds, mainly flavonoids, which is a consequence of the adsorptive character of this fraction.

Chapter 10

The research in this chapter was focused on the development and application of data dependent methods for identification of anthocyanins, anthocynidins and proanthocyanidins in extracts from vinification lees and wines imbibed in them. Advantages were taken from a triple quad mass spectrometer for confirmatory analysis of target analytes without commercial standards, based on three scanning modes: precursor ion, product ion and neutral loss. Then, SRM analysis was used for quantitation of the main compounds that constitute the anthocyans profile in the samples, and semiquantitation of those with no commercial standards was based on the standard of the corresponding aglycone.

The whole instrumental platform was constituted by an SPE automated station coupled in series to a liquid chromatograph and a triple quad mass detector (SPE–LC–MS/MS). Exhaustive optimization of each step involved: first, fragmentation and monitoring with the help of analytical standards, using the

negative ionization mode for identification of proanthocyanidins, and the positive mode for anthocyanidins; then, the chromatographic step was optimized. Sample preparation involved optimization of sorbents with different particle size and cartridge conditioning, sample dilution, retention step, washing solution and nature, volume and flow rate of the eluent. This study, previous to that of stability, ended with validation of the method (using spiked and non spiked samples of imbibed wine and lees extracts) with evaluation of the sensitivity and precision at 3 concentration levels. The obtained limits of detection and quantitation were 15 and 45 pg/mL for imbibed wines, and of 15 to 78 pg/mL for less extracts, respectively. Variability in the day and within days, expressed as relative standard deviation, was between 0.1% and 10.9% and between 0.5 and 10.1%, respectively, for imbibed wines and lees extracts, depending on the target analyte.

Finally, confirmation of identification by data-dependent methods was carried out by LC–MS/MS in high resolution mode, using the same chromatographic method and the QTOF detector.

In general, the concentrations of the target compounds in the extracts from dry lees were higher than in the imbibed wines, particularly for glucoside derivatives. Proanthocyanidin B was always at higher concentrations than that of type A, at levels between 2.0 and 31.5 ng/g in the extracts from dry lees and between 0.3 and 23.5 ng/mL in imbibed wines. The levels detected of anthocyanidins were between 1.1 and 77.5 ng/g in lees extracts and between 0.1 and 7.5 ng/mL in imbibed wines.

Application of quantitative methods made possible comparison of the concentrations of the target analytes in dry lees and imbibed wines in samples from different Spanish wineries. The ability of data dependent methods in combination with high resolution MS to confirm the identity of unknown compounds without the need for commercial analytical standards was the key in this research.

CONCLUSIONES

La investigación que constituye esta Tesis se ha centrado en el estudio de dos desechos derivados del cultivo de la vid y de la industria vinícola con vistas a su potencial aprovechamiento, lo que ha puesto de manifiesto la utilidad de la espectrometría de masas para el análisis de este tipo de muestras y su capacidad para la innovación metodológica, y ha permitido obtener resultados de los que derivan las siguientes conclusiones:

- El estudio en profundidad de los métodos existentes para el análisis de la fracción polar y de polaridad media, de la fracción volátil de los sarmientos de vid y de las virutas comerciales de roble y de las características y propiedades de las diferentes familias que las componen ha permitido:

    1. El desarrollo de la metodología más apropiada para la caracterización de estas fracciones, teniendo en cuenta el tipo de muestra, ha llevado a demostrar que: a) En el caso de compuestos polares y de polaridad media de ambas matrices, la extracción mediante líquidos sobrecalentados es mucho más eficaz que la asistida por otras energías; b) el perfil de compuestos polares y de polaridad media de los extractos de sarmientos de vid es similar al de las virutas de roble comerciales que se utilizan en el proceso de envejecimiento del vino; c) el contenido total de fenoles (determinado mediante el método de Folin-Ciocalteu) y la medida de la capacidad antioxidante de los extractos (determinada mediante el ensayo ORAC) se relacionan directamente con el contenido de compuestos fenólicos individuales.

    2. La comparación de la fracción volátil de distintas variedades de sarmientos de vid y de virutas de roble ha llevado a concluir que: a) La extracción mediante líquidos sobrecalentados proporciona extractos

muy ricos en compuestos volátiles de interés; b) tanto la fracción aromática de extractos de sarmientos y de virutas de roble, como la fracción volatil obtenida por tostado de ambos materiales y el análisis del espacio de cabeza obtenido son muy similares, siendo ambas muy abundantes en compuestos muy apreciados desde el punto de vista enológico.

3. Los sarmientos de vid son residuos muy abundantes y con escaso o nulo valor económico que pueden considerarse una posible fuente de compuestos de interés. La identificación mediante análisis por LC–MS/MS y GC–MS de los compuestos que componen las distintas fracciones de los sarmientos de vid demuestran su semejanza con las virutas de roble, actualmente utilizadas en el proceso de envejecimiento del vino, por lo que son susceptibles de su uso para tal fin.

- El estudio de un vino tinto envejecido en presencia de virutas comerciales de roble o de sarmientos de vid, tostados o no tostados, en condiciones de bodega y durante un espacio de tiempo que va de 3 a 9 meses ha proporcionado una gran cantidad de información sobre la posibilidad del aprovechamiento, lo que ha permitido concluir que:

4. El análisis sensorial de los vinos proporcionado por un panel de 15 catadores expertos ha demostrado que los sarmientos de vid producen vinos con buenas características sensoriales y con bouquets ligeramente diferentes a los obtenidos en presencia/ausencia de virutas de roble. El desarrollo de métodos de comparación multivariante permitió establecer semejanzas entre los vinos obtenidos en función de las condiciones de envejecimiento, poniendo de manifiesto qué sarmientos proporcionan al vino características similares o diferentes a las del envejecimiento en o con madera de roble, lo que permite utilizar los primeros para conseguir un perfil tradicional o innovador en el envejecimiento.

5. La comparación del perfil de los vinos obtenido mediante GC–MS y el apropiado uso de las correspondientes herramientas analíticas permitió elucidar las diferencias significativas en cuanto a la composición de los vinos obtenidos, por lo que se puede concluir que a medida que aumentó el tiempo de envejecimiento del vino hubo una mayor concentración en compuestos muy apreciados organolépticamente en aquellos vinos que se trataron con virutas de roble, no siendo la diferencia significativa con los envejecidos en presencia de sarmientos para vinos envejecidos a tiempos más cortos.

- Las lías de vinificación constituyeron la tercera muestra en estudio, que abarcó el análisis no orientado por LC–MS/MS de alta resolución junto con el uso de las herramientas quimiométricas apropiadas y el análisis orientado para la detección y cuantificación de colorantes, tanto en extractos de lías secas como en el vino embebido en ellas, lo que permitió concluir que:

6. El análisis no orientado de extractos de lías de vinificación por LC–MS/MS en modo de alta resolución ha proporcionado el perfil de compuestos polares y de polaridad media de lías, así como la identificación de las entidades moleculares más representativas mediante comparación de espectros MS/MS, con lo que se han identificado 70 compuestos, todos ellos de gran interés.

7. La anterior estrategia analítica permitió la comparación de los perfiles de compuestos polares y de polaridad media de las lías de vinificación con los obtenidos de los correspondientes vinos embebidos, poniendo de manifiesto que: a) Los extractos de lías secas son mucho más ricos en compuestos de polaridad media que los vinos embebidos, lo que se explica por el carácter adsorbente de las lías. A pesar de poseer una gran cantidad de compuestos comunes, hay diferencias significativas en la composición de cada fracción, ambas ricas en compuestos de interés desde el punto de vista nutracéutico.

8. El análisis del contenido en antocianinas, antocianidinas y proantocianidinas de extractos de lías de vinificación y de vinos embebidos en ellas mediante un espectrómetro de masas de triple cuadrupolo (QqQ) en sus distintas modalidades de trabajo han mostrado que: a) La sensibilidad, selectividad y precisión de la espectrometría de masas la convierten en técnica clave para el análisis confirmatorio y cuantitativo de antocianinas, antocianidinas y proantocianidinas aún en ausencia de patrones analíticos, siendo la versatilidad del triple cuadrupolo en sus distintas modalidades de trabajo ("data-dependent methods") clave para este fin; b) el uso de un sistema automatizado de SPE acoplado a un equipo de LC–MS/MS permite mejorar la detección de antocianos de forma limpia, sencilla y muy reproducible, eliminando los problemas derivados de trabajar con una matriz tan compleja como las lías de vinificación.

CONCLUSIONS

The research in this PhD Book has been focused on the study of two different residues and wastes from vineyards and the wine industry with a view on their potential exploitation. The usefulness of mass spectrometry for the analysis of this type of samples and its possibilities for methodological innovation have allowed obtaining the results from which the following conclusions have been extracted:

- The in-depth study of the methods in the literature for analysis of the polar and mid-polar fraction, of the volatile fraction from vine-shoots and commercial oak chips and the characteristics and properties of the different families in them has allowed:

    1. Development of the proper methodology for characterization of these fractions taking into account the type of sample has led to demonstrate that: a) in the case of polar and mid-polar compounds from both matrices, the extraction by superheated liquids is more efficient than that assisted by other energies; b) the profile of polar and mid-polar compounds in the extracts from vine-shoots is very similar to the profile of commercial oak chips used for ageing wines; c) the total phenol content (as determined by the Folin–Ciocalteu method), and the antioxidant capacity of the extracts (as determined by the ORAC assay) are directly related with the sum of the individual phenols content.

    2. The comparison of the volatile fraction from different vine-shoots and oak chips allowed concluding that: a) the extraction by superheated liquids provides extracts very rich in interesting aromatic compounds; b) the volatile fractions from toasted vine-shoots or oak chips as obtained

by headspace are very similar, both being rich in compounds very appreciated from the oenological point of view.

3. Vine-shoots are very abundant residues with scant or nil economic value, which can be considered a source of interesting compounds. Identification by LC–MS/MS and GC–MS analyses of the compounds that constitute the different fractions of vine-shoots demonstrated their similitude with those from oak chips, presently used in the ageing of wine; therefore, the former are susceptible of being used with the same purpose.

- The study of red wines aged in the presence of either toasted or not toasted commercial oak chips or vine-shoots, under winery conditions for intervals from 3 to 9 months, has provided a huge amount of information on the potential of vine-shoots exploitation, leading to the following conclusions:

4. The sensory analysis of wines by a panel of 15 experts has demonstrated that vine-shoots produce wines with excellent sensory characteristics and with bouquets slightly different to those obtained in the presence/absence of oak chips. The development of multivariate methods for comparison allowed establishing similitude among wines as a function of the ageing conditions, showing that vine-shoots endow wine with characteristics similar or different to those provided by ageing in or with oak wood; thus allowing the use of the former to achieve a traditional or innovator profile in the ageing process.

5. The comparison of wine profiles obtained by GC–MS and the appropriate use of analytical tools allowed elucidating significant differences in the composition of the obtained aged wines, thus concluding that as the ageing time increased, a higher amount of organoleptically appreciated compounds were transferred to wines treated with oak chips, the

difference with wines treated with vine-shoots being insignificant for short times.

- Vinification lees were the third sample under study that encompassed untargeted analysis by high resolution LC–MS/MS together with the use of appropriate analytical tools, and targeted analysis for detection and quantitation of colorants, both in dry lees and in the wine imbibed in them. The conclusions from this study were as follows:

6. Untargeted analysis of the extracts from vinification lees by LC–MS/MS in high resolution mode provided the profile of polar and mid-polar compounds in lees, as well as identification of the most representative molecular entities by comparison of MS/MS spectra. A total of 70 compounds were identified, all them of high interest.

7. The previous analytical strategy allowed comparison of the profiles of polar and mid-polar compounds in lees with those in the corresponding imbibed wines, showing that: a) the extracts from dry lees are much more rich in mid-polar compounds than the imbibed wines, explained by the adsorptive character of lees. Despite the amount of common compounds, there are significant differences between the fractions both rich in compounds of interest from the nutraceutical point of view.

8. The analyses of the content of anthocyanins, anthocyanidins and proanthocyanidins in vinification lees and in wine imbibed in them by a triple quad mass spectrometer (QqQ) in its different working modes have demonstrated that: a) the sensitivity, selectivity and precision of MS make this technique of paramount importance for confirmatory and quantitative analysis of anthocyanins, anthocyanidins and proanthocyanidins, even in the absence of analytical standards, being the versatility of QqQ in its different working modes (data-dependent methods) the key for this purpose; b) the use of an automated SPE station

coupled to LC–MS/MS equipment allows improving the detection of anthocyans in a clean, easy and very reproducible way by overcoming the problems from working with a complex sample as vinification lees are.

ANNEXES

Anexos

ANNEX I

Other publications co-authored by the PhD student

1. Development and application of a quantitative method for determination of steviol glycosides to evaluate the sweetener capability.
   M. Molina-Calle, V. Sánchez de Medina, ***M.P. Delgado de la Torre****, F. Priego-Capote, M.D. Luque de Castro*
   Sent to Food Chemistry

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2. Biological and health promoting activity of vinification by-products produced in Spanish vineyards
   J. Anter, S. Demyda-Peyrás*,* ***M.P. Delgado de la Torre****, J. Campos Sánchez, M.D. Luque de Castro, A. Muñoz-Serrano, A. Alonso-Moraga*
   Published in South African Journal of Enology and Viticulture

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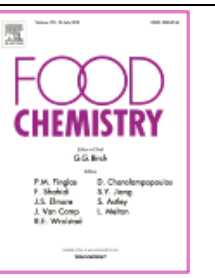
Sent to

Food Chemistry



Development and application of a quantitative method for determination of steviol glycosides to evaluate the sweetener capability of stevia leaves

M. Molina-Calle, [1,2,3] *V. Sánchez de Medina,* , [1,2,3] ***M.P. Delgado de la Torre***, , [1,2,3] *F. Priego-Capote,* [1,2,3]* *M.D. Luque de Castro,* [1,2,3]*

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Development and application of a quantitative method for determination of steviol glycosides to evaluate the sweetener capability of stevia leaves

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Abstract

Stevia plant is currently well-known thanks to the sweetener properties of its leaves. The compounds responsible for this property are the glycoside forms of steviol, considered as natural sweeteners. In this research, an optimized method based on LC–MS/MS by using a triple quadrupole detector has been developed for quantitative analysis of 8 steviol glycosides in extracts from Stevia leaves. The parameters involved in the ionization and fragmentation of the target analytes were optimized to develop a selected reaction monitoring (SRM) method. The resulting method reported detection and quantitation limits ranging from 0.1 to 0.5 ng/mL and from 0.5 to 1 ng/mL, respectively. The panel of steviol glycosides was used to assess the sweetener capacity of extracts from leaves of different varieties of Stevia cultivated in laboratory, greenhouse and field. Stevia varieties cultivated in field presented higher concentration of steviol glycosides than those cultivated in greenhouse; thus, the way of cultivation clearly influences the concentration of these compounds. The inclusion of branches together with leaves as raw material for the extraction of steviol glycosides was also evaluated, showing that branches modify the concentration of certain steviol glycosides.

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and Viticulture

Biological and health promoting activity of vinification by-products produced in Spanish vineyards

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Biological and health promoting activity of vinification by-products produced in Spanish vineyards

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Abstract

The Spanish agricultural system produces several by-products, among them, fresh and vinified grape skins represent an abundant source of phenols and resveratrol with potential nutraceutical value. Fresh grape-skin (FGSE) and vinification grape-skin extracts (VGSE) obtained by a microwave-assisted method have been chemically and biologically characterised. Their role in the maintenance of genetic stability as well as their potential chemopreventive effect have been tested by *in vivo* genotoxic (wing spot test of *Drosophila melanogaster*) and antigenotoxic (HL60 leukaemia cell line *in vitro* model) evaluations. and Total phenolic, anthocyans and resveratrol contents were chemically characterised in the two extracts, showing minimun qualitative differences. Both extracts and resveratrol were not mutagenic in the *Drosophila* somatic mutation and recombination test, and they exerted antigenotoxic activities against hydrogen peroxide. They also showed cytotoxic activity to HL60 leukaemia cells (IC_{50}: 4.5µL/mL, 4.6µL/mL and 98µM, respectively, and induced apoptotic internucleosomic fragmentation in the HL60 cell line.

ANNEX II

Communications to congresses and meetings

1. Use of data dependent methods for profiling analysis of anthocyanins in wine lees by liquid chromatography coupled to electrospray ionization tandem mass spectrometry.

M. P. Delgado de la Torre, C. Ferreiro Vera, F. Priego-Capote, M.D. Luque de Castro

XVIII REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

Úbeda, Jaén, 2013

Tipo de evento: Póster en Congreso **Ámbito:** Nacional

2. Comparison of energy-assisted methods for the extraction of the phenolic fraction from different vine-shoots cultivars.

Delgado de la Torre, M. P., Ferreiro-Vera, C., Priego-Capote, F., Luque de Castro, M. D.

International Congress on Green Extraction of Natural Products – GENP, Avignon, France.

University of Avignon, France, 2013

Tipo de evento: Póster en Congreso **Ámbito:** Internacional

3. Profiling analysis of anthocyanidins, proanthocyanidins, and glucoside derivatives in wine lees by liquid chromatography coupled to electrospray ionization tandem mass spectrometry by data-dependent methods.

M. P. Delgado de la Torre, C. Ferreiro Vera, F. Priego-Capote, M. D. Luque de Castro

XXIII Reunión nacional–VII Congreso Ibérico de Espectroscopía.

Córdoba (España), 2012

Tipo de evento: Póster en Congreso **Ámbito:** Nacional

4. Obtención y aprovechamiento de productos de alto valor añadido a partir de desechos de la vid y de la industria vinícola.

F. Priego-Capote, M. D. Luque de Castro, M. P. Delgado de la Torre.

I Congreso científico de investigadores en formación en agroalimentación del CeiA3

Córdoba, España, 2010

Tipo de evento: Comunicación oral **Ámbito:** Regional

5. Richness and diversity of vine-shoots as a source of antioxidant compounds.

M. P. Delgado de la Torre, C. Ferreiro Vera, F. Priego-Capote, M. D. Luque de Castro

The international conference on natural products

Castres Cedex (Francia), 2011.

Tipo de evento: Póster en Congreso **Ámbito:** Internacional

6. Riqueza y variabilidad de los sarmientos de vid como fuente de antioxidantes.

M. P. Delgado de la Torre, C. Ferreiro Vera, F. Priego-Capote, M. D. Luque de Castro

XII Reunión del grupo regional andaluz de la sociedad española de química analítica.

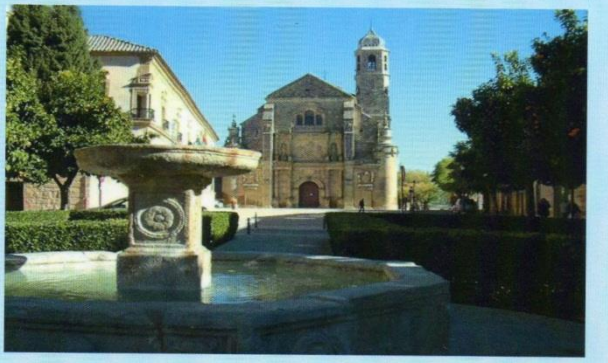
Córdoba (España), 2011

Tipo de evento: Póster en Congreso **Ámbito:** Regional

XVIII REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

2013 SEQA

VI REUNIÓN DE LA SOCIEDAD ESPAÑOLA DE ESPECTROMETRÍA DE MASAS



Úbeda, 16-19 junio 2013

SEQA

S.E.E.M

Use of data dependent methods for profiling analysis of anthocyans in wine lees by liquid chromatography coupled to electrospray ionization tandem mass spectrometry.

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Wine lees are an agricultural residue very abundant in wine producer countries such as Spain and Italy. Its composition is characterized by a high content of anthocyans, the natural pigments responsible for the red color of wines, and also for the colors ranging from red to blue in fruits, vegetables and flowers. Among these compounds, it is worth mentioning anthocyanidins that are characterized by the flavonoid backbone hydroxylated at positions 3, 5, and 7, while the B-ring exhibits a variable hydroxylation-methoxylation pattern. These compounds can also be glycosylated to form anthocyanins which are abundant in skin from *Vitis vinifera* grapes, particularly, glycosides of malvidin (Mv), peonidin (Pn), delphinidin (Dp), petunidin (Pt), and cyanidin (Cy) *(1)*. The relative concentration of these natural compounds in grapes varies widely among varieties *(2)* and is strongly influenced by particular cultivation conditions *(3)* and environmental factors (temperature, moisture, isolation conditions, soil type) *(4–6)*.

The aim of this research was to develop an analytical method for qualitative/quantitative profiling analysis of these natural pigments in wine lees. The analysis was carried out by LC–MS/MS with a triple quadrupole mass analyzer by using data-dependent methods. Qualitative analysis was carried out by: (i)

precursor ion scanning with a common product ion in their fragmentation scheme; (ii) product ion scanning by fragmentation of target precursor ions; and (iii) neutral loss scanning for detection of precursor ion/product ion pairs generated by loss of neutral fragments associated to specific *m/z* values. Quantitative analysis was performed by selected reaction monitoring (SRM) in negative ionization mode. The application of the approach enabled to obtain the anthocyans profile in wine lees collected from different wineries demonstrating the utility of this residue because of its content in these natural colorants. The combination of precursor ion scanning, product ion scanning and neutral loss scanning enabled to confirm the presence of glucoside derivatives in hydroalcoholic extracts from wine lees.

Based on the ESI-MS/MS ionization and fragmentation patterns for the target compounds, positive ESI mode was selected for proanthocyanidins whilst the negative mode was selected for anthocyans. The effect of the addition of different modifiers to the LC mobile phase was studied in terms of the ESI-MS/MS sensitivity and peak shape, being the best results obtained by addition of 0.5% of formic acid to both mobile phases. Under these conditions, tandem mass spectrometry parameters were optimized for efficient isolation of the precursor ions, and their sensitive, selective fragmentation.

As a general trend, the extracts from wine lees were characterized by a high content of anthocyanidins and moderate content of proanthocyanidins, which makes this oenological residue a potential source for isolation of these natural colorants.

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1. Wulf, L.W. and Nagel, C.W., *Am. J. Enol. Vitic.*, **29**, 42-49 (1978)

2. Mazza, G. and Miniati, E. (1993). Anthocyanins in fruits, vegetables and grains, CRC Press, London, United Kingdom, 149.

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6. Ojeda, H. et al. *Am. J. Enol. Vitic.*, **53**, 261-269 (2002)

International Congress on "Green Extraction of Natural Products"

Université d'Avignon et des Pays de Vaucluse, 16-17 April 2013

Comparison of energy-assisted methods for the extraction of the phenolic fraction from different vine-shoot cultivars

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A comparative study of the efficiency of three of the most common energy-assisted extraction techniques —microwave-assisted extraction (MAE), ultrasound-assisted extraction (USAE) and superheated liquid extraction (SHLE)— is presented. They are considered as green extraction techniques thanks to their advantages over traditional extraction techniques in terms of solvent and time consumption. These three approaches have been applied to the characterization of 18 vine-shoot cultivars, an agricultural residue common in wine-producing countries. The extraction efficiency was assessed by measurement of the total phenol compounds using the Folin-Ciocalteu test and by analysis of the extracts by LC–DAD. The influence of the main variables involved in the process was estimated by a multivariate approach.

The superiority of SHLE over MAE and USAE has been demonstrated in this study. Attending to the results, SHLE provided the highest concentration of phenolic compounds expressed as μg of gallic acid per gram of initial solid vine-shoots. Therefore, SHLE seems to be the suited strategy for extraction of phenolic compounds from vine-shoots, which can be considered a potential vegetal source to obtain this valuable fraction.

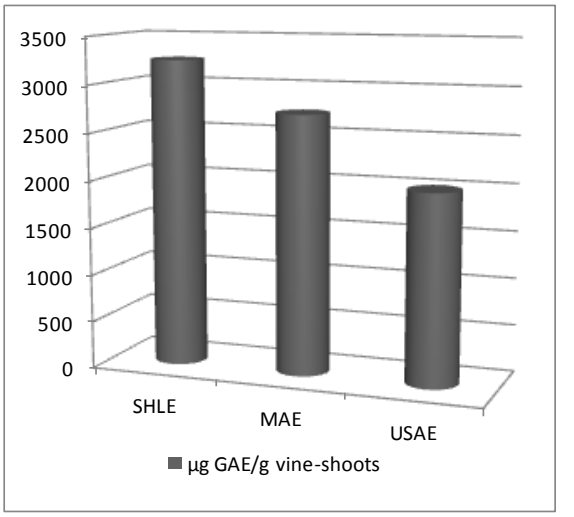

Figure 1. Total concentration of phenols expressed as µg equivalent of gallic acid (µg GAE) per gram of vine-shoots obtained by the Folin-Ciocalteu method by the three extraction approaches.

XXIII REUNIÓN NACIONAL – VII CONGRESSO IBÉRICO
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LIBRO DE COMUNICACIONES

UNIVERSIDAD D CÓRDOBA

CÓRDOBA, 17 AL 20 DE SEPTIEMBRE DE 2012

Profiling analysis of anthocyanidins, proanthocyanidins and glucoside derivatives in wine lees by liquid chromatography coupled to electrospray ionization tandem mass spectrometry by data-dependent methods

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Wine lees are an agricultural residue very abundant in wine producer countries such as Spain, France and Italy. Its composition is characterized by a high content of anthocyans, the natural pigments responsible for the red color of wines, and also for colors ranging from red to blue in fruits, vegetables and flowers. Among these compounds, it is worth mentioning the anthocyanidins that are characterized by the flavonoid backbone hydroxylated at positions 3, 5, and 7 (Figure 1.A), while the B-ring exhibits a variable hydroxylation-methoxylation pattern (Figure 1.B). These compounds can also be glycosylated to form anthocyanins, which are abundant in skin from Vitis vinifera grapes, particularly, glycosides of malvidin (Mv), peonidin (Pn), delphinidin (Dp), petunidin (Pt), and cyanidin (Cy) [1]. One other interesting family of compounds is that formed by proanthocyanidins, which are oligomers and polymers of flavan-3-ols present mainly in grape seeds. The relative concentration of these natural compounds in grapes varies widely among varieties [2 and is strongly influenced by particular cultivation conditions (3) and environmental factors such as temperature, moisture, isolation conditions, soil type [4–6].

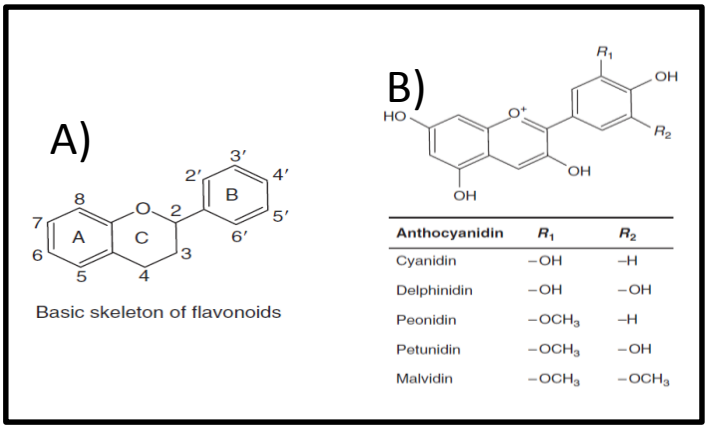
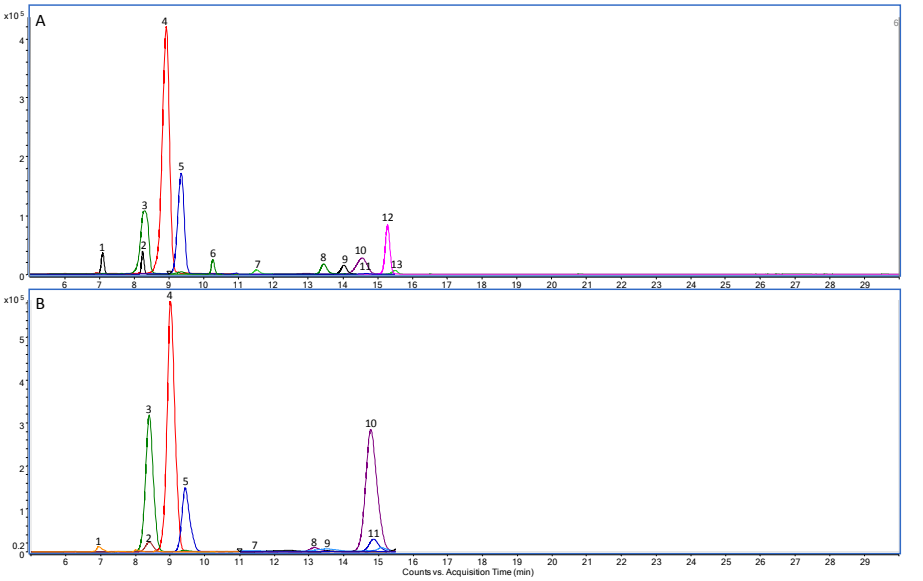


Figure 1. A. Basic structure of flavonoids. B. Common anthocyanins in grapevine.

The aim of this research was to develop an analytical method for qualitative/quantitative profiling analysis of these natural pigments in wine lees. The analysis was carried out by LC–MS/MS with a triple quadrupole mass spectrometer by using data-dependent methods. Qualitative analysis was carried out by: (i) precursor ion scanning with a common product ion in their fragmentation scheme; (ii) product ion scanning by fragmentation of target precursor ions; and (iii) neutral loss scanning for detection of precursor ion/product ion pairs generated by loss of neutral fragments associated to specific m/z values. Quantitative analysis was performed by selected reaction monitoring (SRM) in negative ionization mode. The application of the approach enabled to obtain the anthocyans profile in wine lees collected from different wineries demonstrating the utility of this residue because of its content in these natural colorants. The combination of precursor ion scanning, product ion scanning and neutral loss scanning also made possible confirmation of the presence of glucoside derivatives in hydroalcoholic extracts from wine lees.

Quantitative analysis by SRM was accomplished by an online SPE–LC–MS/MS system —coupling an automated Prospekt-2 SPE workstation (Spark Holland, Emmen, The Netherlands) to an Agilent LC–QqQ system (Palo Alto, USA) equipped with a JetStream Technology electrospray ion source. HySphere

C8 EC cartridges (endcapped silica based octyl phase, particle size 10 μm, 10 × 2mm i.d.) from Spark Holland were used to carry out the SPE step. Response surfaces were developed to optimize volume and flow rate parameters for loading solvent and washing solutions, being the best recoveries found at volumes of 0.5 mL and flow rates of 3.5 and 1.7 mL min-1, respectively. Based on the ESI-MS/MS ionization and fragmentation patterns for the target compounds, the positive ESI mode was selected for proanthocyanidins, whilst the negative mode was selected in the case of anthocyans. The effect of the addition of different modifiers to the LC mobile phase was studied in terms of the ESI-MS/MS sensitivity and peak shape, being the best results obtained by addition of 0.5% formic acid to both mobile phases.



Note: 1, proanthocyanidin B1; 2, proanthocyanidin B2; 3, delphinidin-3-glucoside; 4, petunidin-3-glucoside; 5, peonidin-3-glucoside; 6, proanthocyanidin A2; 7, delphinidin; 8, cyanidin; 9, petunidin; 10, delphinidin-3-rutinoside; 11, peonidin 3,5-diglucoside; 12, peonidin; 13, malvidin.

Figure 2. Extracted ion chromatogram obtained for a spiked sample of wine lees (A), and an extract of wine lees (B).

Under these conditions, tandem mass spectrometry parameters were optimized for efficient isolation of the precursor ions, and their sensitive, selective fragmentation. Figure 2 illustrates the SRM chromatograms provided by an extract from wine lees obtained by microwave-assisted extraction and by the same extract spiked with the target compounds.

As a general trend, the extracts from wine lees were endowed with a high content of anthocyanidins and moderate content of proanthocyanidins, which make this oenological residue a potential source for isolation of these natural colorants.

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El Vicerrector de Estudios de Posgrado y Formación Continua de la Universidad de Córdoba **ACREDITA** que

MARÍA DEL PILAR DELGADO DE LA TORRE

ha asistido al **I Congreso Científico de Investigadores en Formación en Agroalimentación** del eidA3 y **II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba** celebrado en Córdoba los días 8 y 9 de Mayo de 2012.

Y para que así conste, se expide y firma este certificado en Córdoba, a 9 de Mayo de 2012

Fdo: JOSE CARLOS GOMEZ VILLAMANDOS
Vicerrector de Estudios de Posgrado y Formación Continua

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El Vicerrector de Estudios de Posgrado y Formación Continua de la Universidad de Córdoba **ACREDITA** que :

MARÍA DEL PILAR DELGADO DE LA TORRE

ha presentado la comunicación oral que lleva por título :

" OBTENCIÓN Y APROVECHAMIENTO DE PRODUCTOS DE ALTO VALOR AÑADIDO A PARTIR DE DESECHOS DE LA VID Y DE LA INDUSTRIA VINÍCOLA"

en el **I Congreso Científico de Investigadores en Formación en Agroalimentación** de la eidA3 y **II Congreso Científico de Investigadores en Formación de la Universidad de Córdoba** celebrado en Córdoba los días 8 y 9 de Mayo de 2012.

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REUNIÓN DEL GRUPO REGIONAL ANDALUZ DE LA
SOCIEDAD ESPAÑOLA DE QUÍMICA ANALÍTICA

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Córdoba, 10 y 11 de junio

LIBRO DE RESÚMENES

UNIVERSIDAD Ð CÓRDOBA

Reunión del grupo regional andaluz de la Sociedad Española de Química Analítica

Riqueza y variabilidad de los sarmientos de vid como fuente de antioxidantes

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Los sarmientos constituyen un subproducto agrícola muy abundante en regiones como La Rioja, Andalucía y Castilla–La Mancha (millones de Tm anuales). Su valor económico es muy pequeño ya que se utilizan mayoritariamente como combustible o abono. Dado que los sarmientos poseen compuestos fenólicos extraíbles y un contenido medio de lignina del 20% de su peso seco, susceptible de originar también compuestos de este tipo por degradación, una alternativa de explotación de este residuo, que podría incrementar considerablemente su valor, sería su uso como fuente barata de obtención de fibra y, especialmente, de compuestos fenólicos (mayoritariamente polifenoles).

Los polifenoles son una familia de compuestos muy extensa a la que se ha prestado mucha atención en los últimos años debido a sus potenciales efectos beneficiosos sobre la salud, derivados de su condición de atrapadores de radicales libres y de su actividad antioxidante. En el campo enológico tienen un papel clave en la calidad de los vinos, puesto que determinan, en gran medida, su color, sabor y aroma. Los extractos de estos compuestos, fáciles de obtener en corto tiempo mediante líquidos sobrecalentados, pueden tener una aplicación inmediata en el envejecimiento ficticio del vino y como antioxidantes para la fabricación de

alimentos suplementados, de nutracéuticos y/o de cosméticos. Con estos precedentes, los objetivos del presente trabajo han sido: (i) poner de manifiesto la viabilidad de la utilización de sarmientos para la obtención de extractos con un alto contenido fenólico; (2) comprobar que el uso de mezclas etanol–agua sobrecalentadas para este fin es muy superior al de la extracción sólido–líquido convencional. El estudio con líquidos sobrecalentados se llevó a cabo mediante un diseño multivariante para la extracción en régimen estático, que proporcionó los siguientes valores óptimos de las variables que influencian el proceso: composición del extractante, etanol 80 % (v/v); temperatura, 240 ºC; presión, 100 atm. La cinética del proceso mostró que para un tiempo de 60 min de extracción estática se conseguía una eficiencia superior al 100%, considerando este valor el obtenido con la extracción convencional (48 h de extracción con agitación a 25 ºC). La variable respuesta utilizada durante la extracción fue doble: la cantidad total de fenoles extraídos (método de Folin–Ciocalteu) y la evolución del perfil cromatográfico obtenido por cromatografía líquida de alta resolución (HPLC) y detección absorciométrica mediante diodos en fila. La cuantificación se llevó a cabo con patrones de los fenoles conocidos. Los compuestos desconocidos se identificaron mediante detector de masas–tiempo de vuelo (HPLC–Q/TOF). La aplicación del método optimizado a la extracción de los compuestos de interés en diferentes variedades de sarmientos (*e.g*. Cabernet Franc, Airen, Garnacha tinta, Syrah, Cabernet Sauvignon, Merlot) ha puesto de manifiesto que el binomio condiciones de trabajo–variedad de vid permite obtener un extracto de antioxidantes fenólicos “a la carta”.

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RÉGION MIDI-PYRÉNÉES

The International Conference on Natural Products

24-28 Mai 2011, Castres, FRANCE

ISIS, Centre Universitaire J.F.Champollion

Organised by Dr.Patrick Sharrock, Association pour la recherché interdisciplinaire, Toulouse, FRANCE

The International Conference on Natural Products

Richness and diversity of vine-shoots as a source of antioxidant compounds

M. P. Delgado de la Torre, C. Ferreiro Vera, F. Priego-Capote, M. D. Luque de Castro

Department of Analytical Chemistry, C-3 Annex to Marie Curie building, Campus of Rabanales, University of Córdoba, 14071 Córdoba, Spain

Vine-shoots are an agricultural by-product very abundant in wine-producer countries (millions of tons every year). Economic value of vine-shoots is at present very low as they are used mainly as a heating source or cast upon the ground to rot.

The composition of vine-shoots is characterized by a content of lignin around 20% (dry weight), which may be hydrolysed to release aromatic phenolic compounds such as aldehydes, ketones or acids. These phenols have so far not received enough attention despite their potential health benefits as antioxidants because of their natural origin and the ability to act as efficient free radical scavengers. In the oenological field, they could play a key role in wine quality, since they could determine to a large extent their colour, flavor and aroma, acting similarly to wine-ageing either in contact with oak chips or in oak barrels. In addition, these extracts could be used as antioxidants for the manufacture of food supplements, nutraceuticals and/or cosmetics. Based on this background, the objectives of this study were: (i) to demonstrate the feasibility of using vine-shoots to obtain extracts with high phenolic content, (ii) verify that the use of superheated water–ethanol mixtures for this end is by far superior to ultrasound- and microwave-assisted extraction.

Research by superheated liquid extraction was conducted using a multivariate design in static extraction, which gave the following optimal values of the variables that influence the process: extractant composition, ethanol 80% (v/v); temperature, 240 ºC; pressure, 10 atm; extraction time, 60 min. The twofold response variable for the extraction was the total amount of extracted phenols (determined by the Folin–Ciocalteu method) and the chromatographic profile obtained by high-performance liquid chromatography–diode array absorptiometry. Unknown compounds were identified using a time-of-flight mass detector (HPLC–Q/TOF). Application of the optimized method for the extraction of the target compounds in a range of vine-shoots cultivars (e.g. Cabernet Franc, Airen, Garnacha tinta, Syrah, Cabernet Sauvignon, Merlot) has demonstrated that the pair working conditions–vine-shoot cultivar allows obtaining tailor-made phenolic extracts.

Abbreviations

AAPH, [2,2'-azobis(2-amidino-propane) dihydrochloride]

ACN, acetonitrile

ANOVA, analysis of variance

ASE, accelerated solvent extraction

BFF, benzofuran forming fission

BPC, base peak chromatogram

DAD, diode array detector

EEC, European Council Regulation

EFSA, European Food Safety Authority

EI, electron impact ionization

EIC, extracted ion chromatogram

ESI, electrospray ionization

F–C, Folin–Ciocalteu

FDA, Food and Drug Administration

FT, fourier transform

GC, gas chromatography

HMF, 5-hydroxymethylfurfural

HPLC, high performance liquid chromatography

HPSE, high pressure solvent extraction

HRF, heterocyclic ring fission

HS, head space

IL, ionic liquid

IS, internal standard

IR, infrared

IT, ion trap

LC, liquid chromatography

LLE, liquid–liquid extraction

LLOD, lower limit of detection

LOD, limit of detection

LOQ, limit of quantitation

NMR, nuclear magnetic resonance

MAE, microwave assisted extraction

METLIN, Metabolites and Tandem MS Database

MetOH, methanol

MF, molecular featureMPP, mass profiler professional

MS, mass spectrometry

NIST, National Institute of Standards and Technology

OIV, international organisation of vine and wine

ORAC, Oxygen Radical Absorbance Capacity

PCA, principal component analysis

PHSE, pressurized hot solvent extraction

PLE, pressurized liquid extraction

PLS, partial least squares

PLS-DA, partial least squares discriminant analysis

QqQ, triple quadrupole

QTOF, quadrupole–time-of-flight

RDA, retro Diels-Alder

RSD, relative standard deviation

RT, retention time

SD, standard deviation

SHLE, superheated liquid extraction

SFE, supercritical fluid extraction

PLE, pressurized liquid extraction

SPE, solid-phase extraction

SPME, solid-phase microextraction

SSE, subcritical solvent extraction

SIM, selected ion monitoring

SRM, selected reaction monitoring

TIC, total ion current

TOF, time of flight

UAE, ultrasound assisted extraction

USAE, ultrasound assisted extraction

UPLC, ultra performance liquid chromatography