CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

INSTITUTO DE LA GRASA

TESIS DOCTORAL

RECUPERACIÓN DE COMPUESTOS BIOACTIVOS A PARTIR DE SUBPRODUCTOS DEL ACEITE DE OLIVA

Memoria que presenta Dª Fátima Rubio Senent

Para optar al Grado de Doctor

Departamento de Nutrición y Bromatología

Facultad de Farmacia, Universidad de Sevilla

Sevilla, Febrero de 2011

La tesis titulada "**Recuperación de compuestos bioactivos a partir de subproductos del aceite de oliva**" presentada por la Licenciada en Química **Dª Fátima Rubio Senent** para optar al grado de Doctor, ha sido realizada bajo la co-dirección de los Doctores **Juan Fernández-Bolaños Guzmán** y **Guillermo Rodríguez Gutiérrez**, ambos Científicos del Instituto de la Grasa, siendo tutor el Doctor **Francisco José Heredia Mira**, Catedrático de la Facultad de Farmacia de la Universidad de Sevilla. Considerando que se han alcanzado los objetivos inicialmente previstos, y que el trabajo reúne los requisitos necesarios, autorizan su presentación

V. º B.º Los directores

Fdo.: Dr. Juan Fernández-Bolaños Fdo.:Guillermo Rodríguez Gutiérrez

V. º B.º El tutor

Fdo.: Francisco José Heredia Mira

Conforme, la Directora del Departamento de Farmacología,

Fdo.: Dª Ana María Cameán Fernández

A mis padres

Índice

Agradecimientos

AGRADECIMIENTOS

El trabajo aquí presentado ha sido posible gracias a la ayuda de diferentes personas, que de una forma u otra me han enseñado, aconsejado y conducido por el camino correcto, de forma que el resultado final es esta Tesis Doctoral. Por esta razón me gustaría dar las gracias a todas las personas que han hecho posible que este proyecto saliera hacia delante.

A los Drs. D. Juan Fernández-Bolaños Guzmán y D. Guillermo Rodríguez Gutiérrez, mis directores de Tesis. Sin duda alguna los agradacemientos hacia ustedes probablemente ocuparían un volumen más extenso que esta Tesis, ya que es imposible enumerar todas las ocasiones en las que me habéis apoyado, enseñado y animado. Ha sido un placer formarme como investigadora bajo su dirección y espero a lo largo de mi vida aprovechar al máximo todos los conocimientos que me han transmitido, tanto a nivel científico como personal. Gracias por confiar y por apostar por mí.

Al Dr. D. Francisco Heredia, del Departamento de Nutrición y Bromatología de la Facultad de Farmacia de Sevilla. Gracias por colaborar como tutor de este trabajo, por su completa disposición e interés.

A todos los componentes del Grupo de Pared Celular. Al Dr. D. Rafael Guillén por prestarme su ayuda para familiarizarme con el manejo de distintas técnicas instrumentales, sin las cuales gran parte de este trabajo no podría haberse realizado. A los Drs., Dña. Ana Jiménez, Dña. Rocío Rodríguez, D. Antonio Gómez y Dña. Sara Jaramillo y a Olivia Cruz, por haberme prestado su ayuda siempre que lo he necesitado y por su apoyo incondicional. Muchas gracias a todos por hacer que el laboratorio sea un lugar tan agradable, en el que todo el mundo está dispuesto a echar una mano. A la Dr. Aranzazu García, por su ayuda en los estudios de actividad antimicrobiana, muchas gracias por su disposición a la hora realizar los ensayos, gracias por su tiempo, por el interés mostrado por este trabajo y por el cariño demostrado. Gracias por preocuparos por mis preocupaciones y alegraros con mis alegrías.

A la Dra. D. Sara Vázquez, el Dr. D. Antonio Lama, y a Javier Tejero. No hay mejor cosa que pasártelo bien en el trabajo, y sin duda alguna eso ha sido gracias a vosotros, porque hemos trabajado mucho, pero la verdad es que también nos hemos reído y mucho. Gracias por ayudarme cuando lo he necesitado, gracias por los chistes malos, gracias por los buenos almuerzos que hemos echado y gracias por estar siempre ahí. Gracias a Sara Vázquez, por ser mí apoyo en los tres meses de estancia, por convertirse en una gran amiga y por enseñarme a ver las cosas desde otra perspectiva.

A los Drs. D. Garry Duthie y Dña. Baukje de Roos del Rowett Institute of Nutrition and Health de la Universidad de Aberdeen (Escocia, Reino Unido), por haberme permitido una

estancia de tres meses bajo su dirección, gracias por su acogida, disposición, enseñanzas e interés en el trabajo realizado. Gracias a Dña. Sharon Wood por su búsqueda incansable de voluntarios para poder realizar mi trabajo.

A los Drs. D. José Mª Fernández-Bolaños y Sergio Martos Delgado del Departamento de Química Orgánica de la Universidad de Sevilla, por su ayuda en la caracterización estructural de compuestos. Gracias por habernos aconsejado, ayudado y por vuestra completa disposición.

A todos mis compañeros de la sede de Bellavista del Instituto de la Grasa, tanto al personal científico titular, a los becarios, al personal de mantenimiento, etc. No los enumero porque seguramente me dejaría alguien atrás, pero muchas gracias a todos, porque siempre que he necesitado algo de alguien he recibido buenas respuestas y ayuda incondicional.

A la Cooperativa Oleícola El Tejar por acceder al suministro de muestras para realizar este trabajo.

Al Programa Junta para la Ampliación de Estudios (JAE) del Consejo Superior de Investigaciones Científicas (CSIC) y al Fondo Social Europea por financiar la beca que ha hecho posible mi trabajo de investigación.

A mis compañeros de Universidad, en especial a Rocío Martín, Sergio Martos, José Javier Plata y Luis Carlos Vicente. Porque pese a hacer algún tiempo que terminamos la carrera no hemos perdido el contacto, al contrario, seguimos estando igual o incluso más unidos que antes. Que suerte tuve al coincidir con vosotros y ahora que suerte tengo de teneros como grandes amigos.

A mi familia, en especial a mis padres, por apoyarme, aconsejarme y estar siempre a mi lado, ayudándome a ser mejor persona cada día, gracias por aguantarme y por mimarme tanto y tan bien. A mi recién estrenado marido, Eu, por saber entender este complicado mundo de la investigación e interesarse por mi trabajo y por los resultados obtenidos, gracias por apoyarme en todo.

Gracias.

1. Abreviaturas

1. ABREVIATURAS

2,4-D	Ácido 2,4-diclorofenoxiacético
2-MTHF	2-metiltetrahidrofurano
3,4-DHPEA-AC	Acetato de hidroxitirosilo
3,4-DHPEA-DEA	Forma dialdehídica y descarboximetilada del ácido elenólico unido a hidroxitirosol.
3,4-DHPEA-EA	Aglicona de la oleuropeína
ADN	Ácido desoxirribonucleico
ALDH2	Proteína hepática mitocondrial aldehído dehidrogenasa
CSIC	Consejo Superior de Investigaciones Científicas
DES	Solventes eutécticos profundos
DHFG	3,4-dihidroxifenilglicol
DP	Grado de polimerización
EBT	Empresa de base tecnológica
Gal-3	Galectina-3
Glc	Glucosa
HBD	Donador de enlaces de hidrógeno
HCl	Ácido clorhídrico
HDL	Lipoproteínas de alta densidad
HT	Hidroxitirosol
KOH	Hidróxido de potasio
LDL	Lipoproteínas de baja densidad
OMS	Organización Mundial de la Salud
p-HPEA-EA	Aglicona de ligustrósido
p-HPEA-EDA	Forma dialdehídica y descarboximetilada del ácido elenólico unido a tirosol
PMs	Pectinas Modificadas
ROS	Especies reactivas de oxígeno
SVM S.L.	Subproductos Vegetales del Mediterráneo, Sociedad Limitada
Ty	Tirosol

2. Resumen

2. RESUMEN

Numerosas pruebas científicas ponen de manifiesto que la dieta mediterránea, en la cual la principal fuente de grasa es el aceite de oliva, previene la aparición y desarrollo de enfermedades coronarias, desórdenes metabólicos y varios tipos de cáncer (Fabiani y col., 2010; Estruch y col., 2013). Los efectos beneficiosos del aceite de oliva han sido no sólo atribuidos a la composición en ácidos grasos monoinsaturados (mayoritariamente el ácido oleico) sino también a la presencia de componentes minoritarios, entre los que se encuentran los compuestos fenólicos, la vitamina E y los carotenoides (Pérez-Jiménez y col., 2007). Tras el proceso de extracción del aceite de oliva sólo una muy baja cantidad de compuestos fenólicos pasan al aceite, mientras que la mayor parte de ellos (aproximadamente el 98-99%) permanecen en el subproducto.

A pesar de la importancia de estos compuestos por sus propiedades, los subproductos generados en la extracción del aceite de oliva se tratan como residuos contaminantes, cuando en realidad constituyen una fuente barata de estos compuestos bioactivos. En la actualidad la extracción del aceite se realiza principalmente por el sistema de centrifugación, de tres o dos fases. El sistema de tres fases genera dos residuos, el alpechín (fase acuosa) y el orujo (fase sólida), mientras que el sistema de dos fases sólo genera uno, el alperujo (mezcla de alpechín y orujo). En las últimas décadas se ha observado una rápida y generalizada implantación del sistema de dos fases.

Las propiedades fisicoquímicas de este subproducto, el alperujo, causan problemas de gestión en las almazaras que han provocado la transformación de las estrategias tradicionales de valorización. Una de las técnicas actuales más consolidadas en el sector es la combustión, debido a que por el momento la investigación ha tenido poco éxito en encontrar una solución integral económicamente viable. En este contexto, las tecnologías de tratamiento de residuos centradas en la recuperación de compuestos bioactivos a partir de los residuos del aceite de oliva, representan una interesante alternativa.

La dificultad fundamental para la recuperación de estos compuestos a partir del alperujo radica su complejidad y en la necesidad de pre-tratarlo, debido a factores limitantes como la elevada humedad (70%), la separación de sus fases y la solubilización de los compuestos buscados, como consecuencia de la estructura de la matriz de la pared celular del fruto del olivo y a las interacciones entre sus componentes. Dicho problema ha sido solventado por nuestro grupo de investigación tras la aplicación de un novedoso sistema de pre-tratamiento térmico, que ha sido patentado, conjuntamente entre el CSIC y la empresa Oleícola el Tejar, SA (PCT/ES2011/070583). Esta tecnología alternativa para el tratamiento y valorización del alperujo como sustrato para la producción de compuestos valiosos basada en un tratamiento térmico con vapor a alta presión y temperatura, provoca un proceso de autohidrólisis que permite la separación del alperujo de forma sencilla y económica en tres fases (sólida, líquida y

volátil). En la fracción líquida que se obtiene tras el tratamiento térmico sobre el alperujo, no sólo se incrementa la concentración de compuestos como los fenoles o carbohidratos presentes en el alperujo, sino que se forman otros nuevos.

El objetivo principal de esta tesis es el estudio de los diferentes componentes que se solubilizan tras el tratamiento térmico del alperujo y la comprobación de cómo afectan las condiciones del pretratamiento a su composición. Para ello, las fracciones líquidas obtenidas han sido extraídas con acetato de etilo en caliente, de esta forma se han obtenido unas fracciones orgánicas enriquecidas en fenoles y unas fracciones acuosas enriquecidas en oligosacáridos (neutros y pécticos).

Las condiciones de tiempo y temperatura a las cuales se realizan el tratamiento térmico afectan a las características y propiedades de los extractos y fracciones aisladas y estudiadas en este trabajo. Por ello, se ha comprobado como afecta el tiempo de tratamiento térmico empleado (15, 30, 45, 60, 75 y 60 min) a una temperatura fija (160 ºC) a las concentraciones y propiedades de los extractos fénolicos obtenidos tras la extracción con acetato de etilo de la fracción líquida obtenida a partir de alperujo tratado térmicamente, y se han comparado con un control sin tratar. Así mismo se ha comparado la composición de los extractos fenólicos obtenidos en función del pH de la muestra cuando se realiza la extracción con acetato de etilo (pH 4 y 2,5) para el tratamiento térmico de 160ºC y 60 minutos. Se han estudiado las propiedades antioxidantes *in vitro* de los extractos fenólicos tanto a diferentes tiempos como a diferentes pH mediante los ensayos sobre el: poder reductor, captación de radicales ABTS y DPPH, inhibición de la oxidación primaria y secundaria. Se comprueba que la actividad antioxidante de los extractos fenólicos es superior a la observada para el control y que la composición de los extractos fenólicos varía notablemente con la temperatura del tratamiento debido a que algunas especies se liberan o forman con el tratamiento mientras que otras se degradan. También se han realizado ensayos *ex vivo* como la inhibición de la oxidación lipídica sobre microsomas de hígado de ratas y la inhibición a la agregación plaquetaria en sangre humana, observándose por primera vez en ambos casos importantes efectos sinérgicos entre el hidroxitirosol y el acetato de hidroxitirosilo.

El estudio de la composición de los extractos fenólicos nos ha permitido aislar una fracción fenólica polimérica con importantes propiedades de captación de hidroxitirosol. Además, se ha aislado un derivado del ácido glutárico (el ácido *s-trans*-(E)-3-(1-oxobut-2-en-il)glutárico) cuya estructura completa y datos espectroscópicos han sido presentados por primera vez. El origen de la molécula ha sido estudiado, y según los estudios realizados dicha especie se forma y/o libera durante la extracción con acetato de etilo que se realiza sobre el alperujo tratado térmicamente o durante prolongados tiempos de almacenamiento de los alperujos. Así mismo, dichos estudios nos han permitido aislar e identificar los precursores del 3,4-dihidroxifenilglicol, concretamente la 2”-hidroxioleuropeína y los dos diasteroisómeros β-hidroxiacteósido y β-hidroxiisoacteósido. También se ha aislado y caracterizado

estructuralmente por primera vez una nueva molécula que aparece en la fracción acuosa remanente obtenida tras la extracción con acetato de etilo a partir de alperujo tratado térmicamente, la molécula ha sido identificada como el ácido 1-glucosil aciclodihidroelenólico.

Por otro lado, la extracción con acetato de etilo nos proporciona una fracción remanente acuosa la cual se encuentra enriquecida en diferentes oligo y polisacáridos de naturaleza neutra y ácida. Los oligosacáridos neutros son separados de los ácidos previa hidrólisis suave y posterior ultrafiltración a través de una membrana de 3000 Da. La fracción enriquecida en oligosacáridos neutros ha sido caracterizada, permitiendo la identificación de distintos oligosacáridos y de mono y disacáridos enlazados a compuestos fenólicos, observándose que no se encuentran diferencias importantes en las especies identificadas entre diferentes tratamientos. El estudio de la composición ha permitido identificar oligosacáridos como xilanos (Xil_{7-4}), xilanos unidos a ácido metilglucurónico ($Xil_{6-4}MeGlcAc$), glucanos (Glc_{7-3}) y glucoxilanos (Glu_6Xil_3, Glc_5Xil_4 y Glc_3Xil_3), los cuales podrían tener un elevado interés debido a sus propiedades como prebióticos. Así mismo, se ha realizado un estudio detallado de las composiciones y propiedades del material péctico aislado a partir de los diferentes tratamientos térmicos. Los cuales se caracterizan por estar enriquecidos en azúcares neutros, poseer un elevado grado de esterificación y un tamaño medio de 6 kDa. Estas pectinas de bajo peso molecular pueden ser consideradas como pectinas modificadas, las cuales son fácilmente absorbidas y se caracterizan porque se unen y bloquean a la proteína pro-metastásica galectina-3 (Gal-3). Así mismo presentan una elevada capacidad de captación de aceite y se caracterizan por ser capaces de captar ácidos biliares y retardar el índice de diálisis de la glucosa.

También se ha realizado un estudio del material péptico recuperado a partir de un alperujo que ha sufrido un tratamiento térmico suave industrial (50-80 ºC durante 1-2 horas). Este material se caracteriza por estar compuesto por una mezcla de polisacáridos neutros y ácidos de alto peso molecular (110 kDa) unidos covalentemente, con un bajo grado de esterificación. Presenta importantes actividades de captación de aceite, ácidos biliares y retención del índice de diálisis de la glucosa, obteniéndose resultados del mismo orden que los observados para la pectina de limón comercial. Además los polisacáridos pécticos aislados presentan actividad antioxidante, lo que hace que se puedan clasificar como fibra dietética antioxidante.

Finalmente se ha realizado un estudio de la composición y propiedades de las diferentes fracciones recuperadas (celulosa, lignina y hemicelulosa) cuando el tratamiento térmico es llevado a cabo sobre orujo de tres fases y un material puramente lignocelulósico como es el hueso de la aceituna. Los estudios realizados mostraron que la lignina aislada presenta una elevada capacidad de retención de ácidos biliares, cuando el ácido biliar probado es el ácido cólico dicha actividad llega a ser incluso del mismo orden que la presentada por el compuesto colestiramina, que se emplea como fármaco para el tratamiento de la hipercolesterolemia.

Además se comprobó que todas las fracciones presentan un índice de retardo de diálisis de la glucosa similar al observado para la pectina de limón comercial.

En la actualidad hay una gran demanda de compuestos de origen natural. Los consumidores son cada día más sensibles al uso de fitoquímicos de síntesis en la producción y conservación de alimentos, incluidos antimicrobianos. A su vez, la normativa europea que regula su empleo es cada vez más restrictiva. La obtención de compuestos naturales a partir de subproductos de la industria agroalimentaria como el alperujo, supone una prometedora opción para el consumidor, el sector industrial y el medioambiente. El haber hecho posible de forma económica una eficaz separación entre las fases enriqueciéndose cada una de ellas en compuestos bioactivos de interés hace viable la obtención y estudio de cada uno de estos compuestos.

El conjunto de resultados obtenidos justifica la recuperación de compuestos de interés del alperujo, este hecho solucionaría un gran problema medioambiental mediante una valorización completa del alperujo. Además la extracción de los compuestos fenólicos como productos de alto valor añadido podría ser considerada como una interesante alternativa para hacer provechosos los residuos de las almazaras, posibilitando su posterior uso como alimento animal o su biotransformación a biocombustible, compost o fertilizante entre otros, al eliminar gran parte de su contenido fitotóxico.

3. Introducción

3. INTRODUCCIÓN

3.1. La problemática del alperujo

La industria del aceite de oliva representa uno de los sectores más importantes a nivel productivo en nuestro país, siendo España el mayor productor mundial de aceite de oliva (39% de la producción total) (www.internationaloliveoil.org). La tecnología para la extracción del aceite de oliva ha ido evolucionando en España desde principios de los años sesenta hasta la actualidad, pasando en la mayoría de las almazaras de un sistema de centrifugación de tres fases a uno de dos fases. Sin embargo, esta evolución en el proceso de extracción no ha sido suficiente para evitar la generación de subproductos con una elevada carga contaminante.

En los años sesenta apareció el sistema de centrifugación de tres fases (**Figura 1**). Mediante este sistema el proceso de obtención de aceite genera dos subproductos, el primero, un residuo líquido denominado alpechín, y el segundo, un residuo sólido denominado orujo. El alpechín se caracteriza por ser altamente contaminante y estar compuesto por una mezcla de agua de vegetación de las aceitunas más el agua añadida en los diferentes pasos del proceso de extracción, conteniendo azúcares, polialcoholes, pectinas, lípidos y grandes cantidades de compuestos aromáticos, que son responsables de las propiedades fitotóxicas y antimicrobianas que presenta (Muktadirul Bari Chowdhury y col., 2013). El orujo se caracteriza por contener una humedad del 45% y está compuesto por piel, pulpa, hueso y un resto graso.

La eliminación y gestión de los enormes volúmenes generados de estos subproductos representan un antiguo problema medioambiental. El almacenamiento en balsas del alpechín aprovechaba la evaporación natural para concentrar el residuo pero con el tiempo se clasificó como técnica inadecuada ya que contaminaba las aguas subterráneas y producía malos olores por la descomposición de la materia orgánica.

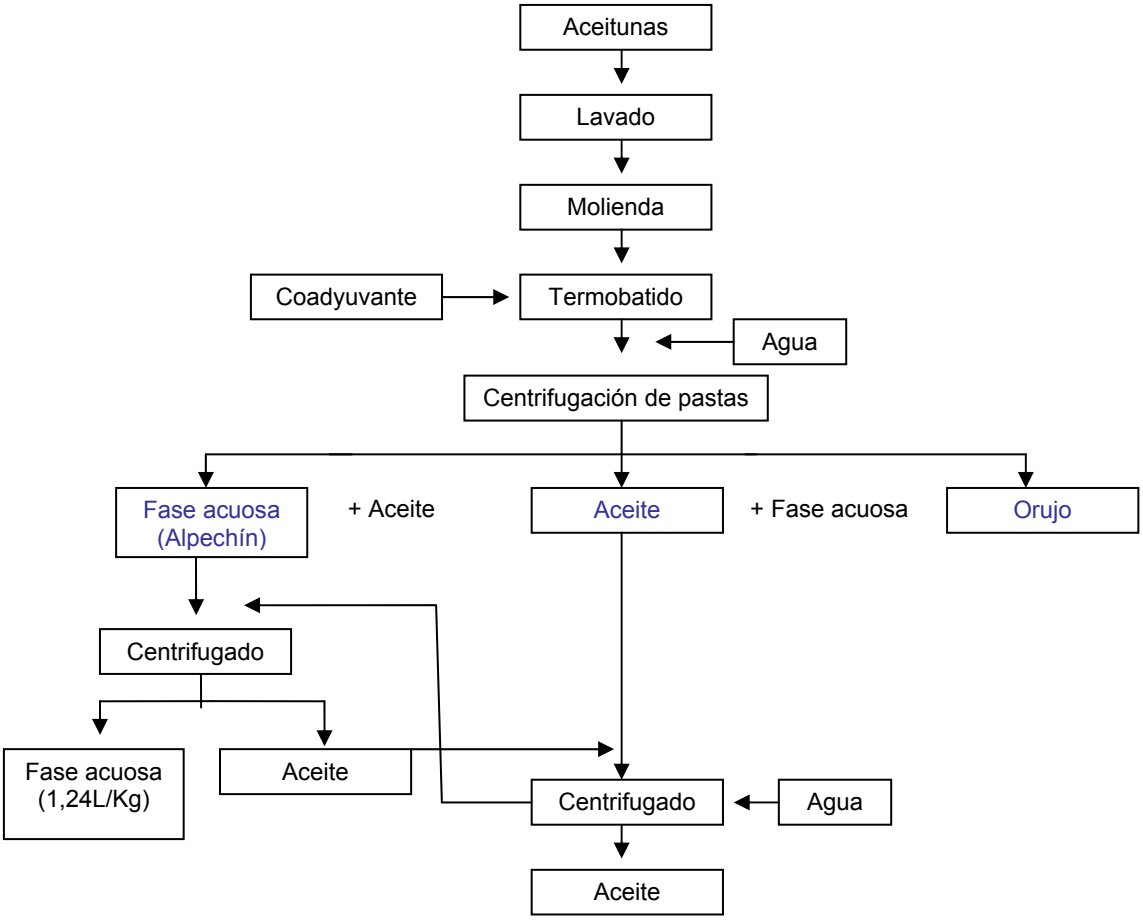


Figura 1: Sistema de centrifugación de tres fases para la elaboración del aceite de oliva virgen (Adaptado de Barranco y col., 2008).

El uso del nuevo tratamiento de dos fases (**Figura 2**), desarrollado durante los años noventa (Alba y col., 1996), ha reducido notablemente el volumen de alpechín, y con ello su carga contaminante (sólidos en suspensión, alta concentración de compuestos orgánicos y sales minerales), pero produce un nuevo subproducto semisólido muy húmedo que es una mezcla del residuo líquido (alpechín) y sólido (orujo), denominado alperujo. En el sistema de extracción de dos fases no se añade agua, de forma que la cantidad de aguas residuales generadas se ve muy reducida en comparación al sistema de tres fases original (de la Casa y col., 2012). La cantidad de efluentes líquidos en el proceso continuo de extracción ha disminuido desde 1.21 L/Kg de aceituna en el sistema de tres fases hasta 0.25 L/Kg de aceituna en el sistema de dos fases (Ochando-Pulido y col., 2013), en este último caso el agua procede del lavado de la aceituna y del aceite fundamentalmente. Aunque el nuevo sistema de centrifugación de dos fases ha supuesto una buena solución para las almazaras frente a los vertidos que ocasionaba el alpechín, que era causa de serios problemas ambientales debido a su elevada demanda química de oxigeno y sus efectos antimicrobianos y fitotóxicos, el alperujo no parece ser más adecuado ni menos contaminante.

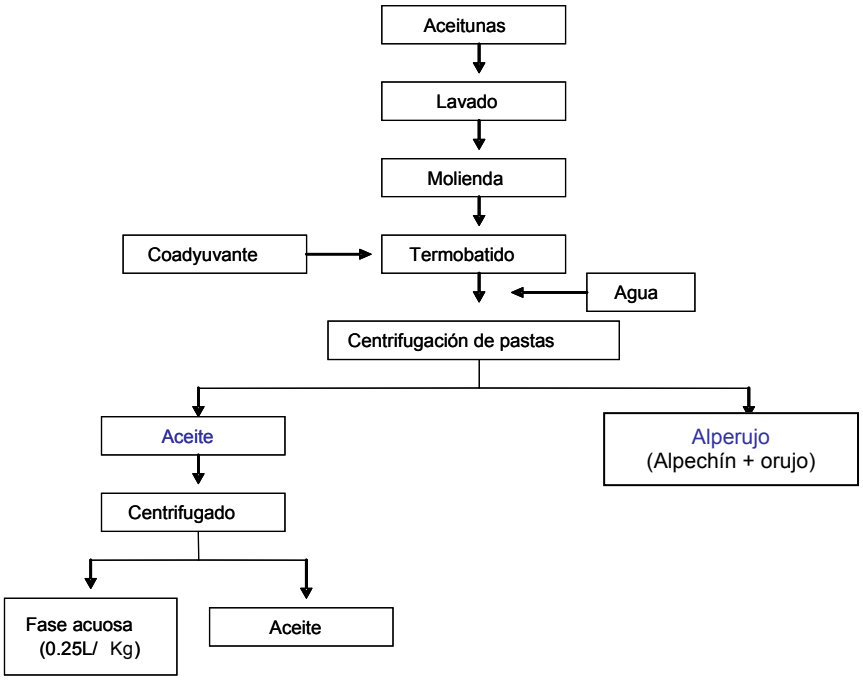


Figura 2: Sistema de centrifugación de dos fases para la elaboración del aceite de oliva virgen (Adaptado de Barranco y col., 2008).

Este subproducto o alperujo, se caracteriza por estar formado por el hueso de la aceituna (15% w/w), la pulpa (20% v/v) y agua de vegetación (65% v/v) (De la Casa y col., 2012). El alperujo no resuelve los problemas de contaminación que presentaba el sistema de extracción de tres fases y plantea otros nuevos, debido a que no sólo contiene más humedad que el orujo de tres fases sino que también posee una elevada carga contaminante debido a su pH ácido, su elevada salinidad tóxica y a que exhibe valores altos de electroconductividad (Ochando-Pulido y col., 2013). Además presenta un alto contenido en sustancias orgánicas, que incluyen carbohidratos, polifenoles, lípidos y proteínas (Tortosa y col., 2012; Rúiz-Méndez y col., 2013). Con respecto al contenido de nutrientes para plantas, el alperujo es especialmente rico en N y K, pero pobre en P, Ca y Mg, el micronutriente principal que presenta es el Fe pero otros micronutrientes como Cu, Mn y Zn se encuentran en bajas concentraciones, estas características junto con la baja concentración de Na, hacen que el alperujo se encuentre en desventaja considerando su uso como fertilizante agrícola frente a otros (Cegarra y col., 2000). También presenta otros inconvenientes como dificultad de manejo por su alto contenido de humedad y consistencia, y mayor severidad y coste de secado en las orujeras, donde se extrae el aceite de orujo, ya que para conseguir el máximo rendimiento debe secarse hasta valores próximos al 8%. La presencia fundamentalmente de azúcares, que en el sistema de tres fases se quedaban en el alpechín, se caramelizan en los secaderos como consecuencia de las altas temperaturas, apelmazando la masa de alperujo, que se seca sólo superficialmente, lo que incide negativamente en el proceso de reducción de la humedad (Antolín y col., 1997).

Además, los componentes orgánicos solidificados en el alperujo seco crean películas envolventes que impiden o dificultan el paso del disolvente, originando problemas de percolación que disminuyen el proceso de extracción. Toda esta problemática ha obligado a las orujeras a realizar costosas modificaciones en sus instalaciones, para adaptarse a las características del nuevo residuo generado por la implantación del sistema de extracción de dos fases.

Debido a la rápida y generalizada implantación del sistema de centrifugación de dos fases en España el 90% de las industrias del aceite emplean este sistema (Plaza y col., 2008). La producción anual de alperujo en nuestro país oscila entre los 5-6 millones de toneladas por campaña (Ministerio de Medio Ambiente y Medio Rural y Marino, 2010. Anuario de Estadística 2010), de las cuales el 90% se obtienen en Andalucía. La gestión y eliminación de los enormes volúmenes generados de este subproducto a partir de la producción del aceite de oliva presentan un gran problema de polución medioambiental que permanece todavía sin resolver. Por lo tanto, el alperujo supone una carga económica para la industria del aceite de oliva y está considerado un subproducto con un impacto negativo sobre el medio ambiente. En concreto, los problemas de su gestión y aprovechamiento están relacionados con la producción de grandes cantidades en un corto periodo estacional, la variabilidad de su composición y su alto contenido en determinados compuestos.

La presencia de compuestos fenólicos de carácter fitotóxicos y antimicrobianos (Tortosa y col., 2012) hace difícil la implementación de métodos de tratamientos biológicos convencionales (aeróbicos y anaeróbicos). El problema se ha trasladado a las industrias orujeras, encargadas del tratamiento del nuevo residuo y que han tenido que adaptarse a su recepción y difícil manejo. El alperujo es a menudo tratado en una segunda centrifugación para extraer el aceite residual o de repaso, esta centrifugación se suele realizar en decanters de dos fases que permiten la extracción del 40-60% del aceite restante. Las orujeras reciben alperujo con un contenido graso medio en torno al 2,5%, pero gran parte de este repasado es llevado a cabo en las almazaras de origen, por lo que en muchos casos llegan partidas con un contenido inferior al 1%, lo cual dificulta su extracción. Este hecho pone de manifiesto la dificultad económica de la extracción con un sólido tan húmedo y agotado. El subproducto resultante de esta segunda extracción se seca hasta llegar al 8% de humedad, y luego es extractado con hexano para producir una cantidad extra de aceite (aceite de orujo). Se han patentado nuevos procedimientos enfocados a favorecer la desecación de los orujos para permitir su uso como combustible en mejores condiciones de aprovechamiento. Estos procesos consisten en realizar una primera centrifugación del orujo, obteniéndose una fracción sólida con poca humedad usada como combustible (orujillo), y una fracción líquida que esta compuesta por alpechín, con un alto contenido de sólidos en suspensión. Ésta segunda fracción es sometida a una nueva centrifugación que permite la separación de un sólido con una elevada proporción de humedad, que es empleado para obtener aceite de orujo y un alpechín con un bajo contenido de sólidos

de suspensión que es concentrado y empleado como fertilizante, alimentación animal o fuente de bioalcohol (Artacho, 2002; Canet, 2007).

El kW/h generado del uso del orujillo como combustible ha estado subvencionado a través del Real Decreto 436/2004 y posteriormente por el Real Decreto 661/2007 y el Real Decreto Ley 9/2013, los cuales han sido modificados por el Real Decreto 413/2014, que regula la actividad de producción de energía eléctrica a partir de fuentes de energía renovables, cogeneración y residuos. El cambio de las ayudas destinadas a la producción de energías renovables ha provocado que la combustión del alperujo no sea una alternativa tan rentable como hace unos años, provocando el interés de usos alternativos del mismo que aumenten su valor y disminuyan sus costes de gestión.

3.2. Usos del alperujo

Actualmente, se están estudiando diferentes métodos para hacer un mejor uso del alperujo, alternativos a la generación de energía eléctrica. Sin embargo, muchos autores coinciden en la presencia de sustancias inhibidoras que pueden reducir el rendimiento de los procesos de estudio y que deben ser tenidas en cuenta para minimizar el potencial efecto negativo. Hasta ahora los esfuerzos para disminuir el impacto medioambiental del alperujo han estado centrados en su detoxificación, ya que no es fácilmente degradable por procesos biológicos naturales. Se han propuestos varios métodos para la eliminación del alperujo.

3.2.1. Biofertilizante y/o compost

Se ha probado como fertilizante orgánico (por su alto contenido en carbono y nutrientes minerales) mediante su aplicación directa sobre suelos agrícolas después de un proceso de compostado. El problema del compostaje del alperujo es su textura, que provoca que la aireación necesaria para que tenga lugar el proceso sea insuficiente, por esta razón se añaden diferentes agentes de carga que facilitan la correcta aireación y se somete a diferentes técnicas de agitación. El material de carga usado, la cantidad añadida para mezclar con el alperujo y el tiempo empleado para que tenga lugar el proceso difiere según los autores.

Cayuela y col. (2008; 2010), Plaza y col. (2008) y Alburquerque y col (2009) realizan diferentes estudios sobre la estabilidad del compost obtenido al mezclar alperujo con diferentes agentes de carga, tales como estiércol de ganado, tallos de uva, o desechos de algodón y olivo durante 40-48 semanas. En todos los casos el proceso del compostaje reduce en un 40-50% la materia orgánica, reduce la relación C/N, disminuye la proporción de metales pesados, aumenta el pH, hace desaparecer la fitotoxicidad, favorece la formación de material húmico y

genera un compost estable con predominio de la matriz lignocelulósica. Baddi y col. (2009) establecen que los polifenoles disminuyen en un 93% durante el proceso de compostado y son estos polifenoles los que contribuyen a la síntesis de sustancias húmicas, observándose que a medida que aumenta la humificacion se produce una disminución de la toxicidad.

El problema de emplear el alperujo como fertilizante radica en que se hace necesario un tratamiento previo de compostaje, que requiere un largo periodo de tiempo. Para intentar solventar este hecho Baddi y col. (2013) realizan una extracción de material húmico desde el alperujo empleando como disolvente extractor hidróxido de potasio (KOH) 1M durante 24 h a 78 ºC, obteniendo un fertilizante líquido que posee un elevado contenido en C total y está enriquecido en sustancias húmicas (80%).

Otra estrategia empleada es el uso de hongos saprofitos. Sampedro y col. (2008) determinan que la toxicidad del alperujo disminuye después de 20 semanas de incubación con estos hongos debido a la eliminación de fenoles, sin disminuir el contenido en N y P.

Por lo tanto el compostado del alperujo es una opción muy prometedora, dado sus bajos costes técnicos y económicos, para preparar fertilizantes orgánicos, el inconveniente de este proceso es el tiempo tan largo necesario para que se complete, lo que implica la acumulación de grandes volúmenes y el riesgo de infiltraciones a las aguas subterráneas. Además tiene la limitación de no ser capaz de eliminar completamente la biotoxicidad de algunos componentes, que interfieren con los procesos oxidativos aeróbicos de la producción de compost.

3.2.2. Alimentación animal

Los subproductos originados durante el proceso de extracción de aceite de oliva también pueden ser empleados como fuente de nutrientes para rumiantes (Molina-Alcaide y Yáñez-Ruiz, 2008). Sin embargo, el proceso de extracción de dos fases, en comparación con el de tres fases, produce un subproducto que incluye la fracción acuosa extraíble, que contiene sustancias solubles en agua, tales como los polifenoles, algunos de los cuales presentan una potente actividad antinutricional, como por ejemplo los taninos condensados. Yáñez-Ruiz y Molina-Alcaide (2007) estudian posibles daños renales o hepáticos en cabras y ovejas, derivados del suministro de una dieta complementada con alperujo, observando que las ovejas presentan una mayor sensibilidad a las dietas que contienen taninos condensados en comparación con las cabras, sin detectar efectos tóxicos en las concentraciones ensayadas. La sensibilidad observada a los taninos se debe a la capacidad que presentan para inmovilizar N, limitando su disponibilidad (Saviozzi y col., 1991; Mangan, 1988).

Un grupo de la unidad de Nutrición Animal de la Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC) ha desarrollado una fórmula para

pienso concentrado de rumiantes a partir de alperujo (López Gallego y col., 2003). Su eficacia ha sido probada de forma experimental en ganado caprino y ovino incorporándose un 10% de alperujo seco a pienso para cordero a base de cebada, maíz y soja, sin observarse anomalías patológicas en el cebo de estos animales tras su ingesta. Molina-Alcaide y col. (2005) y Mandaluniz y col (2010) estudian el efecto en la composición de la leche de cabra y ovejas, respectivamente, al incluir en su dieta alperujo, comprobándose un aumento de la concentración de ácido oleico, ácido linoleico y ácidos grasos insaturados. Faye y col. (2013) suministran subproductos procedentes de la industria del aceite como suplemento alimenticio a camellas, observándose un incremento en la concentración de grasas y proteínas en la leche.

3.2.3. Energía alternativa

También se ha empleado como fuente de energía alternativa, para ello se lleva a cabo una digestión anaeróbica del alperujo con microorganismos bajo condiciones mesófilas, obteniéndose un alto rendimiento en la degradación de la materia orgánica y en la producción de metano (Rincón y col., 2010) que puede ser empleado como combustible en la producción de electricidad. Martínez-García y col. (2009) llevan a cabo un tratamiento previo a la digestión anaeróbica con levaduras que provoca una mejora en el rendimiento del proceso.

La alta concentración de carbohidratos presente en el alperujo hace que este subproducto se pueda emplear también para producir etanol, empleando para ello bacterias o levaduras. Los diferentes polisacáridos presentes pueden ser transformados en etanol siendo necesaria la realización de dos etapas. En la primera tiene lugar una hidrólisis que provoca la liberación de los azucares reductores, y la segunda consiste en el paso de bioconversión en etanol (Asli y Qatibi, 2009; Haagensen y col., 2009). Ballesteros y col. (2002; 2001) aprovechan el material lignocelulósico presente en el alperujo para producir etanol, para este fin realizan un pretratamiento térmico con agitación alcanzándose los niveles máximos de producción de etanol (11.9 g/L) cuando el pretratamiento es realizado a 210 ºC. Por otro lado para producir etanol desde los huesos de las aceitunas se requiere un pretratamiento “steam explosion” catalizado con ácido sulfúrico que llega hasta los 240 ºC durante un corto periodo de tiempo (2-10 min) seguido de una descompresión brusca (Ballesteros y col., 2001).

3.2.4. Obtención de compuestos de alto valor añadido.

Las elevadas cantidades de alperujo que producen las industrias del aceite de oliva, pueden ser usadas para la recuperación de compuestos o sustancias valiosas y la conversión en productos útiles mediante el desarrollo de nuevos procesos. Esto sería de gran relevancia no solo desde el punto de vista medioambiental y económico, sino también para diferentes

sectores de la industria (alimentaría, cosmética y farmacéutica) en los que dichos compuestos pondrían tener aplicación. Debido a su alto contenido en compuestos valiosos, como fenoles y polisacáridos, el alperujo es un prometedor sustrato para la producción de diferentes productos orgánicos. Por lo tanto, la extracción de estos fenoles como productos de alto valor añadido podría ser considerada como una interesante alternativa para hacer provechosos los residuos de las almazaras, posibilitando su posterior uso como suplemento para alimentación animal o su biotransformación para el uso del sólido final como fertilizante, compost o sustrato fermentable, al eliminar gran parte de su contenido fitotóxico (Allouche y col., 2004).

Los polifenoles presentes en el alperujo han demostrado poseer actividad antioxidante, antimicrobiana y antifúngica. Algunos de los antioxidantes comerciales encontrados en el alperujo son el tirosol (Ty), el hidroxitirosol (HT) y en menor concentración la oleuropeína, los cuales pueden ser usados como aditivos alimentarios o cosméticos. Durante los últimos años el interés por la recuperación de los compuestos fenólicos desde los subproductos de la industria del aceite, tanto desde los generados desde el sistema de tres fases (Kalogerakis y col., 2013; Dermeche y col., 2013; Obied y col., 2008), como los generados con el sistema de dos fases (Lesage-Meessen y col., 2001; Rigane y col., 2012; Fernández-Bolaños y col., 2006), ha aumentado drásticamente. El consumo de aceite de oliva y aceitunas de mesa ha mostrado estar asociado con bastantes efectos saludables, incluyendo una disminución en la incidencia de enfermedades cardiovasculares y ciertos tipos de cáncer (Aruoma, 2003; Pérez-Jiménez, 2005; Cicerale y col., 2010; Lopéz-Miranda, 2010; Estruch y col., 2013; Ruiz-Canela y col., 2014; González-Santiago y col., 2006). Un gran número de artículos relacionan los efectos beneficiosos del aceite de oliva con su contenido fenólico (Segura-Carretero y col., 2010; Ghanbari y col., 2012). La recuperación de estos compuestos a partir del alperujo puede cambiar la visión negativa que se tiene de este subproducto, y reconocerlo como una fuente barata y una valiosa materia prima para la producción de compuestos bioactivos y antioxidantes naturales con un amplio espectro de actividades biológicas (Cicerale y col., 2010).

Por otro lado, el alperujo al ser un residuo de naturaleza vegetal también se encuentra enriquecido en los diferentes componentes de la pared celular de la aceituna. La pared celular está compuesta por polisacáridos complejos cuya naturaleza puede ser neutra (hemicelulosas y celulosas) o ácida (pectinas). Recientes estudios han mostrado que una parte sustancial de las hemicelulosas pueden se recuperadas en forma de oligosacáridos mediante un tratamiento auto-hidrolítico desde la fracción soluble en agua del alperujo (Rodríguez y col., 2007). Lama y col. (2012) aíslan a partir del alperujo tratado térmicamente previa extracción de los fenoles con acetato de etilo e hidrólisis suave una serie de oligosacáridos entre los que destacan xiloglucanos y xilo-oligosacáridos. Hsu y col. (2004) observan que los xilooligosacáridos usados como suplementos dietéticos pueden ser beneficiosos para la salud gastrointestinal, y reducen el riesgo de cáncer. Además, los oligosacáridos con grado de polimerización comprendidos entre 3 y 10 unidades se caracterizan por ser prebióticos presentando importantes propiedades

fisiológicas beneficiosas para la salud de los consumidores. Es por esta razón que su uso como ingredientes en alimentos funcionales se ha visto incrementado (Mussatto y Mancilha, 2007).

Así mismo, diferentes autores han sido capaces de aislar pectinas ricas en arabinosas a partir del alperujo (Cardoso y col., 2003a; Coimbra y col., 2010). Comercialmente las pectinas se extraen de dos importantes fuentes, del limón y la manzana. Pero en los últimos años ha aumentado el interés por buscar nuevas fuentes de pectinas, empleando para ello residuos de naturaleza vegetal. Las pectinas se caracterizan porque se pueden emplear como agentes gelificantes, emulsificantes y estabilizantes en la industria alimentaría. Pueden actuar como prebióticos, antiinflamatorios, antidiarreicos, controlan la diabetes y previenen numerosas enfermedades como obesidad y cáncer. (Munarin y col., 2012; Morris y col., 2013; Rodríguez y col., 2006). Además de ser capaces de unirse a ácidos biliares causando su eliminación en las heces y disminuyendo por tanto los valores de colesterol (Eastwood y Halminton, 1968).

3.3. Recuperación de compuestos de interés.

3.3.1. Pretratamientos.

Para aprovechar los principales componentes del alperujo (celulosa, hemicelulosas, pectinas y polifenoles) y a pesar de no tratarse de un material puramente lignocelulósico, se requiere un paso previo para que rompa las barreras físicas y químicas de su complejo entramado estructural, y aumente así la accesibilidad a dichas especies. Este pretratamiento posibilita la separación de las fases (líquida y sólida) y provoca la solubilización de los compuestos de interés, como son los compuestos fenólicos y azúcares, permitiendo así su recuperación. El material lignocelulósico forma parte de la composición de los vegetales y sus residuos, debido a que es el constituyente de las paredes celulares vegetales, y está formado por celulosa, hemicelulosas, lignina y pectinas. La recuperación de compuestos de interés en matrices con naturaleza lignocelulosica se encuentra limitada por la íntima interacción existente entre sus tres principales constituyentes, que son la celulosa, las hemicelulosas y la lignina. Los materiales lignocelulósicos pueden ser fraccionados en dichas especies mediante tratamientos biológicos, físicos, químicos y fisicoquímicos. Es interesante estudiar estos pre-tratamientos para incrementar su digestibilidad y su aprovechamiento (Hendriks y Zeeman, 2009).

- Los pretratamientos biológicos provocan la degradación parcial de la lignina mediante el empleo de microorganismos como hongos y bacterias (Ghosh y Syngh, 1993), pero el proceso es muy lento y solo es económicamente viable mediante su aplicación combinada con otros métodos físicos y/o químicos.

- Los pretratamientos físicos, como la molienda (no aplicable al alperujo) (Rivers y Emert, 1987) y la irradiación con microondas (Zhu y col., 2006), se han empleado para facilitar la hidrólisis del material lignocelulósico debido a que provocan una disminución en la cristanilidad y grado de polimerización de la celulosa (Furcht y Silla, 1990). Dentro de este grupo se engloban también los pretratamientos térmicos que serán estudiados con más detalle a continuación (Sección 3.1.2.).
- Los pretratamientos químicos solubilizan las hemicelulosas y la lignina para exponer la celulosa a hidrólisis ácida y/o enzimática (Wood y Saddler, 1988). Dentro de esta técnica el pretratamiento se puede llevar a cabo con ácido a temperatura ambiente, de forma que se solubiliza las hemicelulosas haciendo la celulosa más accesible (Liu y Wyman, 2003). Con base, provocando la solvatación y saponificación facilitando el ataque de enzimas y bacterias (Fengel y Wagener, 1984). O con especies oxidativas, como el peróxido de hidrógeno, que facilita la eliminación de hemicelulosas y lignina, aumentando la accesibilidad a la celulosa (Hon y Shiraishi, 2001).
- Los pretratamientos físico-químicos, como el pretratamiento térmico con vapor y descompresión explosiva “steam explosion”, el cual se desarrolla a continuación con más detalle (Sección 3.1.1.).
- Disolventes eutécticos: Los disolventes eutécticos profundos (DES) son una nueva clase de solventes iónicos, los cuales están formados por una mezcla de una sal de amonio con un donador de enlaces de hidrógeno (HBD). En estos disolventes el HBD interactúa con el anión, induciendo una disminución del punto de fusión de la mezcla a una temperatura cercana a la temperatura ambiente, y conservan las mismas propiedades de los disolventes iónicos como baja volatibilidad, elevada estabilidad térmica y alta polaridad. Sus mayores atractivos, son su bajo costo, y el hecho de que son considerados como “green solvents”, lo cual puede hacer rentable su aplicación en la industria de procesos. Estos DES actualmente están siendo empleados para el pretratamiento de materiales lignocelulósicos, debido a que son capaces de de separar la lignina y las hemicelulosas de la celulosa (Pablo-Domínguez, 2014; Hertel y col., 2014).

3.3.2. Tratamiento “steam explosion”.

La viabilidad del aprovechamiento y utilización de los subproductos de la extracción del aceite de oliva, como de otros subproductos de origen vegetal, y del alperujo en particular, pasa por encontrar pretratamientos industriales viables y económicamente rentables para alcanzar una eficiente recuperación de sus componentes. Los usos alternativos del alperujo, frente su uso como biocombustible, están recibiendo una especial atención y se centran en dos aplicaciones: la recuperación de componentes naturales de alto valor añadido y la

bioconversión en productos útiles. Varios grupos de investigación están trabajando en la recuperación de compuestos valiosos para dar salida a la creciente cantidad de este subproducto, que a pesar de su uso como combustible sigue teniendo carácter de residuo con un alto potencial contaminante que hay que eliminar.

Para aprovechar los principales componentes del alperujo (celulosa, hemicelulosas y lignina) y los de alto valor añadido (polifenoles y azúcares) se requiere un paso previo para romper las barreras físicas y químicas de su complejo entramado estructural. Existen diferentes métodos de pre-tratamiento, entre los que se encuentra el pre-tratamiento térmico con vapor de agua a alta presión y temperatura, con o sin descompresión rápida (explosiva).

En esta línea, el grupo de Fitoquímicos y Calidad de Alimentos del departamento de Fitoquímica de los Alimentos del Instituto de la Grasa (C.S.I.C.) trabaja desde hace algunos años en el estudio del aprovechamiento de los subproductos de extracción del aceite de oliva. Para este objetivo usan vapor de agua a alta presión mediante un tratamiento "steam explosion" que facilita el aislamiento de compuestos de alto valor añadido, debido a que por esta técnica se solubiliza gran parte de los compuestos fenólicos presentes en la matriz del alperujo (**Figura 3**) (Fernández-Bolaños y col., 2001; Rodríguez y col., 2007). Este pretratamiento "steam explosion" se ha usado para la solubilización de compuestos fenólicos con actividad antioxidante a partir de orujo de aceituna de dos y tres fases, permitiendo la recuperación de HT y otros compuestos de interés (Fernández-Bolaños y col., 1998; Fernández-Bolaños y col 2002; Felizón y col., 2000).

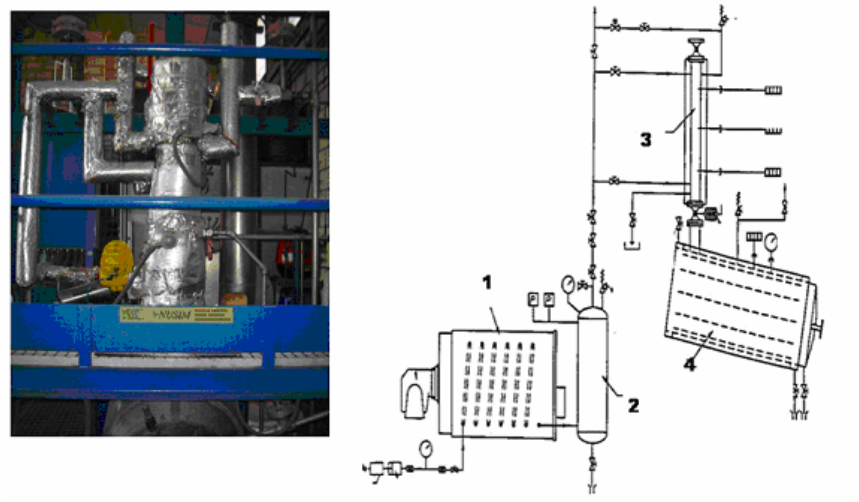


Figura 3: Fotografía y esquema del reactor "steam explosion" del Departamento de Fitoquímica de los Alimentos del Instituto de la Grasa (C.S.I.C.), Sevilla, utilizado en este trabajo de investigación. En el esquema pueden verse los componentes del reactor: (1) caldera de producción de vapor, (2) acumulador de vapor, (3) cámara de reacción y (4) depósito de expansión.

Dentro de los pretratamientos existentes, el tratamiento con vapor de agua a alta presión y temperatura con descompresión rápida (explosiva), es uno de los más usados para el fraccionamiento de los tres principales componentes del material lignocelulósico (Vlasenko y col., 1997; Zhang y col., 2008).

En el tratamiento "steam explosion" la biomasa es tratada con vapor saturado a alta presión seguido de una rápida despresurización, lo que provoca que el material sufra una descompresión explosiva. El efecto del pretratamiento "steam explosion" sobre el material es una combinación de modificaciones físicas y químicas. Las modificaciones físicas se producen como consecuencia de la rápida despresurización, provocando en las regiones más débiles (celulosa amorfa) un resquebrajamiento de la estructura lignocelulósica y la separación de las fibras de celulosa, como consecuencia se produce una reducción del tamaño de partícula y la expansión de los microporos. Las condiciones de presión y temperatura provocan la disociación del agua generando iones hidronio, que junto a los compuestos formados *in situ*, entre los que destaca el ácido acético procedente de la desacetilación de las hemicelulosas, dan lugar a un medio ácido (pH 3-4), y con ello a la acción catalítica en la autohidrólisis (Duff y Murria, 1996), que desencadenan las modificaciones químicas. De esta forma los grupos ácidos catalizan la hidrólisis de las hemicelulosas, favorecen aún más la disminución en el tamaño de los cristales de la celulosa (Mok y Antal, 1992) y se produce una ruptura de los enlaces hemicelulosa-lignina facilitando el acceso de las enzimas a las fibras de celulosa (Mosier y col., 2005). Las elevadas temperaturas alcanzadas en el reactor provocan que la lignina se funda rompiéndose las uniones con los demás componentes de la pared celular, al bajar la temperatura la lignina liberada condensa formando gotas (**Figura 4**). Aunque el ácido acético se ha considerado el principal ácido responsable del efecto catalítico de la autohidrólisis, también se producen otros ácidos como el ácido fórmico y el levulínico que ayudan a la bajada del pH (Ramos, 2003).

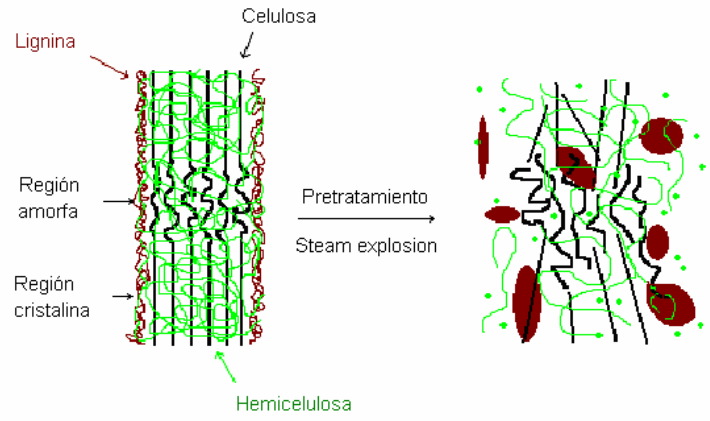


Figura 4: Representación esquemática del efecto del pretratamiento "steam explosion" sobre los materiales lignocelulósicos. (Adaptado de Mosier y col. (2005)).

Los principales factores que afectan al pretratamiento “steam explosion” son el tiempo de residencia y la temperatura, el proceso se puede llevar a cabo a temperaturas dentro del intervalo de 160-240 ºC (correspondiente a presiones comprendidas entre 0,69-4,46 MPa) durante cortos periodos de tiempo, desde varios segundos a pocos minutos antes de que el material sea expuesto a presión atmosférica (Duff y Murria, 1996).

Comparado con los métodos de pretratamiento alternativos, como los procesos de hidrólisis ácida y los procesos oxidativos, el método “steam explosion” destaca debido a que reduce considerablemente el impacto ambiental, los costes económicos y el consumo energético. Además, elimina el uso de cantidades significativas de productos químicos ácidos y/o básicos (Ramos, 2003) y es posible una recuperación más completa de todos los biopolímeros (celulosa, hemicelulosas, lignina) en una forma útil. El pretratamiento puede llevarse a cabo con o sin adición de ácido como catalizador, la función del catalizador ácido es mejorar la hidrólisis enzimática posterior y durante el proceso, y conducir a una más completa eliminación de la hemicelulosas. Si no se añade catalizador ácido el procedimiento “steam explosion” es un procedimiento autocatalítico.

El pretratamiento “steam explosion” se puede aplicar a una gran variedad de biomasas vegetales. Este pretratamiento se ha aplicado sobre huesos de aceitunas para incrementar la susceptibilidad de la celulosa al ataque enzimático e incrementar la solubilidad de las hemicelulosas (Fernández-Bolaños y col., 2001); recuperar compuestos fenólicos de interés, tales como el HT y el Ty, al provocar su solubilización (Fernández-Bolaños y col., 1998); y recuperar lignina y celulosas que pasan a formar parte de la fracción soluble (Fernández-Bolaños y col., 1999). Así mismo el pretratamiento “steam explosion” se ha aplicado sobre residuos procedentes de la industria del aceite de oliva de tres fases y de dos fases. Felizón y col. (2000) realizan el pretratamiento sobre orujo obteniendo una fracción líquida que es extraída con acetato de etilo, permitiendo aislar HT. Por otro lado, Rodríguez y col. (2007) realizan el pretratamiento sobre alperujo, permitiendo la recuperación de azúcares fermentables, y favoreciendo la solubilización de manitol, hemicelulosas de bajo peso molecular e HT. Dicho pretatameinto también se ha aplicado sobre ramas de poda del olivo, favoreciéndose la solubilización de azúcares hemicelulósicos y sus transformación en etanol (Cara y col., 2008), y el aumento de la concentración de compuestos solubles en acetato de etilo, obteniendo un extracto que presenta actividad antioxidante (Conde y col., 2009). Además el pretratamiento se ha aplicado sobre madera de olivo permitiendo obtener extractos enriquecidos en compuestos fenólicos que presentan actividad antioxidante (Castro y col., 2008). Los cambios que tienen lugar durante el tratamiento “steam explosion” permiten una bioutilización más eficiente del contenido de carbohidratos de los materiales lignocelulosicos por parte de los componentes microbiológicos (Castro y col., 1994) y sirve como tratamiento previo para mejorar la digestibilidad de la biomasa lignocelulósica (Hendrikd y Zeeman, 2009).

3.3.3. Fraccionamiento de los componentes de la pared celular vegetal.

Una vez realizado el pretratamiento sobre el material lignocelulósico se hace necesario realizar un fraccionamiento para separar los principales polímeros que forman parte de dicho material. Como ya se ha comentado la temperatura y la presión del agua alteran profundamente la cohesión inter e intrafibrilar, de manera que las muestras quedan desfibriladas siendo posible separarlas en los principales componentes que constituyen dicha pared mediante un proceso de solubilización selectiva.

Hay que destacar que el pretratamiento provoca transformaciones químicas en los diferentes componentes del material lignocelulósico. De tal forma que las hemicelulosas se van liberando a la fase acuosa, pero a medida que aumenta la severidad del tratamiento disminuyen las pentosas recuperadas como consecuencia de su degradación a furfural, de manera que la recuperación máxima de hemicelulosas en las condiciones óptimas de recuperación alcanza el 70%. Por otro lado la lignina es despolimerizada, los enlaces éteres de las unidades de fenilpropano son hidrolizados y tiene lugar una disminución en la cantidad de metoxilos que forman parte de dicha lignina. La celulosa experimenta una disminución de las fuerzas de unión intermicrofibrilares y de algunos puentes de hidrógeno. Todos estos cambios afectan al entramado estructural de la pared celular modificando sus características y facilitando el fraccionamiento.

Una vez que han tenido lugar todos los cambios en los componentes como consecuencia del pretratamiento se consigue separar una fracción líquida y otra sólida, a partir de las cuales se tiene que realizar un fraccionamiento para aislar los diferentes componentes. Unos de los fraccionamientos más empleados para separar dichos componentes del material lignocelulósico una vez realizado un pretratamiento “steam explosion” consiste en realizar un proceso de precipitación/solubilización selectiva que permita aislar a los diferentes componentes (Fernández-Bolaños y col. 1990; da Costa Lopes y col., 2013). La fracción líquida obtenida tras el pre-tratamiento se encuentra compuesta por fenoles y hemicelulosas que son separados mediante una extracción con acetato de etilo, de manera que los fenoles pasan a formar parte de la fracción orgánica. Por otro lado la fracción sólida obtenida tras el pretratamiento es tratada con una solución alcalina que permite la solubilización de la lignina, dejando un residuo enriquecido en celulosa, una vez separa la lignina de la celulosa se puede proceder a su precipitación mediante la acidificación de la solución.

Recientemente otras estrategias de fraccionamiento han sido descritas, de esta forma vom Stein y col. (2011) llevan a cabo un fraccionamiento del material lignocelulósico mediante el empleo de ácidos orgánicos que catalizan una despolimerización de las hemicelulosas. Para este fin en primer lugar el material lignocelulósico es tratado a temperaturas suaves con ácido oxálico que favorece la despolimerización de las hemicelulosas que pasan a azúcares solubles mientras que la celulosa permanece intacta. A continuación se realiza un segundo tratamiento

con 2-MTHF (2-metiltetrahidrofurano) que permite la separación de la lignina. Finalmente la celulosa deslignificada obtenida puede ser sometida a un proceso enzimático de despolimerización. Da Silva y col. (2013), describen un proceso de pretratamiento y fraccionamiento mediante el empleo de líquidos iónicos que permiten la producción de celulosa, hemicelulosas y fracciones ricas en lignina mediante un rápido y simple proceso de fraccionamiento en tres pasos.

3.3.4. Tratamiento térmico con vapor.

A pesar de las ventajas que proporciona en el caso del alperujo el pretratamiento con vapor y descompresión explosiva o "steam explosion", aparecen inconvenientes para su puesta en práctica a nivel industrial. El mayor inconveniente se debe a las condiciones de altas temperaturas (160-240 ºC) y presiones (máxima 4,46 MPa) que son necesarias, seguida de una rápida descompresión, que limita el desarrollo de este sistema en las instalaciones de las industrias orujeras que se encargan de la recepción y del tratamiento del alperujo para extraer el aceite de orujo, y las cuales trabajan a presiones máximas de 0,88 MPa. Por este hecho nuestro grupo de Investigación (Departamento de Fitoquímica de los Alimentos del Instituto de las Grasa, (CSIC)) ha diseñado, construido y puesto en marcha a escala planta piloto un nuevo sistema de tratamiento térmico con vapor discontinuo que funciona a temperaturas y presiones más bajas y sin necesidad de descompresión explosiva. Con este nuevo reactor se pretende mantener las ventajas anteriormente mencionadas para el aprovechamiento del alperujo y reducir la temperatura (máxima 190 ºC) y la presión (máxima 1,18 MPa) eliminando factores críticos como la presencia de oxígeno, gracias a la posibilidad de generar vacío en su interior y la descompresión rápida.

Estudios previos ponen de manifiesto que el tratamiento realizado con el nuevo reactor proporciona las mismas ventajas sobre el alperujo que el reactor "steam explosion" y además la tecnología aplicada se puede adaptar más fácilmente a la industria orujera (Lama, A., 2011).

Los tratamientos térmicos del alperujo se realizan en el nuevo reactor construido a escala piloto por la empresa C.GIL, S.A. (Barcelona, España) (**Figura 5 a y b**). Es un reactor en discontinuo de 100 L de capacidad, de construcción cilíndrico vertical y material de acero inoxidable INOX-316L. Permite el tratamiento del alperujo con vapor en condiciones menos severas y oxidantes que las que se alcanzan en el anterior reactor con vapor y descompresión explosiva ("steam explosion"). El proceso térmico con vapor de agua favorece la solubilización de parte de las hemicelulosas y la liberación de ácidos que a su vez favorecen la autohidrólisis de la misma. Por lo tanto, el tratamiento térmico con vapor hidroliza parcialmente al alperujo liberando algunos enlaces glicosídicos y fragmentando biopolímeros, lo que facilita la disociación de sus componentes y una eficaz separación sólido-líquido. El tratamiento también

solubiliza a una gran cantidad de compuestos bioactivos de interés, quedando un sólido reducido en el que se concentra la grasa. En el aceite extraído a partir del alperujo tratado se produce un considerable incremento en las concentraciones de una serie de componentes minoritarios (esteroles, tocoferoles, alcoholes triterpénicos, etc...) de gran interés para la salud (Lama-Muñoz y col., 2011).

En el caso de la fracción sólida se consigue una reducción drástica de la humedad de más del 20%, obteniéndose un sólido con solo el 30% de humedad. Este hecho, de enorme importancia económica por el ahorro energético en los secaderos, favorece la extracción del aceite y el posterior uso de la biomasa como biocombustible. Además, en la fracción líquida, también se solubiliza una parte muy importante de los compuestos fenólicos de gran interés así como carbohidratos en forma de mono- , oligo- y polisacáridos (Lama-Muñoz y col., 2012).

a)

b)

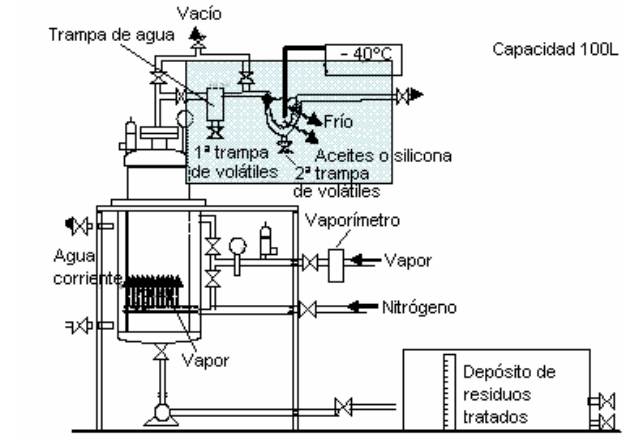


Figura 5 a y b: Esquema (a) y fotografía (b) del nuevo reactor del tratamiento con vapor utilizado y diseñado en el Departamento de Fitoquímica de los Alimentos del Instituto de la Grasa (C.S.I.C.), Sevilla, empleado en el presente trabajo de investigación.

3.4. Compuestos fenólicos.

3.4.1. Compuestos fenólicos en la aceituna.

Los fenoles son un grupo heterogéneo de compuestos naturales presentes en todas las plantas superiores, que poseen la característica química común de poseer al menos un grupo fenol. Entre ellas, el olivo y su fruto han sido reconocidos como una extraordinaria fuente de fenoles. En las plantas, los compuestos fenólicos son metabolitos secundarios y desempeñan funciones fisiológicas: actúan como agentes que reducen el crecimiento de plantas competidoras, por su carácter antimicrobiano son productos de defensa ante microorganismos patógenos como bacterias, hongos y virus, y absorben la radiación ultravioleta en las capas más superficiales protegiendo a los tejidos externos de sus efectos perjudiciales (Ryan y Robards, 1998). Estos compuestos están presentes en todas las diferentes partes de la planta, pero su naturaleza y concentración varia entre los diferentes tejidos (Covas y col., 2006).

La composición media de las aceitunas es de un 50% de agua, 1.6% de proteínas, 22% de aceite, 19.1% de carbohidratos, 5.8% de celulosa, 1.5% de sustancias inorgánicas y de un 1 a un 3% de compuestos fenólicos (Ghanbari y col., 2012). Esta composición fenólica es característica y la existencia y cantidad de fenoles específicos en la aceituna, y por tanto, en el alperujo, dependen de la variedad y estado de madurez, condiciones climáticas, estacionales y geográficas (Bianchi, 2003).

Dentro de los alcoholes fenólicos hay que destacar que los que se encuentran en mayor proporción son el HT y el Ty. En el grupo de los fenoles ácidos se han identificado especies como los ácidos clorogénico, cafeico, p-hidroxibenzoico, protocatéquico, vainíllico, siríngico, p- y o-cumárico, ferúlico, sinápico, benzoico, cinámico y gálico (Ghanbari y col., 2012; Ryan y Robards, 1998). La oleuropeína, un éster glucosídico de 3,4-dihidroxifeniletanol (hidroxitirosol) y ácido elenólico, es el principal compuesto secoiridoide en aceitunas verdes/no maduras; su concentración disminuye con la maduración del fruto. En paralelo con la disminución de la oleuropeína hay un aumento de la desmetiloleuropeína y de la forma dialdehídica del aglicón de la oleuropeína descarboxilmetilada (3,4-DHPEA-DEA) (Romero y col., 2004). Esta última sustancia llega a ser predominante en aceitunas maduras. Otros compuestos secoiridoides encontrados en aceitunas son el glucósido de ácido elenólico, el ligustrósido y la forma dialdehídica de su aglicón (Obied y col., 2007). Entre los compuestos fenólicos que también han sido identificados en la aceituna se encuentran el verbascósido y el salidrósido (glucósido de tirosol), y en concentraciones relativamente altas, los flavonoides apigenín-7-O-glucósido, luteolín-7-O-glucósido, quercitina, rutina y las antiocianinas cianidín-3-O-glucósido y cianidín-3-O-rutinósido, pigmentos que proporcionan el intenso color violeta oscuro a las aceitunas

maduras (Romero y col., 2002). Otros compuestos encontrados en pulpa de aceituna son catecol y metilcatecol **(Figura 6)**.

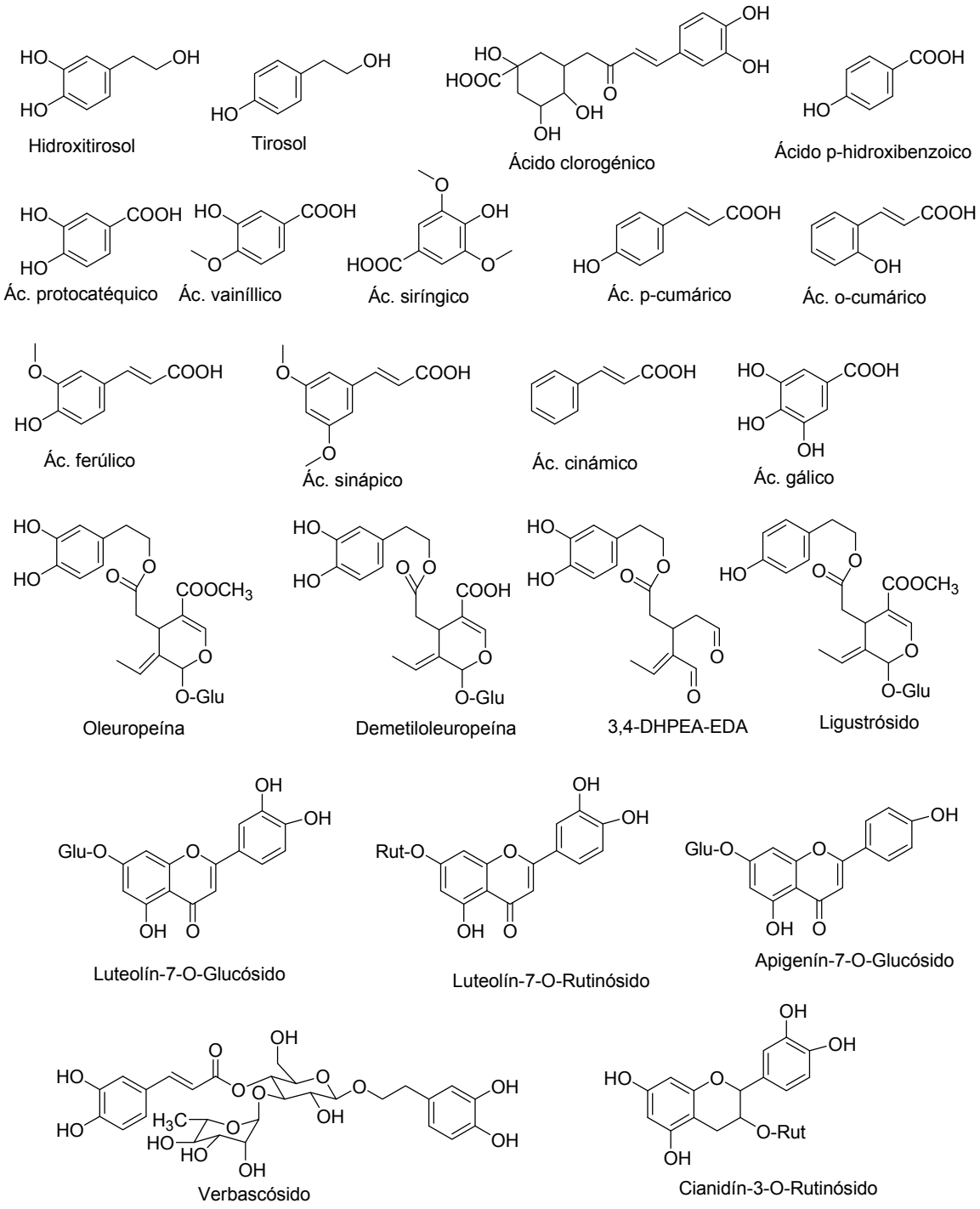


Figura 6: Estructuras de los principales compuestos fenólicos presentes en la aceituna.

Durante el proceso de extracción del aceite de oliva, los fenoles presentes en el fruto se reparten entre la fracción lipídica (aceite de oliva) y los subproductos generados (alpechín y orujo para el sistema de extracción de tres fases, o alperujo en el sistema de extracción de dos fases), en función a su naturaleza hidrofílica o lipofílica. Por lo tanto los fenoles presentes en el

fruto quedan repartidos entre ambas fracciones, en función de sus constantes de reparto (Artajo y col., 2007). La mayoría de los fenoles tienen bajos coeficientes de reparto (aceite/agua) comprendidos entre $6 \cdot 10^{-4}$ y 1,5 (Rodis y col., 2002) por lo que se distribuyen preferentemente en la fase acuosa (Obied y col., 2005a), de forma que sólo el 2% de los fenoles presentes en el fruto pasan a formar parte del aceite de oliva, permaneciendo el 98% restante en el subproducto generado

En el aceite, por tanto, pasan parte de los alcoholes y ácidos fenólicos que hay en el fruto, siendo los más importantes el HT, el Ty, el acetato de hidroxitirosilo (3,4-DHPEA-AC) y los ácidos gálico, protocatéquico, p-hidroxibenzoico, vaníllico, cafeico, elenólico, siríngico, p- y o-cumárico, ferúlico y cinámico (Bakhouche y col., 2013; Enache y col., 2013; Ghanbari y col., 2012; Riachy y col., 2013). El HT es el principal polifenol en el aceite de oliva virgen, y también se puede encontrar en las hojas y frutos de oliva. Aunque el HT es el principal polifenol en aceite de oliva virgen hay que destacar que el 3,4-DHPEA-AC se pude encontrar en iguales o superior concentraciones dependiendo de la variedad de la aceituna (Drira & Sakamoto, 2013). Por otro lado, los secoiridoides más abundantes en el aceite de oliva virgen, provienen de las agliconas de la oleuropeína (3,4-DHPEA-EA) o del ligustrósido (p-HPEA-EA), con el ácido elenólico en su forma dialdehídica y descarboxilmetilado, 3,4-DHPEA-EDA y p-HPEA-EDA, según están unidas a HT o Ty, respectivamente y otros derivados de la aglicona de la oleuropeína, como el 3,4-DHPEA-AC y la demetiloleuropeína (Artajo y col., 2007). También se han detectado en importantes cantidades en el aceite de oliva lignanos, tales como el pinoresinol o el 1-acetoxipinoresinol (Brenes y col., 2000; Suarez y col., 2009). Dentro de las flavonas se han identificado especies como la apigenina y la luteolina y también cabe destacar la presencia de isocromanos (Bianco y col., 2001). Estos compuestos pueden ser sintetizados a partir de HT y compuestos aldehídicos mediante una catálisis ácida (Guiso y col., 2008). De hecho pueden formarse espontáneamente en el aceite debido a que se encuentran los reactivos necesarios, además del ácido oleico que puede actuar como catalizador ácido, sin embargo la concentración de los isocromanos van disminuyendo durante el almacenamiento del aceite, pudiendo ser utilizados como marcador de la calidad del aceite **(Figura 7)**.

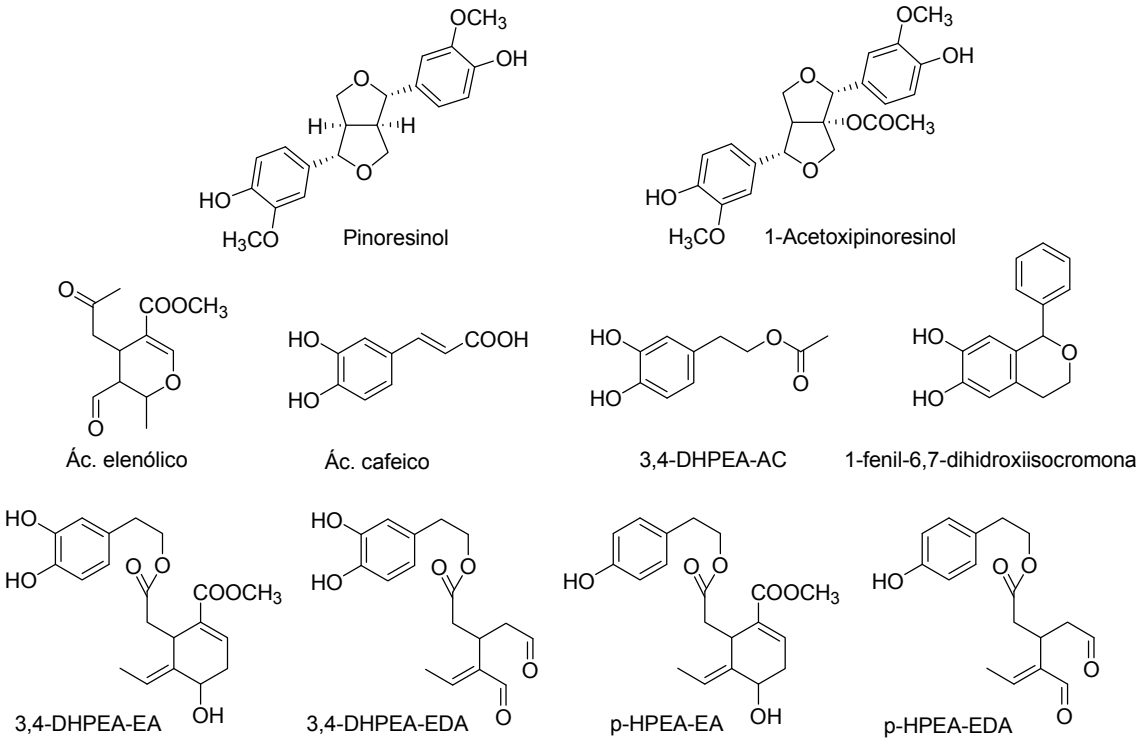


Figura 7: Estructuras de los principales compuestos fenólicos presentes en el aceite de oliva.

El alperujo que resulta durante el proceso de extracción del aceite contiene por tanto el 98% de los fenoles totales de la aceituna (Rodis y col., 2002). El contenido fenólico está estrechamente relacionado con el proceso de extracción, el cual determina el comportamiento de los fenoles y por tanto su distribución entre el aceite y las fracciones residuales. Diversas investigaciones han identificado en el alpechín de tres fases derivados del ácido cinámico como el ácido cafeico, derivados del ácido benzoico como el ácido protocatéquico, y derivados del feniletanol como son el HT, el Ty y el 3,4-dihidroxifenilglicol (DHFG). También se han identificado especies como el ácido vainíllico, ácido ferúlico, ácido p-cumárico, ácido siríngico, ácido 3,4-dihidroxifenilacético, ácido p-hidroxibenzoico, ácido p-hidroxifenilacético, vainillina, siringinaldehido, p-hidroxibenzaldehido, oleuropeína, y algunos flavonoides como apigenín, luteolín, luteolín-7-O-glucósido y quercitina (Ramos-Cormenzana y col., 1996; Allouche y col., 2004; Lesage-Meessen y col., 2001). Por otro lado en el alperujo obtenido como subproducto en el sistema de extracción de dos fases se han identificado especies como el HT, el Ty, el glucósido del hidroxitirosol, el ácido cafeico, el luteolín glucósido y el verbascósido (Obied y col., 2008; 2007). Por lo tanto, el perfil fenólico del alperujo es complejo y hasta la fecha la recuperación de éstos compuestos no ha sido completamente llevada a cabo para la valorización del subproducto.

En el alperujo se han identificado diferentes polímeros fenólicos entre los que cabe destacar la polimerina. Esta fracción polimérica está formada por un complejo entre

polisacáridos (50%), proteínas (10-16%), melamina (17-26%) y sustancias inorgánicas (11%) (Arienzo y Capasso, 2000; Capasso y col., 2002a). La fracción polimérica orgánica (fenoles polimerizados) se caracteriza por estar ligada a iones metálicos los cuales interaccionan con los grupos funcionales de dichas moléculas orgánicas, mediante los grupos carboxilo, sulfhidrilo, hidroxilo, o restos aminos, o fenólicos (Arienzo y Capasso, 2000). Esta fracción denominada polimerina parecer ser responsable de la coloración del alpechín y de alperujos, y es la posible causante de las alteraciones de las determinaciones colorimétricas (Capasso y col., 2002a; Capasso y col., 2004). Por lo tanto esta fracción esta formada por polímeros orgánicos con un elevado contenido en metales.

3.4.2. Propiedades de los compuestos fenólicos.

La dieta Mediterránea caracterizada por un elevado consumo de frutas, vegetales, semillas, legumbres, y cuya principal fuente de grasas es el aceite de oliva ha sido documentada por numerosos estudios epidemiológicos y asociada con un bajo riesgo de enfermedades cardiovasculares, neurodegenerativas, coronarias y algunos tipos de cáncer (Pérez-Jiménez, 2005; Cicerale y col., 2010; Estruch y col., 2013; Ruiz-Canela y col., 2014). Los beneficios de esta dieta se han asociado con el elevado consumo de sustancias captadoras de radicales libres, tales como fenoles y flavonoides, los cuales se encuentran en elevada concentración en el aceite de oliva (Kounturi y col., 2007). Recientes estudios demuestran que los fenoles son poderosos antioxidantes (Kalogerakis y col., 2013; Ziogas y col., 2010; Suárez y col., 2009) y poseen otras actividades biológicas que podrían explicar en parte los efectos saludables observados en la dieta Mediterránea (Martín-Pelaez y col., 2013; Ziogas y col., 2010; Fitó y col., 2008). Estos compuestos también juegan un importante papel en la estabilidad (inhiben la peroxidación lipídica) y en las propiedades químicas y organolépticas de los productos de las aceitunas (aceite de oliva y aceituna de mesa), y tienen significativos efectos nutricionales, fisiológicos y farmacéuticos sobre la salud humana (López-Miranda y col., 2010). La ingesta habitual de aceite de oliva proporciona un continuo aporte de antioxidantes, que pueden reducir el estrés oxidativo en el cuerpo humano. Un gran número de artículos relacionan los efectos beneficiosos del aceite de oliva con su contenido fenólico (Segura-Carretero y col., 2010). El aceite oliva virgen extra contiene gran variedad de antioxidantes fenólicos incluyendo fenoles simples, secoroideos, flavonoides y lignanos (Ocakoglu y col., 2009). Todas estas sustancias son potentes inhibidores del ataque de especies de oxígeno reactivas y en la actualidad, hay evidencias que ponen de manifiesto que las especies oxidantes (oxígeno activo, radicales libres, etc.) están implicadas en la etiología de numerosas enfermedades (Menéndez y col., 2009; Gill y col., 2010; Fabiani y Morozzi, 2010; Visioli y col., 2002).

Se ha demostrado que la aterosclerosis es un proceso inflamatorio crónico que tiene lugar en las paredes de las arterias como respuesta a una agresión sobre el endotelio (Libby, 2002). El desarrollo de este proceso tiene lugar fundamentalmente en la capa íntima arterial donde se desarrolla la capa de ateroma **(Figura 8)**. Esta capa tiene su origen en la capa de lípidos, normalmente las lipoproteínas de baja densidad (LDL) que penetran en las arterias pudiendo interactuar peligrosamente con sustancias reactivas de oxígeno (ROS) que provocan su oxidación. Estas LDL oxidadas son captadas por macrófagos, a través de receptores específicos de eliminación, convirtiéndose en células espumosas, que se encuentran llenas de lípidos (Denis y Wagner, 2007).

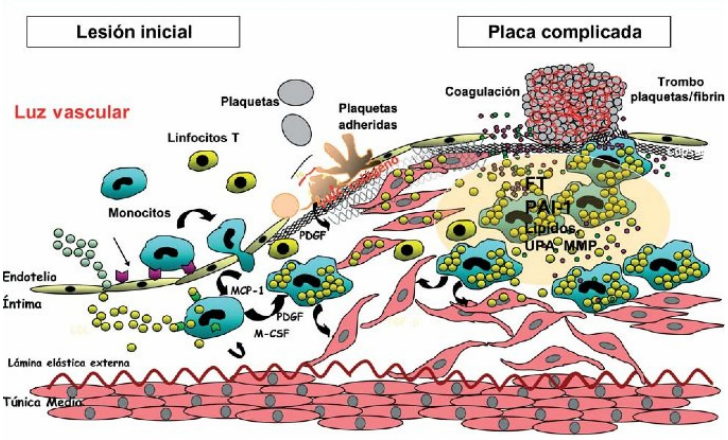


Figura 8: Representación esquemática de la evolución de la placa aterosclerótica (Badimón y col., 2009).

Se ha demostrado que el consumo diario de aceite de oliva provoca la disminución total de colesterol y LDL, los cuales son factores de riesgo para desarrollar aterosclerosis, la principal causa del desarrollo de enfermedad cardiovascular. Además se observa que tiene lugar un aumento de la concentración de lipoproteínas de alta densidad (HDL) que presentan un efecto antiinflamatorio (Gordon y col., 1981), de manera que se favorece la bajada de la presión sanguínea en pacientes hipertensos (Pérez-Jiménez, 2005). Por otro lado el consumo continuado de aceite provoca que se disminuya las concentraciones de LDL oxidado (Castañer y col., 2011). La oxidación de LDL esta considerada unos de los factores de riesgos en el desarrollo de aterosclerosis y enfermedades cardiovasculares, debido a que el LDL oxidado provoca daños en la pared vascular, estimulando la absorción de macrófagos y la formación de células espumosas (Fitó y col., 2005). También se ha demostrado que los fenoles presentes en el aceite de oliva tienen la capacidad de inhibir la activación y agregación plaquetaria (De La Cruz y col., 2000). La activación y posterior agregación de las plaquetas es un factor importante en el desarrollo de aterosclerosis, en esta enfermedad inflamatoria las plaquetas activadas se unen a colágeno y a otras moléculas que son secretadas como respuesta al estimulo inflamatorio y contribuyen a la formación de placas en los vasos sanguíneos (Ruggeri, 2002). Estudios previos han mostrado que los fenoles presentes en el aceite de oliva son capaces de disminuir la concentración de los marcadores de inflamación presentes en la sangre. Estos

agentes inflamatorios son conocidos por producir dolor, enrojecimiento e inflamación y elevadas concentraciones de los mismos son indicadores de un mayor riesgo cardiovascular (Bogani y col., 2007).

Rodríguez y col. (2012) realizan un estudio de los índices y mecanismos de estrés oxidativo y metabólico en ratas. Para ello utilizan ratas alimentadas con una dieta deficiente en vitamina E y posteriormente se suplementa esta dieta con α-tocoferol, un extracto fenólico, HT y DHFG. La biodisponibilidad de los compuestos fenólicos queda demostrada mediante su detección en sangre y tejidos. Estudios proteómicos realizados en este trabajo relevan que el α-tocoferol y los compuestos fenólicos regulan la proteína hepática mitocondrial aldehído dehidrogenasa (ALDH2) que representa un mecanismo antioxidante. Por tanto, se demuestra que los compuestos fenólicos de la aceituna o del aceite de oliva tienen impacto beneficioso sobre la salud cardiovascular.

Son numerosos los estudios que consideran la actividad anticancerosa de los fenoles presentes en el aceite (Gill y col., 2010; Fabiani y col., 2006). El estrés oxidativo producido por las ROS se ha relacionado con un número de enfermedades tales como la aterosclerosis, ciertos cánceres y las enfermedades neurodegenerativas, tales como el Alzheimer o Parkinson (Fabiani y col., 2008; Cooke y col., 2003). Los compuestos fenólicos del aceite de oliva han demostrado tener capacidad secuestrante de las ROS en condiciones naturales y en condiciones de estrés oxidativo simulados químicamente (Owen y col., 2000). El daño oxidativo que sufre el ADN (ácido desoxirribonucleico) es un precursor para la carcinogénesis humana (Cooke y col., 2003), pero debido a que los fenoles actúan como antioxidantes protegen el ADN y evitan su oxidación disminuyendo el riesgo de desarrollar cáncer (Salvini y col., 2006). El consumo de aceite de oliva provoca la preservación de las funciones de las mitocondrias, evitando que sus membranas sufran el ataque de radicales libres, permitiendo que sus funciones de transporte de electrones tenga lugar de forma más favorable y disminuyendo la producción de radicales libres (Pérez-Jiménez y col., 2005). De esta forma se ha demostrado que el HT inhibe la proliferación de las células de leucemia promielocítica HL60 en humanos (Fabiani y col., 2006) y ejerce efectos antiproliferativos contra células de adenocarcinoma de colon humano (Corona y col., 2009). Hashim y col. (2008) observan que existe una relación entre la inhibición del cáncer de colón con el consumo de aceite de oliva. Más recientemente, estudios realizados sobre células cancerosas de mama muestran que los fenoles son capaces de inhibir algunas líneas celulares (Menéndez y col., 2009). Se ha demostrado que en los países en los cuales la población lleva una dieta Mediterránea, como España, Italia y Grecia, donde el aceite de oliva es la principal fuente de grasa, la población que sufre cáncer es menor que en países del norte de Europa (López-Miranda y col., 2010).

En la investigación *in vitro* se ha demostrado que los compuestos fenólicos del aceite de oliva tienen propiedades antimicrobianas. Particularmente, la oleuropeína, el HT y el Ty han demostrado poseer una potente actividad antimicrobiana contra varias cepas de bacterias

responsables de infecciones intestinales y respiratorias (Medina y col., 2006). Romero y col. (2007) observan que la forma dialdehídica del ligustrósido (oleocantal) no se hidroliza en el estómago y por lo tanto ayuda en la inhibición del crecimiento de bacterias que están relacionadas con el desarrollo de úlceras pépticas y algunos tipos de cáncer gástrico (Brenes y col., 2011). El HT y la oleuropeína también han demostrado ser citotóxicos frente a un gran número de cepas bacterianas.

Los fenoles poliméricos aislados del fruto de la oliva también presentan propiedades importantes. Así, la polimerina hasta ahora descrita rica en potasio es fácilmente convertible en otros derivados en los que se remplaza el ión mayoritario, bien por micronutrientes como cobre, zinc, manganeso o hierro, o por otros metales de interés tales como el sodio y el aluminio, empleando dichos derivados como un tipo de ácido húmico enriquecido en metales (Capasso y col., 2002b). Otra aplicación se basa en la captación de herbicidas como el paraquat (dicloro 1,1'-dimetil-4,4'-bipiridinio) y el 2,4-D (ácido 2,4-diclorofenoxiacético), la máxima capacidad de sorción obtenida para el paraquat y el 2,4-D son de 800 mmol y 150 mmol respectivamente por kilogramo de polimerina, en ambos casos los valores de sorción obtenidos son mayores a los observados en el caso en el cual el ácido húmico actúa como adsorbente (Sannino y col., 2008). También se han empleando la polimerina y derivados de polimerina con hidróxido de hierro para la sorción de arsénico (V) y cromo (VI) (Sannino y col., 2009). Así mismo se ha estudiado la capacidad de sorción de la polimerina y de sus derivados K-polimerina y Fe(OH)x-polimerina para retener cromo (III), cobre y zinc presente en aguas contaminadas (Capasso y col., 2004).

3.4.3. Recuperación de compuestos fenólicos.

Los investigadores han empezado a considerar la posibilidad de recuperar los compuestos fenólicos presentes en los subproductos de extracción del aceite de oliva, como compuestos con potenciales propiedades biológicas. Estos compuestos poseen propiedades secuestrantes de radicales libres y quelantes de iones metálicos, y se ha probado *in vitro* que alguno de ellos son más efectivos como antioxidantes que las vitaminas C y E en sistemas lipofílicos (Rice-Evans y col., 1996). El interés de los antioxidantes naturales está aumentando debido a la evidencia de la implicación de los radicales libres y ROS en varios procesos patológicos (enfermedades hepáticas, renales e inflamatorias). El problema que se plantea, es que muchos estudios se ven limitados, debido a que diversos compuestos no están disponibles comercialmente para poder realizar ensayos de interés.

Los métodos de extracción de compuestos fenólicos a partir de los subproductos del aceite de oliva basados en la extracción líquido-líquido o sólido-líquido, emplean diferentes disolventes en distintas proporciones.

Obied y col. (2007), Handen y col. (2009) y De Marco y col. (2007), describen un método de extracción de fenoles del alperujo en el cual emplea una solución metanol/agua al 80% (v/v) a pH 2, dicho método también es empleado por Brenes y col. (2000), Ryan y Robards (1998) y Savarese y col. (2007) para la extracción de compuestos fenólicos del aceite. Suárez y col. (2009) llevan a cabo una extracción sólido-líquido con una mezcla de metanol/agua al 80% (v/v) a altas presiones del orujo, mientras que a partir de las aguas de vegetación realizan una extracción con acetato de etilo.

Diferentes autores emplean acetato de etilo (1:1, v/v) como disolvente extractante acidificado a pH 3 con ácido clorhídrico (HCl). La extracción a partir de los subproductos de la aceituna estabilizados con etanol al 30% evita la oxidación y el ataque enzimático, permitiendo obtener un extracto cuyo contenido total en fenoles totales calculado por el método de Folin-Ciocalteu es 2,1 g de fenoles y 5,4 g de fenoles en el extracto fenólico obtenido a partir de la extracción con acetato de etilo de 1 L de subproducto de dos o tres fases respectivamente (Lesage-Meessen y col., 2001). Con acetato de etilo se consigue extraer los fenoles más fitotóxicos del alpechín que son los de bajo peso molecular (Capasso y col., 1992). Estudios realizados por Della Greca y col. (2001) mostraron que los fenoles más tóxicos del alpechín correspondieron a aquellos que poseen un bajo peso molecular (< 350) como el catecol, el HT y los ácidos cinámicos. Isidori y col. (2005) observan que aquellos fenoles que tienen un peso molecular entre 1000 y 2000 Da llegan incluso a mostrar un efecto estimulador en la germinación de semillas y sobre el metabolismo de la bacteria *Vibrio fischeri*.

Allouche y col. (2004), comparan diferentes disolventes polares como el metil isobutil acetona, metil etil cetona, dietil éter, y acetato de etilo en la extracción de compuestos fenólicos desde el alpechín en un sistema de extracción continua, de forma que la extracción con acetato de etilo proporciona un extracto más rico en fenoles que los demás. Kalogerakis y col. (2013) prueban el poder extractante del acetato de etilo, el dietil éter y de una mezcla de cloroformo/alcohol isopropílico sobre alpechín, recuperándose nuevamente más fenoles en el caso en el cual se emplea acetato de etilo, estos mismos resultados son también observados por Klen y Vodopivec (2011) y El-Abbassi y col. (2012). Obied y col. (2005b), realizan un estudio sobre el poder extractante de diferentes disolventes para obtener un extracto enriquecido en fenoles desde el alperujo. Para ello someten a estudio diferentes mezclas acuosas de metanol, etanol, n-propanol, acetonitrilo, y acetona empleando extracción simple, asistida por Ultra Turrax (homogenizador y dispersador) y mediante agitación magnética. Las mezclas de metanol se prueban en un rango de 30-80% v/v en agua, mientras que el estudio de los otros disolventes es restringido al 50% v/v en agua, y se comparan los resultados con los obtenidos según el procedimiento de Lesage-Meessen y col. (2001), en el cual el residuo procedente de la aceituna a tratar se acidifica con HCl hasta pH 3 y se somete a una extracción con acetato de etilo. Este estudio pone de manifiesto que el acetato de etilo es selectivo para fenoles de pequeño y mediano peso molecular. Los resultados muestran que las mezclas acuosas de los diferentes alcoholes testados poseen una elevada capacidad de extracción

(330-450 mg extracto/g alperujo), aunque los extractos obtenidos poseen un bajo porcentaje de composición fenólica (10%), mientras que el acetato de etilo proporciona menos cantidad de extracto (20 mg extracto/g alperujo), pero se encuentran principalmente compuestos por fenoles (50%).

Se han desarrollado y patentando diferentes técnicas que emplean métodos cromatográficos para obtener HT a partir de subproductos de la extracción de aceite de oliva (Capasso y col., 1999; Fernández-Bolaños y col., 2002). De los diferentes métodos de extracción posibles, las técnicas de absorción-desorción para la extracción de compuestos fenólicos de los alpechines usando resinas iónicas y de adsorción son las más útiles. Siguiendo este proceso Setti y col. (2003) y Johannisbauer y col. (2003) desarrollan procedimientos para aislar compuestos antioxidantes a partir de los subproductos de la industria del aceite de oliva. Para ello el agua de vegetación se hace pasar a través de resinas de intercambio iónico, Amberlite® XAD y Lewait ® EP, quedando los antioxidantes absorbidos. Cuomo y Rabovskiy, (2002) obtienen un extracto fenólico a partir de aceitunas frescas, pulpas, alpechín y aceite de oliva mediante un proceso que requiere una etapa de extracción con un solvente acuoso polar (agua o una mezcla de agua con cualquier solvente polar miscible con ella), y a continuación un pase por resinas Amberlite® XAD o Duolite®.

Uno de los procedimientos descritos y patentado para la purificación de HT de forma simple, práctica y económica, que permite obtener HT a nivel industrial con alto grado de pureza (99,6%), es el método desarrollado en el grupo de Fitoquímicos y Calidad de los Alimentos del Departamento de Fitoquímica de los Alimentos del Instituto de la Grasa (CSIC) (Fernández-Bolaños y col., 2002). A partir de la fracción líquida obtenida tras el tratamiento térmico con vapor nuestro grupo ha aislado y purificado de forma sencilla, práctica y económica, dos antioxidantes fenólicos muy activos presentes en la aceituna, el HT y el DHFG. Los sistemas de purificación de estos dos antioxidantes naturales se han patentado (Fernández-Bolaños y col., 2013; Rodríguez-Gutiérrez y col., 2011) y, en el caso del HT se ha llevado a la industria y hoy se comercializa bajo el nombre de Olivefen®. La empresa SVM (Subproductos Vegetales del Mediterráneo S.L.), de base tecnológica (EBT), surge de nuestro grupo de investigación con el objetivo de llevar a los mercados los principales logros en el campo del aprovechamiento de subproductos alimentarios.

Entre los métodos descritos también para la extracción de fenoles se emplea la extracción líquido-líquido en contracorriente para obtener un extracto fenólico enriquecido en HT y ácido 3,4-dihidroxifenilacético desde el alpechín (Fki y col., 2005); extracción con fluido supercrítico en contracorriente con columnas para obtener HT desde aguas de vegetación de aceitunas (Crea, 2002); técnicas de separación por membranas de ultrafiltración y nanofiltración para conseguir un concentrado de fenoles desde la aceituna utilizando para ello los subproductos generados durante la producción de aceite de oliva (Ibarra y Sniderman, 2005); resinas absorbentes con el fin de purificar HT desde un extracto de hojas de olivo (Beverunge y col., 2005) y adsorción en resinas no iónicas con el fin de obtener un extracto

rico en sustancias antioxidantes a partir de soluciones generadas en el proceso de elaboración de aceitunas de mesa previa ultrafiltración (Brenes y Castro, 2003). Se utilizan técnicas de concentración selectiva por ultrafiltración, ósmosis inversa y extracción con fluidos supercríticos para extraer fenoles desde alpechines (González-Muñoz y col., 2003; Teresa y col., 2006).

3.5. Polisacáridos y oligosacáridos

3.5.1. Composición y estructura de la pared celular vegetal.

La pared celular es uno de los componentes fundamentales de las células vegetales. Es una estructura semirrígida que se encuentra rodeando a la membrana plasmática, y constituye la capa más externa de la célula. Está compuesta fundamentalmente de polisacáridos y pequeñas cantidades de glicoproteínas y compuestos fenólicos, contrastando con la membrana plasmática, de menor espesor, en la que fosfolípidos y proteínas son los componentes principales. La pared celular vegetal realiza funciones vitales para la célula: actúa definiendo la forma y clase de células y tejidos; posee un papel protector, actuando como barrera frente a la acción de microorganismos y otros agentes externos al vegetal; regula el control y desarrollo de plantas y constituye el armazón que proporciona consistencia a la planta.

La pared celular vegetal se encuentra formada por tres capas, la lámina media, la pared celular primaria y la secundaria, que son el resultado de la evolución de una serie de etapas de diferenciación celular (**Figura 9**).

- La primera que se origina es la lámina media, que actúa como tabique de separación entre dos células contiguas, común a ambas y es, por tanto, la parte más externa de la pared. Constituye una masa amorfa, compuesta principalmente por sustancias pécticas, concretamente sales cálcicas de pectinas.
- Tras la formación de la lámina media, se depositan a ambos lados de la misma carbohidratos, algunas glicoproteínas y compuestos fenólicos, originando la pared primaria, de estructura poco compleja, poco ordenada y dinámica. Esta capa se encuentra constituida principalmente por fibras de celulosa embebidas en una mezcla amorfa de polisacáridos y proteínas. La pared celular primaria controla el crecimiento celular y forma la base estructural del esqueleto de la planta.
- Por último, una vez finalizada la etapa de crecimiento celular, la pared puede seguir creciendo en grosor, dando lugar a la pared secundaria, únicamente presente en células diferenciadas con funciones específicas (traqueidas, vasos,

etc…) (Wilson, 1993). A su vez esta formada por tres capas que se distinguen ultraestructuralmente por la distinta orientación de las microfibrillas de celulosa (S_1, S_2 y S_3) (Harris, 1990). Generalmente, la aparición de la pared secundaria va acompañada de cambios en la composición química de la lámina media y de la pared primaria, tales como la lignificación, en esta fase la concentración de lignina es superior en estas dos capas (Jiménez, 1993; Heredia y col., 1993; Heredia y col., 1995). Se considera una pared suplementaria que realiza funciones mecánicas (Monties, 1980).

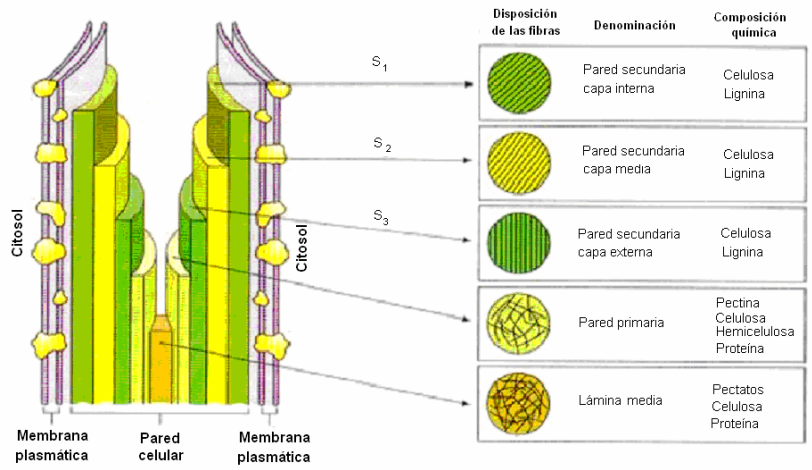


Figura 9: Estructura de la pared celular.

Las paredes primaria y secundaria presentan dos fases, una microfibrilar y otra amorfa (matriz), que no se da en la lámina media. La fase microfibrilar posee un alto grado de cristanilidad y tiene una composición química homogénea constituida principalmente por celulosa. Por lo contrario, la matriz es químicamente compleja.

a) Fase microfibrilar: celulosa

Esta fase está compuesta por microfibrillas largas y delgadas constituidas por moléculas de celulosa, las cuales se alinean paralelamente a su eje longitudinal. La celulosa es un polisacárido lineal de moléculas de glucosa (Glc) unidas por enlaces β(1→4), de alto peso molecular (0.5-1 millón de Daltons), con un grado de polimerización superior a 15.000 en paredes secundarias, e inferior en paredes primarias. Presenta una estructura estable y altamente resistente debido a los puentes de hidrógeno intra- e intermoleculares. La configuración más estable es en forma de silla con los hidroxilos en posición ecuatorial (**Figura 10**).

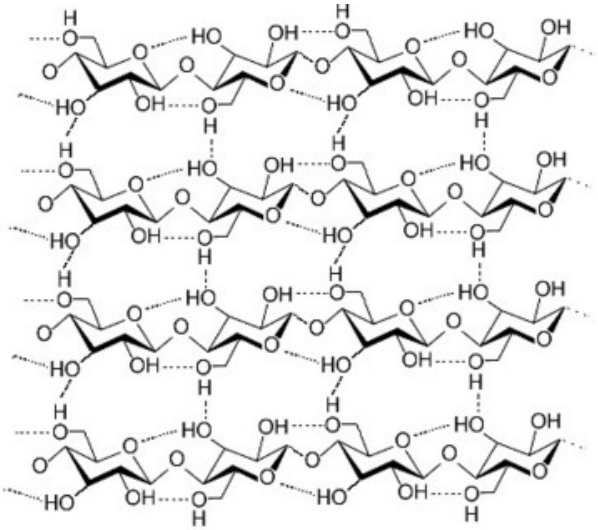


Figura 10: Estructura de la celulosa, puentes de hidrógeno intra e intermoleculares.

Las microfibrillas de celulosa están constituidas por un núcleo muy cristalino y cadenas que forman una zona paracristalina. Las zonas cristalinas están unidas en longitud por otras amorfas que corresponden a defectos de estructura, y diferentes microfibrillas están enlazadas entre sí mediante puentes de hidrógeno o por cadenas aisladas.

b) Fase amorfa o matriz.

Esta fase se caracteriza por estar compuesta por una gran variedad de polisacáridos, junto a proteínas y a compuestos fenólicos (**Tabla 1**). Todos estos compuestos están unidos entre sí y a las microfibrillas por fuerzas de distintos orígenes, y varían su composición según las diferentes partes de la pared celular, tipos de células, y estados del ciclo celular (Brett y Waldron, 1990).

Tabla 1: Cuadro resumen de las distintas pectinas, hemicelulosas, proteínas y fenoles que componen la fase amorfa de la pared celular.

		Unidades
Sustancias pécticas	Homogalacturonanas	α-1,4-galacturónico
	Ramnogalacturonanas I y II	α-1,4- galacturónico; 1,2-ramnosa; 1,3-ramnosa
	Arabinanas	α-1,5-arabinosa α-1,3-arabinosa
	Galactanas	β-1,4-galactosa
	Arabinogalactanas I	β-1,4-galactosa; α-1,5-arabinosa
Hemicelulosas	Xilanas	β-1,4-xilosa
	Glucomananas	β-1,4-glucosa; β-1,4-manosa
	Galactoglucomananas	β-1,4-manosa; α-1,6-galactosa; β-1,4-glucosa
	Mananas	β-1,4-manosa
	Galactomananas	α-1,6-galactosa; β-1,4-manosa
	Glucuronomananas	α-1,2-manosa; β-1,4-glucurónico
	Xiloglucanas	β-1,4-glucosa; α-1,6-xilosa; β-1,2-galactosa; α-1,2-fucosa
	Calosa (β(1→3)-glucanas)	β-1,3-glucosa
	β(1→3)-, β(1→4)-glucanas	β-1,3-glucosa; β-1,4-glucosa
	Arabinogalactanas II	β-1,3-galactosa; β-1,6-galactosa; 1,3-arabinosa; 1,6-arabinosa
Proteínas	Extensina	
	Arabinogalactanas-proteínas	
	Otros, incluyendo enzimas	
Fenoles	Lignina	
	Ácido ferúlico	
	Otros	

Los componentes de la matriz se detallan a continuación.

b.1) Polisacáridos pécticos. Son componentes de la pared celular primaria se caracterizan porque el principal componente es el ácido D-galacturónico. Constituyen una mezcla compleja de polisacáridos helicoidales. Los de alto grado de esterificación, tienen poca capacidad de interacción con otros componentes de la pared celular y por ello pueden extraerse con agua caliente. Los de medio o bajo grado de esterificación están estabilizados por enlaces iónicos con calcio, formando geles con estructura de “caja de huevos” y pueden solubilizarse en agentes quelantes. Otros pueden estar enlazados por enlaces covalentes a

hemicelulosas, celulosa o incluso proteínas y se separan sólo por tratamiento con álcali o con ácido diluido (Van Buren, 1979).

En base a su estructura (Aspinall, 1983), constituyen un grupo de polímeros asociados a residuos de ácido D-galacturónico, unidos por enlaces α(1→4). Además de ácido D-galacturónico, poseen importantes cantidades de ramnosa, arabinosa y galactosa. Generalmente la ramnosa forma parte de la cadena principal, mientras que la arabinosa y la galactosa se encuentran en las cadenas laterales (**Figura 11**).

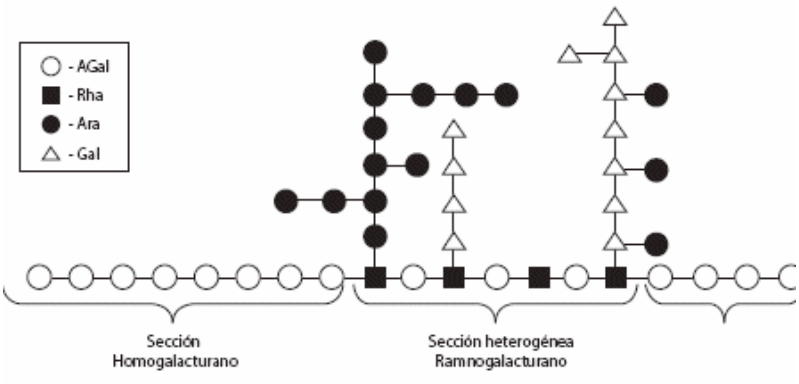


Figura 11: Estructura de la pectina.

b.2.) Hemicelulosas. Son polisacáridos no celulósicos, que se pueden extraer de la pared celular con soluciones acuosas de álcalis diluidas, después de la eliminación de la lignina. A diferencia de la celulosa están compuestas de diferentes azúcares formando cadenas más cortas y con ramificaciones. Los azúcares que la forman se pueden dividir en diferentes grupos, como las pentosas (xilosa y arabinosa), hexosas (glucosa, manosa y galactosa), ácidos hexurónicos (ácido glucurónico, metilglucurónico y galacturónico) y desoxihexosas (ramnosa y mucosa). La cadena principal de una hemicelulosa puede consistir en una sola unidad que se repite (homopolímero), o en dos o más unidades (heteropolímero). La mayoría de las hemicelulosas son heteropolisacáridos complejos que contienen entre dos y cuatro tipos de azúcares (Southgate, 1990). Se caracterizan porque se hidrolizan fácilmente en medio ácido, obteniéndose como principales constituyentes monoméricos D-glucosa, D-manosa, D-galactosa, D-xilosa y L-arabinosa y también pequeñas cantidades de D-glucurónico, 4-O-metil-D-glucurónico y D-galacturónico (Sjöström, 1982). Muchas hemicelulosas se encuentran unidas a las microfibrillas de celulosa por puentes de hidrógeno y también hay evidencias que sugieren uniones covalentes entre hemicelulosas y polisacáridos pécticos y entre éstas y lignina.

b.3.) Lignina y otros componentes fenólicos. La lignina es un polímero estructural que aparece en ciertos tipos de células diferenciadas una vez que la elongación celular ha finalizado. Está constituido por tres redes tridimensionales de unidades de fenilpropano unidas por enlaces C-O-C (éter) y C-C, siendo los primeros mayoritarios (Sjöström, 1982). Los

precursores de la lignina son los alcoholes aromáticos, cumarílico, coniferílico y sinapílico, que se originan a partir de la D-glucosa a través de reacciones enzimáticas complejas, y se unen para formar el polímero final.

La polimerización de la lignina **(Figura 12)** comienza en la lámina media, extendiéndose a la pared primaria y posteriormente a la secundaria, siempre que exista espacio disponible, de modo que tiende a rellenar todo el espacio libre que no está ocupado por macromoléculas, desplazando para ello al agua. El resultado es un entramado hidrofóbico muy fuerte que rodea a los otros componentes de la pared y los mantiene unidos.

La lignina determina las características de la fibra vegetal. Su presencia en la pared es la responsable de la dificultad de degradar la fibra, ya que forma una pantalla que dificulta la accesibilidad de agentes químicos y/o enzimáticos a los otros componentes de la fibra, existiendo una correlación entre el contenido de lignina en la pared celular y la digestibilidad de la misma (Jung y Deetz, 1993). Los componentes más digeribles de la pared son las sustancias pécticas, mientras que las hemicelulosas y celulosas que están protegidas por la barrera de lignina y posiblemente unidas a ellas no son digeribles (Wilson y col., 1989).

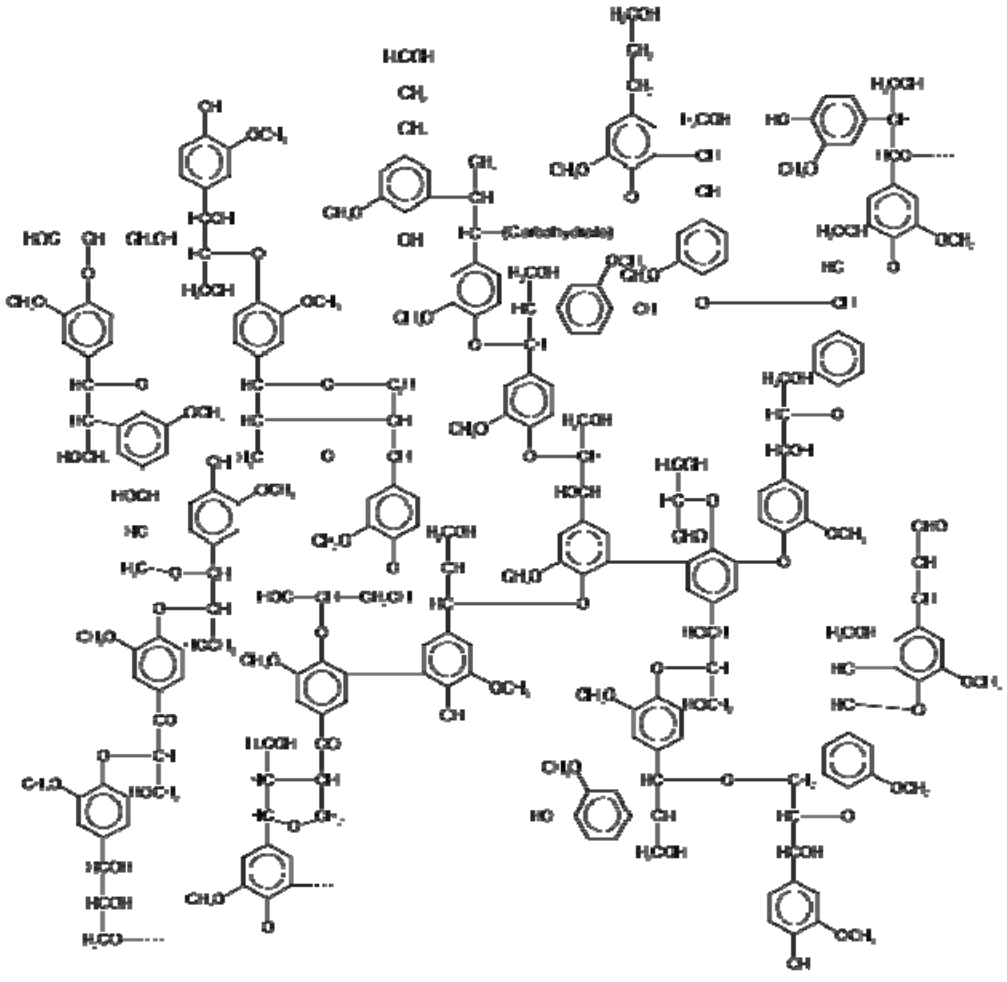


Figura 12: Estructura propuesta de la lignina.

Además de la lignina en la pared celular pueden encontrarse otros fenoles, principalmente el ácido ferúlico, que se halla esterificado con la arabinosa y la galactosa de los polisacáridos pécticos y tiene un papel importante en las uniones transversales de las mismos. También se encuentra el ácido cumárico que principalmente está esterificado y su concentración aumenta con la maduración (Iiyama y col., 1990).

b.4.) Proteínas. Se clasifican según sean estructurales o enzimas. Las proteínas estructurales están glicosiladas y enriquecidas en el aminoácido hidroxiprolina, conteniendo además alanina, serina y treonina (Showalter, 1993). Por otro lado las enzimas se encuentran en menor proporción que las estructurales, en este grupo se incluyen peroxidasas e hidrolasas.

Todos los polímeros descritos están enlazados entre sí por diferentes uniones, siendo éstas las que determinan una estructura que funciona como unidad. El conjunto es mantenido por distintos tipos de enlaces intermoleculares y de interacciones fisicoquímicas que confieren a la célula gran resistencia mecánica y hacen que la pared celular sea resistente a los agentes químicos y fisicoquímicos (Hatfield, 1993). En el seno de este conjunto la celulosa, polímero en parte cristalino y orientado en forma de microfibrillas, constituyen una trama que sirve de sostén a los otros compuestos poliméricos (**Figura 13**).

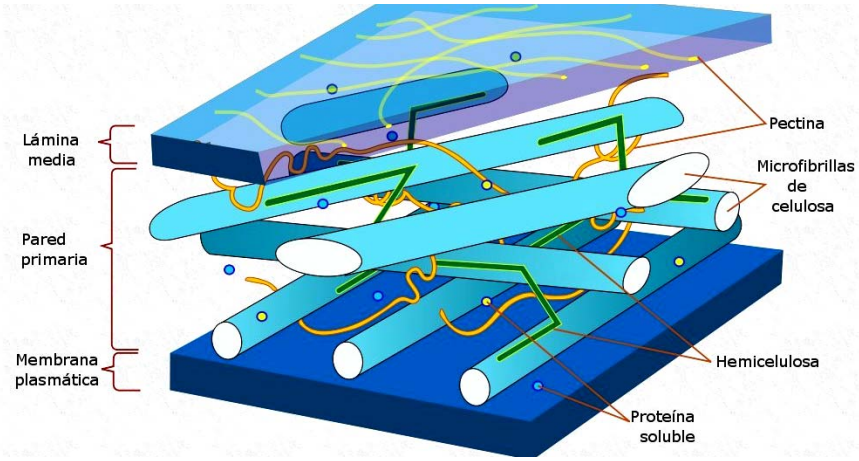


Figura 13: Estructura de la pared celular, disposición de los diferentes componentes.

3.5.2. Pared celular de la aceituna.

La pared celular vegetal consiste por tanto en una mezcla compleja de polisacáridos, los cuales se suelen denominar como fibra alimentaría. La fibra alimentaría del fruto de la oliva incluye pectinas (compuestas a su vez por tres polisacáridos pécticos: arabinanas,

homogalacturanos, y ramnogalacturanos), hemicelulosas (enriquecidas en xilanos, xiloglucanos, glucurunoxilanos y mananos), celulosa y lignina (Coimbra y col., 1994).

La pared celular de la pulpa del olivo contiene sobre un tercio de polisacáridos pécticos con un elevado grado de esterificación (>80%). Las pectinas se encuentran formadas por una fracción neutra compuesta por arabinosa principalmente, mientras que la fracción ácida se encuentra formada por homogalacturanos y ramnogalacturanos (Jiménez y col., 1994). El fruto de la oliva puede estar parcialmente lignificado, principalmente en las zonas cercanas al hueso, en esta zona el contenido de xilanos es elevado mientras que la concentración de pectinas desciende. Igualmente la zona del endocarpio se encuentra altamente lignificada, presentando elevadas concentraciones de celulosa y hemicelulosas (Coimbra y col., 1995). Las pectinas están formadas por una cadena principal de ácido galacturónico enlazados por uniones α(1→4) que presentan de forma intercalada residuos de ramnosa con enlaces α(1→2) que sirven de anclaje para cadenas laterales por el C-4, de residuos de arabinosas enlazados por uniones α(1→2).

Gil Serrano y col. (1986) detectan una hemicelulosa A que contiene xilosa unida por enlaces β(1→4) con residuos de ácido 4-O-metilglucurónico enlazados por los grupos O-2 de la cadena principal, la cual es aislada a partir de la pulpa de la oliva. Por otro lado Gil-Serrano y Tejero-Mateo (1988) aíslan a partir de las aceitunas verdes pequeñas cantidades de xiloglucanos pertenecientes a una hemicelulosa B que tienen en su estructura un esqueleto celulósico de unidades de D-glucosa unidas entre sí por enlaces β(1→4) con ramificaciones 1-6 de unidades de D-xilosa, las cuales a su vez pueden presentar sustituciones con unidades de β(1→2) de D-galactosa. Jiménez y col. (1994) estudian los polisacáridos hemicelulósicos en aceitunas de las variedades “Manzanilla” y “Hojiblanca” detectando la presencia de xilanos, arabinoxilanos (>400 kDa) y xiloglucanos (260 kDa). Coimbra y col. (1994) describen la presencia de xiloglucanos asociados covalentemente con xilanos. En la aceituna los xiloglucanos han sido también encontrados conectados con glucomananos y glicoproteínas ricas en hidroxiprolina, otras uniones encontradas incluyen xilanos-lignina, xilanos-ramnogalacturanos, ramnogalacturanos-xiloglucanos-celulosa y celulosa-xilanos-glucorono-arabinoxilanos (Jiménez y col., 1994). Vierhuis y col. (2001) establecen que los xiloglucanos del fruto del olivo están compuestos principalmente por dos tipos de oligosacáridos. El primero un octa-sacárido constituidos por una cadena de cuatro monómeros de glucosa unidos por enlace β(1→4), donde tres de los monómeros de glucosa están sustituidos por el O-6 por residuos de xilosa y una de las xilosas esta sustituida por el O-2 por un residuo de arabinosa. El segundo descrito es un hepta-sacárido donde dos de los tres residuos de xilosa están sustituidos por el O-2 por arabinosa y galactosa respectivamente. En ambos oligosacáridos algunos de los residuos de arabinosa están sustitutitos por uno o dos grupos acetilos.

3.5.3. Propiedades de los oligosacáridos de la pared celular vegetal.

Los polisacáridos de las pared celular vegetal (celulosa, hemicelulosas y pectinas) son junto con la lignina los principales constituyentes de la denominada fibra alimentaría. Se caracterizan porque no son digeridos por las enzimas humanas, pero sí son parcialmente fermentados por las bacterias del colon pudiendo actuar como prebióticos (Mitchell y Tiihonen, 2003). Existen estudios epidemiológicos que deducen una relación entre el consumo de fibra y la reducción de enfermedades cardiovasculares (Bazzano y col., 2003), cáncer, enfermedades coronarias, accidentes cerebrocardiovasculares, hipertensión, diabetes y obesidad (Willem y col., 2010). Los efectos que puede ejercer la fibra sobre la respuesta fisiológica de un organismo varían en función de la estructura y los componentes de la fibra.

Hay dos tipos de fibra, la soluble y la insoluble. La fibra soluble retiene el agua y se vuelve gel durante la digestión, retarda la digestión y la absorción de nutrientes desde el estómago y el intestino, dentro de este grupo se encuentran las pectinas. Entre tanto, la fibra insoluble parece acelerar el paso de los alimentos a través del estómago y los intestinos y le agrega volumen y consistencia a las heces y esta compuesta por celulosa, hemicelulosas y lignina.

La Organización Mundial de la Salud (OMS) fija un consumo mínimo de fibra de 30 g/persona/día, de la cual el 30% debe ser fibra soluble. La necesidad de consumir unos niveles mínimos radica en que un déficit del consumo de fibra conlleva a la aparición de enfermedades crónicas y funcionales como el estreñimiento, enfermedad inflamatoria intestinal, apendicitis, síndrome del colon irritable y cáncer de colon (Guarner, 2003; Blaut, 2002).

La fibra alimentaría en el estómago, aumenta la viscosidad y retrasa el vaciado gástrico, incrementando la sensación de saciedad (Burton-Freeman, 2000), debido a que entre las propiedades más importantes de esta fibra se encuentra la capacidad de retención de agua (Cleary y Prosky, 2001).

En el intestino delgado, la fibra soluble, hace que disminuya la velocidad de paso de nutrientes como la glucosa y los lípidos a la sangre, ya que ésta es capaz de generar sistemas viscosos (Briceño y col., 2002). La fibra soluble influye en la absorción de lípidos por el organismo, limitando la absorción de grasas debido a su efecto inhibidor de la actividad de la lipasa pancreática (Schneeman, 1987). También disminuye el colesterol total y la lipoproteína de baja densidad en el plasma sanguíneo, que se produce como consecuencia de una menor absorción de lípidos y colesterol debido a la dilución y excreción de ácidos biliares (Gallaher y col., 1992). Eastwood y Halminton (1968) demostraron que las pectinas poseen capacidad de captación de los ácidos biliares, causando su excreción en las heces. El agotamiento continuo de la bilis de esta manera se cree que reduce los niveles de colesterol en suero y por lo tanto reduce el riesgo de enfermedad cardiovascular. Esta unión a las sales biliares también produce

una reducción del nivel excesivo de bilis en el colon, lo que disminuye los riesgos de desarrollar cáncer de intestino. La continua eliminación de ácidos biliares primarios, sintetizados en el hígado, dificulta la formación y concentración de ácidos biliares secundarios que se forman en el colón como metabolitos bacterianos de los ácidos primarios y cuya elevada concentración está relacionada con el riesgo de padecer cáncer (Camire y Dougherty, 2003). Afecta a la disponibilidad de carbohidratos en el tracto intestinal, observándose que la absorción de carbohidratos en individuos diabéticos alimentados con dietas ricas en fibras, especialmente fibras viscosas, provocan un descenso de los niveles de glucosa en sangre. Así mismo existen estudios que relacionan el efecto de la fibra alimentaría con la prevención de diabetes (Kaline y col., 2007).

Finalmente una vez en el colon, la fibra soluble es atacada por la flora bacteriana sufriendo un proceso de fermentación que estimula la proliferación de las células de la mucosa colónica actuando como prebióticos. Dichos efectos evitan la aparición de estreñimiento, previniendo por tanto en gran medida la aparición de enfermedades intestinales y la aparición de cáncer de colon (Davis y Milner, 2009; Laparra y Sanz, 2010).

Los oligosacáridos prebióticos se caracterizan por poseer un grado de polimerización de 3 a 10 unidades de azúcar. Los compuestos prebióticos presentan propiedades fisiológicas beneficiosas para la salud, y por ello se usan como ingredientes en alimentos funcionales. Son ingredientes alimentarios no digeribles que actúan estimulando el crecimiento y/o actividad de una o varias especies de bacterias residentes en el colon, como las bifidobacterias endógenas.

En la actualidad los estudios sobre oligosacáridos con actividad prebiótica son muy numerosos. Muchos de dichos estudios se encuentran centrados en los fructanos, este término es general para describir a todos los oligos o polisacáridos de origen vegetal en los cuales hay uniones fructosil-fructosa. La inulina es un fructano que consiste en mezcla de oligosacáridos formados por uniones β(1→2) fructosil-fructosa, (su grado de polimerización (DP) oscila entre 3 y 60, con un valor promedio aproximado de 10) ésta se encuentra en la raíz de la achicoria, puerro, ajo, banana, cebada, trigo, miel, cebolla espárrago y alcaucil.

Las pectinas son hidrocoloides naturales que se encuentran en las plantas superiores y que han sido ampliamente utilizadas como agentes gelificantes, estabilizantes, emulsionantes y en la industria alimentaría y han aumentado recientemente su interés para aplicaciones tales como la ingeniería de tejidos y el tratamiento del cáncer (Munarin y col., 2012; Morris y col., 2013). Las pectinas actúan como agentes prebióticos, antiinflamatorios, antidiarreicos, y también son beneficiosos para el control de la diabetes y en la prevención de varias enfermedades (estreñimiento, obesidad, cáncer) (Rodríguez y col., 2006; Markov y col., 2011). Olano-Martín y col. (2003) han demostrado que los oligosacáridos pécticos inducen apoptosis en células humanas de colón con adencarcionoma.

Recientemente se ha empezado ha emplear el término “pectinas modificadas” (PMs) para referirse a pectinas de diferentes composición y características comunes. Son fragmentos que se caracterizan por poseen un bajo peso molecular que facilitan su absorción y permiten su unión y consecuente bloqueo a la proteína pro-metastásica galectina-3 (Gal-3) (**Figura 14**). Gal-3 es una proteína que sufre alteraciones en los procesos de cáncer, inflamación, fibrosis, enfermedades cardiacas e infartos. La molécula de Gal-3 normalmente está presente en pequeñas cantidades en todo el cuerpo. Sin embargo, el exceso de Gal-3 en la circulación y su expresión en la superficie de las células cancerosas es un importante mecanismo de unión de las células de cáncer (metástasis), el crecimiento de los tumores, y la atracción y la formación de vasos sanguíneos para alimentar el tumor (angiogénesis) **(Figura 14)**.

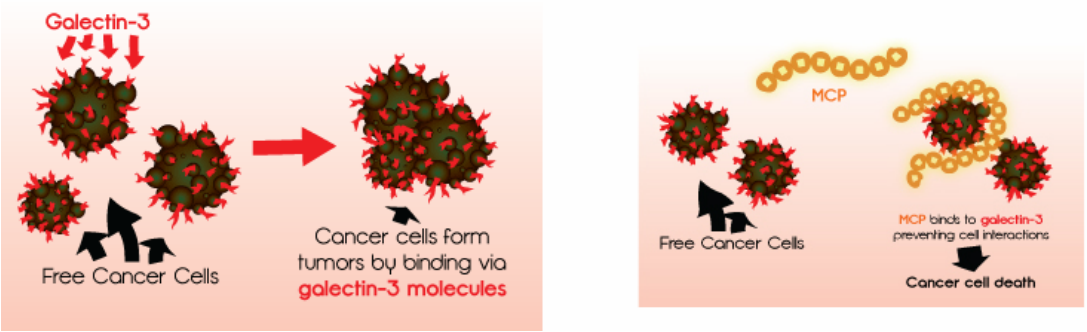


Figura 14: Mecanismo de actuación de las pectinas modificadas sobre la proteína pro-metastásica galectina-3.

Estos efectos inhibidores han suscitado la posibilidad de considerar a las PMs como una tentativa potencialmente segura y no tóxica, para prevenir y reducir la carcinogénesis (Maxwell y col., 2012). Actualmente, se están llevando a cabo numerosas investigaciones cuyos resultados evidencian claramente el retraso de la metástasis de células cancerígenas (Gorelik y col., 2001), la disminución en el tamaño de tumores (Grous y col., 2006), la inducción de la apoptosis de células cancerosas (Jackson y col., 2007), así como efectos positivos en el tratamiento y la prevención de enfermedades inflamatorias y relacionadas con la fibrosis (Forsman y col., 2011; Okaruma y col., 2011). Las características específicas responsables de la unión a Gal-3 así como los mecanismos fundamentales que conducen a la actividad anti-metastática, están aún pendientes de resolverse, sin embargo, a pesar de estas complicaciones, sí parece que son importantes las cadenas de azúcares neutros principalmente arabinosa y galactosa unidas al poligalacturano (Maxwell y col., 2012).

Desde hace aproximadamente una década de ha comenzado a introducir un nuevo concepto dentro de la fibra, “fibra dietética antioxidante”, la cual podría ser considerada como un potente ingrediente funcional (Saura-Calixto, 1998). Al igual que en la fibra dietética, es sabido que los compuestos fenólicos son potentes antioxidantes que previenen el desarrollo de enfermedades cardiovasculares y cáncer (Mainai y col., 2009). La unión de estos dos componentes podría mejorar la calidad de la fibra produciendo un importante efecto sinérgico.

Vitaminas, polifenoles y carotenoides son los principales antioxidantes presentes en la dieta, los polifenoles son un complejo grupo de sustancias con un amplio rango de pesos moleculares que se encuentran en las plantas bien en forma libre o enlazados a los constituyentes de la pared celular (fibra dietética). Se ha comprobado que la presencia de polifenoles y carotenoides asociados a la fibra dietética es una característica común a todos los alimentos vegetales (Saura-Calixto y Díaz-Rubio, 2007). Estos compuestos antioxidantes unidos a la fibra no se disuelven ni absorben en el intestino delgado y llegan inalterados al intestino grueso donde se degradan, actuando la fibra como transportadora de antioxidantes en el tracto intestinal, mientras que las vitaminas son absorbidas en el intestino delgado. De hecho, son muchos los estudios que actualmente han asociado la fibra dietética antioxidante con la prevención de cáncer de colon (Arranz y col., 2010; Saura-Calixto, 2011; López-Oliva y col., 2010).

3.5.4. Recuperación de polisacáridos y oligosacáridos.

Debido al crecimiento de la industria de los alimentos funcionales ha aumentado el interés en los oligosacáridos y polisacáridos procedentes de paredes celulares. Los oligosacáridos no digeribles pueden formar parte de alimentos tales como la remolacha, la fécula, etc. y pueden ser obtenidos mediante extracción usando procesos industriales a partir de estas fuentes naturales, mediante hidrólisis controlada de los polisacáridos (almidón, inulina o xilanos) o también se pueden obtener mediante síntesis química y enzimática a partir de azúcares simples (tales como la sucrosa o lactosa) mediante reacciones de transglicosilación (Mussato y Mancilha, 2007).

Olano-Martín y col. (2001) producen oligosacáridos pécticos en un reactor continuo mediante hidrólisis enzimática controlada de pectinas cítricas con alto (66%) y bajo (8%) grado de metilación. Cardoso y col. (2003b; 2003c) extraen material péctico desde un residuo insoluble procedente del alperujo usando ácido nítrico como extractante. El material recuperado es purificado empleando agentes de quelación y precipitado con etanol, recuperando pectinas con un grado de metilación del 42% con capacidad de gelificar, presentando propiedades reológicas comparables a las observadas para pectinas de limón comerciales con bajo grado de metilación. Galanakis y col. (2010) usan ácido cítrico y etanol para obtener pectinas a partir de alpechín concentrado térmicamente.

Por otro lado, los xilooligosacáridos son producidos a escala industrial a partir de materiales lignocelulósicos, tales como la paja, el maíz, etc., mediante el uso de tres técnicas diferentes. La primera se caracteriza por un fraccionamiento químico para asilar xilanos (o solubilizarlos). La segunda consiste en realizar tratamientos enzimáticos de los polisacáridos hemicelulósicos (xilanos) a xilooligosacáridos. Y la tercera consta de una degradación

hidrolítica mediante procesos hidrotérmicos (o autohidrólisis) con vapor, agua o soluciones diluidas de ácidos minerales (Garrote y col., 2003). Coimbra y col. (1994) obtienen glucuroxilanos y fracciones ácidas de xilanos mediante el tratamiento con álcalis de la semilla de la aceituna, y realizan una posterior hidrólisis parcial ácida para mejorar la producción de azúcares fermentables y oligosacáridos.

Lama y col. (2012) comprueban que el tratamiento térmico con vapor produce la solubilización de los polisacáridos de la pared celular, que son hidrolizados, permitiendo la obtención de oligosacáridos neutros y ácidos con un DP entre 2 y 10 con posibles propiedades bioactivas. Entre los oligosacáridos aislados se identifican xilooligosacáridos neutros (Xil_{10}), xilooligosacáridos sustituido con un grupo 4-*O*-metilglucurónico, Xil_4GlcAMeGlcA, Xil_4MeGlcA y Xil_6MeGlcA, y xiloglucanos no acetilados de fórmula general $(Hexosa)_6(Pentosa)_3$.

Los oligosacáridos aislados mediante estas técnicas no son productos puros, sino mezclas de oligosacáridos con diferentes grados de polimerización, el polisacárido original, azúcares libres y otras impurezas de naturaleza no glucosídica. Para que los oligosacáridos puedan ser usados para aplicaciones alimentarías se necesita que posean una alta pureza, por lo que se requieren múltiples pasos de purificación. Para este fin, diferentes estrategias han sido empleadas, así algunos autores llevan a cabo precipitaciones con soluciones alcohólicas, acetona o 2-propanol (Ku y col., 2003; Moerman y col. 2004), esto les permite fraccionar los componentes de alto y bajo peso molecular. Otro autores emplean técnicas de adsorción para eliminar los monosacáridos presentes (Sanz y col., 2005), o resinas de intercambio iónico que favorecen la desalinización y eliminación de compuestos indeseables (Vázquez y col., 2005). También resultan ser efectivas para la purificación y concentración de oligosacáridos de cualquier naturaleza las tecnologías de membranas, tales como la ultrafiltración y la nanofiltración (Pinelo y col., 2009; Yang y col., 2003; Swennen y col., 2005).

Las pectinas son productos químicos que se obtienen de materias primas vegetales, principalmente frutas, se usan en varias industrias, especialmente la de alimentos, para darle propiedades de gel a los productos y como estabilizantes (Devia-Pineda, 2003). Las pectinas comerciales actualmente se obtienen a partir de limón o manzana, aunque en los últimos años se ha visto incrementado el interés por obtenerlas a partir de otras fuentes vegetales, sobre todo a partir de subproductos de la industria alimentaría. Existen numerosos procesos patentados para obtener pectinas, y en cada uno de ellos se obtienen productos de diferente calidad en función del método de obtención empleado. Glahn (2001) convierte la materia prima en una sal cálcica de la pectina en un medio líquido, para luego secarla, para así obtener un pectinato, que cuando se pone en agua la absorbe para formar partículas estables de un diámetro medio mayor de 100 micrómetros. Ehrlich (1997) hidroliza y extrae la pectina del tejido vegetal, cáscara de naranja, sin adicionar un ácido. De esta forma tienen lugar la solubilización de pectinas con alto contenido de metoxilos y luego las recupera por concentración y secado. Cerda (1996) obtiene un producto enriquecido en pectina, en forma

granular, para usarlo en alimentos y bebidas, poniendo la materia prima en contacto con una proteína comestible, soluble en agua para solubilizar la pectina y luego precipitarla con ayuda de un disolvente. En este caso se puede mejorar el rendimiento agregando un ácido. Fishman, (2000) obtiene pectinas de muy buena calidad a partir de material vegetal aplicándole presión y con calentamiento por microondas. Las pectinas obtenidas se caracterizan por un alto peso molecular y una buena viscosidad, cuando se comparan con las pectinas obtenidas con técnicas convencionales de calentamiento.

Las pectinas modificadas son pectinas de diferentes composición y características comunes, que se caracterizan por poseen un bajo peso molecular lo que facilitan su absorción y permiten su unión y consecuente bloqueo a la proteína pro-metastásica galectina-3 (Gal-3). En la actualidad este tipo de pectinas se están obteniendo a partir de cítricos mediante tratamientos químicos, con ácidos o álcalis a altas temperaturas, enzimas e incluso con ultrasonidos (Zhang y col., 2013), que producen una despolimerización efectiva y/o β-eliminación de las cadenas de homogalacturanos. No se tienen completamente definidas las moléculas de pectina que son efectivas, aunque sí parece que tienen un papel importante las cadenas de azúcares neutros, arabinosa y galactosa principalmente, unidos al poligalacturano.

4. Bibliografía

4. BIBLIOGRAFÍA

A

Alba, J., Hidalgo, F., Ruiz, M.A., Martínez, F., Moyano, M.J., Cert, A., Pérez, M.C., Ruiz, M.V. 1996. Características de los aceites de oliva de primera y segunda centrifugación. *Grasas y aceites, 47*, 163-181.

Alburquerque, J. A., Gonzálvez, J., Tortosa, G., Baddi, G.A., Cegarra, J. 2009. Evaluation of ‘‘alperujo’’ composting based on organic matter degradation, humification and compost quality. *Biodegradation*, *20*, 257–270.

Allouche, N., Fki, I., Sayadi, S. 2004. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. *Journal of Agricultural and Food Chemistry*, *52*, 267-273.

Antolín, G., Lara, A., Peran, J.R. 1997. Depuración y aprovechamiento integral de las aguas residuales de la industria del aceite de oliva (alpechín). *Información Tecnológica*, *8*, 95-102.

Arienzo, M., & Capasso, R. 2000. Analysis of metal cations and inorganic anions in olive oil mill waste waters by atomic absorption spectroscopy and ion chromatography. Detection of metals bound mainly to the organic polymeric fraction. *Journal Food Chemistry*, *48*, 1405-1410.

Arranz, S., Silván, J.M., Saura-Calixto, F. 2010. Non extractable polyphenols, usually ignored are the mayor part of dietary polyphenols: a study on the Spanish diet. *Molecular Nutrition and Food Research*, *81*, 230-242.

Artacho, A. 2002. Procedimiento para la desecación y tratamiento de orujos de aceituna. Patente de invención, ES 2 156 716 B1

Artajo, L.S., Romero, M.P., Suárez, M., Motilva, M.J. 2007. Partition of phenolic compounds during the virgin olive oil industrial extraction process. *European Food Research and Technogy, 225*, 617–625.

Aruoma, O.I. 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research*, *523-524*, 9-20.

Asli, A.E., & Qatibi, A.-I. 2009. Ethanol Production from Olive Cake Biomass Substrate. *Biotechnology and Bioprocess Engineering*, *14*, 118-122.

Aspinall, G.O. 1983. Classification of polysaccharides. En: The Polysaccharides 2. Aspinall, G.O. *Ed. Academic Press.* New York. 1-9.

B

Baddi, G.A., Alburquerque, J.A., Tortosa, G., Winterton, P., Hafidi, M., Cegarra, J. 2013. Characterization of Potential Liquid Organic Fertilizers Obtained by Alkaline Extraction from Two-Phase Olive-Mill Waste Composts. *Environmental Progress & Sustainable Energy*. (Vol.32, No.4) DOI 10.1002/ep.

Baddi, G.A., Cegarra, J., Merlina, G., Revel, J.C., Hafidi, M. 2009. Qualitative and quantitative evolution of polyphenolic compounds during composting of an olive-mill waste–wheat straw mixture. *Journal of Hazardous Materials*, *165*, 1119–1123.

Badimón, L., Vilahur, G., Padró, T. 2009. Lipoproteínas, plaquetas y aterotrombosis. *Revista Española de Cardiología*, *62*, (10), 1161-1178.

Bakhouche, A., Lozano-Sánchez, J., Beltrán-Debón, R., Joven, J., Segura-Carretero, A., Fernández-Gutiérrez, A. 2013. Phenolic characterization and geographical classification of commercial Arbequina extra-virgin olive oils produced in southern Catalonia. *Food Research International, 50*, 401–408.

Ballesteros, I., Oliva, J.M., Negro, J.M., Manzanares, P., Ballesteros, M. 2002. Ethanol production from olive oil extraction residue pretreated with hot water. *Applied Biochemistry and Biotechnology*, *98-100*, 717-732.

Ballesteros, I., Oliva, J.M. Saez, F., Ballesteros, M. 2001. Ethanol production from lignocellulosic byproducts of olive oil extraction. *Applied Biochemistry and Biotechnology. 91-93*, 237-252.

Barranco, D., Fernández-Escobar, R., Rallo, L. 2008. El cultivo del olivo, págs. 678-679. Ediciones Mundi-Prensa y Junta de Andalucía, Madrid.

Bazzano, L.A., He, J., Orden, L.G., Loria, C.M., Whelton, P. K. 2003. Dietary fibre intake and reduced risk of coronary heart disease in US men and women: The national health and nutrition examination survey I epidemiologic follow-up study. *Archives of Internal Medicine, 163*, 1897-1904.

Beverungen, C., Prous, R., Kempers, P., Buchwald-Werner, S. 2005. Process for obtaining hydroxytyrosol from olive leaves extracts. *European Patent Application*, EP 1 582 512 A1.

Bianchi, G. 2003. Lipids and phenols in table olives. *European Journal of Lipid Science and Technology, 105*, 229–242.

Bianco, A., Coccioli, F., Guiso, M., Marra, C. 2001. The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans. *Food Chemistry*, *77*, 405-411.

Blaut, M. 2002. Relationship of prebiotics and food to intestinal microflora. *European Journal of Nutrition*, *41*, 11-16.

Bogani, P., Galli, C., Villa, M., Visioli, F. 2007. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis*, *190*, 181–186.

Brenes, M., & de Castro, A. 2003. Obtención de sustancias antioxidantes a partir de soluciones del proceso de elaboración de aceitunas de mesa. *Oficina Española de Patentes y Marcas*, ES 2 186 467 A1.

Brenes, M., García, A., de los Santos, B., Medina, E., Romero, C., de Castro, A., Romero, F. 2011. Olive glutaraldehyde-like compounds against plant pathogenic bacteria and fungi. *Food Chemistry*, *125,* 1262–1266.

Brenes, M., Hidalgo, J.H., García, A., Rios, J.J., García, P., Zamora, R., Garrido, A. 2000. Pinoresinol and 1-Acetoxypinoresinol, two new phenolic compounds identified in olive oil. *Journal of the American Oil Chemist's Society*, *77*, 715-720.

Brett, C., & Waldron, K. 1990. Physiology and biochemistry of plant cell wall. Ed. Black, M. y Chapman, J. Unwin Hyman Boston.

Briceño, R., Gómez, M., Dadan, M. 2002. Fibra dietaria. Temas Pediátricos. *Nestlé Nutrition*, *19* (1), 5-24.

Burton-Freeman, B. 2000. Dietary fiber and energy regulation. *Journal of Nutrition*, *130*, 272-275.

C

Camire, M.E., & Dougherty, M.P. 2003. Raisin dietary fiber composition and in vitro bile acid bindig. *Journal of Agricultural and Food Chemistry*, *51*, 834-837.

Canet, M. 2007. Procedimiento para la industrialización de subproductos de almazaras y producto obtenido. Solicitud de patente ES 2 277 490 A1.

Capasso, R., Cristinzio, G., Evidente, A., Scognamiglio, F. 1992. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry*, *12*, 4125-4128.

Capasso, R., De Martino, A., Arienzo, M. 2002a. Recovery and characterization of the metal polymeric organic fraction (Polymerin) from olive oil mill wastewaters. *Journal of Agricultural and Food Chemistry*, *50*, 2846-2855.

Capasso, R., De Martino, A., Cristinzio, G. 2002b. Production, Characterization, and effects on tomato of humic acid-like polymerin metal derivatives from olive oil mill waste waters. *Journal of Agricultural and Food Chemistry*, *50*, 4018-4024.

Capasso, R., Evidente, A., Avolio, S., Solla, F. 1999. A highly convenient synthesis of hidroxytyrosol and its recovery from agricultural waste waters. *Journal of Agricultural and Food Chemistry*, *47*, 1745-1748.

Capasso, R., Pigna, M., De Martino, A., Pucci, M., Sannino, F., Violante, A. 2004. Potential remediation of waters contaminated with Cr(III), Cu, and Zn by sorption on the organic polymeric fraction of olive mill wastewaters (polymerin) and its derivates. *Environmental Science & Technology*, *38*, 5170-5176.

Cara, C., Ruiz, E., Ballesteros, M., Manzanares, P., Negro, M.J., Castro, E. 2008. Production of fuel ethanol from steam-explosion pretreated olive tree pruning. *Fuel, 87*, 692–700.

Cardoso, S. M., Coimbra, M.A., Lopes da Silva, J.A. 2003b. Calcium-mediated gelation o fan olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Polymer*s, *52*, 125-133.

Cardoso, S.M., Coimbra, M.A., Lopes da Silva, J.A. 2003c**.** Temperature dependence of the formation and meeting of pectin-Ca^{2+} networks: a rheological study. *Food Hydrocolloids*, 17, 801-807.

Cardoso, S.M., Silva, A.M.S., Coimbra, M.A. 2003a. Structural characterisation of the olive pomace pectic polysaccharide arabinan side chains. *Carbohydrate Research*. *337*, 917–924.

Castañer, O., Fitó, M., López-Sabater, M.C., Poulsen, H.E., Nyyssönen, K., Schröder, H., Salonen, J.T., De la Torre-Carbot, K., Zunft, H.-F., De la Torre, R., Bäumler, H., Gaddi, A.V., Saez, G.T., Tomás, M., Covas, M.I. 2011. The effect of olive oil polyphenols on antibodies against oxidized LDL. A randomized clinical trial. *Clinical Nutrition*, *30*, 490-493.

Castro, E., Conde, E., Moure, A., Falqué, E., Cara, C., Ruiz, E., Domínguez, H. 2008. Antioxidant activity of liquors from steam explosion of Olea europea wood. *Wood Science & Technology, 42*, 579–592.

Castro, F.B., Hotten, P.M. Orskov, E.R. 1994. Inhibition of rumen microbes by compounds formed in the steam treatments of wheat straw. *Bioresource Technology*, *50*, 25-30.

Cayuela, M.L., Mondini, C. Sánchez-Monedero, M.A., Roig, A. 2008. Chemical properties and hydrolytic enzyme activities for the characterisation of two-phase olive mill wastes composting. *Bioresource Technology, 99*, 4255–4262.

Cayuela, M.L., Sánchez-Monedero, M.A., Roig, A. 2010. Two-phase olive mill waste composting: enhancement of the composting rate and compost quality by grape stalks addition. *Biodegradation*, *21*, 465–473.

Cegarra, J., Amor, J.B., Gonzálvez, J., Bernal, M.P., Roig, A. 2000. Characteristics of a new solid olive-mill by-product ("alperujo") and its suitability for composting. *Proceedings of the International Composting Symposium*, *1*, 124-140.

Cerda, J.J., Burgin, C.W. (1996). Preparation and use of a protein-enriched pectin composition. U.S. Patent 5,514,666.

Cicerale, S., Lucas, L., Keast, R. 2010. Biological Activities of Phenolic Compounds Present in Virgin Olive Oil. *International Journal of Molecular Science, 11*, 458-479; doi:10.3390/ijms11020458.

Cleary, B.V., & Prosky, L. 2001. Advanced dietary fibre technology. *Ed Blackwell Science. Great Britany*. 168-185.

Coimbra, M.A., Cardoso, S.M. Lopes-da-Silva, J.A. 2010. Olive Pomace, a Source for Valuable Arabinan-Rich Pectic Polysaccharides. *Topics in Current Chemistry.* DOI: 10.1007/128_2010_60.

Coimbra, M.A., Waldron, K.W., Selvendran, R.R. 1994. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europea L*.) *Carbohydrate Research*, *252*, 245-262.

Coimbra, M.A., Waldron, K.W., Selvendran, R.R. 1995. Isolation and characterisation of cell wall polymers from olive pulp (*Olea europea*) seed hull. *Carbohydrate Polymers*, *27*, 285-294.

Conde, E., Cara, C., Moure, A., Ruiz, E., Castro, E., Domínguez, H. 2009. Antioxidant activity of the phenolic compounds released by hydrothermal treatments of olive tree pruning. *Food Chemistry*, *114*, 806-812.

Cooke, M.S., Evans, M.D., Dizdaroglu, M., Lunec, J. 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *Federation of American Society for Expeimental Biology, 17*, 1195–1214.

Corona, G., Deiana, M., Incani, A., Vauzour, D., Dessi, M.A., Spencer, J.P.E. 2009. Hydroxytyrosol inhibits the proliferation of human colon adeocarcinoma cells through inhibition of ERK1/2 and cycling D1. *Molecular Nutrition & Food Research*, *53*, 897–903.

Covas, M.I., Ruiz-Gutiérrez, V., de la Torre, R., Kafatos, A., Lamuela-Raventós, R.M., Osada, J., Owen, R.W., Visioli, F. 2006. Minor Components of Olive Oil: Evidence to Date of Health Benefits in Humans. *Nutrition Reviews,* Vol. *64*, No. 10, S20- S30.

Crea, R. 2002. Method of obtaining a hydroxytyrosol-rich composition from vegetation water. *United States Patent*, US 6,416,808 B1.

Cuomo, J., & Rabovskiy, A.B. 2002. Antioxidant composition extracted from wastewater from olive oil production. *Unites States Patent US* 6,361,803 B1.

D

Da Costa Lopes, A.M., Joao, K.G., Rubik, D.F., Bogel-Lukasik, E., Duarte, L.C., Andreaus, J., Bogel-Lukasik, R. 2013. Pre-treatment of lignocellulosic biomass using ionic liquids: Wheat straw fractionation. *Bioresource Technology 142*, 198–208.

Da Silva, S.P.M., da Costa Lopes, A.M., Roseiro, L.B., Bogel-Lukasik, R. 2013. Novel pre-treatment and fractionation method for lignocellulosic biomasa using ionic liquids. *RSC Advances, 3*, 16040-16050.

Davis, C.D., & Milner, J.A. 2009. Gastrointestinal microflora, food components and colon cancer prevention. *The Journal of Nutritional Biochemistry, 20*, 743-752.

De la Casa, J.A., Romero, I., Jiménez, J., Castro E. 2012. Fired clay masonry units production incorporating two-phase olive mill waste (alperujo). *Ceramics International, 38*, 5027–5037.

De La Cruz, J.P., Villalobos, M.A., Carmona, J.A., Martin-Romero, M., Smith-Agreda, J.M., de la Cuesta, F.S. 2000. Antithrombotic potential of olive oil administration in rabbits with elevated cholesterol. *Thrombosis Research*, *100*, 305–315.

Della Greca, M., Monaco, P., Pinto, G., Pollio, A., Previtera, L., Tamussi, F. 2001. Phytotoxicity of low-molecular- weight phenols from olive mill waste waters. *Letters in Applied Microbiology*, *42*, 405-411.

Denis, C.V., & Wagner, D.D. 2007. Platelet adhesion receptors and their ligands in mouse models of thrombosis. *Arteriosclerosis, Thrombosis and Vascular Biology*, *27*, 728- 739.

De Marco, E., Savarese, M., Paduano, A., Sacchi, R. 2007. Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. *Food Chemistry*, *104*, 858–867.

Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., Michaud, P. 2013. Olive mill wastes: Biochemical characterizations and valorisation strategies. *Process Biochemistry, 48*, 1532–1552.

Devia Pineda, J. E. 2003. Procesos para producer pectinas cítricas. Universidad EAFIT, *29*, 21-29.

Drira, R., & Sakamoto, K. 2013. Modulation of adipogenesis, lipolysis and glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes by hydroxytyrosol acetate: A comparative study. *Biochemical and Biophysical Research Communications, 440*, 576-581.

Duff, S.J.B., & Murray, W.D. 1996. Bioconversion of forest products industry waste cellulosics to fuel ethanol: A review. *Bioresource Technology, 55*, 1-33.

E

Eastwood, M.A., & Hamilton, D. 1968. Studies of the adsorption of bile salts to non-absorbed components of diet. *Biochemical et Biophysica Acta*, *152*, 165-173.

Ehrlich, R. M. (1997). Methods for making pectin and pectocellulosic products. U. S. Patent 5,656,734.

El-Abbassi, A., Kiai, H., Hafidi, A. 2012. Phenolic profile and antioxidant activities of olive mill wastewater. *Food Chemistry*, *132* (1), 406-412.

Enache, T.A., Amine, A., Brett, C.M.A., Oliveira-Brett, A.M. 2013. Virgin olive oil ortho-phenols-electroanalytical quantification. *Talanta*, *105*, 179–186.

Estruch, R., Ros, E., Salas-Salvado, J., Covas, M.I., Corella, D., Aros, F., Gomez-Gracia, E., Ruiz-Gutierrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R.M., Serra-Majem, L., Pinto, X., Basora, J., Munoz, M.A., Sorli, J.V., Martinez, J.A., & Martinez-Gonzalez, M.A. 2013. Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. *New England Journal of Medicine, 368*, 1279-1290.

F

Fabiani, R., De Bartolomeo, A., Rosignoli, P., Servili, M., Selvaggini, R., Montedoro, G.F., Di Saverio, C., Morozzi, G. 2006. Virgin olive oil phenols inhibit proliferation of human promyelocytic leukemia cells (HL60) by inducing apoptosis and differentiation. *Journal of Nutrition*, *136*, 614–619.

Fabiani, R., & Morozzi, G., 2010. Anticarciogenic properties of olive oil phenols: effects on proliferation, apoptosis and differentiation. *Olive and Olive Oil Health and Disease Prevention*, *105*, 981-988.

Fabiani, R., Rosignoli, P., De Bartolomeo, A., Fuccelli, R., Servili, M., Montedoro, G.F., Morozzi, G. 2008. Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. *Journal of Nutrition, 138,* 1411–1416.

Faye, B., Konuspayeva, G., Narmuratova, M., Serikbaeva, A., Musaad, A.M., Mehri, H. 2013. Effect of crude olive cake supplementation on camel milk production and fatty acid composition. Diary Science & Technology, *93*, 225-239.

Felizón, B., Fernández-Bolaños, J., Heredia, A., Guillén, R. 2000. Steam-explosion pretreatment of olive cake. *Journal of the American Oil Chemist's Society*, *77*, 15-22.

Fengel, D., & Wegener, G. 1984. Wood: Chemistry, Ultrastructure, Reactions. *De Gruyter*, Berlin.

Fernández-Bolaños, J., Felizón, B., Brenes, M., Guillén, R., Heredia A. 1998. Hidroxitirosol and Tyrosol as the Main Compounds Found in the Phenolic Fraction of Steam-Exploded Olive Stones. *Journal of the American Oil Chemists' Society*, *75,* 1643-1649.

Fernández-Bolaños, J., Felizón, B., Heredia, A., Guillén, R., Jiménez, A. 1999. Characterization of the lignin obtained by alkaline delignification and of the cellulose residue from steam-exploded olive atones. *Bioresource Technology*, *68*, 121-132.

Fernández-Bolaños, J., Felizón B., Heredia, A., Rodríguez, R., Guillén, R., Jiménez, A. 2001. Steam-explosion of olive stones: hemicellulose solubilization and enhancement of enzymatic hydrolysis of cellulose. *Bioresource Technology*, *79*, 53-61.

Fernández-Bolaños, J., Guillén, R., Heredia, A., Jiménez, A., Sánchez, C. 1990. El hueso de la aceituna como fuente de material lignocelulósico. *Alimentación, Equipos y Tecnología*, 109-115.

Fernández-Bolaños, J., Rodríguez, G., Lama-Muñoz, A., Rubio-Senent, F., Fernández-Bolaños, J.M.G., Castilla-Maya, I., López-López, O., Castro-Marset, A. 2013. Procedimiento para la obtención de extracto de hidroxitirosol, extracto mezcla de hidroxitirosol y 3,4-dihidroxifenilglicol, y extracto de acetato de hidroxitirosilo, a partir de subproductos del olivo, y su purificación. WO213/007850 A1.

Fernández-Bolaños, J., Rodríguez, G., Rodríguez, R., Guillén, R., Jiménez, A. 2006. Extraction of interesting organic compounds from olive oil waste. *Grasas y Aceites*, *57* (1), Enero-Marzo, 95-106, 2006, ISSN: 0017-3495.

Fernández-Bolaños, J., Rodríguez, G., Rodríguez, R., Heredia, A., Guillén, R., Jiménez, A. 2002. Production in large quantities of highly purified hidroxitirosol from liquid-solid waste of two-phase olive oil processing or “alperujo”. *Journal of Agricultural and Food Chemistry*, *50*, 6804-6811.

Fishman, M.L., & Chau, H. K. (2000). Extraction of pectin by microwave heating under pressure. U.S. Patent 6,143,337.

Fito, M., Cladellas, M., de la Torre, R., Marti, J., Alcantara, M., Pujadas-Bastardes, M., Marrugat, J., Bruguera, J., Lopez-Sabater, M.C., Vila, J., Covas, M.I. 2005. Antioxidant effect of virgin olive oil in patients with stable coronary heart disease: a randomized, crossover, controlled, clinical trial. *Atherosclerosis*, *181*, 149–158.

Fitó, M., Cladellas, M., de la Torre, R., Martí, J., Muñoz, D., Schröder, H., Alcántara, M., Pujadas-Bastardes, M., Marrugat, J. López-Sabater, M.C., Brugera, J., Covas, M-I. 2008. Anti-inflammatory effect of virgin olive oil in stable coronary disease patients: a randomized, crossover, controlled trial. *European Journal of Clinical Nutrition*, *62*, 570-574.

Fki, I., Allouche, N., Sayadi, S. 2005. The use of polyphenolic extract, purified hydroxytyrosol and 3,4-dixydroxyphenyl acetic acid from olive mill wastewater for the stabilization of refined oils: a potential alternative to synthetic antioxidant. *Food Chemistry*, *93*, 197-204.

Forsman, H., Islander, U., Andreasson, E., Andersson, A., Onnheim, K., Karlstrom, A., Sävman, K., Magnusson, M., Brown, K.L., Karlsson, A. 2011. Galectin-3 aggravates joint inflammation and destruction in antigen-induced arthritis. *Arthritis Rheumatism*, *63*, 445-454.

Furcht, P.W., & Silla, H. 1990. Comparison of simultaneous wet milling and enzymatic hydrolysis of cellulose in ball and attrition mill reactors. *Biotechnology and Bioengineering*, *35*, 630-645.

G

Galanakis, C.M., Tornberg, E., Gekas, V. 2010. A study of the recovery of the dietary fibres from olive mill wastewater and the gelling ability of the soluble fibre fraction. *LWT-Food Science and Technology*, *43*, 1009-1017.

Gallaher, D.D., Locket, P.L. Gallaher, C.M. 1992. Bile acid metabolism in rats fed two levels of corn oil and brans of oat, re and barley and sugar beet fibre. *Journal of Nutrition, 122*, 473-481.

Garrote, G., Cruz, J.M., Domínguez, H., Parajó, J. C. 2003. Valorisation of waste fractions from autohydrolysis of selected lignocellulosic materials. *Journal of Chemical Technology and Biotechnology*, *78*, 392-398.

Ghanbari, R., Anwar, F., Alkharfy, K.M., Gilani, A.-H., Saari, N**.** 2012**.** Valuable Nutrients and Functional Bioactives in Different Parts of Olive (Olea europaea L.)—A Review. *International Journal of Molecular Science, 13*, 3291-3340.

Ghosh, P., & Singh, A. 1993. Physicochemical and biological treatments for enzymatic/microbial conversion of lignocellulosic biomass. *Advances in Applied Microbiology*, *39*, 295-333.

Gil-Serrano, A., Mateos-Matos, N.I. Tejero-Mateo, M.P. 1986. Acidic xylan from olive pulp. Phytochemistry, *25,* 2653-2654.

Gil-Serrano, A., & Tejero-Matos, P. 1988. A xyloglucan from olive pulp. Carbohydrate Research, *181*, 278-281.

Gill, C.I.R., Hasmin, Y.Z.H-Y., Servili, M., Rowland, I.R. 2010. Olive oil and its components and their effects on early- and late-stage events in carcinogenesis. *Olive and Olive Oil in Health and Disease Prevention, 108*, 1005-1012.

Glahn, P.E. (2001). Pectin process and composition. U.S. Patent 6,207,194 y U.S. Patent 6,159,503.

González-Muñoz, M.J., Luque, S., Álvarez, J.R., Coca, J. 2003. Recovery of phenol from aqueous solutions using hollow fibre contactors. *Journal of Membrane Science*, *213,* 181-193.

Gordon, T., Kannel, W.B., Castelli, W.P., Dawber, T.R. 1981. Lipoproteins, cardiovascular disease, and death. The Framingham study. *Archives of Internal Medicine, 141*, 1128–1131.

Gorelik, E., Galili, U., Raz, A. 2001. On role of cell surface carbohydrates and their binding protein (lectins) in tumor metastasis. *Cancer and Metastasis Review*, *20*, 245-277.

Grous, J.J., Redfern, C.H., Mahadevan, D., Schlinder, J. 2006. GCS-100, a galectin-3 antagonist, in refractory solid tumors: a phase I study. *Journal of Clinical Oncology*, ASCO *Annual Meeting Proceedings* part I, 24 (18S).

Guarner, F. 2003. Gut flora in health and disease. *The Lancet*, *360*, 512-19.

Guiso, M., Marra, C., Arcos, R.R. 2008. An investigation on dihydroxy-isochromans in extra virgin olive oil. *Natural Product Research*, *16*, 1403-1409.

H

Haagensen, F., Skiadas, I.V., Gavala, H.N., Ahring, B.K. 2009. Pre-treatment and ethanol fermentation potential of olive pulp at different dry matter concentrations. *Biomass and bioenergy*, *33*, 1643-1651.

Handen, K., Allouche, N., Damak, M., Elfeki, A. 2009. Hypoglucemic and antioxidant effects of phenolic extracts and purified hydroxytyrosol from olive mill waste in vitro and in rats. *Chemico-Biological Interactions*, *180,* 421-432.

Harris, P.J. 1990. Plant cell wall structure and development. En: Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Rumiants. Alkin, E. D. (ed.). *Elservier Sci. Publ. Co*. Nueva York, pp. 71-90

Hashim, Y.Z., Rowland, I.R., McGlynn, H., Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., Montedoro, G., Kaisalo, L., Wahala, K., Gill, C.I. 2008. Inhibitory effects of olive oil phenolics on invasion in human colon adenocarcinoma cells in vitro. *International Journal of Cancer, 122*, 495–500.

Hatfield, R.D. 1993. Cell wall polysaccharide interaction and degradability. En: Forage Cell Wall Structure and Digestibility. Ed. Jung, H. G., Buxton, D. R., Hatfield, D. R., Ralph, J. ASA/CSSA/SSSA. *Madison, Wisconsin*, USA. 285-313

Hendriks, A.T.W.M., & Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresource Technology*, *100*, 10-18.

Heredia, A., Guillén, R., Jiménez, A., Fernández-Bolaños, J. 1993. Plant cell wall structure. *Revista Española de Ciencia y Tecnología de Alimentos, 33*, 113-131.

Heredia, A., Jiménez, A., Guillén, R. 1995. Composition of plant cell wall. *Z Lebensm. Uniter Forsch*, *200*, 24-31.

Hertel, M., Bommarius, S., Realff, J., Kang, Y. 2014. Deep eutectic solvent systems and methods. International Publication Number WO 2012/145522 A3.

Hon, D.N.S., & Shiraishi, N. 2001. Wood and Cellulosic Chemistry, second ed. *Dekker*, New York.

Hsu, C.-K., Liao, J.-W., Chung, Y.-C., Hsieh, C.-P., Chan, Y.-C. 2004. Xylooligosaccharides and Fructooligosaccharides Affect the Intestinal Microbiota and Precancerous Colonic Lesion Development in Rats. *American Society for Nutritional Science*, 1523-1528.

I

Ibarra, A., & Sniderman, N. 2005. Obtaining olive polyphenol concentrate from a by-product of olive oil extraction using membrane separation techniques. *UK Patent Application*, GB 2 415 136 A.

Iiyama, K., Lam, T.B., Stone, B.A. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemestry*, *29*, 733-737.

Isidori, M., Lavorgna, M., Nardelli, A., Parrela, A. 2005. Model study on the effect of 15 phenolic olive mill wastewaters constituents on seed germination and Vibrio fischeri metabolism. *Journal of Agricultural and Food Chemistry*, *43*, 8414-8417.

J

Jackson, C.L., Dreaden, T.M., Theobald, L.K., Tran, N.M., Beal, T.L., Eid, M. 2007. Pectin induces apoptosis in human prostate cancer cells: correlation of apoptotic function with pectin structure. *Glycobiology*, *17,* 805-819.

Jiménez, A. 1993. Modificación de la pared celular de aceitunas durante su elaboración. *Tesis doctoral*.

Jiménez, A., Guillén, R., Fernández-Bolaños, J., Heredia, A. 1994. Cell wall composition of olives. *Journal of Food Science*, *59*, 1192-1196.

Johannisbauer, W., Bonakdar, M., Richard-Elsner, C. 2003. Procedimiento para el aislamiento de antioxidantes. *ES 2 231 635 T3.*

Jung, H.G., & Deetz, D.A. 1993. Cell wall structure and digestibility. En: *Forage Cell Wall Structural and Digestibility*. Ed. Jung, H. G., Buxton, D. R., Hatfield, R. D., Ralph, J. ASA/CSSA/SSSA. Inc. Madison, Wisconsin, USA. 315-346.

K

Kaline, K., Bornstein, S.R., Bergmann, A., Hauner, H., Schwarz, P.E.H. 2007. The importance and effect of dietary fiber in diabetes prevention with particular consideration of whole grain products. *Hormone and Metabolic Research*, *39*, 687-693.

Kalogerakis, N., Polito, M., Foteinis, S., Chatzisymeon, E., Mantzavinos, D. 2013. Recovery of antioxidants from olive mill wastewaters: A viable solution that promotes their overall sustainable management. *Journal of Environmental Management, 128*, 749-758.

Klen, T.J., & Vodopivec, B.M. 2011. Ultrasonic extraction of phenols from olive mill wastewater: comparison with conventional methods. *Journal of Agricultural and Food Chemistry, 59* (24), 12725e12731

Kountouri, A.M., Mylona, A., Kaliora, A.C., & Andrikopoulos, N.K. 2007. Bioavailability of the phenolic compounds of the fruits (drupes) of Olea europaea (olives): Impact on plasma antioxidant status in humans. *Phytomedicine*, *14*, 659–667.

Ku, Y., Jansen, O., Oles, C.J., Lazar, E.Z., Arder, J.I. 2003. Precipitation of inulins and oligoglucoses by ethanol and other solvents. *Food Chemistry*, *81*, 125-132.

L

Lama A. 2011. Obtención y caracterización de compuestos y fracciones bioactivas a partir de alperujo tratado térmicamente. Estudio de un poderoso antioxidante natural (3,4-dihidroxifenilglicol, DHFG): Propiedades y purificación industrial. *Tesis Doctoral*.

Lama-Múñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Fernández-Bolaños, J. 2011. New hydrothermal treatment of alperujo enhances the content of bioactive minor components in crude pomace olive oil. *Journal of Agricultural and Food Chemistry, 59*, 1115-1123

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Fernández-Bolaños, J. 2012. Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids*, *28*, 92-104.

Laparra, J.M., & Sanz, Y. 2010. Interactions of gut microbiota with functional components and nutraceuticals. *Pharmacological Research, 61*, 219-225.

Lesage-Meessen, L., Navarro, D., Maunier, S., Sigoillot, J-C., Lorquin, J., Delattre, M., Simon, J-L., Asther, M., Labat, M. 2001. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chemistry*, *75*, 501-507.

Libbi, P. 2002. Inflammation in atherosclerosis. *Nature*, 420, 868-874.

Liu, C., & Wyman, C.E. 2003. The effect of flow rate of compressed hot water on xylan, lignin and total mass removal from corn stover. *Industrial & Engineering Chemistry Research, 42*, 5409– 5416.

López Gallego, F., Rodríguez, P.L., Espejo, M., Sanz, E., Cortés, P. 2003. Efecto de la utilización del alperujo en el pienso de cebo de corderos merinos, sobre la calificación Corderex. *XXVIII Jornadas Científicas y VII Internacionales de la SEOC*, Badajoz, 110-113.

López-Miranda, J. 2010. Olive oil and health: Summary of the II International conference on olive oil and health consensus report. Jaen and Córdoba (Spain). *Nutrition, Metabolism& Cardiovascular Diseases*, *20*, 284-294.

López-Oliva, M.E., Agis-Torres, A., Goñi, I., Muñoz-Martínez, E. 2010. Grape antioxidant dietary fibre reduced apoptosis and induced a pro-reducing shift in the glutathione redox state of the rat proximal colonia mucosa. *British Journal of Nutrition, 103*, 1110-1117.

M

Mainai, G., Periago, M.J., Catasta, G., Toti, E., Goñi, I., Bysted, A., Böhm, V., Mayer, E., Behsnilian, D., Schelemer, U. 2009. Carotenoids: actual knowledge on food sources, intakes, stability and bioavility and their protective role in humans. *Molecular Nutritional Food Research, 53*, S194-S218.

Mandaluniz, N., Larronde, E., Amores, G., Virto, M., Barron, L.J.R., Abilleira, E., Arranz, J., Ruiz-de-Gordoa, E.J.C., Najera, A., Ruiz, R. , Albisu, M., Perez-Elortondo, F.J., Renobales, M. 2010. Supplementation of sheep diets with olive mill waste results in healthier milk. *Alimentación, Equipos y Tecnologías, 254, 18-22.*

Mangan J.L. 1988. Nutritional effects of tannins in animal feeds. *Nutrition Research Reviews*, *1*, 209-231.

Markov, P.A., Popov, S.V., Nikitina, I.R., Ovodova, R.G., Ovodov, Y.S. 2011. Anti-Inflammatory activity of pectins and their galacturonan backbone. *Russian Journal of Bioorganic Chemistry*, *37*, 817-821.

Martinez-Garcia, G., Johnson, A.C., Bachmann, R.T., Williams, C.J., Burgoyne, A., Edyvean, R.G.J. 2009. Anaerobic treatment of olive mill wastewater and piggery effluents fermented with *Candida tropical. Journal of Hazardous Materials*, *164*, 1398–1405.

Martín-Peláez, S., Covas, M.I., Fitó, M., Kusar, A., Pravst, I., 2013. Health effects of olive oil polyphenols: Recent advances and possibilities for the use of health claims. *Molecular Nutritional and Food Research, 57*, 760–771.

Maxwell, E.G., Belshaw, N.J., Waldron, K.W., Morris, V.J. 2012. Pectin-An emerging new bioactive food polysaccharide. *Trends in Food in Science, 24*, 64-73.

Medina, E., de Castro, A., Romero, C., Brenes, M. 2006. Comparison of the concentrations of phenolic compounds in olive oils and other plant oils: correlation with antimicrobial activity. *Journal of Agricultura and Food Chemestry*, *54*, 4954–4961.

Menéndez, J.A., Vázquez-Martín, A., Oliveras-Ferraros, C, García-villalba, R., Carrasco-Pancorbo, A., Fernández-Gutiérrez, A., Segura-Carretero, A. 2009. Extra-virgin olive oil polyphenols inhibit HER2 (erbB-2)- induced malignant transformation in human breast epithelial cells: relationship between the chemical structures of extravirgin olive oil secoiridoids and lignans and their inhibitory activities on the tyrosine kinase activity of HER2. *International Journal of Oncology, 34*, 43-51.

Mitchell, H., & Tiihonen, K. 2003. Prebiotics. *Nutra. Cos*., 20-24.

Moerman, F.T., van Leeuwen, M.B., Delcour, J.A. 2004. Enrichment of higher molecular weight fractions in inulin. *Journal of Agricultural and Food Chemistry*, *52*, 3780-3783.

Mok, W.S.-L., & Antal, M.J.A., 1992. Uncatalyzed solvolysis of whole biomass hemicellulose by hot compressed liquid water. *Industrial & Engineering Chemistry Research, 31*, 1157– 1161.

Molina-Alcaide, E., Yáñez-Ruiz, D. R. 2008. Potential use of olive by-products in ruminant feeding: A review. *Animal Feed Science and Technology*, *147*, 247-264.

Molina-Alcaide, E., Morales García, E. Y., Martín García, A. I. 2005. Effect of feeding multinutrient blocks on rumen fermentation, intake, digestibility and milk yield and composition in dairy goats. En: Proceedings of the 11th Seminar of the Sub-Network FAO-CIHEAM on Sheep and Goat Nutrition, 77.Catania, Italy.

Monties, B. 1980. Les polymers vegetaux. Polymères pariétaux et alimentaires non azotes. *Collection Biochinie Appliqué*. Ed. Gauthier-Villars, París.

Morris, V.J., Belshaw, N.J., Waldron, K.W. & Maxwell, E.G. 2013. The bioactivity of modified pectin fragments. *Bioactive Carbohydrates and Dietary Fibre*, *1*, 21-37.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., Ladish, M. 2005. Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresource Technology*, *96*, 673-686.

Muktadirul Bari Chowdhury, A.K.M., Akratos, C.S., Vayenas, D.V., Pavlou, S. 2013.Olive mill waste composting: A review. *International Biodeterioration & Biodegradation*, *85*, 108-119.

Munarin, F., Tanzi, M.C., & Petrini, P. 2012. Advances in biomedical applications of pectin gels. *International Journal of Biological Macromolecules*, *51*, 681-689.

Mussatto, S.I., Mancilha, I.M. 2007. Non-digestible oligosaccharides: A review. *Carbohydrate Polymers*, *68*, 587-597.

O

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, Jr. P.D., Robards, K. 2005b. Investigation of australian olive mill waste for recovery of biophenols. *Journal of Agricultural and Food Chemistry*, *53*, 9911-9920.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D., Robards, K., Stockmann, R**. 2005a**. Bioactivity and analysis of biophenols recovers from olive mill waste. *Journal of Agricultural and Food Chemistry, 53*, 823-837.

Obied, H.K., Bedgood, D.R., Prenzler, P.D., Robards, K. 2007a. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Analytica Chimica Acta*, *603*, 176-189.

Obied, H.K., Karuso, P., Prenzler, P.D., Robards, K. 2007b. Novel secoiridoids with antioxidant activity from Australian olive mill waste. *Journal of Agricultural and Food Chemistry, 55*, 2848-2853.

Obied, H.K., Prenzler, P.D., Robards, K. 2008. Potent antioxidant biophenols from olive mill waste. *Food Chemistry*, *111*, 171–178.

Ocakoglu, D., Tokatli, F., Ozen, B., Korel, F. 2009. Distribution of simple phenols, phenolic acids and flavonoids in Turkish monovarietal extra virgin olive oils for two harvest years. *Food Chemistry*, *113*, 401-410.

Ochando-Pulido, J.M., Hodaifa, G., Victor-Ortega, M.D., Rodriguez-Vives, S., Martinez-Ferez, A. 2013. Effective treatment of olive mill effluents from two-phase andthree-phase extraction processes by batch membranes in series operation upon threshold conditions. *Journal of Hazardous Materials 263*P, 168-176.

Okamura, D.M., Pasichnyk, K., Lopez-Guisa, J.M., Collins, S., Hsu, D.K., Liu, F.T. et al. 2011. Galectin-3 preserves renal tubules and modulates extracelular matrix remodelling in progressive fibrosis. *American Journal of Physiology Renal Physiology*, *300*, F245-F253.

Olano-Martín, E., Mountzouris, K.C., Gibson, G.R., Rastall, R.A. 2001. Continuous production of pectic-oligosaccharides in an enzyme membrane reactor. *Journal of Food Science*, *66,* 966-971.

Olano-Martín, E., Rimbach, G.H., Gibson, G.R., Rastall, R.A. 2003. Pectin and pectic-oligosaccharides induce apoptosis in *in vitro* human colonia adenocarcionoma cells. *Anticancer Research*, *23*, 341-346.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Spiegelhalder, B., Bartsch, H. 2000. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *European Journal of Cancer, 36*, 1235–1247.

P

Pablo Domínguez, M. 2014. Recent trends in (lingo)cellulose dissolution using neoteric solvents: switchable, distillable and bio-based ionic liquids. *Journal of Chemical Technology and Biotechnology, 89*, 11-18.

Pérez-Jiménez, F. 2005. International conference on the healthy effect of virgin olive oil. *European Journal of Clinical Investigación*, *35*, 421-424.

Pinelo, M., Jonson, G., Meyer, A. S. 2009. Membrane technology for purification of enzymatically produced oligosaccharides: Molecular and operational features affecting performance. *Separation and Purification Technology*, *70*, 1-11.

Plaza, C., Nogales, R., Senesi, N., Benitez, E., Polo, A. 2008. Organic matter humification by vermicomposting of cattle manure alone and mixed with two-phase olive pomace. *Bioresource Technology*, *99*, 5085–5089.

R

Ramos, L.P. 2003. The chemistry involved in the steam treatment of lignocellulosic materials. *Quimica Nova*, *26*, 863-871.

Ramos-Cormenzana, A., Juárez-Jiménez, B., García-Pareje, M.P. 1996. Antimicrobial activity of olive mill waste waters (alpechin) and biotransformed olive oil mill wastewaters. *International Biodeterioration and Biodegradation*, *38*, 283-290.

Riachy, M., Priego-Capote, F., Rallo, L., Luque-de-Castro, M.D., León, L. 2013. Phenolic composition of virgin olive oils in cultivars for narrow hedgerow olive orchards. *European Journal of Lipid Science and Technology, 115*, 800–810.

Rice-Evans, C.A., Miller, N.J., Paganga, G. 1996. Structure-antioxidant activity relationship of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, *20*, 933-956.

Rigane, G., Bouaziz, M., Baccar, N., Abidi, S., Sayadi, S., Salem, R.B. 2012. Recovery of hydroxytyrosol rich extract from two-phase chemlali olive pomace by chemical treatment. *Journal of Food Science*, *77*, Nr. 10, C1077-C-1083

Rincón, B., Borja, R., Martín, M.A., Martín, M. 2010. Kinetic study of the methanogenic step of a two-stage anaerobic digestion process treating olive mill solid residue. *Chemical Engineering Journal*, *160*, 215–219.

Rivers, D.B., & Emert, G.H. 1987. Lignocellulose pre-treatment: a comparasion of wet and dry ball attrition. *Biotechnology Letters*, *9*, No 5, 365-368.

Roberfroid M.B. 2005. Introducing inulin-type fructans. *British Journal of Nutrition*, Apr;93 Suppl 1:S13-25.

Rodis, P.S., Karathanos, V.T., Mantzavinou, A. 2002. Partitioning of olive oil antioxidants between oil and water phase. *Journal of Agricultural and Food Chemistry*, *50*, 596-601.

Rodriguez-Gutierrez, G., Duthie, G.G., Wood, S., Morrice, P., Nicol, F., Reid, M., Cantlay, L.L., Kelder, T., Horgan, G.W., Guzman, J.F.B., de Roos, B. 2012. Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats proteomics and network analysis approach. *Molecular Nutrition & Food Research, 56*, 1131-1147.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., Heredia, A. 2006. Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Science and Technology*, *17*, 3-15.

Rodríguez-Gutíerrez, G., Jiménez-Araujo, A., Rodríguez-Arcos, R., Lama-Muñoz, A., Fernández-Bolaños, J., Guillén-Bejarano, R. 2011. Procedimiento de purificación de 3,4-dihidroxifenilglicol (DHFG) a partir de productos vegetales. ES2341526 B1

Rodríguez, G., Rodríguez, R., Jiménez, A., Guillén, R., Fernández-Bolaños, J. 2007. Effect of steam treatment of alperujo on the composition, enzymatic saccharification, and in vitro digestibility of alperujo. *Journal of Agricultural and Food Chemistry*, *55*, 136-142.

Romero, C., Brenes, M., Yousfi, K., García, P., García, A., Garrido, A. 2004. Effect of cultivar and processing method on the contents of polyphenols in table olives. *Journal of Agricultural and Food Chemistry*, *52*, 479-484.

Romero, C., García, P., Brenes, M., García, A., Garrido, A. 2002. Phenolic compounds in natural black Spanish olive varieties. *European Food Researchand Technology*, *215*, 489-496.

Romero, C., Medina, E., Vargas, J., Brenes, M., De Castro, A. 2007. In vitro activity of olive oil polyphenols against Helicobacter pylori. *Journal of Agricultural and Food Chemistry, 55*, 680–686.

Ruggeri, Z. M. 2002. Platelets in atherothrombosis. *Nature Medicine, 8*, 1227–1234.

Ruiz-Canela, M., Estruch, R., Corella, D., Salas-Salvado, J., & Martinez-Gonzalez, MA. 2014. Association of mediterranean diet with peripheral artery disease: The PREDIMED randomized trial. *Journal of the American Medical Association*, 311-415.

Ruiz-Méndez, M.V., Romero, C., Medina, E., García, A., de Castro, A., Brenes, M. 2013. Acidification of Alperujo Paste Prevents Off-Odors During Their Storage in Open Air. *Journal of the American Oil and Chemists' Society, 90*, 401–406.

Ryan, D., & Robards, K. 1998. Phenolic compounds in olives. *The Analyst*, *123*, 31-44.

S

Salvini, S., Sera, F., Caruso, D., Giovannelli, L., Visioli, F., Saieva, C., Masala, G., Ceroti, M., Giovacchini, V., Pitozzi, V., Galli, C., Romani, A., Mulinacci, N., Bortolomeazzi, R., Dolara, P., Palli, D. 2006. Daily consumption of a high-phenol extra-virgin olive oil reduces oxidative DNA damage in postmenopausal women. *British Journal of Nutrition, 95*, 742–751.

Sampedro, I., Aranda, E., Díaz, R., García-Sanchez, M., Ocampo, J.A., García-Romera, I. 2008. Saprobe fungi decreased the sensitivity to the toxic effect of dry olive mill residue on arbuscular mycorrhizal plants. *Chemosphere*, *70*, 1383-1389.

Sannino, F., Iorio, M., De Martino, A., Pucci, M., Brown, C.D., Capasso, R. 2008. Remediation of waters contaminated Ruth ionic herbicides by sorption on polymerin. *Water research*, *42*, 643-652.

Sannino, F.M., De Martino, A., Pigna, M., Violante, A., Di Leo, P., Mesto, E., Capasso, R. 2009. Sorption of Arsenate and dichromate on polymerin, Fe(OH)x-polymerin complex and ferrihydrite. *Journal of Hazardous Materials*, *166*, 1174-1179.

Sanz, M.L., Polemis, N., Morales, V., Corzo, N., Drakoularakou, A., Gibson, G. R., Rastall, R.A. 2005. In vitro investigation into the potential prebiotic activity of Money oligosaccharides. *Journal of Agricultural and Food Chemistry*, *53*, 2914-2921.

Saura-Calixto, F. 1998. Antioxidant dietary fiber product: a new concept and a potencial food ingredient. *Journal of Agricultural and Food Chemistry, 46*, 4303-4306.

Saura-Calixto, F. 2011. Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. *Journal of Agricultural and Food Chemistry, 59*, 43-49.

Saura-Calixto, F., & Díaz-Rubio, M.E. 2007. Polyphenols associated with dietary Wbre in wine A wine Polyphenols gap?. *Food Research International, 40*, 613–619.

Savarese, M., De Marco, E., Sacchi, R. 2007. Characterization of phenolic extracts from olives (Olea europaea cv. Pisciottana) by electrospray ionization mass spectrometry. *Food Chemistry*, *105*, 761–770.

Saviozzi, A., Levi-Minzi, R., Riffaldi, R., Lupetti, A. 1991. Effects of waste-water from olive processing on agricultural soil. *Agrochimica*, *35*, 135-148.

Scheneeman, B.O. 1987. Soluble vs insoluble fibre-different physiological responses. *Food Technology, 41*, 81-82.

Segura-Carretero, A., Menéndez-Menéndez, J., Fernández-Gutiérrez, A. 2010. Polyphenols in olive oil: The importance of phenolic compounds in the chemical composition of olive oil. *Olives and Olives Oil in Health and Disease Prevention*, *19*, 167-175.

Setti, L., Pifferi, P., Bordado, J. 2003. Plant for the treatment of waste water, WO 03/004419 A1.

Showalter, A. M. 1993. Structure and function of plant cell wall proteins. *Plant Cell,* 5, 9-23.

Sjösttröm, E. 1982. Wood chemistry. Fundamentals and Applications. *Academic Press. London*. pp. 15-50.

Southgate, D. A. T. 1990. Determination of Carbohydrates. *Elservier Applied Science*. Londres.

Suárez, M., Rometo, M-P, Ramo, T., Macia, A., Motilva, M-J. 2009. Methods for Preparing Phenolic Extracts from Olive Cake for Potential Application as Food Antioxidants. *Journal of Agricultural and Food Chemistry*, *57*, 1463–1472.

Swennen, K., Courtin, C.M., Van der Bruggen, B., Vandecasteele, C., Delcour, J. A. 2005. Ultrafiltration and ethanol precipitation for isolation of arabinoxylooligosaccharides with different structures. *Carbohydrate Polymers*, *62*, 283-292.

T

Teresa, M., de Freitas, O., Ferreira, L.M., Carvalho, J. 2006. Extraction of 2-(4-hydroxyphenyl) etanol from aqueous solution by emulsion liquid membranes. *Journal of Membrane Science*, *269*, 161-170.

Tortosa, G., Alburquerque, J.A., Ait-Baddi, G., Cegarra, J. 2012. The production of commercial organic amendments and fertilisers by composting of two-phase olive mill waste ("alperujo"). *Journal of Cleaner Production, 26*, 48-55.

V

Van Buren, J. P. 1979. The chemistry of texture in fruits and vegetables. *Journal of Texture Studies*, *10*, 1-23.

Vázquez, M.J., Garrote, G., Alonso, J.L., Domínguez, H., Parajó, J.C. 2005. Refining of autohydrolysis liquors for manufacturing xylooligosaccharides: evaluation of operacional strategies. *Bioresource Technology*, *96*, 889-896.

Vierhuis, E., Schols, H.A., Beldman, G., Voragen. **2001**. Structural characterisation of xyloglucan and xylans present in olive fruit (*Olea europaea* cv koroneiki). *Carbohydrate Polymers*, *44*, 51-62.

Visioli. F., Poli, A., Gall, C. (2002). Antioxidant and other biological activities of phenols from olives and olive oil. *Medicinal Research Reviews, 22*, 65-75.

Vlasenko, E.Y., Ding, H., Labavitch, J.M, Shoemaker, S.P. 1997. Enzymatic hydrolysis of pretreated rice straw. *Bioresource Technology*, *59*, 109-119.

Vom Stein, T., Grande, P.M., Kayser, H., Sibilla, F., Leitner, W., Domínguez de María, P. 2011. From biomass to feedstock: one-step fractionation of lignocellulose components by the selective organic acid-catalyzed depolymerization of hemicellulose in a biphasic system. *Green Chemistry, 13*, 1772-1777.

W

Willem, J., Jones, J., MacCleary, B., Topping, D. 2010. Dietary Fibre: New frontiers for food and health. *Wageningen Academic Publisher: Wageningen, The Netherlands*. 1-586.

Wilson, J. R. 1993. Organization of forage plant tissues. En: *Forage Cell Wall Structure and Digestability*. Jung, H. G., Buxton, D. R:, Hatfield, R. D:, Ralph, J. (eds). Madison, Wisconsin, pp. 1-32.

Wilson, W.D., Jarvis, M.C., Duncan, H.J. 1989. In-vitro digestibility of kale (brassica oleracea) secondary xylem and parenchyma cell wall and their polysaccharide components. *Journal of the Science of Foodand Agriculture, 48*, 9-14.

Wood, T.M., & Saddler, J.N. 1988. Increasing the availability of cellulose in bio mass materials. *Methods in Enzymology*, *160*, 3-11.

Y

Yang, F.G., Fang, Z., Xu, Y., Yao, C.C., Yu, S.Y., Zhu, Q.X. 2003. Separation of oligosaccharides from enzymatic hydrolytes using membrane reactor. *Journal of Central South University of Technology*, *10*, 122-125.

Yáñez-Ruiz, D.R., & Molina-Alcaide, E. 2007**.** A comparative study of the effect of two-stage olive cake added to alfafa on digestion and nitrogen losses in sheep and goats. *Animal*, *1*, 227-232.

Z

Zhang, L.H., Li, D., Wang, L.-J., Wang, T.-P., Zhang, L., Chen, X.D., Mao, Z.H**.** 2008. Effect of steam explosion on biodegradation of lignin in wheat straw. *Bioresource Technology*, *99*, 8512–8515.

Zhang, L., Ye, X., Ding, T., Sun, X., Xu, Y., Liu, D. 2013. Ultrsound effects on the degradation kinetics, structure and rheological properties of apple pectin. *Ultrasonics Somochemistry*, *20*, 222-231.

Zhu, S., Wu, T., Yu , Z., Zhang, X., Li, H., Gao, M. 2006. The effect of microwave irradiation on enzymatic hydrolysis of rice straw. *Bioresource Technology*, *97*, 1964–1968.

Ziogas, V., Tanou, G., Molassiotis, A., Diamantidis, G. 2010. Antioxidant and free radical-scavenging activities of phenolic extracts of olive fruits. *Journal Food Chemistry*, *120*, 1097-1103.

5. Objetivos

5. OBJETIVOS

El objetivo principal de este trabajo de investigación se centra en el aprovechamiento integral del subproducto generado en el proceso de extracción del aceite de oliva. El alperujo es tratado térmicamente permitiendo la solubilización de una gran cantidad de compuestos de interés que pasan a formar parte de una fracción líquida. Esta tesis se centra en el estudio de los compuestos fenólicos y componentes de la pared celular tales como los oligosacáridos neutros y ácidos liberados, cuyas características y composición se ven modificadas en función de las condiciones del tratamiento térmico empleado. Para afrontar este objetivo principal se han desarrollado los siguientes objetivos específicos:

1.- Fraccionamiento y caracterización de los nuevos extractos fenólicos obtenidos a partir del alperujo tratado térmicamente y comparación con un extracto obtenido a partir de alperujo sin tratar. Se evalúa como afecta el tiempo de tratamiento térmico a una temperatura fija, así como el pH de la fracción líquida obtenida durante la extracción con acetato de etilo realizada a la composición fenólica de los extractos obtenidos.

2.- Determinación de las propiedades de dichos extractos fenólicos, mediante estudios de las propiedades antioxidantes *"in vitro"* (poder reductor, captación de radicales ABTS y DPPH, inhibición de la oxidación primaria y secundaria), así como estudios llevado a cabo "*ex vivo*" tales como la inhibición de la agregación plaquetaria e inhibición de la oxidación de microsomas.

3.- Identificación de los precursores básicos de uno de los fenoles más activos encontrados, el DHFG, que incrementan su cantidad en forma libre cuando se lleva a cabo un tratamiento térmico sobre el alperujo.

4.- Determinar la composición de los oligosacáridos neutros liberados como consecuencia del tratamiento térmico.

5.- Estudiar la composición y propiedades (tamaño, grado de esterificación, capacidad de retención de agua/aceite, capacidad de emulsificación, estabilidad de la emulsión, capacidad de retención de ácidos biliares e índice de retardo de difusión de la glucosa) de los polisacáridos ácidos liberados durante el tratamiento térmico, considerando las variaciones que tienen lugar como consecuencia de los diferentes tiempos (30, 45 y 60 min) de tratamiento térmico aplicados sobre el alperujo a una temperatura determinada (160 ºC).

6.- Estudiar la composición y propiedades (tamaño, grado de esterificación, capacidad de retención de agua/aceite, capacidad de emulsificación, estabilidad de la emulsión, capacidad de retención de ácidos biliares, índice de retardo de difusión de la glucosa y actividad

antioxidante) de los polisacáridos ácidos recuperados a partir de un alperujo que ha sufrido un tratamiento térmico suave industrial (50-80 ºC, 1-2 horas).

7.- Estudio de la composición y propiedades de las diferentes fracciones recuperadas (celulosa, lignina y hemicelulosa) cuando el tratamiento térmico es llevado a cabo sobre un material puramente lignocelulósico como es el hueso de la aceituna y orujo de tres fases.

6. Resultados y Discusión

6. RESULTADOS Y DISCUSIÓN

Los resultados obtenidos durante está Tesis Doctoral van a ser presentados mediante el conjunto de artículos científicos publicados. Dichos artículos se pueden agrupan en dos bloques según la naturaleza de los compuestos estudiados.

6.1. Compuestos fenólicos y secoiridoides.

6.1.1. Capítulo 1:

New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

Journal of Agricultural and Food Chemistry, 2012, 60, 1175-1186.

6.1.2. Capítulo 2:

Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

LWT- Food Science and Technology, 2013, 54, 114-124.

6.1.3. Capítulo 3:

Influence of pH on the extraction and antioxidant activity of phenolic extracts obtained with ethyl acetate from hydrothermally treated alperujo.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., García, A., Fernández-Bolaños, J.

6.1.4. Capítulo 4:

Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

Food Research International, 2013, 54, 2122-2129.

6.1.5. Capítulo 5:

Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E deficient rats.

Rubio-Senent, F., de Roos, B., Duthie, G., Fernández-Bolaños, J., Rodríguez-Gutiérrez, G.

European Journal of Nutrition, DOI: 10.1007/s00394-014-0807-8.

6.1.6. Capítulo 6:

Isolation and characterization of a secoiridoids derivative from two-phase olive waste (alperujo).

Rubio-Senent, F., Martos, S., García, A., Fernández-Bolaños, J.G., Rodríguez-Gutiérrez, G., Fernández-Bolaños, J.

J. Agric. Food Chem. DOI: 10.1021/jf5057716.

6.1.7. Capítulo 7:

Isolation and identification of minor secoiridoids and phenolic components from thermally treated olive oil by-products.

Rubio-Senent, F., Martos S., Lama-Muñoz, A., Fernández-Bolaños, J.G., Rodríguez-Gutíerrez, G., and Fernández-Bolaños J.

6.1.8. Capítulo 8:

A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste.

Lama-Muñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Palacios-Díaz. R., Fernández-Bolaños, J.

Food Chemistry, 2013, 140, 154-160.

6.2. Polisacáridos y oligosacáridos

6.2.1. Capítulo 9:

Production of oligosaccharides with low molecular weights, secoiridoids and phenolic glycosides from thermally treated olive by-products.

Fernández-Bolaños, J., Rubio-Senent, F., Lama-Muñoz, A., García, A., Rodríguez-Gutiérrez, G.

Oligosaccharides: Food Sources, Biological Roles and Health Implications, Cap 6, pag 173-208. Editors: Lori S. Schweizer and Stanley J. Krebs. Enero, 2014. ISBN: 978-1-62948-328-3.

6.2.2. Capítulo 10:

Pectin extracted from thermally treated olive oil by-products: characterization, physico-chemical properties, *in vitro* bile acid and glucose binding.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

Food Hydrocolloids, 2015, 43, 311-321.

6.2.3. Capítulo 11:

Novel pectin present in new olive mill wastewater with similar emulsifiers and better biological properties than citrus pectin.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., García, A., Fernández-Bolaños, J.

6.2.4. Capítulo 12:

Properties of lignin, cellulose, and hemicelluloses isolated from olive cake and olive stones: binding of water, oil, bile acids, and glucose

Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Lama-Muñoz, Aranzazu García, and Juan Fernandez-Bolaños.

Journal of Agricultural and Food Chemistry, 2014, 62, 8973-8981.

6.1. Compuestos fenólicos y secoiridoides

6.1. Compuestos fenólicos y secoiridoides.

El tratamiento térmico realizado sobre el alperujo facilita la solubilización de una gran cantidad de compuestos atrapados en el entramado de la matriz de la pared celular vegetal, así como la hidrólisis de moléculas complejas, provocando el aumento de la concentración de los compuestos fenólicos, oligosacáridos y polisacáridos en la fracción acuosa. Los fenoles solubilizados son fácilmente extraíbles con acetato de etilo, permitiendo la obtención de un extracto fenólico.

En un primer paso se ha estudiado como afectan el tiempo de tratamiento térmico a una temperatura fija a la composición (cualitativa y cuantitativamente) y la capacidad antioxidante de los extractos fenólicos (Capítulos 1 y 2), así como la influencia del pH al cual se realiza la extracción con acetato de etilo (Capítulo 3). El estudio detallado de los extractos fenólicos nos ha permitido aislar una fracción fenólica polimérica, cuya composición, propiedades antioxidantes y capacidad de sorción/desorción de hidroxitirosol han sido evaluadas (Capítulo 4).Por otro lado, se ha evaluado la actividad antiagregante y la capacidad de inhibición de la oxidación de microsomas procedentes de ratas deficientes en vitamina E de algunos de los compuestos y extractos fenólicos aislados a partir de alperujo tratado térmicamente (Capitulo 5).

Además se ha aislado y determinado la estructura de un derivado del ácido glutárico que se encuentra en elevadas concentraciones en los extractos fenólicos obtenidos (Capítulo 6). También se ha aislado un compuesto secoiridoide presente en la fracción acuosa remanente una vez realizada la extracción con acetato de etilo de la fracción líquida obtenida tras tratar térmicamente el alperujo (Capítulo 7). Finalmente se ha realizado un proceso de aislamiento, purificación e identificación de los precursores del 3,4-dihidroxifenilglicol (DHFG), este compuesto se caracteriza por poseer una elevada capacidad antioxidante y su concentración se ve incrementada cuando el alperujo es sometido a tratamientos térmicos suaves (Capítulo 8).

6.1.1. Capítulo 1.

New phenolic compounds hydrothermally extracted from the olive oil by-Product alperujo and their antioxidative activities.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

Journal of Agricultural and Food Chemistry, 2012, 60, 1175-1186.

La industria del aceite de oliva es muy importante en la región mediterránea, de manera que España produce más del 30% del aceite de oliva del mundo. En los últimos años en España se ha implementado de forma generalizada el sistema de extracción de dos fases, el cual se caracteriza porque durante el proceso no se añade agua. Este sistema genera un nuevo subproducto llamado alperujo, que es una combinación de residuos líquidos y sólidos, el cual contiene celulosa, hemicelulosas, pectina, gomas, taninos, polifenoles y minerales.

Se ha demostrado que el consumo de aceite de oliva virgen, con un alto contenido en ácidos grasos monoinsaturados y con una fracción saponificable rica en componentes minoritarios tales como polifenoles, esteroles, y tocoferoles, está relacionada con una baja incidencia de enfermedades crónicas tales como enfermedades cardiovasculares y cáncer. Los polifenoles presentes en el aceite de oliva actúan como antioxidantes naturales con propiedades anti-inflamatorias, anti-ateroscleróticas y afectan a la función plaquetaria humana. Sin embargo, después de la extracción del aceite, el 98-99% de los compuestos fenólicos presentes en el fruto de la aceituna permanecen en el alperujo. Para posibilitar la recuperación de los compuestos fenólicos presentes en el subproducto se realiza un tratamiento térmico patentado (PCT/ES2011/ 070583) por nuestro grupo de investigación que provoca la ruptura del entramado de la matriz celular vegetal facilitando la liberación de los compuestos de interés.

El objetivo de este trabajo ha sido determinar la composición de los diferentes extractos fenólicos obtenidos a partir de alperujo tratado térmicamente y estudiar la influencia del tiempo de tratamiento a una temperatura fija en la composición de los extractos fenólicos obtenidos. Así como estudiar las actividades antioxidantes de los extractos aislados.

Los resultados de este trabajo han mostrado que los extractos obtenidos tras los diferentes tratamientos térmicos poseen un mayor contenido en fenoles y actividad antioxidante que el control empleado (un extracto fenólico obtenido a partir de alperujo no tratado térmicamente).

New Phenolic Compounds Hydrothermally Extracted from the Olive Oil Byproduct Alperujo and Their Antioxidative Activities

Fátima Rubio-Senent, Guillermo Rodríguez-Gutíerrez, Antonio Lama-Muñoz, and Juan Fernández-Bolaños

ABSTRACT

The application of a novel process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final liquid phase that contained a high concentration of simple phenolic compounds. This study evaluated the effects of time (15-90 min) on the composition of the phenolic compounds isolated at a fixed temperature of 160 °C. Phenolic compounds were extracted with ethyl acetate. Both qualitative and quantitative HPLC analyses of the extracts showed variation of the concentrations of phenolic compounds with time. In addition, new phenols that were not present in the untreated control have been characterized. The antioxidant activities of different phenolic extracts was measured by various assays conducted *in vitro*: antiradical capacity (using DPPH and ABTS radicals), ferric reducing power (P_R), inhibition of primary and secondary oxidation in lipid systems, and other tests, such as inhibition of tyrosinase activity. The results show that the phenolic extracts inhibited oxidation in aqueous and lipid systems to a significantly greater extent than the untreated control, and they performed as well as or better than vitamin E in this capacity.

KEYWORDS: alperujo, olive oil wastes, phenols, antioxidant, hydroxytyrosol, tyrosinase, ethyl acetate extract

Introduction

The olive oil industry is important in the Mediterranean region, and Spain produces >30% of the world's olive oil. Traditionally, the three-phase mill uses large volumes of water to aid the separation of oil and generates two by products. The first one is a liquid waste, which is known as olive mill wastewater, vegetation water, or alpechin. The second by product, a solid waste, is a combination of olive pulp and stones and is called pomace or orujo. The use of a modern two-phase processing technique, in which no water is added, generates a new by product called alperujo, which is a combination of liquid and solid waste. This two-phase centrifugation process is used for the separation of the oil from the vegetable material, which includes all of the mineral celluloses, hemicelluloses, pectins, gums, tannins, and polyphenols. In Spain, a massive change

from the traditional three-phase process to the new two-phase process has taken place, and large volumes of waste, approximately 3.5-6 million tons/year, are generated.[1]

The Mediterranean diet has been documented by a large number of epidemiological studies.[2] It has been shown that the consumption of virgin olive oil, with high content in monounsaturated fatty acids and with an un saponifiable fraction rich in minor components such as polyphenols, sterols, and tocopherols, leads to an increase in the total phenolic content of low-density lipoprotein (LDL) to prevent the LDL oxidation in the arterial intima.[3] Phenolic compounds from virgin olive oil may delay the progression of atherosclerosis by this mechanism. The antioxidative effects of phenolic compounds present in olive products also may contribute to the prevention of chronic diseases such as cardiovascular disease and cancer.[4] These phenolic compounds protect organisms against oxidative damage and prevent the deterioration of food by inhibiting lipid oxidation. The dialdehydic form of one of the secoiridoids, deacetoxy-ligstroside aglycone (oleocanthal), was also recently found in the olive oil. Oleocanthal has the ability to inhibit cyclooxygenase (COX-1 and COX-2), showing an anti-inflammatory effect similar to that seen with ibuprofen.[5]

After the extraction of oil, 98-99% of the phenolic compounds present in the fruit of the olive remain in the alperujo. To explore the possibility of obtaining simple phenolic compounds in high yield from two-phase olive waste, a series of hydrothermal treatments was performed. A process that allows for easy separation of the solid and liquid phases of alperujo has been developed. The process also allows for the recovery of value-added compounds in the water-soluble fraction. In this treatment, which has been patented (PCT/ES2011/070583), an autohydrolytic process occurs, resulting in the solubilization of the alperujo. Usually, whenever a lignocellulosic material is treated with water or steam at temperatures from 160 to 240 °C, an autohydrolysis process occurs.[6] Depending on the conditions used, there may be a depolymerization of polysaccharides (mainly of hemicelluloses) and a breaking of lignin structures, resulting in the solubilization of lignin fragments of low weight.

As a consequence of our hydrothermal treatment, the byproduct was partially solubilized. The simple phenols, such as hydroxytyrosol, tyrosol, and 3,4-dihydroxyphenylglycol, increased in concentration as a result of the breakdown of complex molecules, such as oleuropein, demethyloleuropein, verbascoside, and others, that contain them in their structure.[7] Interest in antioxidants for the prevention and treatment of human diseases has been sustained for at least two decades. It has been suggested that the consumption of certain foods that contain bioactive compounds, including fruits, vegetables, and red wine, may help to prevent cardiovascular diseases.[8] Several studies conducted *in vitro* have shown that this beneficial effect may be explained in part by the presence of polyphenols.[2]

The first objective of this work was to determine the composition of the different phenolic extracts obtained from treated alperujo and to study the influence of reaction time on the

composition of the phenolic compounds extracted. The second objective was to characterize the specific antioxidative potentials of individual extracts obtained from treated alperujo and to compare their antioxidative capacities with those of untreated alperujo and antioxidants such as hydroxytyrosol, 3,4-dihydroxyphenylglycol, and vitamin E.

Materials and Methods

Materials. The sample of alperujo (a semisolid residue composed of olive peels, pulp, seeds, and ground stones) was obtained in March of 2009 from Picual olives processed at a Spanish oil mill (Almazara Experimental, Instituto de la Grasa, Seville). The alperujo was processed in the pilot reactor without removal of the stones.

Standard Compounds. Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, caffeic acid, chlorogenic acid, 2,6-dihydroxybenzoic acid, 4-methylcatechol, syringicacid, vanillin, and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol and 3,4-dihydroxyphenylacetic acid were obtained from Fluka (Buchs, Switzerland). Oleuropein, verbascoside, and luteolin-7-O-glucoside were obtained from Extrasynthese (Lyon Nord, Geney, France). Hydroxytyrosol was obtained according to the method described by Fernández-Bolaños et al.[9]

Thermal Treatment. The hydrothermal treatment used has been patented (PCT/ES2011/070583). It was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor has a stainless steel reservoir (100 L capacity) that can operate at temperatures between 50 and 190 °C and at a maximum pressure of 1.2 MPa.

Either 10 or 20 kg of alperujo was loaded into the reactor, according to the treatment. Fresh alperujo samples were treated for 15-90 min at 160 °C. Then the wet material was centrifuged at 4700g (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, 10 L of the liquid phase of each treatment was concentrated to 1 L by rotary evaporation in a vacuum at 30 °C.

Phenol Extraction. The liquid portions obtained after treatment were washed with hexane to remove the lipid fraction: 1 L of liquid was mixed with 500 mL of hexane; the mixture was shaken vigorously, and then the phases were separated by decantation and washed twice.

Extraction of phenolic compounds was carried out with ethyl acetate (500 mL per 200 mL of sample). The liquid-liquid extraction was performed with ethyl acetate (refluxed at 77 °C) in a continuous extractor of a heavier liquid (water) by lighter liquid (ethyl acetate) for 8 h. The aqueous and organic phases were separated, and the organic phase was rotary-evaporated under vacuum at 30 °C, producing a viscous dark brown extract.

Phenolic Extract Obtained from Control by Conventional Extraction. Phenols were extracted from the alperujo control to assess the effects of heat treatment on the composition of the extracted phenolic compounds. We used the method reported by Obied et al.,[10] with some modifications. Alperujo was extracted with aqueous methanol (80% v/v; 15 mL/10 g of alperujo) for 30 min at ambient temperature. The extraction was repeated twice more using 10 mL/10 g of alperujo. The combined liquid fraction was filtered, and then the phenols were extracted with ethyl acetate under the above-mentioned conditions.

Determination of the Total Phenolic Content. The phenolic content was measured according to the Folin-Ciocalteu method[11] and expressed as grams of gallic acid equivalents per kilogram of fresh alperujo.

Chromatographic Fractionation of the Ethyl Acetate Extracts. Approximately 12 g of each extract was dissolved in 60 mL of H_2O/MeOH (80:20). First, the extracts were passed through a column 3.5 cm in diameter and 40 cm in height filled with Amberlite XAD16. The elution was performed with 1 L of H_2O, 30% EtOH (v/v), 50% EtOH (v/v), and 95% EtOH (v/v). Ten fractions of 100 mL each were collected. Fractions were analyzed by HPLC, and those with similar compositions were mixed, concentrated to 50 mL, and passed through a second column. The second column was 3.5 cm in diameter and 45 cm in height, and it was filled with polyamide (particle size = 50-160 μm, Fluka Analytica). The elution was performed with 500 mL of H_2O, 25% MeOH (v/v), 50% MeOH (v/v), 75% MeOH (v/v), and 100% MeOH (v/v). Ten fractions of 50 mL each were collected and monitored by HPLC. Fractions with similar compositions were combined. Only a few fractions were further passed through a third column to purify their components, helping their identification. The third column was 3.0 cm in diameter and 40 cm in height, and it was filled with reverse phase Amberchrom CG161 M (particle size = 75 μm, pore size = 150 Å, Rohm and Haas). The elution was performed with 100 mL of H_2O and 100 mL of 5% EtOH in water (v/v) increased from 5 to 100%, at 5% intervals, with 100 mL each. Ten fractions of 10 mL each were collected in a collector of fractions RadiFrac (Pharmacia Biotech). In each fraction, the different phenols were identified by HPLC-DAD and HPLC-MS, and they were quantified by HPLC-DAD.

Isolation of Compounds of Interest. Compounds of interest that are not commercially available were purified. The fractions containing these compounds were purified on silica gel preparative TLC (Merck 60F254) and eluted with a mixture of chloroform and methanol (8:2, v/v). The different bands were identified by their absorption at different wavelengths (visible light, 254 and 366 nm).

HPLC-DAD. The different phenols were quantified using a Hewlett- Packard 1100 liquid chromatography system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm i.d., 5 μm). The system was equipped with a diode array detector (DAD; the wavelengths used for quantification were 254, 280, and 340 nm) and Rheodyne injection valves (20 μL loop). The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile utilizing the following

gradient over a total run time of 55 min: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, and 95% A at 52 min until the run was completed. Quantification was carried out by integration of peaks at different wavelengths with reference to calibrations made using external standards. The linearity of standards curve was expressed in terms of the determination coefficients plots of the integrated peaks area versus concentration of the same standard. These equations were obtained over a wide concentration range in accordance with the levels of these compounds in the samples. The system was linear in all cases ($r > 0.99$). Three replicates on the same day were carried out.

HPLC-MS. The phenolic compounds present in the different fractions were identified by electron impact mass data collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters Inc., Manchester, U.K.). Electron spray ionization (ESI) mass spectra were obtained at ionization energies of 50 and 100 eV in negative mode and of 50 eV in positive mode; the capillary voltage was 3 kV, the desolvation temperature was 200 °C, the source temperature was 100 °C, and the extractor voltage was 12 V. The flow was maintained at 1 mL min^{-1} in split mode (UV detector MS) for each analysis. A Tracer Extrasil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm) (Teknokroma, Barcelona, Spain) was used. The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile utilizing the gradient used in HPLC-DAD.

Antiradical Activity: 2,2-Diphenyl-1-picrylhydrazyl (DPPH). Free radical-scavenging capacity was measured using the DPPH method described in a previous work.[12] The method is based on the measurement of the free radical-scavenging capacity of the antioxidant against the stable radical $DPPH^{\bullet}$. An iMark microplate absorbance reader model 550 (Bio-Rad, Hercules, CA) was used for the absorbance measurements. $DPPH^{\bullet}$ has an absorption band at 515 nm, which disappears upon reduction by antiradical compound. For each test compound and standard, the decrease in absorbance (expressed as a percentage of the initial absorbance) was plotted against the concentration of the antioxidant in the reaction mixture. The radical-scavenging capacity of each antioxidant was expressed as the EC_{50} (effective concentration, mg/mL), which is the amount of antioxidant necessary to decrease the initial absorbance by 50%, calculated from a calibration curve by linear regression for each antioxidant. The values of parameters for the regression analyses of the extracts were as follows: control ($y = -8.51x + 97.66$, $R^2 = 0.969$), 160 °C/15 min ($y = -32.25x + 102.78$, $R^2 = 0.993$), 160 °C/30 min ($y = -28.68x + 92.65$, $R^2 = 0.990$), 160 °C/ 45 min ($y = -22.08x + 74.45$, $R^2 = 0.990$), 160 °C/60 min ($y = -24.76x + 80.43$, $R^2 = 0.896$), 160 °C/75 min ($y = -24.76x + 76.34$, $R^2 = 0.985$), and 160 °C/90 min ($y = -26.64x + 95.95$, $R^2 = 0.988$).

Antiradical Activity: 2,2-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The ABTS assay was performed according to the method of Gonçalves et al.[13] This assay is based on the scavenging of ABTS radical ($ABTS^{\bullet+}$) by antioxidants compared to the antioxidant potency of Trolox that is used as standard (water-soluble α-tocopherol analogue). The concentration of ABTS radical was adjusted with ethanol 80% (v/v) to an initial absorbance of 0.700 ± 0.020 at 734 nm.

Aliquots of 13 µL of each phenolic extract, the standards (hydroxytyrosol (HT), 3,4-dyhydroxyphenyl glycol (DHPG), and vitamin E), and their dilutions were added to 187 µL of the $ABTS^{\bullet+}$ solution in a 96-well microplate in triplicate. For each sample, a blank with ethanol instead of $ABTS^{\bullet+}$ solution was included. A delay of 30 min was programmed into the reader before readings at 414, 655, and 750 nm. The results were expressed as the average of the ratios of the slopes of the lines obtained for each sample with Trolox calculated for each of these three wavelengths. The results were expressed in terms of Trolox equivalent antioxidant capacity (TEAC) in mg/mL. The values of parameters for the regression analyses of the extracts were as follows: control (y = 302.19x + 6.81, R^2 = 0.951), 160 °C/15 min (y = 727.07x + 6.22, R^2 = 0.991), 160 °C/30 min (y = 521.01x + 8.78, R^2 = 0.907), 160 °C/45 min (y = 705.56x + 3.88, R^2 = 0.992), 160 °C/60 min (y = 722.82x + 4.14, R^2 = 0.989), 160 °C/ 75 min (y = 589.39x + 7.90, R^2 = 0.964), and 160 °C/90 min (y = 721.13x + 4.30, R^2 = 0.992).

Reducing Power. The reducing power assay was carried out according to the procedure described in a previous work.[12] The antioxidative potentials of the extracts were estimated for their ability to reduce $FeCl_3$. All of the analyses were made using a microplate reader, and the absorbance was measured at 490 nm. The values of parameters for the regression analyses of the extracts were as follows: control (y = 0.13x + 0.02, R^2 = 0.994), 160 °C/15 min (y = 0.45x − 0.01, R^2 = 0.998), 160 °C/30 min (y = 0.35x − 0.01, R^2 = 0.990), 160 °C/45 min (y = 0.47x − 0.02, R^2 = 0.968), 160 °C/60 min (y = 0.50x − 0.01, R^2 = 0.995), 160 °C/75 min (y = 0.44x − 0.01, R^2 = 0.997), and 160 °C/ 90 min (y = 0.48x − 0.01, R^2 = 0.964). The assay was calibrated using Trolox. To express the results, a calibration curve was established by plotting A_{490} against known concentrations of Trolox (0.059-0.56 mg/mL). Reducing power (P_R) was expressed as Trolox equivalents (TE) in mg/mL from the equation as determined from linear regression:

$$P_R = 1932 \times A_{490} - 177.48$$

(correlation coefficient R^2 = 0.994)

Inhibition of Primary Oxidation. Evaluation of the inhibition of primary oxidation of lipid was based on the ferric thyocianate (FTC) method of Sánchez-Moreno et al.[14] with modifications. Twenty-five microliters of each fraction of phenolic extracts, reference compounds (HT, DHPG, and vitamin E) and their dilutions, 5 µL of 0.07 M 2,2′- azobis(2-amidinopropane) dihydrochloride (ABAP) (water-soluble free radical initiator), and 2 mL of 2.6 mM linoleic acid (LA) emulsion (0.1 M of SDS in aqueous solution of 0.01 M Na_2HPO_4, pH 7.4) were placed in different tubes in triplicate. For each sample, a blank without LA was included. The absorbance of LA without antioxidant solution was used as the standard for 100% oxidation. Test tubes were incubated at 50 °C for 15.5 h. After cooling at room temperature, 100 µL of 30% NH_4SCN in water and 100 µL of 0.02 M $FeCl_2$ in 3.5% HCl were added to each tube. Accumulation of hydroxyperoxides due to the oxidation of LA was measured at 490 nm. The results, calculated from the regression curves, are expressed as EC_{50} (mg/mL). The values of parameters for the regression analyses of the extracts were as follows: control (y = 81.71 − 35.46$\sqrt{x}$, R^2 = 0.886), 160 °C/15 min (y = 81.26 − 31.96$\sqrt{x}$, R^2 =

0.846), 160 °C/30 min (y = 89.52 − 36.96$\sqrt{x}$, R^2 = 0.897), 160 °C/45 min (y = 88.42 − 39.69$\sqrt{x}$, R^2 = 0.873), 160 °C/60 min (y = 93.89 − 41.28$\sqrt{x}$, R^2 = 0.886), 160 °C/75 min (y = 82.20 − 31.96$\sqrt{x}$, R^2 = 0.896), and 160 °C/90 min (y = 84.19 − 33.42$\sqrt{x}$, R^2 = 0.795).

Inhibition of Secondary Oxidation. Evaluation of the inhibition of secondary oxidation was based on the method of Moon and Shibamoto[15] with some modifications. This assay was based on thiobarbituric acid-reactive substances (TBARS) to measure the antioxidant ability of the tested samples with a lipid peroxidation inducer, using LA emulsion as lipid-rich media. Twenty-five microliters of each fraction of phenolic extracts, reference compounds (HT, DHPG, and vitamin E) and their dilutions, 5 µL of 0.07 M ABAP, and 1 mL of 5.2 mM LA emulsion (see primary oxidation) were placed in different tubes in triplicate. For each sample, a blank without LA was included. The absorbance of LA without antioxidant solution was used as the standard for 100% oxidation. Test tubes were vortexed and then incubated at 50 °C for 24 h. One hundred microliters of each reaction mixture was mixed with 100 µL of 2.8% (w/v) trichloroacetic acid in water and 100 µL of 0.8% thiobarbituric acid in 1.1% SDS. This mixture was vortexed and then heated at 90 °C for 20 min. After cooling at room temperature, 0.5 mL of 1-butanol was added, and the samples were stirred and centrifuged at 8050g for 3 min. The absorbance of the butanol layer was measured at 540 nm. The results are expressed as EC_{50} (mg/mL). The values of parameters for the regression analyses of the extracts were as follows: control (y = 87.54 − 28.01$\sqrt{x}$, R^2 = 0.940), 160 °C/15 min (y = 105.62 − 46.03$\sqrt{x}$, R^2 = 0.988), 160 °C/30 min (y = 99.11 − 42.89$\sqrt{x}$, R^2 = 0.984), 160 °C/ 45 min (y = 82.38 − 31.52$\sqrt{x}$, R^2 = 0.915), 160 °C/60 min (y = 77.90 − 28.46$\sqrt{x}$, R^2 = 0.876), 160 °C/75 min (y = 102.22 − 44.50$\sqrt{x}$, R^2 = 0.974), and 160 °C/90 min (y = 104.56 − 42.66$\sqrt{x}$, R^2 = 0.985).

Inhibition of Tyrosinase Activity. Inhibition was tested according to the method of Prasad et al.[16] with some modifications. Polyphenol oxidase (PPO) catalyzes two successive reactions involving molecular oxygen; the hydroxylation of monophenols leads to the formation of o-diphenols, followed by their subsequent oxidation into o-quinones, which are in turn polymerized into brown, red, or black pigments. The inhibition in enzyme activity is followed by a decrease in the formation of products that absorb radiation at 490 nm. The decrease in absorbance is indicative of a decrease in the product formed and hence a greater inhibition of enzyme activity.

The mushroom tyrosinase used for this bioassay was purchased by Sigma (St. Louis, MO) and was used without further purification. Tyrosinase functions as both a monophenolase and an o-diphenolase. In the current experiment, L-tyrosine (monophenolase assay) was used as a substrate. For each sample, a blank without L-tyrosine was included. The absorbance without a sample solution was used as the standard for 100% oxidation. Fifty microliters of 5 mM L-tyrosine was added to 100 µL of phosphate buffer (0.05 M, pH 6.6) in a 96-well microplate. After 10 min of incubation at 30 °C, 50 µL of the sample solution and 50 µL of the aqueous solution of the

mushroom tyrosinase (50 units/mL) were added to the mixture solution. The absorbance at 490 nm was recorded every 20 s for 15 min using the microplate reader. For the inhibition of tyrosinase activity, a time point of 300 s was fixed. Percent of anti-tyrosinase activity was calculated using the following formula: ((optic density (OD) of blank − OD of sample)/OD of blank) × 100.

Statistical Analysis. Statgraphics Plus software was used for statistical analysis. Correlation coefficients were determined using regression analysis at the 95% confidence level. Comparisons among samples were made with one-way analysis of variance (ANOVA) and the LSD method at the same confidence level.

Result and discussion

Hydrothermal Treatment. The study of the hydrothermal processing of alperujo was undertaken at temperature of 160 °C for treatment times of 15, 30, 45, 60, 75, and 90 min. This process led to a solution that contained a high quantity of compounds that are easily solubilized (including carbohydrates, organic acid, phenols, polyphenols) or that are formed from thermal degradation (including hydroxymethylfurfural).

As can be seen from **Table 1**, different amounts of alperujo were introduced into the reactor, providing different volumes of liquid fractions. Ten litters of each liquid fraction was collected and concentrated to 1 L. The extraction of phenols was carried out with ethyl acetate. The amounts of phenolic extract obtained per kilogram of fresh alperujo are shown in **Table 1** for different treatments and the control.

Table 1. Yield of Phenolic Extracts and Total Phenolic Content Obtained by Hydrothermal Treatment of Alperujo at 160 °C for Increasing Treatment Time and Comparison with an Untreated Control.

		Hydrothermal treatment at 160ºC					
control	0 min	15 min	30 min	45 min	60 min	75 min	90 min
amount of alperujo treated (kg)	2.7	10	10	20	20	20	10
liquid volume fraction (L)	9.4	16.5	21	42	51	63	29
g of phenolic extract/kg fresh alperujo	5.8	4.1	7	8.3	7.1	11.2	9.1
total phenolic content (gallic acid equivalent (g/kg fresh alperujo))	0.65	1.62	2.03	3.06	1.74	3.43	2.21

The amount of phenolic extract increased with increasing treatment time, reaching a maximum at 75 min of 11.2 g/kg fresh alperujo that doubles the amount found in the control. At 90 min, the amount of phenolic extract was slightly lower than the maximum, possibly due to degradation or polymerization reactions as a result of such prolonged treatment.

Three groups of results were obtained. In the first group (the control and treatments of 15 min), the amount of extract was 4-5 g/kg of fresh alperujo; in the second group (treatments of 30, 45, and 60 min), the amount of extract was 7-8 g/kg; and in the third group (treatments of 75 and 90 min), the amount of extract was 9-11 g/kg.

Fractionation and Characterization of the Phenolic Extracts. The extracts, which are a highly diverse and complex mixture of phenolic compounds (**Figure 1**), need to be fractionated with different resins to purify their components for further identification (**Figure 2**). The preliminary identification of phenolic compounds present in the phenolic extracts (control and treatments at 160 °C of 30, 60, and 90 min) was performed by HPLC-DAD; the relative retention times and UV spectra were compared against standard solutions. The final identification was made by HPLC-MS; for those compounds that lacked pure standards, identification was based on the search for pseudomolecular $[M-H]^-$ ions, using ion mass chromatograms, together with the interpretation of collision-induced dissociation (CID) fragments. When standards were available, identification was carried out by comparing retention times and mass spectra with those standards.

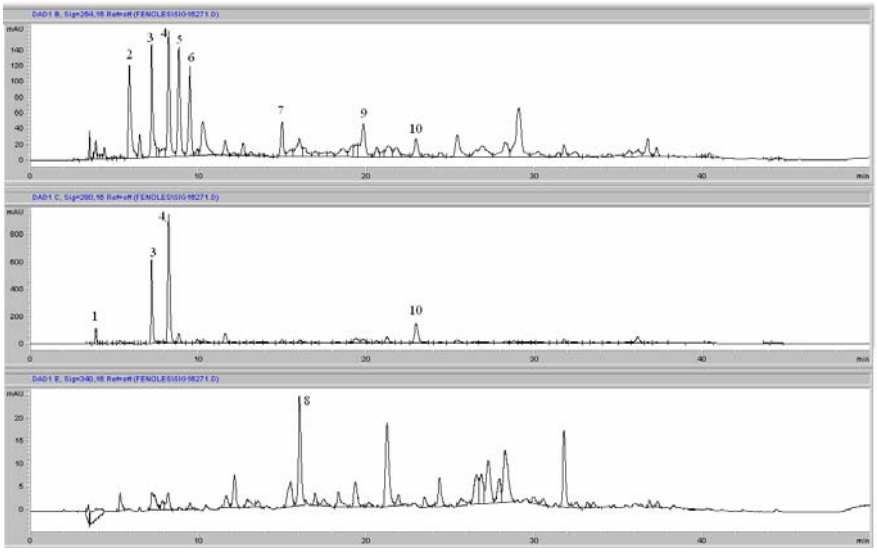


Figure 1. Representative HPLC-DAD chromatograms (at 254, 280, and 340 nm) of phenolic extracts obtained from steam-treated alperujo at 160 °C for 60 min. Identified compounds: (1) 3,4-dihydroxyphenylglycol; (2) unknown; (3) hydroxymethylfurfural; (4) hydroxytyrosol; (5) protocatechuic acid; (6) elenoic acid derivative; (7) vanillic acid; (8) caffeic acid; (9) elenoic acid derivative; (10) hydroxytyrosol acetate

Figure 2. Flow diagram of the fractionation protocol followed for the ethyl acetate extracts from the liquid phase generated during hydrothermal treatment of alperujo.

The concentrations of phenolic compounds in the extracts were calculated by comparing HPLC-DAD peak areas with the corresponding external standards (**Table 2**). The HPLC-DAD equipment was calibrated using external standards for those species not available commercially but proceeded to their isolation.

Table 2. Wavelength of the Maximum Absorption, Concentration Range of Quantification, and Regression Equation of the Calibration Curve for Standard Compounds Identified in Extracts.

compounds	λ quantification (nm)	concentration range (mg/mL)	Lineal regression[a]	R^{2b}
3.4-dihydroxyphenylglycol	280	0.4-0.01	y = 16415 x + 49.663	0.9998
hydroxytyrosol	280	0.63-0.0315	y = 14298 x - 54.05	0.9991
tyrosol	280	1-0.01	y = 12520 x + 120.95	0.9993
vanillin	280	0.5-0.05	y = 72734 x - 125.89	0.9997
4-methylcatechol	280	2.5-0.1	y = 19637x - 1060.7	0.9989
hydroxymethilfurfural	280	0.1-0.005	y = 145360 x + 52.662	0.9996
3.4-dihydroxyphenylacetic acid	280	5-0.2	y = 16433 x - 391.64	0.9996
protocatechuic acid	254	0.25-0.005	y = 63627 x + 125.13	0.9987
caffeic acid	340	1-0.05	y = 65738 x + 60.021	0.9972
4-hydroxybenzoic acid	254	0.2-0.005	y = 109834 x + 79.529	0.9998
p-coumaric acid	280	0.2-0.01	y = 91146 x + 5.75	0.9996
chlorogenic acid	340	2.5-0.25	y = 41912 x + 49.776	0.9992
syringic acid	280	5-0.5	y = 40581 x - 1265.6	0.999
vanillic acid	254	0.2-0.005	y = 65622 x - 22.725	0.9988
oleuropein	280	0.75-0.05	y = 48117 x + 437.26	0.9941
elenoic acid derivative A	254	4.4-0.176	y = 2517.4 x + 5.9867	0.999
elenoic acid derivative B	254	10.7-0.535	y = 2538.1 x- 152.17	0.9939
luteolin-7-O-Glucoside	340	0.1-0.005	y = 352117 x - 556.12	0.9978
hydroxytytrosol acetate	280	4.5-0.45	y = 11161 x - 1397.1	0.9985
1-phenil-6.7-dihydroxyisochroman	280	4.5-0.225	y = 8271.9 x - 40.043	0.9981
verbascoside	280	0.1-0.01	y = 19632 x - 16.416	0.9995
comsegoloside	280	2.68-0.134	y = 15167 x + 461.21	0.9993

[a]y, peak area of standard compound; x, mg standard compound. [b]R, correlation coefficient.

The results obtained for the different extracts are shown in **Tables 3** and **4**. The hydrothermal treatment caused a significant increase in the concentration of phenolic alcohols. This is mainly due to increased concentrations of hydroxytyrosol and 3,4-dihydroxyphenylglycol in free form. The treatments caused the rupture of more complex molecules that contain simple phenols, and the treatments enhanced their solubilization. [17] Tyrosol, which is found in olive seeds, also increased in concentration because the treatment caused the rupture of complex molecules containing tyrosol and their solubilization.[7]

Increased concentrations of hydroxymethylfurfural were obtained for longer treatment times. This species has not been previously described in alperujo. It formed in the reactor due to the degradation of hexoses during the treatment.

Phenolic acids increased slightly in concentration with respect to the control. In the extracts obtained after 30 and 60 min of treatment, protocatechuic acid was obtained in high concentrations; after 90 min, chlorogenic acid and 3,4-dihydroxyphenylacetic acid were obtained in high concentrations.

The concentration of oleuropein decreased over time due to its breakdown into simpler molecules. The concentration of demethyloleuropein, which comes from the degradation of

oleuropein by loss of a methyl group, a process favored in the treatment, increased. Overall, the extract obtained after 30 min of treatment contained the highest concentrations of derivatives of oleuropein. This may be because the shorter treatment favors the solubilization of these compounds without degrading them.

Elenoic acid derivatives decreased in concentration, compared with the control, with increasing temperature, probably because the treatment promotes the breakdown of elenoic acid into simpler molecules.

The treatment also favours the formation of demethylligstroside, as happened with the oleuropein and demethyloleuropein.

In the extract were also identified two lignans, 1-acetoxypinoresinol (**Figure 3a**) and pinoresinol, which were not present in the extract control. These species were identified in the oil due to their liposoluble natures.[18] These molecules have been described in alperujo only in one recent paper,[19] probably due to their low concentration. These compounds can be used as antioxidative, antiviral, antibacterial, insecticidal, and fungicidal agents.[20]

The treatments promoted the formation of 1-phenyl-6,7- dihydroxyisochroman (**Figure 3b**). This molecule is part of the phenolic composition of olive oil, and it is formed spontaneously during storage of the oil by the reaction of benzaldehyde and hydroxytyrosol, with oleic acid acting as a catalyst.[21] The high pressure and temperature used together with the abundant presence of free hydroxytyrosol and the aldehyde formation in acid medium make possible the synthesis of the species during the treatment. This molecule acts as an inhibitor of platelet aggregation and helps prevent cardiovascular disease.[8]

The concentration of comsegoloside decreased compared with the control with regard to the treatment time because the molecule breaks down into simple structures such as caffeic acid.

In addition, the ethyl acetate extraction process after the hydrothermal treatment favors the formation of a polymeric phenolic fraction, the concentration of which increases with increasing treatment time. This fraction has the ability to absorb hydroxytyrosol. The results of research on this new polymeric phenolic fraction will be shown in a forthcoming paper.

Table 3. Phenolic Compounds Identified in Different Extracts Obtained (Control and Treatments at 160 °C for 30, 60, and 90 min) from Alperujo by HPLC-DAD and HPLC-DAD-MS.

Compound	retention time (min)	mol wt	λ max.	m/z -	ref
phenolic alcohol					
3.4-dihydroxyphenylglycol	4.3	170	214, 234 and 278	169	10
hydroxytyrosol	8.9	154	214, 234 and 278	153 and 123	22
tyrosol	11.7	138	200, 218 and 276	137	23
vanillin	17.8	152	225 and 320	151	22
4-methylcatechol	19.9	124	236		10
hydroxytytrosol acetate	23.1	196	214, 234 and 278	195	
degradation product of sugar					
hydroxymethylfurfural	7.3	126	194,228 and 284		
phenolic acid					
3.4-dihydroxyphenylacetic acid	10.2	168	214, 234 and 278	151,123, 109 and 59	10
protocatechuic acid	8.3	154	206, 218, 260 and 294	109 and 45	10
caffeic acid	16.2	180	202, 218, 240 and 324	179, 163, 135 and 45	24
4-hydroxybenzoic acid	14.4	138	194 and 256		10
protocatechuic acid derivative	8.7	154	206, 218, 260 and 294	109 and 45	10
p-coumaric acid	20.5	164	194, 210, 226 and 310	119	19
p-coumaric acid derivative	13.4	536	230 and 310	205, 145 and 117	25
chlorogenic acid	15.2	354	200 and 345	247, 163, 135, 133 and 109	
syringic acid	14.5	198	218 and 276		10
vanillic acid	14.1	168	200, 218, 255 and 298		22
oleuropein derivative					
oleuropein aglycone hemiacetal	17.6	352	198, 224 and 275	707 [2M-H], 351, 137 and 119	26
	29.2	340	214, 234 and 278	539, 337,201, 157 and 139	
oleuropein derivative	36	538	214, 234 and 278	537, 403, 361, 223, 151 and 123	
	36.4	556	214, 234 and 278	555, 381, 245, 201 and 183	
oleuropein	30.7	540	198, 232 and 282	539, 377, 307, 275 and 223	23
demethyloleuropein	17.3	526	242	525, 509, 389, 243, 211, 181, 137 and 123	27
secologanoside	10.4	390	206, 218, 260 and 294	389, 345, 167, 123 and 108	10
oleuropein aglycone derivative	32.6	378	200, 222 and 280	377, 307, 275, 149 and 139	10
10-hydroxymethyloleuropein	29.3	556	214, 234 and 278	555, 223, 151 and 123	28
caffeoyl-6'-O-secologanoside	27.1	552	198 and 328	551, 507, 389, 281, 251, 179 and 161	10
6'-O-[(2E)-2.6-dimethyl-8-hydroxy-2-octenoyloxy]secologanoside	37.6	558	214, 234 and 278	557, 539, 345, 167	28
elenoic acid derivatives					
elenoic acid derivative A	8.5		230	453, 423 and 241	10
elenoic acid derivative C	21.2		242	243, 211 and 151	29
elenoic acid derivative B	30.1		210 and 264	241 and 251	10
ligstroside derivatives					
ligstroside	32.3	524	220, 224 and 280	523, 361, 291 and 259	24
demethylligstroside	20.4	510	250	509 (m/z +: 511, 211 and 181)	27
flavonoids					
luteolin-7-O-Rutinoside	26.6	594	200, 254 and 349	593, 447, 285 and 151	30
apigenin-7-O-Rutinoside	25.7	578	212 and 264	577, 371 and 269	30
luteolin-7-O-Glucoside	26.4	448	206, 256, 266 and 350	447 and 285	30
cianidine-3-O-Rutinoside	29.1	596	200, 225 and 280	(m/z +: 597, 521, 405, 345 and 137)	30
lignans					
1-acetoxypinoresinol	38.6	416	200, 230 and 280	415, 151 and 123	18.19
pinoresinol	37.9	357	200, 225 and 280	357, 151 and 123	18.19
Others					
1-phenil-6.7-dihydroxyisochroman	10.8	242	200, 230 and 280	241 and 136	10
verbascoside	24.5	624	198 and 328	623, 461, 161	30
comsegoloside	32.1	536	192, 230 and 312	535, 205 and 145	10
nüzhenide	28.9	686	240 and 280	685, 523, 299 and 223	30
3-hydroxymethyl-2.3-dihydro-5-(methoxycarbonyl)-2-methyl-2H-pyran-4-acetic methyl ester	29.1	210	198, 232 and 282	257, 210, 151 and 123	28

Table 4. Concentrations[a] of Identified Phenolic Compounds in the Extracts from Hydrothermally Treated Alperujo and Control Untreated by HPLC-DAD

	mg /Kg fresh alperujo			
compound	phenolic extract untreat	160ºC/30	160ºC/60	160ºC/90
3.4-dihydroxyphenylglycol [b]	24.80±0.41	123.44±4.07	70.19±0.45	71.63±3.53
hydroxytyrosol [b]	15.73±0.39	1624.83±6.05	776.53±14.68	1127.53±5.43
tyrosol [b]	14.97±1.22	108.68±1.37	80.62±2.11	167.70±5.97
vanillin [b]	n.d.	0.09±0.01	n.d	11.12±0.04
4-methylcatechol [b]	n.d.	1.92±0.01	n.d	n.d
hydroxytyrosol acetate [b]	n.d.	154.66±0.92	60.13±1.27	174.93±1.09
total phenolic alcohol	**55.50±2.02**	**1858.96±11.51**	**927.34±17.24**	**1377.98±14.97**
hydroxymethilfurfural[b]	n.d.	1.32±0.02	4.67±0.15	21.29±0.53
degradation product of sugar	**n.d.**	**1.32±0.02**	**4.67±0.15**	**21.29±0.53**
3.4-dihydroxyphenylacetic acid [b]	n.d.	15.56±0.38	n.d	19.58±0.46
protocatechuic acid [b]	9.17±0.04	24.47±0.46	18.35±0.58	2.74±0.02
caffeic acid [b]	n.d.	7.01±0.21	2.65±0.03	0.96±0.05
4-hydroxybenzoic acid [b]	0.40±0.06	1.65±0.02	1.44±0.01	1.07±0.02
protocatechuic acid derivative [c]	n.d.	5.14±0.07	n.d	n.d
p-coumaric acid [b]	2.93±0.04	n.d	1.49±0.02	n.d
p-coumaric acid derivative [c]	n.d.	n.d	0.05±0.01	n.d
chlorogenic acid [b]	n.d.	n.d	n.d	19.52±0.17
syringic acid [b]	n.d.	n.d	n.d	1.78±0.10
vanillic acid [b]	3.36±0.09	n.d	5.01±0.05	2.09±0.03
total phenolic acid	**15.86±0.23**	**53.83±1.14**	**28.99±0.70**	**47.74±0.85**
oleuripein aglycone hemiacetal [d]	n.d	n.d	0.02±0.01	n.d
oleuropein derivative [d]	n.d	8.36±0.13	1.39±0.01	n.d
oleuropein [b]	2.74±0.01	0.92±0.02	0.46±0.01	n.d
demethyloleuropein [d]	n.d	n.d	n.d	14.50±0.38
secologanoside [f]	n.d	7.36±0.06	n.d	n.d
oleuropein aglycone derivative [d]	n.d	n.d	0.20±0.01	n.d
10-hydroxymethyloleuropein [d]	n.d	4.27±0.12	n.d	n.d
caffeoyl-6'-O-secologanoside [h]	n.d	8.75±0.48	n.d	0.81±0.06
6'-O-[(2E)-2.6-dimethyl-8-hydroxy-2-octenoyloxy]secologanoside [d]	n.d	2.14±0.06	n.d	n.d
total Oleuropiein derivative	**2.74±0.01**	**31.80±0.87**	**2.07±0.04**	**15.31±0.44**
elenoic acid derivative A [e]	1339.95±22.03	664.38±6.59	191.29±1.17	527.40±5.59
elenoic acid derivative C [f]	n.d	294.47±3.90	123.56±0.08	n.d
elenoic acid derivative B [e]	n.d	31.79±0.33	0.57±0.01	9.38±0.45
elenoic acid derivative	**1339.95±22.03**	**990.64±10.82**	**315.42±1.26**	**536.78±6.04**
ligstroside [d]	n.d	n.d	0.04±0.01	n.d
demethylligstroside [d]	n.d	18.71±0.61	2.20±0.01	111.39±0.68
ligstroside derivatives	**n.d**	**18.71±0.61**	**2.24±0.02**	**111.39±0.68**
luteolin-7-O-Rutinoside [g]	n.d	1.33±0.01	0.07±0.01	0.12±0.01
apigenin-7-O-Rutinoside [g]	n.d	n.d	0.18±0.01	n.d
luteolin-7-O-Glucoside [d]	3.69±0.07	n.d	0.58±0.01	n.d
cianidina-3-O-Rurioside [g]	n.d	0.06±0.01	n.d	n.d
flavonoids	**3.69±0.07**	**1.39±0.02**	**0.83±0.03**	**0.12±0.01**
1-phenil-6.7-dihydroxyisochroman [e]	n.d	5.09±0.05	39.92±0.08	21.05±0.03
verbascoside [b]	n.d	2.02±0.08	8.43±0.08	n.d
comsegoloside [e]	184.42±3.47	58.94±0.24	22.68±0.24	5.12±0.16
nüzhenide [d]	n.d	2.43±0.06	n.d	n.d
3-hydroxymethyl-2.3-dihydro-5-(methoxycarbonyl)-2-methyl-2H-pyran-4-acetic methyl ester [f]	n.d	207.18±13.70	n.d	n.d
others	**184.42±3.47**	**275.66±14.85**	**71.03±0.40**	**26.17±0.19**
polymeric phenolic fraction[i] (PPF)	**n.d**	**515.82±13.47**	**77.66±4.98**	**1286.41±14.72**

[a]Mean ± SD (standard deviation) of three determinations. [b]Compounds were identified and quantified with commercial standards. [c]Compounds were quantified with a calibration of protocatechuic acid and p-coumaric acid. [d]Compounds were quantified with a calibration of oleuropein. [e]Compounds were quantified with isolated compounds. [f]Compounds were quantified with a calibration of elenoic acid derivative C. [g]Compounds were quantified with a calibration of luteolin-7-O-glucoside. [h]Compounds were quantified with a calibration of comsegoloside. [i]PPF was calculated by gravimetrically from the fraction eluted in the polyamide column with 50% of methanol in water.

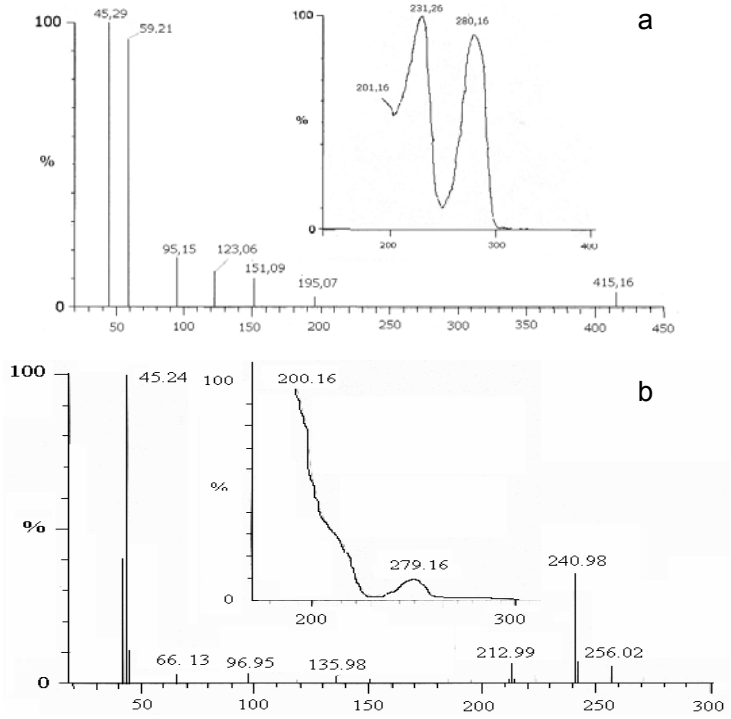


Figure 3. Mass spectra in negative ionization mode m/z^- and absorption (nm) spectra of 1-acetoxypinoresinol (**a**) and 1-phenyl-6,7- dihydroxyisochroman (**b**).

Antiradical Activity and Reducing Power. Statistical analyses of the results show that the dose response adjusted to a linear model for three of the assays, with $R^2 \geq 0.98$ (data not shown).

Individual regression equations were used to calculate the antioxidative capacities of each sample. The values for each phenolic extract obtained after the different treatments (160 °C for 15, 30, 45, 60, 75, and 90 min) were compared to the values for the phenolic extract control (untreated) and to the values of species that have potent antioxidant activity, such as HT, DHPG, and vitamin E. Antiradical activity was evaluated with radicals from two different sources, ABTS and DPPH (**Table 5**). Antiradical activity against the DPPH radical is expressed as EC_{50}, and antiradical activity against the ABTS radical is expressed as TEAC. The results were similar in both cases; all of the phenolic extracts obtained after the treatments had activities similar to that of vitamin E, somewhat lower in the case of DPPH assay, and to each other, and all had higher activities than the control extract.

The results for reducing power (**Table 5**) are expressed as Trolox equivalents in mg/mL; high concentrations indicate high activity. In this case, all of the phenolic extracts obtained after treatments had similar activities, and all were more effective reducing agents than vitamin E. Thus, the hydrothermal treatment promotes the solubilization/ formation of compounds that increase antiradical activity against the two types of radicals studied, ABTS and DPPH, also increasing reducing power.

Table 5. Radical Scavenging Capacities (DPPH and ABTS Radicals) and Reducing Power of Extracts Obtained from Hydrothermally Treated Alperujo, Control Untreated, and Standards (HT, DHPG, and Vitamin E)[a]

	antiradical activity		reducing power
	DPPH (EC_{50})	ABTS (TEAC)	Trolox equiv (mg/mL)
control	5.59	0.22	0.15
160 ºC/15 min	1.64	0.53	0.44
160 ºC/30 min	1.50	0.46	0.34
160 ºC/45 min	1.11	0.51	0.48
160 ºC/60 min	1.23	0.54	0.49
160 ºC/75 min	1.06	0.42	0.42
160 ºC/90 min	1.72	0.51	0.49
HT	0.11	1.53	1.60
DHFG	0.17	0.99	1.96
vitamin E	0.83	0.50	0.15

[a]The results are expressed as EC_{50} (mg/mL), TEAC (mg/mL), and Trolox equivalent (mg/mL), respectively. The tests were performed in triplicate.

Inhibition of Lipid Oxidation. In the primary oxidation assay, the accumulation of hydroxyperoxide in the oxidation of linoleic acid was measured. These were unlikely to display antiradical activity or reducing power, and the regression analyses of the dose-response lines confirmed that they were described not by a lineal model but by a "square root of x" ($y = a + bx^{-2}$), with $R^2 \geq 0.91$. In the secondary oxidation assay, all of the samples are described by a "square root of x" regression model ($R^2 \geq 0.94$). The results of both assays are expressed as EC_{50} (**Table 6**). The capacities of all extracts to inhibit the primary oxidation were 1.1-1.9-fold higher than the control and showed less activity than HT, DHPG, and vitamin E. The results obtained for the inhibition of secondary oxidation showed that the extracts had values similar to the control and lower than the reference standards.

Table 6. Inhibition of Primary and Secondary Oxidation by the Different Extracts Obtained from Hydrothermally Treated Alperujo, Control Untreated, and Standards (HT, DHPG, and Vitamin E) Expressed as EC_{50} [a].

	EC_{50} (mg/mL)	
	primary oxidation	secondary oxidation
Control	1.80	1.04
160 ºC/15 min	1.46	0.93
160 ºC/30 min	1.31	1.19
160 ºC/45 min	1.06	0.93
160 ºC/60 min	0.96	0.87
160 ºC/75 min	1.38	1.07
160 ºC/90 min	1.64	1.12
HT	0.68	0.07
DHFG	0.83	0.03
vitamin E	0.52	0.23

[a]The tests were performed in triplicate.

Theoretical Activity of HT and DHPG in Phenol Extracts. The theoretical contribution of HT and DHPG to reducing power, antiradical activity (ABTS), and inhibition of primary and secondary oxidation was calculated on the basis of the concentrations of free-form HT and DHPG in each of the extracts. These values were compared with the experimental values. The concentrations (in mg/mL) of HT and DHPG present in 1 mg/mL of extract were determined, and an estimate of activity for these concentrations was established from the graphs obtained for standards (HT and DHPG) related to the activity with a given concentration in milligrams per millilitre of HT and DHPG. The sum of the activities obtained for HT and DHPG provides theoretical activity (theor) without considering possible synergistic or antagonistic effects. This value was compared with the activity provided by 1 mg/mL of each extract, giving the experimental value (exptl). The theoretical and experimental results were compared (**Table 7**).

Table 7. Combined Action of HT and DHPG: Comparison of Experimental and Theoretical Values for the Different Antioxidant Assays

		control	160 ºC / 30 min	160 ºC / 60 min	160 ºC / 90 min
composition (%)	HT	0.27	28.08	10.93	9.58
	DHFG	0.43	1.75	0.99	0.79
reducing power (Trolox equivalent, mg/mL)	HT (theor)	-	0.30	0.13	0.12
	DHFG (theor)	-	-	-	-
	HT+DHFG (theor)	-	0.30	0.13	0.12
	extract (exptl)	0.14	0.34	0.49	0.50
antiradical activity, DPPH (% inhibition oxidation)	HT (theor)	-	79.53	53.34	45.84
	DHFG (theor)	0.59	4.39	2.20	1.63
	HT+DHFG (theor)	0.59	83.92	55.54	47.50
	extract (exptl)	10.86	35.98	44.33	30.69
inhibition primary oxidation (% inhibition oxidation)	HT (theor)	2.06	28.55	19.28	17.97
	DHFG (theor)	14.86	17.61	16.25	15.82
	HT+DHFG (theor)	16.92	46.16	35.53	33.79
	extract (exptl)	40.47	43.78	50.56	38.09
inhibition secondary oxidation (% inhibition oxidation)	HT (theor)	11.15	88.07	61.17	57.38
	DHFG (theor)	40.92	43.16	41.87	41.53
	HT+DHFG (theor)	52.07	100	100	98.91
	extract (exptl)	53.77	47.45	47.4	49.23

The results show that the extracts at 60 and 90 min had more reducing power than the individual standards, as the experimental Trolox equivalents are higher than the theoretical ones. This indicates that there are species in the extract that may promote this activity, and/or there is a synergistic effect between HT, DHPG, and other components. The theoretical values for HT and DHPG for antiradical activity (DPPH) of the three extracts were higher than the experimental values, but not for the case of the control. This may be due to antagonistic effects between the HT, DHPG, and other phenolic species present in the extract, which lowered the activity. For primary oxidation, the data show that the extracts, including control, had a greater ability to inhibit primary

oxidation than the standards (theor), except in the extract obtained at 30 min. Therefore, some species in the extracts promote this activity, and/or there is a synergistic effect between HT, DHPG, and other components. Finally, values from the inhibition of secondary oxidation showed that both HT and DHF (theor) were similar to the experimental value in the case of extract control but had higher capacities for inhibition of secondary oxidation than in the case of the extracts. This may be due to possible antagonistic effects between the HT, DHPG, and other phenolic species present in the extracts.

Inhibition of Tyrosinase Activity. Tyrosinase activity (monophenolmono-xygenase, EC 1.14.18.1) was measured using L-tyrosine as the substrate. All phenolic extracts obtained after hydrothermal treatment showed similar inhibition capacities after 300 s (**Figure 4**) in a concentration-dependent manner (**Table 8**), and all were higher than that of the control. This untreated extract control did not inhibit tyrosinase at the concentrations tested. From a concentration of 0.75 mg/mL of extract the activity was enhanced. The same test was conducted to determine the ability to inhibit the enzyme by the HT. To obtain results similar to those of the treated extracts, the HT concentration must be at least 316 mg/mL. Therefore, the phenolic extracts hydrothermally treated were more effective at inhibiting tyrosinase activity than HT. However, kojic acid, a reference inhibitor, showed a strong tyrosinase inhibitory activity with 50% inhibition for 0.01 mg/mL.

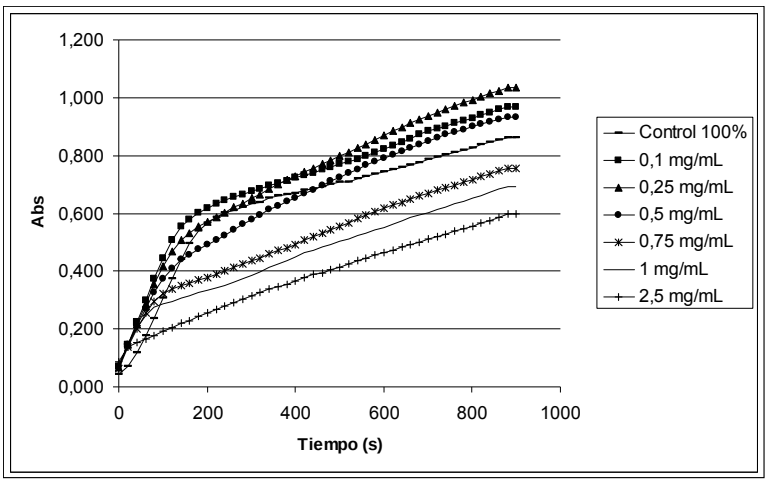


Figure 4. Progress curves for the inhibition of mushroom tyrosinase by different concentrations of phenolic extract (dissolved in a solution of H_2O and MeOH (8:2)) obtained after treatment at 160 °C for 60 min. All analyses were run in quadruplicate, and the average values are presented, the coefficient of variation being <10% in all cases.

Table 8. Tyrosinase Inhibitory Activity of Extracts Isolated from Hydrothermally Treated Alperujo and Untreated Control Using L-Tyrosine as a Substrate

	tyrosinase inhibitory activity (%)		
	0.75 mg/mL	1 mg/mL	2.5 mg/mL
control	0	0	0
160 ºC/15 min	25.7	38.1	47.3
160 ºC/30 min	11.1	23.9	41.9
160 ºC/45 min	31.6	36.5	45.1
160 ºC/60 min	31.3	39.0	49.9
160 ºC/75 min	30.1	39.0	46.8
160 ºC/90 min	15.0	21.8	27.2

General Comments. The results of the present work showed liquors obtained from a new hydrothermal process of alperujo and extracted with ethyl acetate that have higher phenolic content and stronger antioxidant/free radical-scavenging and antityrosinase activities than the control obtained with conventional extraction method. In addition, the great diversity and complexity of the natural mixtures of phenolic compound in the extracts was indicated. This thermal treatment promotes the solubilization and/or formation of new compounds not present in untreated alperujo, such as 1-acetoxypinoresinol, pinoresinol, and 1-phenyl-6,7-dihydroxyisochroman, that contribute significantly to the health benefits associated with the consumption of virgin olive oils. Of all treatments and compounds determined, hydroxytyrosol was found in the highest concentration, and it was mainly responsible for the *in vitro* antioxidant activity of the extracts. However, antagonist or synergistic interaction between phenolic components of extracts should be considered. In addition, the positive results prove this low-cost procedure could be an alternative to the conventional extraction method for obtaining antioxidant phenolic extracts from alperujo. These antioxidants could be used as food additives or for applications in the pharmaceutical and cosmetic industries, revaluing the byproduct of virgin olive oil processing. However, the biological properties of these compounds in vivo will depend on the extent to which they are absorbed and metabolized in the gastrointestinal tract and even the interaction with other food components.

Funding

F.R.-S. and G.R.-G. thank the Consejo Superior de Investigaciones Cientif́ icas (CSIC) of Spain for their research contracts (JAE Pre_2010_00425 and JAE Doc_2009_00666).

This work was partially financed by Ministerio de Ciencia e Innovación of Spain (Grant AGL2009-12352) and Consejería de Innovación, Ciencia y Empresa de la Junta de Andalusí (Proyecto de Excelencia P08-AGR-03751).

References

(1) Aragón, J. M.; Palancar, M. C. Improlive 2000. Present and Future of Alpeorujo; Ed.ial Complutense: Madrid, Spain, 2001; pp 242−300.

(2) Pérez-Jiménez, F. International conference on the healthy effect of virgin olive oil. Eur. J. Clin. Invest. 2005, 35, 421−424.

(3) Gimeno, E.; Fitó, M.; Lamuela-Raventós, R. M.; Castellote, A. I.; Covas, M.; Farré, M.; de la Torre-Boronat, M. C.; López- Sabater, M. C. Effect of ingestion of virgin olive oil on human low-density lipoprotein composition. Eur. J. Clin. Nutr. 2002, 56, 114−120.

(4) Tuck, K. L.; Hayball, P. J. Major phenolic compounds in olive oil: metabolism and health effects. J. Nutr. Biochem. 2002, 13, 636− 644.

(5) Beauchamp, G. K.; Keast, R. S. J.; Morel, D.; Lin, J.; Pika, J.; Han, Q.; Lee, C.-H.; Smith, A. B. III; Breslin, P. A. S. Ibuprofen-like activity in extra-virgin olive oil: enzymes in an inflammation pathway are inhibited by oleocanthal, a component of olive oil. Nature 2005, 437, 45−46.

(6) Fernández-Bolaños, J.; Felizón, B.; Brenes, M.; Guillén, R.; Heredia, A. Hydroxytyrosol and tyrosol as the main compounds found in the phenolic fraction of steam-exploded olive stones. J. Am. Oil Chem. Soc. 1998, 75, 1−7.

(7) Romero, C.; Brenes, M.; García, P.; Garrido, A. Hydroxytyrosol- 4-β-D-glucoside, an important phenolic compound in olive fruits and derived products. J. Agric. Food Chem. 2002, 50, 3835−3839.

(8) Togna, G. I.; Togna, A. R.; Franconi, M.; Marra, C.; Guiso, M. Olive oil isochromans inhibit human platelet reactivity. J. Nutr. 2003, 133, 2532−2536.

(9) Fernández-Bolaños Guzmán, J.; Heredia Moreno, A.; Rodríguez Gutiérrez, G.; Rodríguez Arcos, R.; Jiménez Araujo, A.; Guillén Bejarano, R. Method for obtaining purified hydroxytyrosol from products and by-products derived from the olive tree.U.S. Patent 6,849,770 B2, 2005.

(10) Obied, H. K.; Bedgood, D. R. Jr.; Prenzler, P. D.; Robards, K. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. Anal. Chim. Acta 2007, 603, 176−189.

(11) Singleton, V. L.; Rossi, J. A. Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 1965, 16, 144−158.

(12) Rodríguez, R.; Jaramillo, S.; Rodríguez, G.; Espejo, J. A.; Guillén, R.; Fernández-Bolaños, J.; Heredia, A.; Jiménez, A. Antioxidant activity of ethanolic extracts from several asparagus cultivars. J. Agric. Food Chem. 2005, 53, 5212−5217.

(13) Goncçalves, B.; Falco, V.; Moutinho-Pereira, J. M.; Bacelar, E.; Peixoto, F.; Correia, C. Effects of elevated CO2 on grapevine (Vitis vinifera L.): Volatile composition, phenolic content, and *in vitro* antioxidant activity of red wine. J. Agric. Food Chem. 2009, 57, 265− 273.

(14) Sánchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. Food Res. Int. 1999, 32, 407−412.

(15) Moon, J. K.; Shibamoto, T. Antioxidant assays for plant and food components. J. Agric. Food Chem. 2009, 57, 1655−1666.

(16) Prasad, K. N.; Yang, B.; Shi, J.; Yu, Ch.; Zhao, M.; Xue, S.; Jiang, Y. Enhanced antioxidant and antityrosinase activities of longan fruit pericarp by ultra-high-pressure-assisted extraction. J. Pharm. Biomed. Anal. 2010, 51, 471−477.

(17) Fernández-Bolaños, J.; Rodríguez, G.; Rodríguez, R.; Heredia, A.; Guillén, R.; Jiménez, A. Production in large quantities of highly purified hydroxytyrosol from liquid-solid waste of two-phase olive oil processing or “alperujo”. J. Agric. Food Chem. 2002, 50, 6804− 5811.

(18) Brenes, M.; Hidalgo, F. J.; García, A.; Rios, J. J.; García, P.; Zamora, R.; Garrido, A. Pinoresinol and 1 Acetoxypinoresinol, two new phenolic compounds identified in olive oil. J. Am. Oil Chem. Soc. 2000, 77, 715−720.

(19) Suárez, M.; Romero, M. P.; Ramo, T.; Macià, A.; Motilva, M. J. Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. J. Agric. Food Chem. 2009, 57, 1463− 1472.

(20) Maldini, M.; Montoro, P.; Piacente, S.; Pizza, C. Phenolic compounds from Bursera simaruba Sarg. bark: phytochemical investigation and quantitative analysis by tandem mass spectrometry. Phytochemistry 2009, 70, 641−649.

(21) Guiso, M.; Marra, C.; Arcos, R. R. An investigation on dihydroxy-isochromans in extra virgin olive oil. Nat. Prod. Res. 2008, 22, 1403−1409.

(22) Suárez, M.; Macià, A.; Romero, M. P.; Motilva, M. J. Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. J. Chromatogr., A 2008, 1214, 90−99.

(23) De la Torre-Carbot, K.; Jauregui, O.; Gimeno, E.; Castellote, A. I.; Lamuela-Raventós, R. M.; López-Sabater, M. C. Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. J. Agric. Food Chem. 2005, 53, 4331−4340.

(24) Savarese, M.; De Marco, E.; Sacchi, R. Characterization of phenolic extracts from olives (Olea europaea cv. Pisciottana) by electrospray ionization mass spectrometry. Food Chem. 2007, 105, 761−770.

(25) Romero, C.; García, P.; Brenes, M.; García, A.; Garrido, A. Phenolic compounds in natural black Spanish olive varieties. Eur. Food Res. Technol. 2002, 215, 489−496.

(26) De Nino, A.; Mazzotti, F.; Perri, E.; Procopio, A.; Raffaelli, A.; Sindona, G. Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from Carolea and Coratina cultivars by ionspray ionization tandem mass spectrometry. J. Mass Spectrom. 2000, 35, 461−467.

(27) Sivakumar, G.; Bati, C. B.; Uccella, N. HPLC-MS screening of the antioxidant profile of Italian olive cultivars. Chem. Nat. Compd. 2005, 41, 588−591.

(28) Obied, H. K.; Prenzler, P. D.; Ryan, D.; Servili, M.; Taticchi, A.; Esposto, S.; Robards, K. Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from Olea europaea L. Nat. Prod. Rep. 2008, 25, 1167−1179.

(29) De Nino, A.; Mazzotti, F.; Morrone, S. P.; Perri, E.; Raffaelli, A.; Sindona, G. Characterization of Cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. J. Mass Spectrom. 1999, 34, 10−16.

(30) Ryan, D.; Antolovich, M.; Herlt, T.; Prenzler, P. D.; Lavee, S.; Robards, K. Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. J. Agric. Food Chem. 2002, 50, 6716−6724.

6.1.2. Capítulo 2.

Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

LWT- Food Science and Technology, 2013, 54, 114-124.

Muchos estudios epidemiológicos muestran que la dieta mediterránea protege contra una amplia variedad de enfermedades patológicas crónicas, incluyendo la enfermedad coronaria del corazón, el cáncer y las enfermedades neurodegenerativas. Este efecto se puede atribuir a muchos componentes de la dieta, incluyendo los fenoles que exhiben actividad de captación de radicales libres y protegen al organismos contra el daño oxidativo. Sin embargo, después de la extracción de aceite de oliva, sólo un pequeño porcentaje (1-2%) de los fenoles totales presentes en el fruto de la oliva permanecen en el aceite, mientras que el resto queda remanente en el alperujo (subproducto generado por el sistema de extracción de dos fases) que puede ser utilizado como una fuente prometedora de compuestos fenólicos.

Para extraer los fenoles con un elevado rendimiento desde el alperujo se realiza un tratamiento térmico que provoca un proceso autohidrolítico (PCT/ES2011/070583). El objetivo de este estudio fue fraccionar el extracto fenólico aislado con acetato de etilo después del tratamiento hidrotérmico del alperujo a 160 ºC durante 60 min, para evaluar la actividad antioxidante de cada fracción y poder relacionarla con la composición de cada una de las fracciones.

El presente estudio demostró que entre todos los compuestos detectados, el hidroxitirosol es el que se encuentra en mayor concentración seguido de los derivados del ácido elenólico producidos durante el tratamiento térmico. Se observó que había una serie de fracciones cuyo comportamiento en todos los ensayos antioxidantes era bastante bueno, estas fracciones se encontraban enriquecidas en DHFG, hidroxitirosol, tirosol, ácido protocatéquico, verbascósido, 4-hidroxibenzoico, ácido vainíllico, acetato de hidroxitirosilo, la fracción fenólica polimérica, comsegolosido o/y ácido p-cumárico. En el caso de la inhibición de la oxidación primaria se observa un efecto sinérgico entre el ácido protocatéquico con el ácido vainíllico y con el ácido p-hidroxibenzoico. Mientras que las fracciones que mostraron una menor capacidad de inhibición de la oxidación se encontraban enriquecidas en hidroximetilfurfural y/o derivados del ácido elenólico.

Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity

Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños

ABSTRACT

Alperujo or olive oil waste was hydrothermally treated at 160 ºC for 60 min to increase the phenols in the liquid phase. The extract obtained from the liquid using ethyl acetate extraction was divided into 27 phenolic fractions using adsorbent and polyamide resins. Phenolic alcohols and acids, secoiridoid molecules, elenolic acid derivatives, flavonoids, verbascoside, degradation products of sugar and a polymeric phenolic fraction (PPF) were characterized using HPLC-ESI-MS. The antiradical activity, ferric reducing power and the inhibition of primary and secondary oxidation were examined for each fraction. Hydroxytyrosol was the most abundant phenol in the ethyl acetate extract and possibly the main component responsible for the *in vitro* antioxidant activity of the entire phenol extract. However, other phenolic and secoiridoid molecules with interesting biological properties were also identified, and the crude extract could be considered a potential source of natural antioxidants.

KEYWORDS: Alperujo, Phenols, 3,4-Dihydroxyphenylglycol, Hidroxitirosol, Antioxidant

1. Introduction

Many epidemiological studies show that the Mediterranean diet provides protection against a wide array of common chronic pathological conditions, including coronary heart disease, cancer and neurodegenerative disorders (Pérez-Jiménez, 2005). This effect may be attributed to many components of the diet, including the phenols that exhibit free radical scavenging activity and protect organisms against oxidative damage (Covas et al., 2006). Thus, the daily consumption of olive oil, rich in phenolic compounds improves health by reducing oxidative damage to the human body. However, after olive oil extraction, only a small percentage (1-2%) of the total phenols present in the olive fruit remain in the oil, with the majority remaining in the olive mill wastes like alperujo (two phase extraction system) that may be used as a promising source of these phenolic compounds.

To increase the concentration of these phenols and to extract them in high yield from alperujo, an environmentally friendly process based on hydrothermal treatment in which an autohydrolytic process occurs has been developed (PCT/ES2011/070583). In a previous study, phenols were selectively extracted from the autohydrolysis liquid using ethyl acetate, yielding extracts with antioxidant activity levels comparable to vitamin E (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012).

The aim of this study was to determine the phenolic composition of the ethyl acetate extract obtained after hydrothermal treatment of the alperujo at 160 ºC for 60 min. Fractionation of the extract is used to facilitate the characterization and identification of the main phenolic and secoiridoid components. A second purpose of this study was to assess the antioxidant activity of each fraction to determine the relative contribution of the various compounds to the above activities for the entire phenolic extract. This study will help to evaluate the use of this extract as a potential source of natural antioxidants.

2. Materials and methods

2.1. Materials

The sample of alperujo was obtained in March 2009 from Picual olives processed at a Spanish oil mill (Instituto de la Grasa, Seville).

2.2. Standard compounds

Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, caffeic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol and 3,4-dihydroxyphenylacetic acid were obtained from Fluka (Buchs, Switzerland). Oleuropein, verbascoside and luteolin-7-O-glucoside were obtained from Extrasynthese (Lyon Nord, Geney, France). (±)-α-Tocopherol ≥95% purchased from Sigma-Aldrich was used as reference compound. Hydroxytyrosol was obtained using the method described by Fernández-Bolaños et al. (2002).

2.3. Thermal treatment

In total, 20 kg of alperujo was treated for 60 min at a fixed temperature of 160 ºC in the patented reactor (100 L). Then, the wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain), obtaining 51 L of liquid, and an aliquot of 10 L was collected for the experiment and was concentrated to 1 L.

2.4. Determination of the total phenolic content

The phenolic content was measured according to the Folin-Ciocalteu method and expressed as grams of gallic acid equivalents per kilogram of fresh alperujo (Singleton, & Rossi, 1965).

2.5. Phenol extraction

The liquid portions (1 L) obtained after treatment were washed with hexane (500 mL) to remove the lipid fraction. The mixturewas shaken vigorously and the phases were then separated by decantation and washed twice (De Marco, Savarese, Paduano, & Sacchi, 2007).

Phenolic compounds were extracted with ethyl acetate (500 mL per 200 mL of sample) using a continuous extraction at 77 ºC for 8 h. The organic phase was rotary-evaporated under vacuum at 30 ºC.

2.6. Chromatographic fractionation of the ethyl acetate extract (EtOAc extract)

Approximately 12 g of EtOAc extract was dissolved in 60 mL of H_2O:MeOH (80:20). The extract was passed through an open column of 3.5 cm in diameter and 40 cm in height packed with the non-ionic resin Amberlite XAD16 (Vivaqua, Spain). The elution was performed using 1 L of H_2O, EtOH 30% (v/v), EtOH 50% (v/v) and EtOH 95% (v/v); ten fractions of 100 mL each were collected and analyzed using HPLC, and those with similar compositions were mixed, concentrated to 50 mL and passed through a second column. This second column was 3.5 cm in diameter and 45 cm in height and filled with polyamide (particle size = 50-160 µm, Fluka Analytica). The elution was performed using 500 mL of H_2O, MeOH 25% (v/v), MeOH 50% (v/v), MeOH 75% (v/v) and MeOH 100% (v/v). Ten fractions of 50 mL each were collected and monitored using HPLC. Fractions with similar composition were combined (**Fig. 1**). In each fraction, the phenols were identified using HPLC-DAD and HPLC-DAD-MS, and quantified using HPLC-DAD.

2.7. Isolation of compounds of interest

The compounds that are not commercially available were purified on a silica gel preparative TLC (Merck 60F254) and eluted with a mixture of chloroform and methanol 8:2 (v/v). The different bands were identified by their absorption at 254 nm and 366 nm.

2.8. HPLC-DAD

Quantitative evaluation of phenols content was carried out as described by Rubio-Senent et al. (2012). Quantification was performed in triplicate using external standards. Standards curve were linear in all cases ($R^2 > 0.99$).

2.9. HPLC-DAD-MS

The phenolic compounds were separated by HPLC as described above and identified by the electrospray ionization mass data collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters Inc.; Manchester, U.K.). Electrospray ionization (ESI) mass spectra were obtained at ionization energies of 50 eV and 100 eV in negative mode and 50 eV in positive mode; the capillary voltage was 3 kV, the desolvation temperature was 200 ºC, the source temperature was 100 ºC, and the extractor voltage was 12 V. The flow was maintained at 1 mL/min in split mode (UV detector MS) for each analysis.

2.10. Antiradical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical-scavenging capacity was measured using the DPPH method described in a previous study (Rodríguez et al., 2005) and expressed as EC_{50} (effective concentration, mg/mL), calculated from a calibration curve using linear regression for each antioxidant.

2.11. Antiradical activity: 2,20-azino-bis(3-ethylbenzothiazoline-6- sulphonic acid) (ABTS)

The ABTS assay was performed according to the method of Gonçalves et al. (2009), with some modifications described in a previous work (Rubio-Senent et al., 2012). The results were expressed in terms of the Trolox equivalent antioxidant capacity (TEAC) in mg/mL.

2.12. Reducing power

The reducing power assay was performed according to the procedure described in a previous study (Rodríguez et al., 2005). The assay was calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed in mg/mL TE (Trolox equivalent). To express the results, a calibration curve was created by plotting A_{490} against the known concentration of Trolox (0.059-0.56 mg/mL) [correlation coefficient (R) = 0.9936].

2.13. Inhibition of primary oxidation

The method is based on the ferric thiocyanate (Sánchez-Moreno, Larrauri, & Saura-Calixto, 1999) with some modifications described in a previous work (Rubio-Senent et al., 2012). The accumulation of hydroxyperoxides due to the oxidation of linoleic acid was measured at 490 nm. The results are expressed as EC_{50} (mg/mL).

2.14. Inhibition of secondary oxidation

The evaluation of the inhibition of secondary oxidation was based on thiobarbituric acid method of Sánchez-Moreno et al. (1999) with modifications. This assay was based on

thiobarbituric acid-reactive substances (TBARS) to measure the antioxidant ability of the tested samples with a lipid peroxidation inducer. The results are expressed as EC_{50} (mg/mL).

2.15. Statistical analysis

Three replicates were performed for each assay. STATGRAPHICS® Plus software was used for statistical analysis. The correlation coefficients were determined using regression analysis at the 95% confidence level. Comparisons among samples were made with one-way analysis of variance (ANOVA) and the LSD method at the same confidence level.

3. Results and discussion

3.1. Hydrothermal treatment and extraction with ethyl acetate

We have previously characterized the antioxidant extracts recovered with ethyl acetate from autohydrolysis liquids of alperujo steam-treated at a fixed temperature of 160 ºC for 15-90 min. The results indicated that the phenol extracts were effective antioxidants to a significantly greater extent than the untreated control and comparable or better than α-tocopherol (Rubio-Senent et al., 2012). The present study was performed to further characterize the antioxidants present in the most active extract, which corresponds to alperujo treated at 160 ºC/60 min and extracted for 8 h with ethyl acetate. The yield of the phenol extract was 7.1 g/kg of fresh alperujo with a total phenolic content of 1.74 g gallic acid equivalent/kg of fresh alperujo. In an initial approach a fractionation method was used for characterization (**Fig. 1**) and the antioxidant activity was evaluated for each fraction.

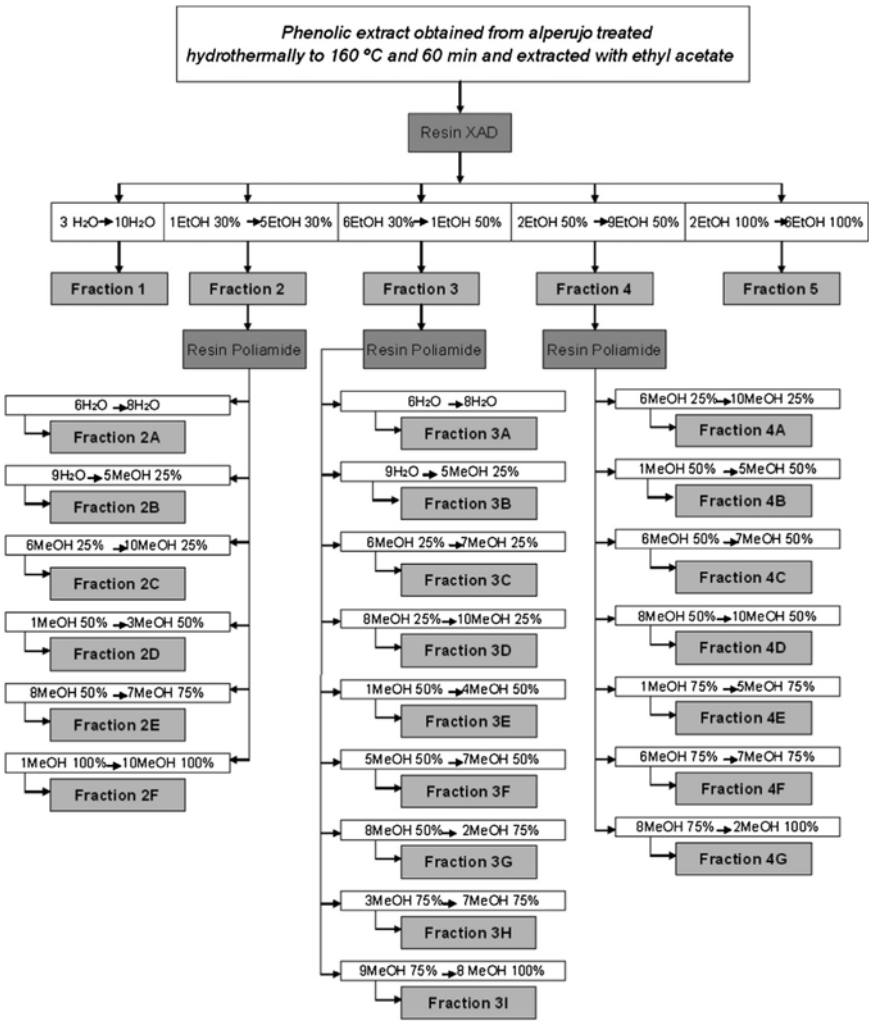


Fig. 1. Steps of fractioning of ethyl acetate extract. The fractions corresponding to the elution through the fist resin Amberlite XAD with one liter of H_2O, EtOH 30% (v/v), EtOH 50% (v/v) and EtOH 95% (v/v) divided in ten fractions of 100 mL each one, and elution through the second resin polyamide with 500 mL of H_2O, MeOH 25% (v/v), MeOH 50% (v/v), MeOH 75% (v/v) and MeOH 100% (v/v) divided in ten fractions of 50 mL each one. The intervals of the eluted fractions and collected fractions were specified.

3.2. Fractionation and characterization of the phenolic extract

Fractionation of the ethyl acetate extract was performed using a system easy to scale up for industries that consist of resin Amberlite XAD-16 and further desorption with water and followed by increasing the concentration of ethanol. Five fractions (1-5) were collected, which were characterized by HPLC analysis on a C_{18} reverse column. **Fig. 2** showed the absorbance profiles of the 5 fractions obtained after fractioned the ethyl acetate extract through the XAD resin to two different wavelengths; 280 nm, where the phenolic compounds absorbed, and 254 nm, where secoiridoids derivatives absorbed. In the first fraction, a very polar compound was identified as 3,4-dihydroxyphenylglycol (DHPG), which was present almost exclusively (Fraction 1). The fractions 2-4 had were further fractionated in a polyamide resin. Six (2A-2F), nine (3A-3I) and seven fractions (4A-4G) were collected from subfractions 2, 3 and 4 respectively.

Fraction 5 was eluted with 100% ethanol and represents a new polymeric phenol fraction (PPF) constituted by several unseparated peaks (30-48 min).

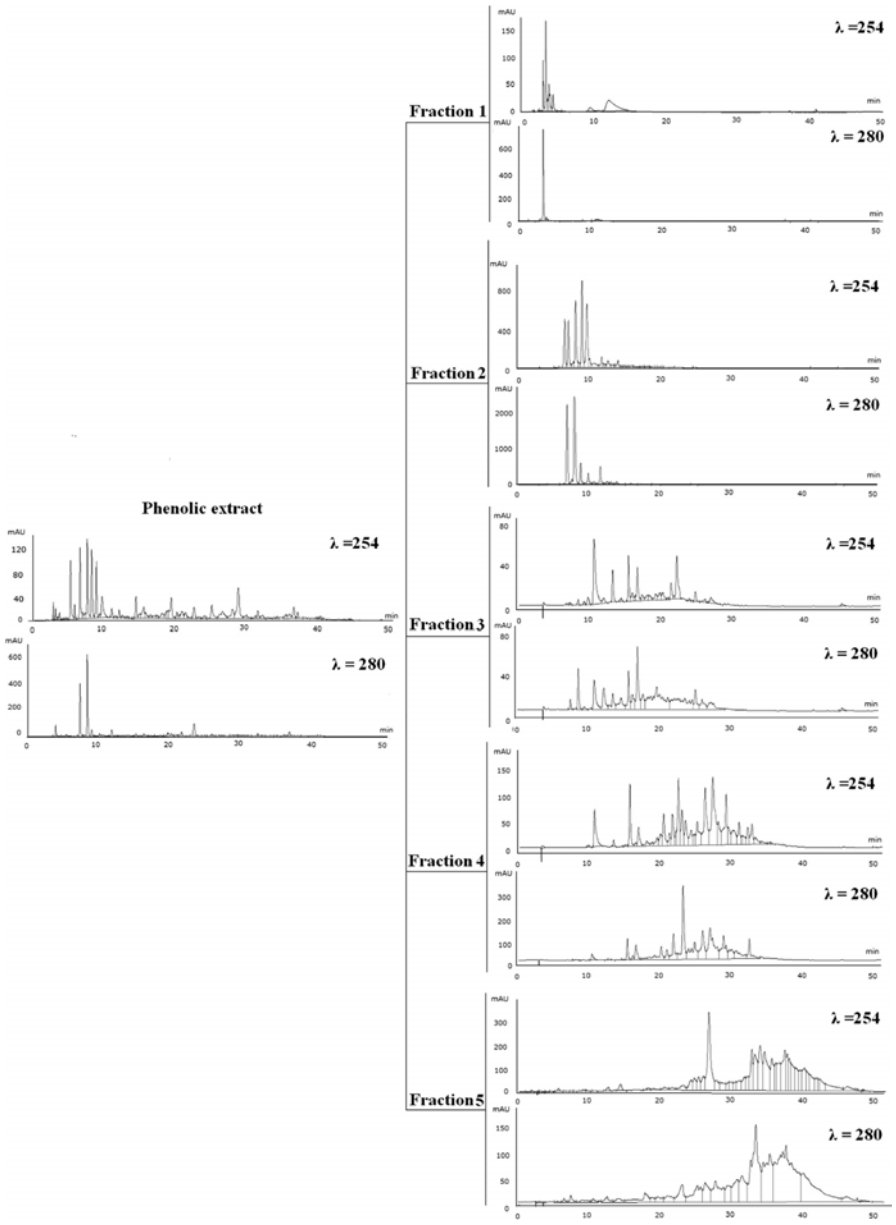


Fig. 2. HPLC-DAD chromatographic profile at 254 and 280 nm of the ethyl acetate extract collected from steam-treated alperujo and the different fractions obtained after the first step using the XAD resin.

The identification of the phenols in each fraction was performed using HPLC-DAD-MS, the retention time and UV spectra (**Table 1**). **Table 2** summarizes the concentrations of the phenolic compounds of each of the fractions and the mg dry extract/mL of the fraction. The analysis of the ethyl acetate extract demonstrated the wide structural diversity of the compounds (**Fig. 3**).

Table 1
Compounds identified in the acetate ethyl extract obtained after the hydrothermal treatments and ethyl acetate extraction.

Compounds	Retention time (min)	Molecular weight	λ_{max} (nm).	*m/z* (mode negative)	mg /kg fresh alperujo	Reference
3,4-Dihydroxyphenylglycol [a]	4.3	170	214, 234 and 278	169	70.19±0.45	Obied et al., 2007
Hydroxymethylfurfural[a]	7.3	126	194,228 and 284		4.67±0.15	--
Protocatechuic acid [a]	8.3	154	206, 218, 260 and 294	109 and 45	18.35±0.58	Obied et al., 2007
Elenolic acid derivative A[b]	8.5		230	423, 241, 197 and 137	191.29±1.17	Obied et al., 2007
Hydroxytyrosol[a]	8.9	154	214, 234 and 278	153 and 123	776.53±14.68	Suárez, Macià, Romero & Motilva, 2008
Tyrosol[a]	11.7	138	200, 218 and 276	137	80.62±2.11	De la Torre-Carbot et al., 2005
p-Coumaric acid derivative[c]	13.4	536	230 and 310	205, 145 and 117	0.05±0.01	Romero et al., 2002
Vanillic acid[a]	14.1	168	200, 218, 255 and 298		5.01±0.05	Suárez et al., 2008
4-Hydroxybenzoic acid [a]	14.4	138	194 and 256		1.44±0.01	Obied et al., 2007
Caffeic acid [a]	16.2	180	202, 218, 240 and 324	179, 163, 135 and 45	2.65±0.03	Savarese, De Marco & Sacchi , 2007
Oleuropein aglycone hemiacetal [d]	17.6	352	198, 224 and 275	707 [2M-H], 351, 137 and 119	0.02±0.01	De Nino et al., 2000
p-Coumaric acid [a]	20.5	164	194, 210, 226 and 310	119	1.49±0.02	Suárez, Rometo, Ramo, Macià & Motilva, 2009
Elenolic acid derivative C[b]	21.2		242	243, 211 and 151 in mode positive	123.56±0.08	De Nino et al., 1999
Hydroxytyrosol acetate[b]	23.1	196	214, 234 and 278	195	60.13±1.27	
Verbascoside[a]	24.5	624	198 and 328	623, 461 and 161	8.43±0.08	Savarese et al., 2007
Apigenin-7-O-Rutinoside[e]	25.7	578	212, 264 and 350	577, 371 and 269	0.07±0.01	Ryan et al., 2002
Luteolin-7-O-Glucoside[a]	26.4	448	206, 256, 266 and 350	447 and 285	0.18±0.01	Ryan et al., 2002
Luteolin-7-O-Rutinoside[e]	26.6	594	200, 254 and 349	593, 447, 285 and 151	0.58±0.01	Ryan et al., 2002
Elenolic acid derivative B[f]	30.1		210 and 264	241 and 251	0.57±0.01	Obied et al., 2007
Oleuropein[a]	30.7	540	198, 232 and 282	539, 377, 307, 275 and 223	0.46±0.01	Fu, Segura-Carretero et al., 2009
Comsegoloside[b]	32.1	536	192, 230 and 312	535, 205 and 145	22.68±0.24	Obied et al., 2007b
Ligstroside [d]	32.3	524	220, 224 and 280	523, 361, 291 and 259	0.04±0.01	De la Torre-Carbot et al., 2005
Oleuropein aglycone derivative[d]	32.6	378	200, 222 and 280	377, 307, 275, and 149	0.20±0.01	Fu, Arráez-Román, Menéndez, Segura-Carretero 6 Fernández-Gutiérrez., 2009
Oleuropein derivative[d]	36	538	214, 234 and 278	211, 139 and 123	1.39±0.01	Obied et al., 2007
1-Phenyl-6,7-dihydroxyisochroman	38.1	242	200, 230 and 280	241, 211 and 136	n.q.[g]	Bianco et al., 2001
Polymeric phenolic fraction[h]	30-48				77.66	

[a] The compounds were identified and quantified by corresponding commercial standards.
[b] The compounds were identified with isolated compounds.
[c] The compounds were quantified with calibration of p-coumaric acid.
[d] The compounds were quantified with calibration of oleuropein.
[e] The compounds were quantified with calibration of luteolin-7-O-rutinoside.
[f] The compounds were quantified with calibration of elenolic acid derivative A.
[g] Not quantified.
[h] Quantified by evaporation to dryness.

Table 2
Composition of each of the fractions that compose the acetate ethyl extract obtained after treating alperujo at 160 ºC for 60 min. The nature of the compounds present in each fraction and their concentration in µg/mL are specified.

Fraction	mg/mL (Extract dry)	mg/mL (Total phenol)	Compound	µg Compound/mL fraction	% Individual compound
1	14.24±0.25	2.01±0.18	3,4-Dihydroxyphenylglycol	2457.38±14.83	100
2A	15.17±0.65	2.23±0.13	3,4-Dihydroxyphenylglycol	74.44±0.66	7.99
			Hydroxymethylfurfural	93.02±3.02	9.98
			Hydroxytyrosol	99.88±6.69	10.72
			Tyrosol	664.21±3.84	71.3
2B	44.81±2.32	36.75±1.71	3,4-Dihydroxyphenylglycol	284.16±1.44	0.91
			Hydroxymethylfurfural	53.51±2.32	0.17
			Hydroxytyrosol	29890.67±519.13	96.14
			Tyrosol	861.22±9.27	2.77
2C	2.02±0.02	1.01±0.01	Protocatechuic acid	534.62±21.94	100
3A	7.92±0.57	0.47±0.01	Hydroxymethylfurfural	38.75±0.44	100
3B	15.02±0.65	0.94±0.09	Hydroxymethylfurfural	2.95±0.01	0.08
			Hydroxytyrosol	9.55±0.06	0.26
			Elenolic acid derivative A	3639.06±2.93	99.64
			Oleuropein aglycone hemiacetal	0.76±0.04	0.02
3C	7.32±0.25	3.72±0.07	Hydroxytyrosol	888.20±54.84	29.98
			Elenolic acid derivative A	582.15±9.39	19.65
			Tyrosol	1489.29±66.26	50.27
3D	6.79±0.05	3.45±0.03	Hydroxytyrosol	51.95±2.01	1.76
			p-Coumaric acid derivative	1.96±0.18	0.07
			Tyrosol	224.51±1.26	7.6
			Elenolic acid derivative A	2631.37±29.13	89.03
			1-Phenyl-6,7-dihydroxyisochroman	-	-
			Verbascoside	45.88±0.08	1.55
3E	2.24±0.15	0.82±0.01	Protocatechuic acid	53.51±0.35	6.5
			4-Hydroxybenzoic acid	46.91±0.22	5.7
			Elenolic acid derivative A	625.75±1.35	76
			Luteolin-7-O-Rutinoside	2.91±0.03	0.35
			Vanillic acid	94.31±0.46	11.45
3F	1.32±0.08	0.31±0.01	Protocatechuic acid	150.14±0.84	93.37
			4-Hydroxybenzoic acid	10.66±0.03	6.63
3G	1.82±0.02	0.18±0.01	Caffeic acid	106.86±1.32	100
4A	9.27±0.02	0.19±0.05	3,4-Dihydroxyphenylglycol	5.45±0.66	2.34
			Hydroxytyrosol	12.28±0.01	5.28
			Tyrosol	2.78±0.45	1.19
			Elenolic acid derivative A	211.81±5.03	91.18
4B	4.58±0.25	0.5±0.01	Hydroxytyrosol	78.82±0.24	1.54
			Elenolic acid derivative C	4967.18±32.34	97.36
			Oleuropein derivative	56.11±0.25	1.1
4C	4.56±0.15	3.26±0.29	Hydroxytyrosol	78.82±1.26	3.33
			Hydroxytyrosol acetate	2260.07±50.67	95.48
			Oleuropein	18.55±0.52	0.78
			Ligstroside	1.71±0.56	0.07
			Oleuropein aglycone derivative	7.87±1.14	0.33
4D	1.37±0.08	0.41±0.01	Hydroxytyrosol	64.10±2.44	26.68
			Elenolic acid derivative B	23.19±0.59	9.65
			Hydroxytyrosol acetate	153.01±0.42	63.67
4E	3.78±0.95	0.85±0.02	Polymeric phenolic fraction (PPF)		100
4F	3.65±0.45	1.86±0.16	Hydroxytyrosol	42.64±3.91	9.47
			Vanillic acid	107.21±1.80	23.82
			Apigenin-7-O-Rutinoside	7.19±0.22	1.6
			Verbascoside	293.09±3.19	65.11
4G	2.67±0.18	0.55±0.02	p-Coumaric acid	60.17±0.96	6.04
			Luteolin-7-O-glucoside	23.29±0.07	2.34
			Comsegoloside	911.89±6.62	91.61
5			Polymeric phenolic fraction (PPF)		100

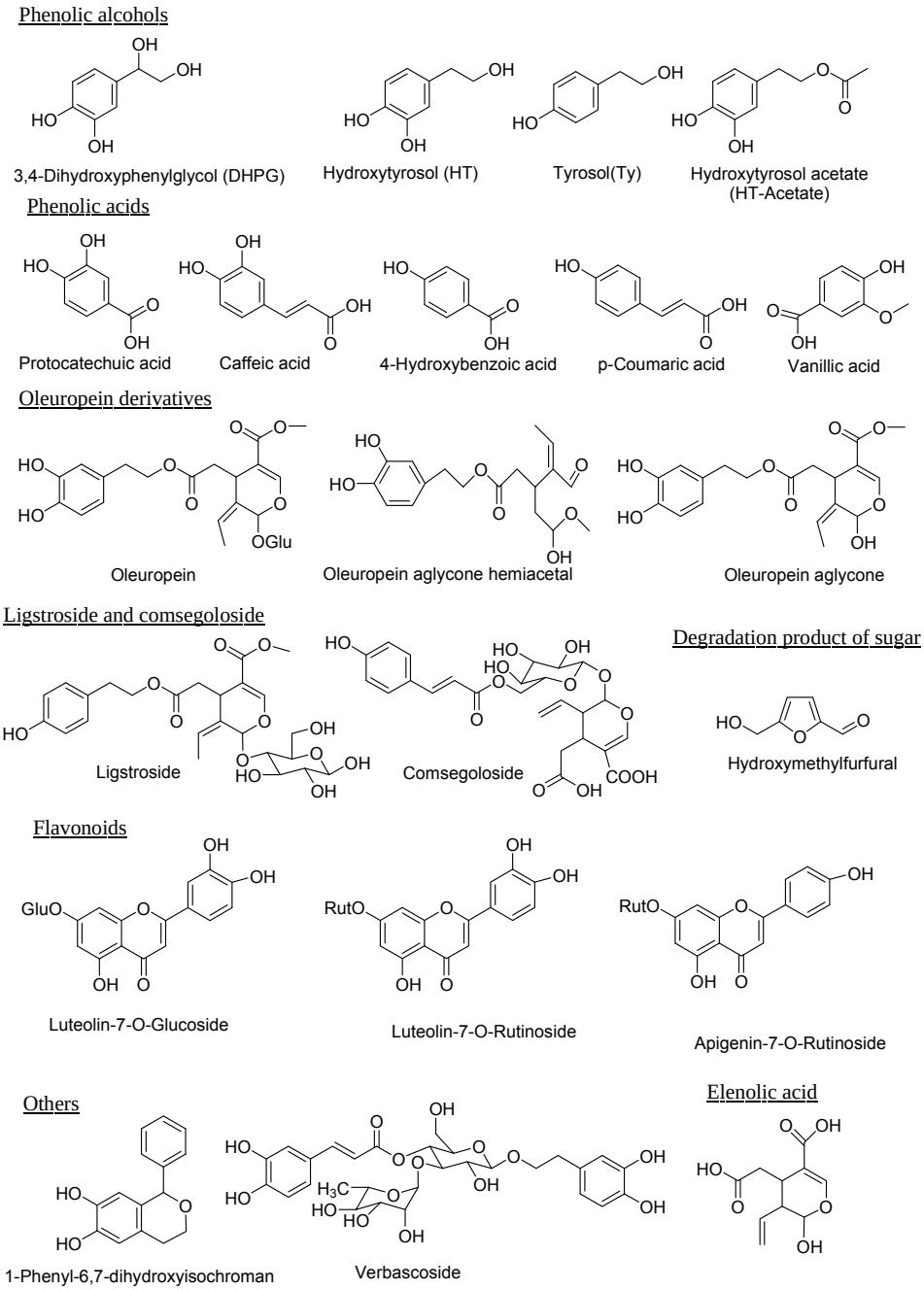


Fig. 3. Structures of the species identified.

Among the phenolic alcohols detected, hydroxytyrosol was the main compound. The thermal treatments caused the rupture of the more complex molecule that contained simple phenols and enhanced their solubilization (Fernández-Bolaños et al., 2002). Hydroxytyrosol has been associated with important antioxidant, anti-inflammatory, antiproliferative and antifungal activities (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010). Other phenolic alcohol compounds such as DHPG, tyrosol and hydroxytyrosol acetate were also detected.

Phenolic acid and oleuropein family compounds were also identified in the extract. The concentration of the oleuropein and oleuropein derivatives also is low, possibly because the treatment promotes the breakdown of oleuropein into simpler molecules.

Three elenolic acid derivatives were tentatively identified in the extract being their spectral characteristics and *m/z* values coincident with those given in the literature (Obied, Bedgood, Prenzler, & Robards, 2007). The first, termed A (Rt = 8.5 min), is characterized by a maximum absorbance in the UV-vis spectrum at 230 nm. In its mass spectrum, a possible molecular ion is present at *m/z* 423 that has not been identified; however, its fragmentation in negative mode produces a profile of fragments associated with the elenolic acid structure, with the presence of the fragment at *m/z* 241 [elenolic acid-H]$^-$ and the fragments at *m/z* 197 [elenolic acid-COO]$^-$ and *m/z* 137 [197-$COOCH_3$]$^-$. This same signal at *m/z* 241 was identified in the mass spectrum of the derivative B (Rt = 30.1 min); this derivative is present in a minor amount and is characterized by the presence of absorbance maxima at 210 and 264 nm. The derivative C (Rt = 21.2 min) has a UV-vis spectrum characteristic of elenolic derivatives, with a maximum absorbance at 242 nm. This molecule produces fragmentation, in positive mode, with a profile signal similar to elenolic acid, as described by De Nino et al. (1999), with fragments at *m/z* 243 [elenolic acid]$^+$, *m/z* 211 [elenolic acid- CH_3-H_2O]$^+$ and *m/z* 151 [211-CH_3COOH]$^+$.

Verbascoside and the flavones apigenin-7-O-rutinoside, luteolin- 7-O-glucoside and luteolin 7-O-rutinoside previously reported in olive fruit (Ryan et al., 2002) were also detected in the ethyl acetate extract.

The molecular ion of 1-phenyl-6,7-dihydroxyisochroman ([M-H]$^-$ at *m/z* 241) was detected together with two characteristic fragments, at *m/z* 211 and 136, in the mass spectrum (Bianco, Coccioli, Guiso, & Marra, 2001). This molecule is not present in olive fruit; however, it is formed during the storage of olive oil. It has not been previously detected in alperujo although in this study has been found in very low amounts. Because of the hydrothermal treatment conditions (high temperature and pressure) of the alperujo and the abundant presence of free HT, the aldehyde formation and the acidity condition of the extract could promote the formation of this compound.

3.3. Antioxidant activity

The antioxidant value potential of each phenolic fraction obtained after the fractionation was compared with the values for the unfractionated phenolic extract and with the values of other known antioxidant compounds such as HT, DHPG, α-tocopherol and Trolox, the water-soluble α-tocopherol analog.

3.3.1. Free radical scavenging capacity (DPPH and ABTS assays)

Data available in the literature on DPPH and ABTS assays indicate that they are not always well correlated and they don't often give the same results because it deal with two different action mechanisms using two different radicals. It is for this reason that both assays were considered. Individual regression equations were used to calculate the antiradical activity of each sample. Statistical analyses of the results indicate that the dose response adjusted to a linear model for two of the assays, with $R^2 \geq 0.90$. Lower EC_{50} values for DPPH assay and higher Trolox equivalent values for ABTS assay mean high antioxidant activity.

The results of the antiradical activity against the DPPH radical (**Fig. 4a**) indicate that fractions rich in DHPG, HT, tyrosol, protocatechuic acid, vanillic acid, hydroxytyrosol acetate, caffeic acid, comsegoloside, verbascoside, and polymeric phenolic fraction, have a higher antiradical capacity and do not exhibit significant differences compared with the three standards (HT, DHPG and α-tocopherol). The fractions 4A and 3B with a high percentage of elenolic acid derivative A (91 and 99%, respectively) exhibit very minimal activity.

The results of antiradical activity against the ABTS radical (**Fig. 4b**) indicate that fractions 2B, 4F and 4G have a high capacity for ABTS antiradical activity, with capacities between those of HT and DHPG. The fraction 2B is characterized by a high percentage of HT (96.14%), the fraction 4F contains HT and other compounds such as vanillic acid (23.82%) and verbascoside (65.11%), and the fraction 4G is characterized by a high percentage of comsegoloside (91.61%). All of these compounds are most likely responsible for the antiradical activity. However, fractions 2C, 3C, 3E, 4C, 4D and 4E have ABTS antiradical activity similar or greater than that of α–tocopherol because of the presence of compounds such as HT, tyrosol, protocatechuic acid, vanillic acid, hydroxytyrosol acetate and polymeric phenolic fraction (PPF). The activity of fraction 3E with a high percentage of elenolic acid derivative A is not attributable to the compound because fraction 4A and 3B, which are among the richest in this compound, exhibit no activity.

The different comportment between fraction 1, composed almost exclusively of the phenol DHPG, and the standard DHPG is most likely explicable by the low purity of fraction 1 of only approximately 18% of total phenol/weight total. The same result was observed in the reducing power and secondary oxidation assays discussed above.

3.3.2. Reducing power

The reducing power results (**Fig. 4c**) indicate that there are two fractions, 2B rich in HT (96.14%) and 2C is composed exclusively of protocatechuic acid (100%), whose reducing values are higher than Trolox. There is a group of fractions rich in HT, tyrosol, verbascoside, protocatechuic acid, vanillic acid and hydroxytyrosol acetate (3D, 3E, 3F, 4C, 4D and 4F) with Trolox equivalent values between 0.8 and 1. In fraction 3D, the major component is the elenolic acid derivative A (89.03%); however, this species cannot be attributed to the reducing power because fractions 3B and 4A are also rich in this species (99.64 and 91.18%, respectively) and have very low reducing power. The dose response adjusted to a lineal model ($R^2 \geq 0.90$).

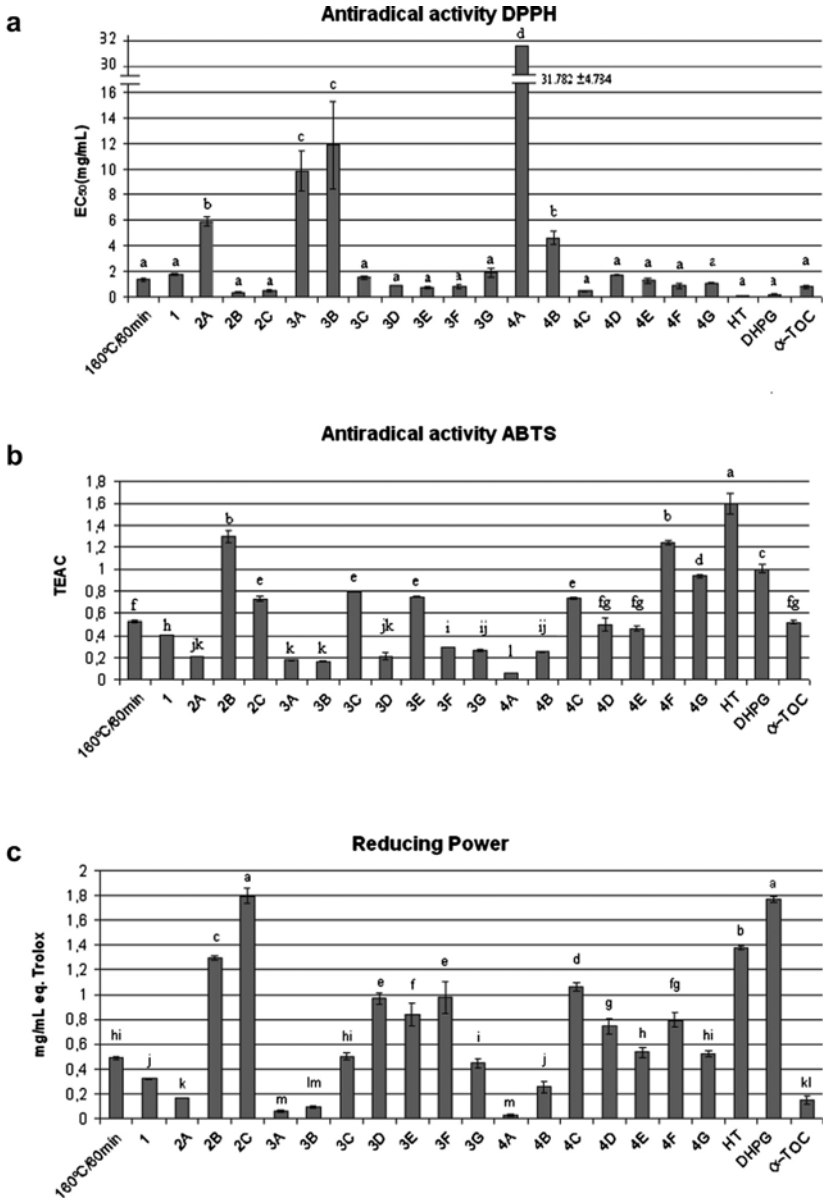


Fig. 4. Results for the different fractions obtained after the hydrothermal treatments for the fractionated, unfractionated and standard samples. (**a**) and (**b**) Antiradical activity values expressed as EC_{50} and TEAC for the DPPH and ABTS, respectively. (**c**) Reducing power values expressed as mg/mL Trolox. The data are presented as means ± SDs. The different letters indicate significantly different results ($p < 0.05$).

3.3.3. Inhibition of lipid oxidation

In the primary oxidation assay the response was not linear but depended on the square root of x ($y = a + bx^{-2}$), with $R^2 \geq 0.882$. In the secondary oxidation assay (TBARS), all of the samples are also described by a "square root x" regression model ($R^2 \geq 0.807$).

In fraction 1, 2B and 3C, the positive effect shown in the primary oxidation assay (**Fig. 5a**) may be due to the high concentrations of HT and DHPG. The fraction 4C is rich hydroxytyrosol acetate (95.5%), the antioxidant effect is most likely due to this compound, which is the hydroxytyrosol derivative more liposoluble according to Trujillo et al. (2006). In other cases, the activity may be due the presence of vanillic acid, verbascoside, comsegoloside and PPF, as in the previous cases. If we compare the fraction composition of 3F, 3E and 2C, we see that 2C consists exclusively of protocatechuic acid, whereas 3E also contains vanillic acid (11.46%) and 3F also contains p-hydroxybenzoic acid (6.63%) and is most active with respect to 2C inhibition primary oxidation, most likely because these species act synergistically.

The antioxidant properties of these fractions were also tested with a peroxidation inducer by thiobarbituric acid-reactive substance (TBARS) assay (**Fig. 5b**). Almost all of the fractions exhibit a value of inhibition of secondary oxidation similar to that observed for HT and DHPG, and, in some cases, even better than that of α-tocopherol. The fractions that exhibited less activity are rich in tyrosol and hydroxymethylfurfural that should not have an excess capacity to inhibit the secondary oxidation. However, fractions 3B, 4A and 4B are high in elenolic acid derivatives (99.64, 91.18 and 97.36%, respectively), which should not inhibit the secondary oxidation.

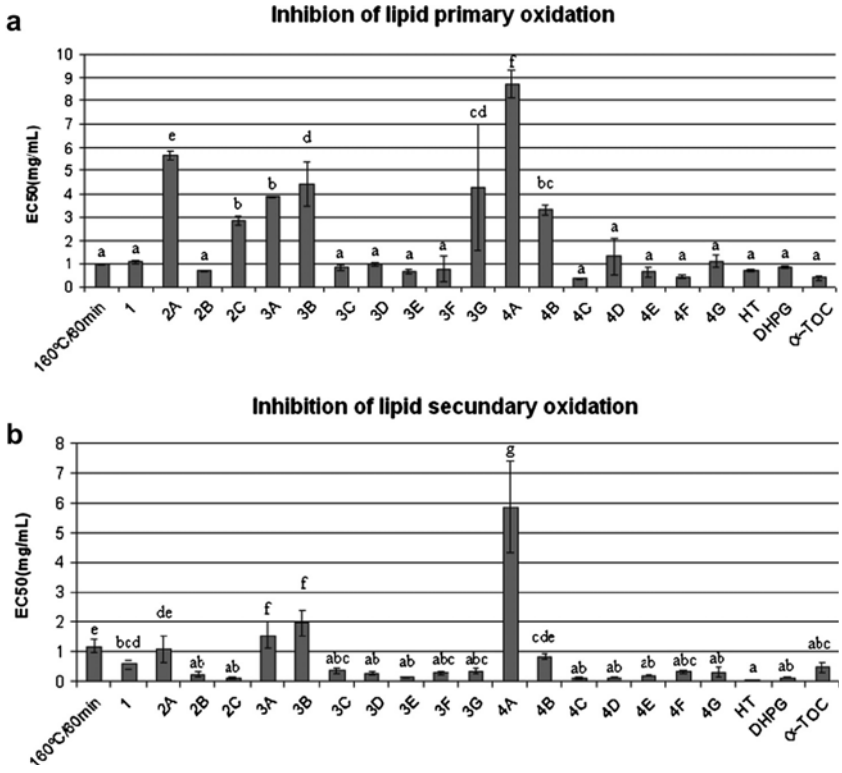


Fig. 5. Inhibition of lipid primary and secondary oxidation of the different extracts, control and standard tested, expressed as EC_{50} (mg/mL). The data are presented as means ± SDs. The different letters indicate significantly different results ($p < 0.05$).

3.4. Conclusions

The present study demonstrated the great diversity and complexity of the mixture of phenolic compounds present in the ethyl acetate extract of steam-treated alperujo. Among all of the compounds detected, hydroxytyrosol was observed in the highest concentration, followed by the elenolic acid derivatives produced during the process, which possess the core structure of secoiridoid compounds. Tyrosol, 3,4-dihydroxyphenylglycol, hydroxytyrosol acetate, comsegoloside, protocatechuic acid, along with a new polymeric phenolic fraction (PPF), must also be considered because its concentration and its contribution to the antioxidant/free radical-scavenging activity. A significant contribution was made by PPF, exhibiting a strong antioxidant activity in a lipid system as an emulsion, similar to α-tocopherol.

It was noted that there were a number of fractions whose behaviour in all tests were quite good, these fractions are the 2B, 2C, 3C, 3D, 3E, 3F, 4C, 4D, 4E, 4F and 4G, which are rich in DHFG, hydroxytyrosol, tyrosol, protocatechuic acid, verbascoside, 4-hydroxybenzoic acid, vainillic acid, hydroxytyrosol acetate, polymeric phenolic fraction, comsegoloside or/and p-coumaric acid. Furthermore it was observed that there are also a number of fractions to the test whose results were bad in all cases studied; these fractions were 3A, 3B, 4A and 4B rich in hydroxymethylfurfural, elenolic acid derivative A and/or elenolic acid derivative B.

These findings might be helpful for the production of a natural antioxidant extract for use in the food, cosmetic or pharmaceutical industries. Although further studies about their toxicity, *in vivo* activity and bioavailability are necessary. Thus, hydrothermal treatment, a technology being scaled to the industrial level, along with ethyl acetate extraction and the use of adsorbent resin, may enable the use of alperujo, which should not be considered a polluting residue but a precious and inexpensive source of natural antioxidants.

Acknowledgments

We thank the Ministerio de Economía y Competitividad of Spain for financial support (Grant no. AGL2009-12352), Spain Program JAE-DOC (CSIC-FSE) (to G.R.G) and JAE-PRE (CSIC-FSE)(to F.R.S), and Junta de Andalucía (project P08-AGR-03751).

References

Bianco, A., Coccioli, F., Guiso, M., & Marra, C. (2001). The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans. Food Chemistry, 77, 405-411.

Covas, M. I., de la Torre, K., Farré-Albaladejo, M., Kaikkonen, J., Fitó, M., López-Sabater, C., et al. (2006). Postpandrial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. Free Radical Biology and Medicine, 40, 608-616.

De la Torre-Carbot, K., Jáuregui, O., Gimeno, E., Castellote, A. I., Lamuela- Raventós, R. M., & López-Sabater, M. C. (2005). Characterization and quantification of phenolic compounds in olive oil by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. Journal of Agricultural and Food Chemistry, 53, 4331-4340.

De Marco, E., Savarese, M., Paduano, A., & Sacchi, R. (2007). Characterization and fractionation of phenolic compounds extracted from olive oil mill wastewaters. Food Chemistry, 104, 858-867.

De Nino, A., Mazzotti, F., Morrone, S. P., Perri, E., Raffaelli, A., & Sindona, G. (1999). Characterization of Cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. Journal of Mass Spectrometry, 34, 10-16.

De Nino, A., Mazzotti, F., Perri, E., Procopio, A., Raffaelli, A., & Sindona, G. (2000). Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from Carolea and Coratina cultivars by ionspray ionization tandem mass spectrometry. Journal of Mass Spectrometry, 35, 461-467.

Fernández-Bolaños, J., Rodríguez, G., Rodríguez, R., Heredia, A., Guillén, R., & Jiménez, A. (2002). Production in large quantities of highly purified hydroxytyrosol from liquid-solid waste of two-phase olive oil processing or “alperujo”. Journal of Agricultural and Food Chemistry, 50, 6804-6811.

Fu, S., Arráez-Román, D., Menéndez, J. A., Segura-Carretero, A., & Fernández-Gutiérrez. (2009). Characterization of isomers of oleuropein aglycon in olive oils by rapad-resolution liquid chromatography coupled to electrospray time-offlight and ion trap tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 23, 51-59.

Fu, S., Segura-Carretero, A., Arráez-Román, D., Menéndez, J. A., De la Torre, A., & Fernández-Gutiérrez, A. (2009). Tentative characterization of novel phenolic compounds in extra virgin olive oils by rapid-resolution liquid chromatography coupled with mass spectrometry. Journal of Agricultural and Food Chemistry, 57, 11140-11147.

Gonçalves, B., Falco, V., Moutinho-Pereira, J., Bacelar, E., Peixoto, F., & Correia, C. (2009). Effects of elevated CO_2 on grapevine (Vitis vinifera L.): volatile composition, phenolic content, and *in vitro* antioxidant activity of red wine. Journal of Agricultural and Food Chemistry, 57, 265-273.

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Sanchez-Rovira, P., & Ramirez-Tortosa, M. C. (2010). Hydroxytyrosol: from laboratory investigations to future clinical trials. Nutrition Review, 68, 191-206.

Obied, H. K., Bedgood, D. R., Prenzler, P. D., & Robards, K. (2007). Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. Analytical Chimica Acta, 603, 179-189.

Pérez-Jiménez, F. (2005). International conference on the healthy effect of virgin olive oil. European Journal of Clinical Investigation, 35, 421-424.

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández-Bolanos, J., et al. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. Journal of Agricultural and Food Chemistry, 53, 5212-5217.

Romero, C., García, P., Brenes, M., García, A., & Garrido, A. (2002). Phenolic compounds in natural black Spanish olive varieties. European Food Research and Technology, 215, 489-496.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil byproduct alperujo and their antioxidative activities. Journal of Agricultural and Food Chemistry, 60, 1175-1186.

Ryan, D., Antolovich, M., Herlt, T., Prenzler, P. D., Lavee, S., & Robards, K. (2002). Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. Journal of Agricultural and Food Chemistry, 50, 6716-6724.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1999). Free radical scavenging capacity and inhibition of lipid oxidation of wines, grape juices and related polyphenolic constituents. Food Research International, 32, 407-412.

Savarese, M., De Marco, E., & Sacchi, R. (2007). Characterization of phenolic extracts from olives (Olea europaea cv. Pisciottana) by electrospray ionization mass spectrometry. Food Chemistry, 105, 761-770.

Singleton, V. L., & Rossi, J. A., Jr. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144-158.

Suárez, M., Macià, A., Romero, M. P., & Motilva, M. J. (2008). Improved liquid chromatography tandem mass spectrometry method for the determination of phenolic compounds in virgin olive oil. Journal of Chromatography A, 1214, 90-99.

Suárez, M., Rometo, M. P., Ramo, T., Macià, A., & Motilva, M. J. (2009). Methods for preparing phenolic extracts from olive cake for potential application as food antioxidants. Journal of Agricultural and Food Chemistry, 57, 1463-1472.

Trujillo, M., Mateos, R., Collantes de Terán, L., Espartero, J. L., Cert, R., Jover, M., et al. (2006). Lipophilic hydroxytyrosol esters. Antioxidant activity in lipid matrices and biological systems. Journal of Agricultural and Food Chemistry, 54, 3779-3785.

6.1.3. Capítulo 3.

Influence of pH on the extraction and antioxidant activity of phenolic extracts obtained with ethyl acetate from hydrothermally treated alperujo.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., García, A., Fernández-Bolaños, J.

En la bibliografía se observa que para la extracción de compuestos fenólicos desde subproductos de la industria de la aceituna los autores utilizan diferentes disolventes extractantes. Existen trabajos concretos que evalúan y comparan la capacidad extractora de diferentes disolventes, comprobándose que es el acetato de etilo el que posee un mayor rendimiento para la extracción, actuando de forma selectiva para fenoles de pequeño y mediano peso molecular. Se ha comprobado que el pH al cual se realiza la extracción con acetato de etilo también difiere mucho entre distintos autores, de forma que algunos no modifican el pH de la muestra antes de realizar la extracción, mientras que otros acidifican la muestra hasta un pH 2-3 con HCl, para posteriormente realizar dicha extracción.

En el trabajo desarrollado en este capítulo se considera como afecta el pH a la extracción con acetato de etilo, para ello se realizan dos extracciones diferentes, la primera sin modificar el pH de la muestra de alperujo tratado térmicamente (pH 4.5), y la segunda se realiza sobre la muestra de alperujo tratada térmicamente y acidificada hasta un pH 2.5. Los extractos obtenidos fueron caracterizados, su composición fenólica fue estudiada y sus propiedades antioxidantes fueron consideradas con el objetivo de evaluar como afecta el pH al proceso de extracción.

Los resultados demuestran que el pH al que se realiza la extracción afecta el extracto fenólico obtenido. El extracto obtenido a pH 4,5 presentó un mayor porcentaje de fenoles totales y los fenoles mayoritarios fueron diferentes a los observados en el extracto obtenido a pH 2.5. Además, el extracto fenólico obtenido a pH 4,5 mostró una mayor capacidad antioxidante.

Influence of pH on the extraction and antioxidant activity of phenolic extracts obtained with ethyl acetate from hydrothermally treated alperujo.

Fátima Rubio-Senent, Guillermo Rodríguez-Gutíerrez, Antonio Lama-Muñoz, and Juan Fernández-Bolaños

ABSTRACT

The application of a novel process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final liquid phase that contained a high concentration of simple phenolic compounds. In this study the effect of pH on phenol extraction with ethyl acetate from the aqueous phase of hydrothermally treated alperujo at 160ºC/60 min (without modification, pH 4.5, and adjusted to pH 2.5) was evaluated. For each pH value, the corresponding extract was analyzed quantitatively by HPLC showing the variation of the concentration of phenolic compounds. The antioxidant activities were measured by two assays conducted *in vitro*: antiradical capacity (using the DPPH radical) and ferric reducing power (P_R). The phenolic extract obtained at pH 4.5 presented a higher proportion of total and individual phenols and better antioxidant activity than the extract obtained at pH 2.5.

KEYWORDS: alperujo; phenols; hydroxytyrosol; antioxidant; olive oil wastes; ethyl acetate extract.

Introduction

The Mediterranean diet, characterized by a high consumption of olive oil, fruits, vegetables, grains and legumes, reduces the incidence of major cardiovascular events and is associated with a lower risk of peripheral artery disease.[1-3] High concentrations of free radical-scavenging like polyphenols and flavonoids have been attributed as an important contributory factor for the health benefits of the Mediterranean diet. Virgin olive oil is rich in unsaponifiable minor components such as sterols, tocopherols, and polyphenols. The polyphenols are natural antioxidants that not only contribute to the stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties.[4] After olive oil extraction, only a low percentage of the total phenolic compounds present in the olive fruits are found in the virgin olive oil. The remaining phenolics (98-99 %) end up in alperujo, a by-product of the modern two-phase processing technique used in olive oil production.[5] As such, this material should be considered as an important source of polyphenols. Several studies have evaluated the extraction capacity of

different solvents to obtain phenolic extracts from olive oil by-products. Allouche et al.[6] compared various polar solvents such as methyl isobutyl, acetone, methyl ethyl ketone, diethyl ether and ethyl acetate in the extraction of phenolic compounds from the alperujo by a continuous extraction system. The results showed that extraction with ethyl acetate yields more enriched extract phenols than the others solvents. Obied et al.[7] conducted a study on different solvents to obtain an extract enriched in phenols from the alperujo. Acetate ethyl and various aqueous mixtures of methanol, ethanol, n-propanol, acetonitrile, and acetone were tested, and the results showed that acetate ethyl extraction is selective for small and medium molecular weight phenols.

The pH of extraction with ethyl acetate used was highly variable among the different studies, with some studies utilising ethyl acetate acidified with HCl to pH 3 to extract phenols from olive oil solid residues,[7-9], and others using ethyl acetate at pH 4.5,[10] and pH 2,[11] to extract phenol from olive mill waste water.

Our research group developed a hydrothermal treatment [12] that allows the recovery of simple phenolic compounds in high yield from two-phase olive waste. This process produces a separation of alperujo into two phases (solid and liquid) and results in a high solubilisation of simple phenolic compounds in the liquid phase due to their breakdown from complex molecules.

In previous work, the effect of the length of hydrothermal treatment on the composition of phenolic extracts and their properties was studied.[13] In this work, the influence of the pH of ethyl acetate extraction on the composition and antioxidant characteristics of phenolic extracts has been evaluated, for two different pHs. These two different pHs have been sectioned considering the most employed by different author. So usually two different actions were realized when an extraction with ethyl acetate was carried out, or the sample was acidified at pH 2-3 with HCl, or the pH not is modified leaving the sample with its original value of pH (4.5). Furthermore, we confirmed the reproducibility of the hydrothermal treatment together with the phenolic extraction.

Materials and Methods

Raw material. The sample of alperujo (a semi-solid residue composed of olive peels, pulp, seeds, and ground stones) was obtained in February of 2011 (end of the olive oil season) from Picual olives processed at a Spanish oil mill (Almazara Experimental, Instituto de la Grasa, Seville). The alperujo was processed in the pilot reactor without removal of the stones.

Standard compounds. Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, 4-methylcatechol, catechol, 3,4-dihydroxyhydrocinnamic acid, homovainillic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol was obtained from Fluka (Buchs, Switzerland). Oleuropein was obtained from

Extrasynthese (Lyon Nord, Geney, France). Hydroxytyrosol was obtained by the method described by Fernández-Bolaños et al.[14]. Hydroxytyrosol acetate was isolated following the process described by Rodríguez-Gutíerrez et al.[15].

The compound (E)-3-(1-Oxobut-2-en-2-yl)glutaric acid that are not commercially available was purified on a silica gel preparative TLC (Merck 60F254) and eluted with a mixture of chloroform and methanol 8:2 (v/v). The different bands were identified by their absorption at 254 nm and 366 nm.

Thermal treatment. The hydrothermal treatment [12] was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). 10 kg of fresh alperujo were treated for 60 min at 160 ºC, and the wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, 10 L of the liquid phase of each treatment was concentrated to 1 L by rotary evaporation in a vacuum at 30 ºC. The process was realized in duplicate (termed process A and process B).

Phenol extraction. The liquid portions obtained after treatment were washed with hexane to remove the lipid fraction. Half of the sample was subjected to extraction with ethyl acetate without modifying the pH (4.5), while the other half was acidified with HCl to pH 2.5. The same method was performed for the samples from process A and process B. Extraction of phenolic compounds was carried out with ethyl acetate (500 mL per 200 mL of sample), following the process described by Rubio-Senent et al.[13], where the aqueous and organic phase were separated.

Determination of the total phenolic content. The phenolic content was measured according to the Folin-Ciocalteu method[16], and expressed as grams of gallic acid equivalents per kilograms of fresh alperujo.

Chromatographic fractionation of the ethyl acetate extracts. For facility determination and quantification of phenols in each extract a fractionation had been realized. Each extract were passed through a column 3.5 cm in diameter and 40 cm in height filled with Amberlite® XAD16. The elution was performed with 1 L of H_2O, 30% EtOH (v/v), 50% EtOH (v/v) and 95% EtOH (v/v). Ten fractions of 100 mL each were collected. Fractions were analyzed by HPLC, and those with similar compositions were mixed. In each fraction, the different phenols were identified and quantified by HPLC-DAD.

HPLC-DAD. The method used was described by Rubio-Senent et al.[13] The different phenols were quantified using a Hewlett-Packard 1100 liquid chromatograph system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250 mm x 4.6 mm i.d. 5 µm). The system was equipped with a diode array detector (DAD; the wavelengths used for quantification were 254, 280 and 340 nm) and Rheodyne injection valves (20 µL loop). The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile utilizing the following gradient over a total run time

of 55 min: 95 % A initially, 75 % A at 30 min, 50 % A at 45 min, 0 % A at 47 min, 75 % A at 50 min, 95 % A at 52 min until the run was completed. Quantification was carried out by integration of peaks at different wavelengths with reference to calibrations made using external standards.

Antiradical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH). The free radical-scavenging capacity was measured using the DPPH method described in a previous study [13] and expressed as EC_{50} (effective concentration, mg/mL), calculated from a calibration curve using linear regression for each antioxidant.

Reducing power. The reducing power assay was performed according to the procedure described in a previous study.[13] The assay was calibrated using 6-hydroxy 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed in mg/mL TE (Trolox equivalent). To express the results, a calibration curve was created by plotting A_{490} against the known concentration of Trolox (0.059-0.56 mg/mL) [correlation coefficient $(R)^2$ 0.9936].

Statistical analysis. Results were expressed as mean values ± standard deviations. To assess the differences between samples a comparison was performed using the Statgraphics Plus program version 2.1. Multivariate analysis of variance (ANOVA), followed by Duncan's comparison test was performed. Results were considered statistically significant for $p < 0.05$.

Results and discussion

Hydrothermal treatment and phenolic extract. The results obtained after hydrothermal treatment of fresh alperujo at 160ºC/60 min and extraction of the aqueous fraction with ethyl acetate at pH 4.5 or adjusted to 2.5 are shown in **Table 1**. Double the amount of phenolic extract (organic fraction evaporated to dryness) per kg of fresh alperujo was obtained when the extraction was performed at pH 2.5 (30 g for pH 2.5 vs. 15 g for pH 4.5). However, the phenolic content in the extracts showed that at pH 4.5 the extracts contained 62% of phenol while at pH 2.5 only 46% of the extract had phenolic nature, and therefore contained many other compounds with no phenolic nature (16 g for pH 2.5 vs 6 g for pH 4.5).

Table 1. Yield of phenolic extracts and phenolic content obtained by hydrothermal treatment of alperujo at 160 ºC/60 min and extracted with ethyl acetate with two different pH (without modification, pH 4.5, and with acidification, pH 2.5).

	Replicate A		Replicate B	
pH	4.5	2.5	4.5	2.5
g of phenolic exctract/kg fresh alperujo	13.99	29.58	15.67	30.92
g phenol in organic fraction[a]/100 g extract	60.41±2.43 [b]	44.26±3.44	64.20±5.71	47.43±2.24
g phenol in aqueous fraction[a]/100 g extract	10.70±0.01	8.01±0.59	8.79±0.76	9.09±0.15
g phenol in organic fraction[a]/kg fresh alperujo	11.45±0.34	13.68±1.61	11.63±1.32	14.66±1.31
g phenol in aqueous fraction[a]/kg fresh alperujo	7.66±0.04	5.26±0.14	6.7±0.58	5.25±0.12

[a] Determined by Folin-Ciocalteu's method as gallic acid equivalent.
[b] Mean ± SD (Standard deviation) of three determinations.

Extraction with ethyl acetate at pH 4.5 caused a small increase of phenols in the aqueous fraction (g/ 100 g aqueous extract) compared to extraction at pH 2.5. Previous studies conducted by our research group have shown that in the aqueous fraction at pH 4.5 the phenolic compounds present were bound to oligosaccharides, and others were identified as phenolic glycosides (i.e. glucosides of hydroxytyrosol and tyrosol), verbascoside and secoiridoids.[17]

Characterization of phenolic extract. The compositions of phenolic extracts from the organic fraction were analyzed by chromatographic fractionation using Amberlite XAD-16,[13] and the different phenols identified and quantified by HPLC-DAD in each extract are listed in **Table 2**.

Firstly the data show that there are not important differences between the two replicates for each of the treatments.

Some differences were found in the phenolic composition of extracts depending on the pH of extraction with ethyl acetate. Changing the pH did not cause a significant difference in the concentration of some compounds, such as protocatechuic acid, p-hydroxybenzoic acid, vainillic acid, oleuropein derivatives and the polymeric phenolic fraction (PPF). However, many other compounds quantified showed significant differences in concentration as a function of pH. The phenolic alcohols (3,4-dihydroxyphenylglycol, hydroxytyrosol, tyrosol), the phenolic acid 3,4-dihydroxyhydrocinnamic acid, oleuropein, and the elenolic acid derivatives, including the (E)-3-(1-Oxobut-2-en-2-yl)glutaric acid, showed a higher concentration in the extract obtained at pH 4.5 than at pH 2.5. This last compound, and the second most abundant, is a novel elenolic derivative whose structural determination and nature will be presented by our group in a forthcoming paper. Other species, such as catechol, 4-methylcatechol, hydroxytyrosol acetate and homovainillic acid were found in greater amounts in the extract obtained at pH 2.5 than at

pH 4.5. In addition, the extract obtained at pH 2.5 had higher amounts of degradation products of sugars (hydroxymethylfurfural). These differences in concentration may be due to the instability of some compounds at the lower pH. Alternatively, the degradation of some compounds may cause the release of different units that form their structures, as for the case of oleuropein, which is more concentrated in the extract at pH 4.5 than at pH 2.5. The concentration of hydroxytyrosol acetate and hydroxytyrosol showed an inverse relationship between the two extracts at different pH, probably because the acetylation for the formation of hydroxytyrosol acetate is favored in the presence of ethyl acetate at low pH (2.5).

Table 2. Concentration[a] of identified phenolic compounds in the extracts from hydrothermally treated alperujo with ethyl acetate at different pHs by HPLC-DAD.

	mg/Kg fresh alperujo				
pH	4.5			2.5	
Treatment	A	B		A	B
phenolic alcohol					
3,4-dihydroxyphenylglycol[b]	20.16±1.44	19.43±0.89	*	9.73±0.98	7.50±1.47
Hydroxytyrosol[b]	1508.91±29.01	1412.55±88.22	*	799.82±16.37	832.69±35.60
Tyrosol[b]	196.74±4.33	149.41±1.21	*	115.43±13.23	69.56±4.43
Catechol[b]	39.90±2.55	40.65±3.13	*	66.60±3.60	63.77±1.81
4-methylcatechol[b]	268.26±0.72	222.46±2.26	*	978.44±80.18	936.85±17.48
hydroxytyrosol acetate[b]	686.43±13.75	562.31±22.68	*	866.34±16.37	764.85±4.80
degradation product of sugar					
Hydroxymethylfurfural[b]	36.34±2.42	48.20±0.97	*	59.56±2.31	49.79±6.36
phenolic acid					
protocatechuic acid[b]	40.49±1.83	40.44±1.20		44.38±1.39	38.36±2.32
3,4-dihydroxyhydrocinnamic acid[b]	40.24±1.16	38.78±5.17	*	n.d.	n.d.
homovainillic acid[b]	n.d.	n.d.	*	18.67±0.55	12.56±0.23
p-hydroxybenzoic acid[b]	13.80±0.11	10.29±0.41		7.35±0.58	6.86±0.25
vainillic acid[b]	25.15±0.26	20.56±0.10		23.97±0.26	21.62±0.78
oleuropein derivatives					
Oleuropein[b]	12.96±0.06	14.67±1.68	*	8.47±0.39	7.62±0.32
oleuropein derivatives[c]	36.99±4.55	27.58±2.24		38.12±4.82	39.20±7.41
elenoic acid derivatives					
(E)-3-(1-Oxobut-2-en-2-yl)glutaric acid[b]	1124.38±64.52	950.28±52.14	*	776.48±18.64	770.00±44.16
elenoic acid derivatives[d]	787.60±30.89	895.82±85.93	*	n.d.	n.d.
polymeric phenolic fraction					
polymeric phenolic fraction	2689.00±32.45	2855.00±35.48		2360.00±78.52	2056.00±87.52
total	**7127.36±123.82**	**7243.40±285.93**		**6112.88±238.18**	**5696.09±214.93**

[a] Mean ± SD (Standard deviation) of two determinations. [b] Compounds were identified and quantified with their corresponding standards. [c] Compounds was quantified with a calibration of oleuropein. [d] Compounds was quantified with a calibration of (E)-3-(1-Oxobut-2-en-2-yl)glutaric acid. [e] PPF was calculated by gravimetrically. n.d. not detected. * Significantly different from concentration of individual phenols in the different pH of liquid fraction (p >0.05).

With respect to the total balance, although the method of Folin-Ciocalteu quantified more total phenols in the extract obtained at pH 2.5 in comparison with the extract obtained at pH 4.5 (14 g or 11.5 g phenol per kg of fresh alperujo, respectively), a greater amount of individual phenols were identified and quantified by HPLC-DAD in the extract obtained at pH 4.5 (7.4 g vs. 5.9 phenol per kg of fresh alperujo).

In relation to PPFs, which were formed during the ethyl acetate extraction process and have been studied in a previous work,[18] there were no significant differences between the amounts of PPF obtained at the different pHs of extraction. However, an important difference in the composition of PPF was detected. The PPF obtained at pH 2.5 presented in their composition only 10.5% of total phenols, whereas the PPF obtained without modification of pH (4.5) presented in their composition 70 % of total phenols. Therefore, the extract obtained with modification of pH was enriched in other components different to phenols that affected the composition of the PPF fraction. This observation is consistent with the properties of adsorption of the phenol of PPF[18] that reaches its maximum at pH 4.5, or at least these phenols adsorbed are those quantitatively detectable.

Antiradical activity and reducing power. The results obtained for the antiradical activity and reducing power of the organic and aqueous fractions from the liquid-liquid extraction with ethyl acetate at the two pH tested were related with phenol content. **Figure 1A** shows the antiradical activity against DPPH radicals (EC_{50}), and **Figure 1B** the reducing power (expressed as Trolox equivalents in mg/mL) for the fractions from each hydrothermal treatment in duplicate (Treatment A and B).

Both the antiradical activity (**Figure 1A**) and reducing power (**Figure 1B**) showed a positive relationship with the total phenolic content in the fractions studied. Interestingly, the organic fractions obtained at pH 4.5, which had a higher proportion of total and individual phenols (**Tables 1** and **2**) than at pH 2.5, presented an antiradical activity similar to widely known natural antioxidants such as HT, DHPG and α-tocopherol, as deduced from the EC_{50} value. The reducing power values, expressed as Trolox equivalents in mg/mL, indicated that the organic extract obtained at pH 4.5 exhibited higher activity than at pH 2.5, although they presented lower reducing power values than for HT and DHPG, although they were more effective than α-tocopherol.

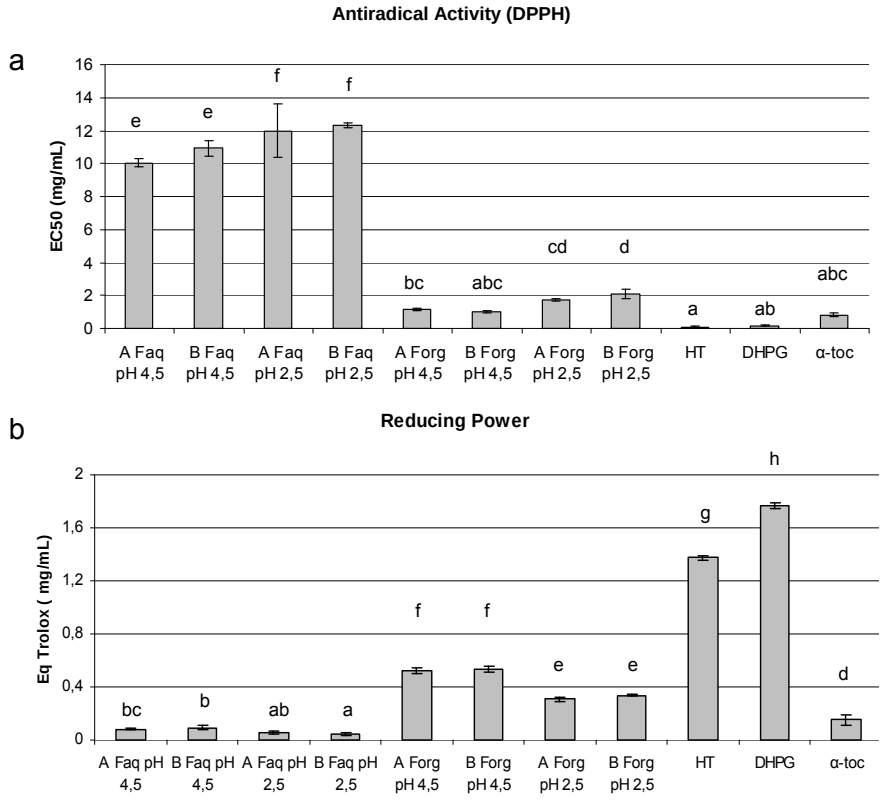


Figure 1. **A.** Radical scavenging capacity (DPPH radical) and **B.** reducing power of the extracts obtained from hydrothermally treated alperujo and extraction with ethyl acetate (organic and aqueous phases) using two different pHs (2.5 and 4.5) and standards (HT, DHFG, and α-tocopherol). Antiradical activity DPPH is expressed as EC_{50} (mg/mL) and reducing power in Trolox equivalent (mg/mL). The data are presented as means ± SD (Standard deviation) of three determinations. The different letters indicate significantly different results ($p < 0.05$). Faq, aqueous fraction; Forg, organic fraction.

The results show that the aqueous fraction presented lower antioxidant properties than the organic fraction for both pHs tested. These low activities have been related with antioxidant phenols that remain in the aqueous fraction and are bound to oligosaccharides.[19] Furthermore, the aqueous fractions could act as antioxidant soluble fiber with bioactive properties assigned to a phenol moiety.

In addition, we confirmed that there were no significant differences in either the composition or activity of both the organic and aqueous extracts obtained from the two treatments at 160 ºC/60 min (treatment A and B). Consequently, we can conclude that the hydrothermal treatment for the same raw material is reproducible.

General Comments. The results demonstrate that the pH at which the extraction is performed affects the phenolic extract obtained. The extract obtained at pH 4.5 presented a greater percentage of total phenols and it was composed mainly of hydroxytyrosol and an

elenolic acid derivative. In the extract obtained at pH 2.5 however, other molecules such as 4-methylcatechol and hydroxytyrosol acetate were in greater proportion, followed by hydroxytorosol and the elenolic acid derivative. Moreover, the phenolic extract obtained at pH 4.5 showed higher antioxidant/free radical-scavening activity than the extract obtained at pH 2.5, with an activity similar to HT, DHPG and α-tocopherol. The PPF isolated was also more enriched in phenolic compounds at pH 4.5. Therefore, this study reveals that the pH of the extraction with ethyl acetate is critical since it determines both the characteristics and the composition of the extract obtained. We also confirmed that the hydrothermal treatment of alperujo and the liquid-liquid solvent extraction process is reproducible, with no significant differences observed in the composition of the extracts obtained in duplicate in any of the tests performed.

Acknowledgments

Funding was received from the Ministerio de Economía y Competitividad of Spain and co-funded by European Social Fund (ESF) (project AGL2013-48291-R). F.R.S. received funding from the Spanish JAE-PRE program (CSIC-ESF).

References

(1) Estruch, R.; Ros, E., Salas-Salvado, J.; Covas, M. I.; Corella, D.; Aros, F.; Gomez-Gracia, E.; Ruiz-Gutierrez, V.; Fiol, M.; Lapetra, J.; Lamuela-Raventos, R. M.; Serra-Majem, L.; Pinto, X.; Basora, J.; Munoz, M. A.; Sorli, J. V.; Martinez, J. A.; Martinez-Gonzalez, M.A. Primary prevention of cardiovascular disease with a mediterranean diet. *N. Engl. J. Med*. **2013**, *368*, 1279-1290.

(2) De Lorgeril, M.; Salen, P.; Martin, J.L.; Monjaud, I.; Delaye, J.; Mamelle, N. Mediterranean diet, traditional risk factors, and the rate of cardiovascular complications after myocardial infarction: final report of the Lyon Diet Heart study. *Circulation.* **1999***, 99,* 779-785.

(3) Ruíz-Canela, M.; Estruch, R.; Corella, D.; Salas-Salvado, J.; Martínez-González, MA. Association of Mediterranean diet with peripheral artery disease: The PREDIMED Randomized Trial. *JAMA.* **2014***, 311*, 415-417.

(4) González-Santiago, M.; Martin-Bautista, E.; Carrero, J.J.; Fonolla, J.; Baro, L.; Bartolomé, M.V; Gil-Loyzaga, P.; López-Huertas, E. One-month administration of hidroxitirosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improve blood lipid profile, antioxidant status and reduces atherosclerosis development. *Atherosclerosis.* **2006***, 188*, 35-42.

(5) Fernández-Bolaños, J.; Rodríguez, G.; Gómez, E.; Guillén, R.; Jiménez, A.; Heredia, A.; Rodríguez, R. Total recovery of the waste of two-phase olive oil processing: isolation of added-value compounds. *J. Agric. Food Chem.* **2004**, *52*, 5849-5855.

(6) Allouche, N.; Fki, I.; Sayadi, S. Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. *J. Agric. Food Chem.* **2004***, 52*, 267-273.

(7) Obied, H. K.; Allen, M. S.; Bedgood, D. R.; Prenzler, Jr. P. D.; Robards, K. Investigation of Australian olive mill waste for recovery of biophenols. *J. Agric. Food Chem.* **2005***, 53*, 9911-9920.

(8) Lesage-Meessen, L.; Navarro, D.; Maunier, S.; Sigoillot, J-C.; Lorquin, J.; Delattre, M.; Simon, J-L.; Asther, M.; Labat, M. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chem.* **2001**, *75*, 501-507.

(9) Mulinacci, N.; Innocenti, M.; La Marca, G.; Mercalli, E.; Giaccherini, C.; Romani, A.; Erica, S.; Vincieri, F.F. Solid olive residues: Insight into their phenolic composition. *J. Agric. Food Chem.* **2005***, 53*, 8963-8969.

(10) Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F. Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. *Phytochemistry.* **1992***, 12*, 4125-4128.

(11) El-Abbassi, A.; Kiai, H.; Hafidi, A. Phenolic profile and antioxidant activities of olive mill wastewater. *Food Chem.* **2012***, 132*, 406–412.

(12) Fernández-Bolaños, J.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Sánchez-Moral, P. Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención de aceite de oliva. Patent ES 2374675 A1, 2012.

(13) Rubio-Senent, F.; Rodríguez-Gutíerrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. New phenolic compounds hydrothermally extracted from the olive oil byproduct Alperujo and their antioxidative activities. *J. Agric. Food Chem.* **2012***, 60*, 1175−1186.

(14) Fernández-Bolaños Guzmán, J.; Heredia Moreno, A.; Rodríguez Gutiérrez, G.; Rodríguez Arcos, R.; Jiménez Araujo, A.; Guillén Bejarano, R. Method for obtaining purified hydroxytyrosol from products and by-products derived from the olive tree.U.S. Patent 6,849,770 B2, 2005.

(15) Rodríguez-Gutiérrez, G.; Fernández-Bolaños Guzmán J.; Lama Muñoz, A.; Fernández-Bolaños Guzmán, J.M.; Maya Castilla, I.; Rubio Senent, F.; López López, O.; Marset Castro A. Procedimiento para la obtención de extracto de hidrositirosol, extracto mezcla de hidroxitirosol y 3,4-dihidroxifenilglicol, y extracto de acetato de hidroxitirosilo, a partir de subproductos del olivo y su purificación. P201131161, 2011.

(16) Singleton, V. L.; Rossi, J. A. Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Viti.* 1965, *16*, 144−158.

(17) Rubio-Senent, F.; Rodríguez-Gutíerrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. *Food Res. Int.* **2013***, 54*, 2122–2129.

(18) Rubio-Senent, F.; Lama-Muñoz, A.; Rodríguez-Gutíerrez, G.; Fernández-Bolaños, J. Isolation and identification of phenolic glucosides from thermally treated olive oil byproducts. *J. Agric. Food Chem.* 2013, *61*, 1235-1248.

(19) Fernández-Bolaños, J.; Rubio-Senent, F.; Lama-Muñoz, A.; García, A.; Rodríguez-Gutiérrez, G. Production of oligosaccharides with low molecular weights, secoiridoids and phenolic glycosides from thermally treated olive by-products. *In Oligosaccharides: Food Sources, Biological Roles and Health Implications*. Ed. L.S. Schweizer and S.J. Krebs, Nova Science Publisher, New York (pp 173-208).

6.1.4. Capítulo 4.

Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

Food Research International, 2013, 54, 2122-2129.

El pretratamiento térmico realizado sobre el alperujo provoca la solubilización de gran parte de los compuestos fenólicos presentes en la muestra, los cuales han sido analizados y cuantificado en capítulos previos. El análisis realizado sobre los extractos fenólicos obtenidos nos ha permitido identificar una nueva fracción fenólica polimérica.

Muchos compuestos fenólicos poliméricos han sido identificados en el fruto de la oliva con anterioridad, y sus actividades antioxidantes han sido descritas previamente. Este hecho se debe a que durante el proceso de extracción del aceite de oliva o durante la maduración de la fruta tienen lugar reacciones de polimerización entre los fenoles presentes en el fruto. Una fracción orgánica polimérica formada a partir de fenoles, polisacáridos, proteínas y sustancias inorgánicas, ha sido previamente descrita y denominada como polimerina.

El trabajo desarrollado en este capítulo se centra en aislar y estudiar la fracción fenólica polimérica presente en el extracto fenólico obtenido mediante extracción con acetato de etilo de un alperujo tratado térmicamente, comprobándose que la fracción fenólica polimérica aislada es totalmente diferente a la polimerina descrita previamente por otros autores. Además, se considera la capacidad de esta fracción fenólica polimérica para absorber y desorber HT, su capacidad antioxidante y su uso como resina para purificar hidroxitirosol.

Los resultados mostraron que la fracción fenólica polimérica aquí aislada se encuentra compuesta por hidratos de carbono, proteínas y ceniza, y que se forma durante el proceso de extracción con acetato de etilo. Su capacidad para absorber y desorber hidroxitirosol hace que esta fracción polimérica sea una buena herramienta para la purificación HT.

Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste

Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños

ABSTRACT

Polymeric phenolic fraction (PPF) was isolated by ethyl acetate extraction from hydrolyzed liquids from steam-treated alperujo and characterized. PPF is composed mainly of phenolic compounds with small amounts of carbohydrates, protein and ash. Phenols such as hydroxytyrosol (HT) and tyrosol were bound to PPF during the ethyl acetate extraction. Acid hydrolysis (3 N HCl for 10 min at 100 °C) was used to liberate HT from the PPF. The properties of both sorption and desorption of the PPF were studied to enhance the purification of HT, up to 100%. The antioxidant activities of the PPF were evaluated *in vitro* using ferric reducing power and DPPH radical scavenging, showing activity but lower than HT and TROLOX. The inhibition of primary oxidation of PPF was similar to both HT and vitamin E. These antioxidant activities of the PPF were not a result of the presence of HT or similar phenols.

KEYWORDS: Alperujo; Antioxidant; Autohydrolysis; Ethyl acetate extracts; Hydroxytyrosol; Polymeric phenols; Sorption.

1. Introduction

The olive oil industry has received an increased attention in the last several decades with the growing interest in the consumption of olive oil as an inherent part of a Mediterranean diet, which has been linked with low incidence of cardiovascular disease and cancer. These effects have been ascribed to the high content of phenolic compounds and other antioxidants, such as tocopherols (Pérez-Jiménez, 2005). The phenolic compounds in olives are potentially bioactive. Olive oil contains only 1-2% of the total phenol content of the entire olive. The remaining 98-99% is present in the olive mill waste, which is available in large quantities. The residue obtained from olive oil extraction by a two-phase technique, termed “alperujo”, is a valuable starting material for the production of phenols characterized by a wide array of biological activities (antioxidant, cardioprotective, antimicrobial, antihypertensive, anti-inflammatory and anticancerigenic) that could be used in the pharmaceutical, cosmetic and food industries.

These phenolic compounds present in alperujo can be recovered by a novel process based on hydrothermal treatment (Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, & Sánchez, 2011). This treatment, which is performed in a discontinuous reactor using steam at high pressures and temperatures (170 °C, 8 kg/cm^2), allows the separation of alperujo into two phases (liquid and solid). While it is practically impossible without the pretreatment process, this operation produces a high solubilization of phenolic compounds into the liquid phase.

In a previous study, phenolic compounds were extracted with ethyl acetate from the aqueous fraction of steam-treated alperujo. This process was selective for small and medium molecular weight phenols. These compounds were fractionated by an adsorption column to characterize the composition and to determine the specific antioxidant potential of the extracts in each fraction. A fraction of phenolic polymerics has been previously identified in these extracts (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012).

Many different polymeric phenolic compounds have been identified in the fruit of *Olea europaea* (Oleaceae). The antioxidative abilities of these oligomeric (oxidized phenolics) have also been characterized (Cardinali et al., 2010). The pigments from olive mill waste have been identified as catechol-melanin macromolecules derived from a polymerization of the phenolic compounds linked to sugars, proteins and fatty acids (Obied, Allen, Bedgood, Prenzler, & Robards, 2005). Phenolic antioxidants generally undergo a polymerization during the oil extractive process or even during ripening of the fruit (Bianco et al., 2006). A polymeric organic fraction formed from phenols, polysaccharides, proteins and inorganic substances, which was named “polymerin”, has also been isolated from olive oil mill waste water (Capasso, De Martino, & Arienzo, 2002).

Among the phenolic compounds present in olives, olive oil and olive by-products, hydroxytyrosol (HT) is the greatest by quantity; moreover, it possesses noteworthy antioxidant activities and potentially beneficial health properties (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010; Killeen, Pontoniere, & Crea, 2011). The European Food Safety Authority (EFSA) has recently issued a positive scientific opinion regarding health claims for the dietary consumption of HT (up to a dose of 5 mg/day) from olive fruit and oil with the resulting protection of blood lipids from oxidative damage. This oxidative damage has been demonstrated to adversely affect cardiovascular health (EFSA, 2011). Several methods have been developed to produce HT (chemical synthesis, enzymatic synthesis, etc.), with considerable attention placed on acquiring this compound from olive waste. These techniques, including solvent extraction, membrane separation, centrifugation and chromatographic procedures, can be used individually or in combination. An effective and patented system for purifying HT from this natural source has been developed by our group using ion-exchange resins and adsorbent polymers (Fernández-Bolaños et al., 2008). Using this system, HT is currently produced commercially as an additive for the nutraceutical, cosmetic and pharmaceutical industries.

This work characterizes the composition and properties of the polymeric phenol fraction (PPF) isolated from the ethyl acetate extract obtained after a hydrothermal treatment of an alperujo. The ability of this PPF to sorb and desorb HT as well as a characterization of its antioxidant capacity is discussed. A simple and effective approach to using this PPF in the purification of HT is also discussed.

2. Materials and methods

2.1. Materials

A sample of alperujo (a semisolid residue composed of olive peels, pulp, seeds, and ground stones) was obtained in March 2009 from Picual olives processed at a Spanish oil mill (Almazara Experimental, Instituto de la Grasa, Seville). The alperujo was processed in a pilot reactor without removal of stones.

2.2. Thermal treatment

The hydrothermal treatment used in this process has been patented (Fernández-Bolaños et al., 2011). This treatment was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor includes a 100 L capacity stainless steel reservoir that can operate at temperatures between 50 and 190 °C with a maximum pressure of 1.2 MPa.

Fresh alperujo samples (10 kg) were treated for 30, 60, 75 and 90 min at a temperature of 160 °C. The wet material was then centrifuged at 4700 *g* (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, 10 L of liquid phase from each sample was concentrated to 1 L by rotary evaporation under vacuum at 30 °C.

2.3. Phenol extraction

The liquid portions obtained after the treatment were washed with hexane to remove the lipid fraction with the following process: 1 L of liquid fraction was mixed with 500 mL of hexane; the mixture was vigorously shaken; and phases were separated by decantation and washed twice.

Extraction of phenolic compounds was performed with ethyl acetate (500 mL per 200 mL of sample). The liquid-liquid extraction was performed with hot ethyl acetate refluxed at 77 °C in a continuous extractor for 8 h. The ethyl acetate phase was rotary evaporated under vacuum at 30 °C, producing an extract rich in phenols.

2.4. Chromatographic fractionation of ethyl acetate extracts and isolation of the polymeric phenolic fraction (PPF)

The phenolic compounds were fractionated by an Amberlite® XAD-16 resin, as described by Rubio-Senent et al. (2012). Fractions were analyzed by HPLC. Those fractions with similar compositions were combined. The fractions rich in PPF were eluted with 100% EtOH and identified by HPLC-DAD.

2.5. Characterization of the PPF

An ash analysis was performed according to the AOAC procedure (AOAC, 1990). Protein was determined by the micro Kjeldahl method using the N × 6.25 conversion factor. Total sugars were quantified by the anthrone-sulfuric acid colorimetric assay, using glucose as a standard (Dische, 1962). Total phenol content was determined by the Folin-Ciocalteu spectrophotometric method (Singleton & Rossi, 1965) using gallic acid as the reference standard.

2.6. HPLC-DAD analysis

HPLC analyses were performed using a Hewlett Packard Series 1100 liquid chromatography system equipped with a diode array detector and a Rheodyne injection valve (20 µL-loop). A Spherisorb ODS-2 column (250 × 4.6 mm i.d.; particle size 5 µm) (Teknokroma, Barcelona, Spain) was used at room temperature. A linear gradient elution was performed at a flow rate of 1.0 mL/min, using ultrapure water (pH 2.5, adusted with trifluoroacetic acid) and acetonitrile as a mobile phase from 5 to 25% of acetonitrile over 30 min. The system was equilibrated between runs for 5 min using the starting mobile phase composition. The detection of the PPF was performed at 254, 280 and 340 nm. Quantitative evaluation of both free HT and tyrosol was based on comparison of retention times using reference compounds recorded at a UV spectra wavelength of 280 nm. Measurements were performed in triplicate, producing data that were analyzed using a five-point regression curve of the individual stock solutions of HT (r^2 ≥ 0.99) and of tyrosol (r^2 ≥ 0.99).

2.7. Assay of hydrolysis and extraction from the PPF

2.7.1. Assay of hydrolysis

An aliquot of 20 mg of the PPF was digested with 1.5 mL of 3 N HCl on a heating block at 100 °C for 8 h. In a second test, 20 mg of the PPF was completely dissolved with 300 µL of MeOH and further hydrolyzed using 1.2 mL of 3 N HCl at 100 °C for 8 h.

2.7.2. Assay of extraction

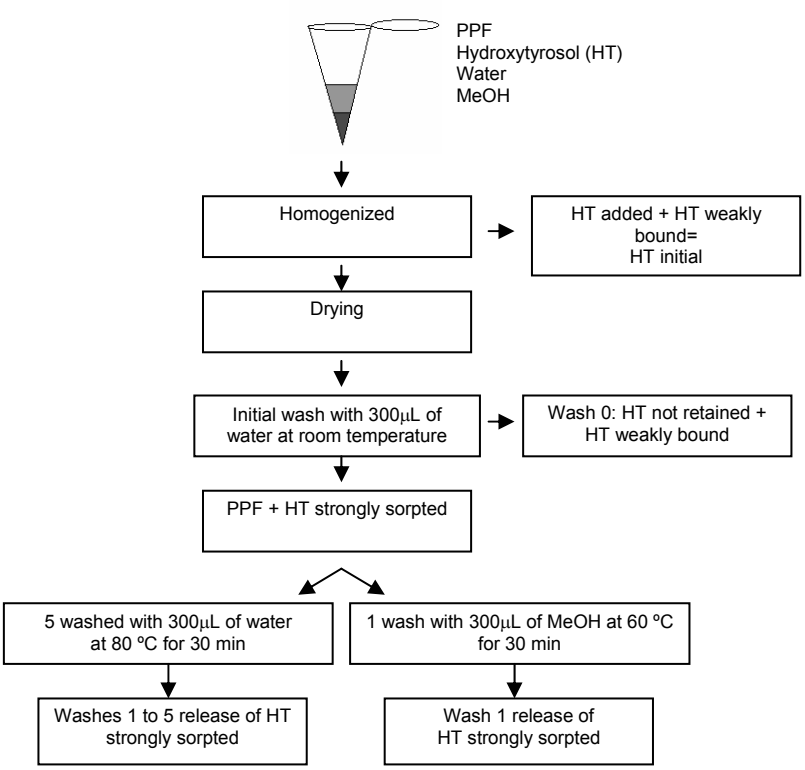
An aliquot of 20 mg of the PPF was solubilized with 1 mL of MeOH, and the mixture was heated at 60 °C on a heating block for 8 h. In a second test, 20 mg of the PPF was suspended

in 1 mL of distilled water and held at 80 °C for 8 h. For each assay, aliquots of 50 µL were taken at 10, 30, 60, 120, 180, 240, 300, 360, 420 and 480 min. For the soluble samples, the aliquots were pipetted. For insoluble samples, the suspension was centrifuged first at 7000 g for 5 min. The concentration of released HT was determined by HPLC-DAD with correction for the periodic removal of the sampling volume. Released HT was expressed as µg of HT/mg of PPF.

2.8. Sorption and desorption assays

For the sorption assay, 130 mg of the sorbent agent (the PPF obtained from the alperujo steam-treated at 160 °C for 30 and 90 min, termed PPF-30 and PPF-90, respectively) was dissolved in 1 mL of MeOH. Using a stock solution of 4 mg/mL of the HT in water (98% of purity), aliquots with 0.2, 0.4 and 0.8 mg of HT were added to 100 µL of the sorbent. To facilitate the dissolution, 100 µL of MeOH was added with an additional 500 µL of water to produce concentrations of HT of 0.4, 0.8 and 1.2 mg/mL. The mixtures were homogenized by agitation in a vortex. The samples were analyzed to quantify the amounts of added HT together with the amount of HT present in the PPF, which is weakly attached to the PPF and can pass easily to the equilibrium solution, a joint value that was assumed to be the amount of HT initially present in the mixture (µg of HT initial/mg of PPF). The mixture was dried by air flow to facilitate an intimate interaction between the PPF and the externally added HT. The dried mixture was washed with 300 µL of distilled water at room temperature, shaken in a vortex, and centrifuged at 7000 g for 5 min. The amount of HT retained in the PPF was determined by the difference between the quantity of HT initially present in the mixture (the externally added plus the weakly bound) and the amount of HT present in the washed solution.

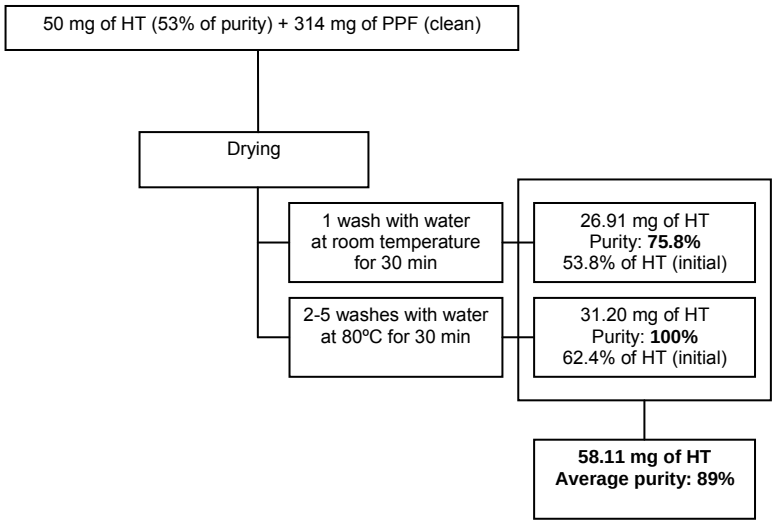
In the desorption experiments, the strongly bound HT was assayed using two procedures. In the first procedure, the PPF was subjected to five successive washings with 300 µL of water at 80 °C for 30 min followed by a centrifugation at 7000 g for 5 min. In the second procedure, the PPF was dissolved in 300 µL of MeOH. The amount of HT released was determined for both procedures (Scheme 1).



Scheme 1. Sorption and desorption of hydroxytyrosol (HT) assay.

2.9. HT purification assay

The assay for the purification of HT used a sample of the PPF-90 (641 mg obtained from 160 °C/90 min treatment) that was first washed with 2.5 mL distilled water under stirring for 30 min at room temperature followed by five washings with 2.5 mL of distilled water under stirring at 80 °C for 30 min. After each wash, the mixture was centrifuged at 7000 g for 5 min and the phases separated, leaving a clean PPF of 314 mg. The clean PPF (314 mg) was dissolved in 2 mL of MeOH and mixed with 1 mL of a solution of 50 mg/mL of HT (purity of 53% w/w) (Scheme 2). The mixture was vigorously homogenized by shaking in a vortex and then dried by air flow to facilitate the interaction between the PPF and the HT solution. The mixture was then washed with 1.25 mL of distilled water at room temperature under stirring for 30 min and centrifuged at 7000 g for 5 min. The solubilized fraction was analyzed by HPLC to determine the amount of HT. The purity was determined by weighing the samples. This procedure was performed in four successive cycles of washing to 80 °C and 30 min. The solubilized fractions were collected and analyzed by HPLC to determine the amount of recovered HT. An aliquot was weighed to determine purity.



Scheme 2. Hydroxytyrosol (HT) purification assay.

2.10. Antioxidant capacity assays

2.10.1. Reducing power

The reducing power assay was performed using the procedure described in a previous study (Rodríguez et al., 2005). The assay was calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the results are expressed in mg/mL TE (Trolox equivalent). A calibration curve was created by plotting A_{490} as a function of the known concentration of Trolox (0.059–0.56 mg/mL) [correlation coefficient (r^2) = 0.9936].

2.10.2. Anti-radical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The free radical-scavenging capacity was measured using the DPPH method described in a previous study (Rodríguez et al., 2005). The radical-scavenging capacities of each antioxidant were expressed as EC_{50} (effective concentration, mg/mL) values calculated from a calibration curve using linear regression for each antioxidant.

2.10.3. Inhibition of primary oxidation

This method is based on the ferric thiocyanate assay reported by Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) with some modifications, as described in a previous work (Rubio-Senent et al., 2012). Accumulation of hydroxyperoxides due to oxidation of linoleic acid was measured at 490 nm. These results, calculated from the regression curves, are expressed as EC_{50} (mg/mL).

2.11. Statistical analysis

STATGRAPHICS® Plus software was used for statistical analysis. The correlation coefficients were determined using regression analysis at the 95% confidence level.

Comparisons among samples were made with one-way analysis of variance (ANOVA) and the LSD method at the same confidence level.

3. Results and conclusions

3.1. Polymeric phenolic fraction (PPF)

The PPF was formed during an ethyl acetate extraction process from the autohydrolysis liquids of steam-treated alperujo. This PPF was recovered from the ethyl acetate extract by selective adsorption on a neutral resin, Amberlite XAD, followed by desorption with 100% ethanol (Rubio-Senent et al., 2012). This process was followed by HPLC on a C_{18} reverse column resulting in detection of phenolic compounds of a curve at long retention times under the assay chromatographic conditions. This fraction was characterized by several un-separated broad peaks in the chromatogram at three selected UV absorbance wavelengths (**Fig. 1**). This chromatographic comportment is similar both to the polymerin behavior described by Arana et al. (2007) for a dry olive mill residue and the polymerin standard recovered from olive oil mill waste waters by Capasso et al. (2002). PPF is characterized by a complete solubility in EtOH, with only a partial solubility in water. The polymerin is completely soluble in water. In addition, chemical analyses suggested a considerable difference between both complexes. The polymerin, a complex metal-polymeric organic mixture, contained polysaccharides (52.4%), melanins (26.1%), proteins (10.4%) and metals (11.1%) (Capasso et al., 2002). The composition of the two polymeric fractions obtained from the hydrothermal processing of the alperujo (at 160 °C for 30 and 90 min of treatment, PPF-30 and PPF-90, respectively) had very small amounts of polysaccharides, proteins and ash (in which metals are present) as well as a high proportion of phenolic compounds (**Table 1**).

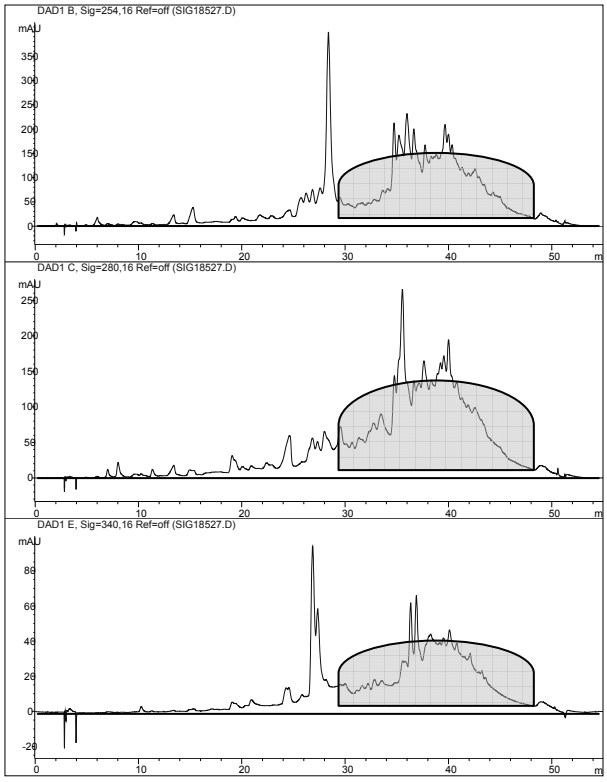


Fig. 1. HPLC-UV chromatographic profiles at the three wavelengths 254, 280 and 340 nm of the polymeric phenolic fraction (PPF) obtained from ethyl acetate extract of steam-treated alperujo. These data were detected as a curve in the range of 30-48 min (shaded area).

Table 1
Percentage composition of the polymeric phenolic fraction (PPF) of two samples of alperujo steam-treated at 160 °C for 30 min (PPF-30) and 90 min (PPF-90).

	PPF-30	PPF-90
	g/100 g PPF	
Carbohydrates	2,99 ± 0.15[a]	3,44±0.48
Proteins	0,85 ± 0.01[b]	3,11±0.01
Ash	0,27 ± 0.02	0,21±0.01
Melanin	95,89[c]	93,24
Phenol + non-phenol aromatic unit	6,43 ± 0.35[c1] + 89,46[c2]	5,28±0.28[c1]+87,96[c2]
Total	100	100
Hydrolysable phenols	8.32 ± 0.40[d1] + 1.43 ± 0.05[d2]	8.07 ± 0.56[d1] + 3.37 ± 0.18[d2]
Compounds extractable with chloroform	63,29 ± 2.31	16,09 ± 1.85

[a]Determined by an anthrone reagent. [b]Determined by the micro Kjeldahl method using the N × 6.25 conversion factor. [c]Determined by a complement to 100 of the sum of the other components.[c1]Determined by Folin–Ciocalteu's method as gallic acid equivalents. [c2]Determined by the difference between [c] and [c1]. [d1] and [d2] HT and tyrosol values, respectively, obtained after hydrolysis of the PPF with 3 N HCl at 100 °C for 10 min, as quantified by HPLC (to see Section 2.6).

These differences in solubility and chemical composition suggest that this PPF was formed during the ethyl acetate process of extraction of phenolic compounds from the water-soluble fraction obtained after the hydrothermal treatment of alperujo, in contrast with the

soluble polymerin complex derived from olive mill waste waters. In addition, another phenolic polymer with a complex profile with similar characteristics to the chromatograms observed in our study has been reported for an extract obtained from an ethyl acetate continuous counter-current extraction from olive mill waste waters (Allouche, Fki, & Sayadi, 2004). In addition, the concentration of hydroxytyrosol (HT) (g HT/kg of fresh alperujo), the most important phenol present in the ethyl acetate extract, increased with increasing treatment times in the aqueous fraction after steam treatment (160 °C/for 15 to 90 min) and before ethyl acetate extraction (**Fig. 2**). This observation is consistent with previous reports in the literature suggesting that increased severity of the alperujo treatment produced important increases in HT (Fernández-Bolaños et al., 2002). However, this fact is in contrast to the decrease in HT concentration from 1.62, 1.46 to 1.13 g HT/kg fresh alperujo for alperujo extracts treated with 160 °C/during 30, 60 and 90 min, respectively, after the organic extraction. This decrease occurred despite increases in HT concentration in the ethyl acetate extraction with respect to HT concentration in the aqueous fraction before ethyl acetate extraction, especially in the sample PPF-30, this effect can be produced because during the extraction with ethyl acetate refluxed at 77 °C a rupture of the more complex molecule that contained hydroxytyrosol is produced, enhancing their solubilization. This decrease also coincided with the increases in the amount of PPF formed during ethyl acetate extraction that increased from 0.52 g/kg fresh alperujo for the PPF-30, 1.28 g/kg fresh alperujo for the PPF-60 to 1.29 g/kg fresh alperujo for the PPF-90 (calculated gravimetrically after drying the fraction eluted with 100% ethanol) and showing therefore the PPF-90 complex a high affinity for HT.

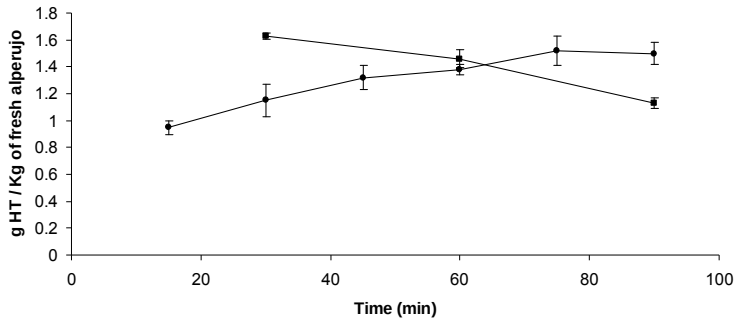


Fig. 2. Effect of steam treatment at 160 °C at different times (15, 30, 45, 60, 75 and 90 min) on hydroxytyrosol (HT) content recovered into the aqueous phase (●) from steam-treated alperujo. Effect of the three steam treatments, 160 °C/30 min, 160 °C/60 min and 160 ºC/ 90 min and 160 °C/90 min, with a subsequent ethyl acetate extraction on recovered HT (■). Values are expressed in g/kg of fresh alperujo. Standard deviations are shown.

Several assays on the hydrolysis and/or extraction of the PPF were performed to determine the retention of HT within this polymeric fraction. The PPF-90 sample, with a possible high retention or capture of HT, was hydrolyzed with 3 N HCl at 100 °C for 8 h, both with and without the presence of the solubilizing agent MeOH. The PPF-90 was also extracted with water at 80 °C for 8 h and with MeOH at 60 °C for 8 h, followed by an analysis of HT content. The

results (**Fig. 3**) showed that HT was present in the PPF-90 with the maximal liberation of the HT from the hydrolysis with 3 N HCl at 100 °C/10 min, producing approximately 52 µg of HT/mg PPF, followed by a decrease in concentration with increased exposure to acidic conditions. For the water treatment at 80 °C, approximately 40 µg of HT/mg PPF was produced after 8 h. Tyrosol was also liberated from the PPF hydrolyzed with 3 N HCl.

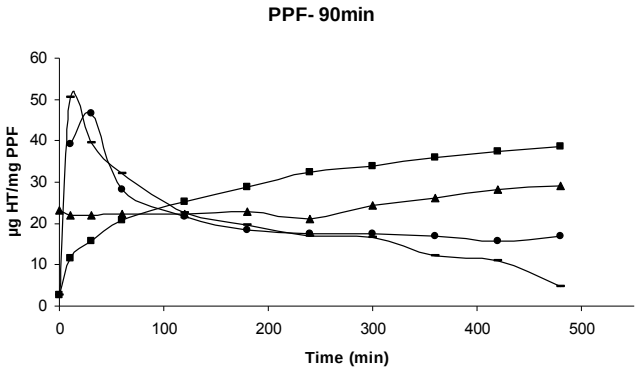


Fig. 3. Liberation of hydroxytyrosol from the polymeric phenolic fraction (PPF-90) using different processes of hydrolysis (-) 3 N HCl at 100 °C, (●) 3 N HCl + MeOH at 100 °C and/or extraction (▲) MeOH at 60 °C and (■) water at 80 °C.

A second assay (Scheme 1) on the possible sorption and desorption of HT (added or external) using both PPF-30 and PPF-90 suggested that different comportments existed between them. Three amounts of HT (15.4, 30.8 and 61.6 µg of HT/mg PPF) were added to both PPFs dissolved in MeOH. The mixture was homogenized, and an aliquot was analyzed by HPLC to quantify the HT being higher than the HT added because the solubilization of HT weakly linked to the PPF. This value was assumed to be the amount of HT initially present in the mixture (µg of HT/mg PPF). The mixture was dried by air flow to facilitate an intimate interaction between HT and the PPF making possible the sorption. A subsequent washing with water at room temperature extracted the HT that was not retained in the matrix of PPF as well as the weakly bound HT. The amount of the sorpted HT was determined by the difference between initial HT and HT that was not retained (**Table 2**). The results showed that the PPF-90 had a greater capacity to sorption of HT than the PPF-30 at approximately 40% and 30% of the added HT, respectively.

Table 2
Sorption capacity of the PPF-30 and PPF-90 on external or added hydroxytyrosol (HT). Percentage of bound HT.

PPF-30			PPF-90		
µg HT initial/mg of PPF[a]	µg HT sorbed/mg of PPF[b]	% HT adsorbed	µg HT initial/mg of PPF[a]	µg HT sorbed/mg of PPF[b]	% HT adsorbed
17,35 ± 0,29	4,94 ± 0,23	28,46 ± 1,32	17,51 ± 0,95	6,96 ± 0,41	39,77 ± 2,34
32,37 ± 0,47	10,14 ± 0,85	31,31 ± 2,63	35,25 ± 2,47	12,55 ± 0,97	35,60 ± 2,75
64,35 ± 3,27	19,55 ± 1,37	30,38 ± 2,13	73,78 ± 1,32	34,40 ± 1,87	46,62 ± 2,53

[a] Added HT (15.4, 30.8 and 61.6 µg HT/mg of PPF) plus the weakly bound HT.
[b] Value calculated by the difference between initial HT and HT that was not retained.

For the desorption assays, two procedures were used to determine the recovery of retained HT (Scheme 1). In the first procedure, the PPF-30 and the PPF-90, both with the sorpted HT, were subjected to successive washings with water (5 times) at 80 °C for 30 min to extract the maximum amount possible of the strongly sorpted HT. In the second procedure, both complexes were solubilized with MeOH and heated at 60 °C for 30 min, followed by an extraction and quantification of HT. Both procedures were also used on the PPF-30 and PPF-90 without the externally added HT. This released HT was quantified and considered to be the initial concentration of HT (μg of HT/mg PPF). **Table 3** shows the amount of recovered HT and **Table 4** the material balances with the initial concentration of HT and corresponding amounts of the retained HT from each PPF complex treated with the two solvents used in the desorption (water and MeOH) assay. The initial concentration of HT was greater in the PPF-90 than the PPF-30 for the extractions with both water and MeOH. These data suggest, as observed in the previous experiments, that the PPF-90 had a higher ability to sorpted HT than the PPF-30, retaining significant quantities of the HT. The first water wash facilitated recovery of HT. Considering the theoretical and experimental values the recovery from the PPF-30 was approximately 100%, in contrast to the PPF-90 values at approximately 85%. The theoretical values is the sum of initial HT contained in PPF and the value of the sorpted HT, being the sorpted one the difference between the HT added and the HT removed with the first wash using cold water. The experimental value is the amount of recovered HT after total extraction (5washes with hot water). Although the recovery in the PPF-90 was lower than the values observed with the PPF-30, capture capacity was higher than for the PPF-30. The desorption with MeOH resulted in a desorption exceeding 100% of the theoretical value (in general approximately 140%), indicating that this solvent extracted all of the externally added HT as well as a portion of the initial HT contained in PPF. The only exception to these observations was for PPF-90 with a sorpted HT recovery of approximately 91%. Nevertheless, this extraction still resulted in a large amount of recovered HT.

Table 3
Desorption of the sorpted HT on the PPF-30 and PPF-90 with repeated washings with water at 80 °C/30 min and by extraction with MeOH at 60 °C for 30 min.

Extraction with water at 80 ºC							Extraction with MeOH at 60 ºC	
PPF-30							PPF-30	
µg HT sorbed /mg of PPF	µg HT extracted/mg of PPF					µg HT extracted total/mg of PPF	µg HT sorbed/mg of PPF	µg HT extracted total/mg of PPF
	Washing 1	Washing 2	Washing 3	Washing 4	Washing 5			Washing 1
0	11,36 ± 1,02	2,34 ± 0,35	0,86 ± 0,02	0,37 ± 0,03	0,12 ± 0,25	15,06[a] ± 167	0	4,15[a] ± 0,35
4,94±0,23	16,77 ± 0,55	2,88 ± 0,57	0,78 ± 0,12	0,41 ± 0,08	0,22 ± 0,09	21,06 ± 1,41	4,94 ± 0,23	12,76 ± 0,03
10,14±0,85	19,79 ± 1,22	3,50 ± 0,32	0,89 ± 0,03	0,40 ± 0,15	0,28 ± 0,08	24,86 ± 1,78	10,14 ± 0,85	20,66 ± 0,55
19,55±1,37	32,34 ± 2,01	5,10 ± 0,04	1,16 ± 0,03	0,44 ± 0,13	0,24 ± 0,08	39,29 ± 2,29	19,55 ± 1,37	30,93 ± 2,17
PPF-90							PPF-90	
µg HT sorbed/mg of PPF	µg HT extracted/mg of PPF					µg HT extracted total/mg of PPF	µg HT sorbed/mg of PPF	µg HT extracted total/mg of PPF
	Washing 1	Washing 2	Washing 3	Washing 4	Washing 5			Washing 1
0	13,39 ± 1,25	4,04 ± 0,03	1,26 ± 0,17	0,40 ± 0,07	0,29 ± 1,53	19,50[a] ± 1,53	0	8,28[a] ± 0,35
6,96±0,41	18,03 ± 0,97	3,15 ± 0,45	0,76 ± 0,15	0,24 ± 0,09	0,19 ± 1,70	22,38 ± 1,70	6,96 ± 0,41	21,46 ± 1,27
12,55±0,97	21,51 ± 2,03	4,03 ± 0,07	1,06 ± 0,09	0,29 ± 0,03	0,22 ± 2,29	27,11 ± 2,29	12,55 ± 0,97	29,08 ± 2,55
34,40±1,87	34,20 ± 2,25	8,85 ± 0,17	2,31 ± 0,04	0,66 ± 0,04	0,30 ± 2,51	46,32 ± ±2,51	34,40 ± 1,87	38,74 ± 3,17

[a] Value of recovered HT (strongly bound) from the PPF-30 and the PPF-90 without externally added HT. Weakly bound HT removed by washing with water at room temperature was not considered. The sum of both is the order of that obtained in the desorption test, as depicted in **Fig. 3**.

Table 4
Material balance of the sorption and the desorption processes of hydroxytyrosol on PPF-30 and PPF-90.

Extraction with water 80 ºC

PPF-30	Material balance				PPF-90	Material balance			
µg HT initial/mg of PPF[a]	µg HT sorbed/mg of PPF	HT heoretical[b]	HT experimental[c]	%[d]	µg HT initial/mg of PPF[a]	µg HT sorbed/mg of PPF	HT heoretical[b]	HT experimental[c]	%[d]
15,06 ± 1.67	0	15,06 ± 1.67	15,06 ± 1.67	100	19,5 ± 1.53	0	19,5 ± 1.53	19,5 ± 1.53	100
15,06 ± 1.67	4,94 ± 0.23	19,99 ± 0.85	21,06 ± 0.85	105,3	19,5 ± 1.53	6,96 ± 0.41	26,47 ± 1.94	22,38 ± 1.94	84,6
15,06 ± 1.67	10,14 ± 0.85	25,19 ± 0.69	24,87 ± 0.69	98,7	19,5 ± 1.53	12,55 ± 0.97	32,05 ± 2.50	27,11 ± 2.07	84,6
15,06 ± 1.67	19,55 ± 1.37	34,61 ± 1.74	39,29 ± 1.74	113,5	19,5 ± 1.53	34,4 ±1.87	53,9 ± 3.40	46,32 ± 1.35	85,9

Extraction with MeOH 60 ºC

PPF-30	Material balance				PPF-90	Material balance			
µg HT initial/mg of PPF[a]	µg HT sorbed/mg of PPF	HT heoretical[b]	HT experimental[c]	%[d]	µg HT initial/mg of PPF[a]	µg HT sorbed/mg of PPF	HT heoretical[b]	HT experimental[c]	%[d]
4,15 ± 0.35	0	4,15 ± 0.35	4,15 ± 0.35	100	8,28 ± 0.35	0	8,28 ± 0.35	8,28 ± 0.35	100
4,15 ± 0.35	4,94 ± 0.23	9,09 ± 0.58	12,76 ± 1.58	140,4	8,28 ± 0.35	6,96 ± 0.41	15,24 ± 0.76	21,46 ± 0.46	140,8
4,15 ± 0.35	10,14 ± 0.85	14,29 ± 1.20	20,66 ± 1.64	144,6	8,28 ± 0.35	12,55 ± 0.97	20,83 ± 1.32	29,08 ± 1.51	139,7
4,15 ± 0.35	19,55 ± 1.37	23,7 ± 1.72	30,93 ± 2.03	130,5	8,28 ± 0.35	34,4 ± 1.87	42,67 ± 2.22	38,74 ± 2.04	90,8

[a] Value of recovered HT (strongly bound) from PPF-30 and PPF-90 without externally added HT.
[b] Theoretical HT (µg/mg of PPF) is the sum of the initial value of HT and the value of sorpted HT.
[c] Experimental HT (µg/mg of PPF) is the value of total HT extracted in the total washings.
[d] The percentage was determined by the ratio of experimental to theoretical HT values.

3.2. Use of the isolated PPF to purify HT

The high capacity of the PPF for HT sorption combined with an ability to release this molecule represents a potential tool for the purification of HT. In olives, olive oil and olive by-products, hydroxytyrosol is the most important phenol in terms of quantity with remarkable properties, hence PPF could be used to purify industrially this phenol. With a greater sorption capacity than the PPF-30, PPF-90 was examined after washing with water at 80 °C/ 30 min (5 times) to remove the greatest possible amount of the HT intimately linked to the PPF as well as other impurities. After this exhaustive cleaning, only 49% of the PPF remained, suggesting that 51% of a mixture of HT and other molecules was removed during the washing process.

The HT used for this purity test with the clean PPF was determined to have a degree of purity of 53% (w/w), as measured from an effluent of a system developed for the purification of HT and patented by our research group (Fernández-Bolaños et al., 2008).

To facilitate interaction between the aqueous solution of HT to be purified and the clean PPF, the mixture (dissolved in MeOH) was dried using air flow. The HT release was performed using water, as PPF is partially insoluble in water and the HT is highly soluble, facilitating the separation of HT from the PPF.

Scheme 2 shows the diagram of the HT purification process using 314 mg of the clean PPF and 50 mg of the HT with a purity of 53% (w/w). The first washing with water at room temperature recovered 26.9 mg of HT, a value that is 53.8% of the added HT (purity of 75.8%). Four subsequent washings with water, at 80 °C for 30 min, were collected to produce a solution with 31.2 mg of HT, a value that represents 62.4% of the added HT with a purity of 100%. This recovery exceeded 100%, specifically at 116%, suggesting that the HT bound to the cleaned PPF continued to be progressively released. This result supported the hypothesis mentioned above of a high retention of HT by the PPF.

This new purification system recovered all of the added HT without any losses with an average purity of 89%, representing more than one-half of the total calculated HT with a purity of 100%. These results were significantly better than those obtained previously by our research group using a method that purified HT in the final step using an XAD adsorption resin, as described in the patented system (Fernández-Bolaños et al., 2008). The previous system was only able to recover 73% of the loaded HT with an average purity of 90%, with only two fractions that represented 37% of the loaded HT at a purity of approximately 100%. Chemically, using PPF was an appropriate method for the purification of the HT. Further studies to determine the economic viability of this process are needed to produce a commercial product at an industrial scale.

The development of this application at a large scale and the purification of other molecules of interest that are currently being investigated by our group could potentially represent new uses for this PPF.

3.3. Analysis of the antioxidant activities of PPF

The antioxidant activities of PPF-60 and PPF-75 (PPFs obtained after a steam treatment of alperujo of 160 °C for 60 min and 75 min respectively) were assessed. To determine the antioxidant effects of the PPF and the bound HT, the antioxidant potential for PPF-75 was compared with the value of a clean PPF-75 (PPFC-75) that had been cleaned with five successive water washes at 80 °C for 30 min to release both the weakly and strongly bound HT. The results obtained in both cases were compared with both the standard HT and the vitamin E values (**Fig. 4**).

The reducing power results (**Fig. 4a**), expressed as Trolox equivalents in mg/mL, indicated that the reducing values for both the PPF-60 and the PPF-75 were lower than the values measured for HT and Trolox, with no significant difference between the PPF-75 and the PPFC-75. The antiradical activity against the DPPH radical (**Fig. 4b**), expressed as EC_{50} (the half maximal effective concentration in mg/mL), suggested that the PPF-60 complexes have higher antiradical capacities for DPPH than the PPF-75 complexes, with no significant differences compared with PPFC-75. The PPF complexes had antiradical activities lower than the two standards (HT and vitamin E). In the primary oxidation inhibitor assay, inhibition of hydroxyperoxide accumulation during the oxidation of linoleic acid was measured (**Fig. 4c**). The results, expressed as EC_{50}, revealed a high capacity for inhibition by the PPF-60 without exhibiting significant differences compared with the standard HT and vitamin E, as analyzed using an ANOVA test with 95% LSD. These results are consistent with the results obtained previously by Cardinali et al. (2010), demonstrating that the high molecular weight phenolic fraction present in olive mill waste water had high antioxidant capacities. The data for both PPF-75 and PPFC-75 also demonstrated that the antioxidant effect was a result of the polymeric phenols without additional influence by the presence of HT.

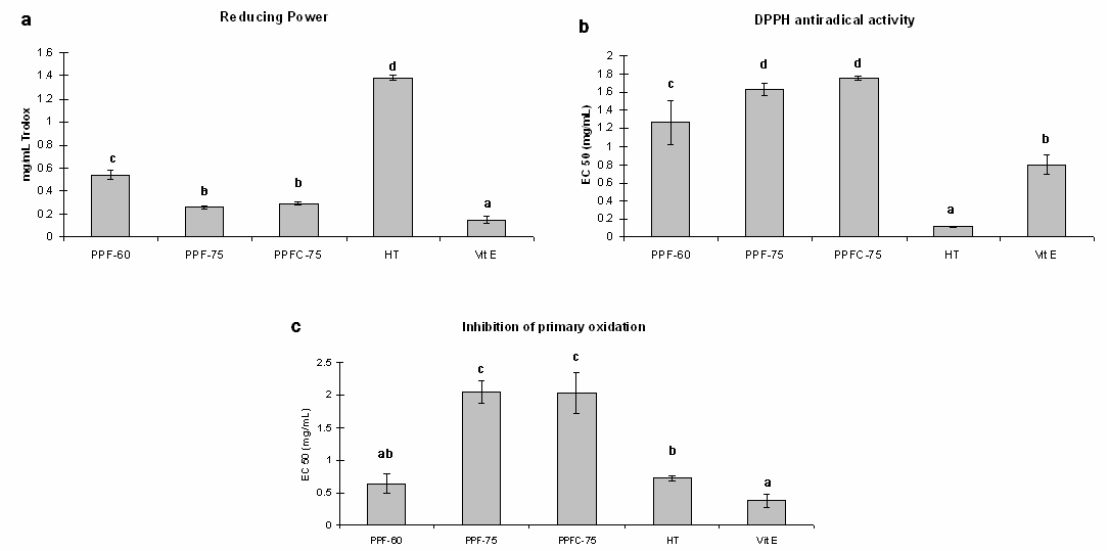


Fig. 4. Reducing power (**a**), radical scavenging capacity on DPPH (**b**) and inhibition of the primary oxidation (**c**) of the polymeric phenolic fraction (PPF) obtained after treatment of the alperujo at 160 °C for 60 min (PPF-60) and 75 min (PPF-75). A cleaned polymeric phenolic fraction (PPFC-75) was also assessed. Data are presented as the means ± SDs. Different letters indicate significantly different results ($p < 0.05$).

4. Conclusions

Polymeric phenolic fraction, composed mainly of phenolic compounds with small amounts of carbohydrates, protein and ash, was formed during an ethyl acetate extraction process from the autohydrolysis liquids of steam-treated alperujo. The interest of the new PPF is based on its antioxidant and physical activities. PPF has shown antioxidant activities *in vitro* in aqueous less than HT and in lipid matrix similar to both HT and vitamin E for PPF-60, and its activity not only depends on the HT present in the PPF matrix. The capacity of PPF for sorption and desorption of hydroxytyrosol and other phenols, makes this polymeric fraction a good tool for HT purification, which is present in the majority of phenol in olives, olive oil and olive by-products. Further studies are needed to understand the mechanism of sorption of phenols, and to determine the real biological activity with or without sorpted phenols.

Acknowledgments

This research was funded by the Ministerio de Economía y Competitividad (AGL2009-12352) of the Spanish Government. Dr. Guillermo Rodríguez-Gutiérrez and Fátima Rubio-Senent would like to express their appreciation to the JAE-Doc and JAE-Pre Programme (CSIC) co-funded by European Social Fund (ESF). They also wish to thank the Junta de Andalucía (Consejería de Innovación, Ciencia y Empresa) (P08-AGR-03751) for financial support.

References

Allouche, N., Fki, I., & Sayadi, S. (2004). Toward a high yield recovery of antioxidants and purified hydroxytyrosol from olive mill wastewaters. Journal of Agricultural and Food Chemistry, 52, 267–273.

AOAC (Official Methods of Analysis) (1990). (15th ed.). Food composition, vol. 1. (pp. 915–919)Arlington, Virginia: AOAC International.

Arana, E., García-Romera, I., Ocampo, J. A., Carbone, V., Mari, A., Malorni, A., et al. (2007). Chemical characterization and effects on Lepidium sativum of the native and bioremediated components of dry olive mil residue. Chemosphere, 69, 229–239.

Bianco, A., Bonadies, F., Romeo, G., Scarpati, M. L., Soriero, A., & Uccella (2006). The recovery of biophenols from wastewaters of olive oil production. Natural Product Research, 20, 259–264.

Capasso, R., De Martino, A., & Arienzo, M. (2002). Recovery of the metal polymeric organic fraction (polymerin) from olive oil mill waste waters. Journal of Agricultural and Food Chemistry, 50, 2846–2855.

Cardinali, A., Cicco, N., Linsalata, V., Minervini, F., Pati, S., Pieralice, M., et al. (2010). Biological activity of high molecular weight phenolics from olive mill wastewater. Journal of Agricultural and Food Chemistry, 58, 8585–8590.

Dische, Z. (1962). Colour reactions of carbohydrates. In R. L. Whistler, & M. L. Wolfram (Eds.), Methods in carbohydrates chemistry (pp. 477–512). NewYork: Academic Press.

European Food Safety Authority (2011). EFSA Journal, 9, 2033 (http://www.efsa.europa.eu/ en/efsajournal/pub/2033.htm)

Fernández-Bolaños, J., Heredia, A., Rodríguez, G., Rodríguez, R., Jiménez, A., & Guillén, R. (2008). Methods for obtaining purified hydroxytyrosol from products and by-products derived from the olive tree. (Patent No. EP1369407B1).

Fernández-Bolaños, J., Rodríguez, G., Rodríguez, R., Heredia, A., Guillén, R., & Jiménez, A. (2002). Production in large quantities of highly purified hydroxytyrosol from liquid–solid waste of two-phase olive oil processing or “alperujo”. Journal of Agricultural and Food Chemistry, 50, 6804–6811.

Fernández-Bolaños, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Sánchez, P. (2011). Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de oliva. (Patent request No. PCT/ES2011/070583).

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Sanchez-Rovira, P., & Ramirez-Tortosa, M. C. (2010). Hydroxytyrosol: From laboratory investigations to future clinical trials. Nutrition Reviews, 68, 191–206.

Killeen, M. J., Pontoniere, P., & Crea, R. (2011). Hydroxytyrosol: An examination of its potential role in cardiovascular disease, inflammation, and longevity. Agro Food Industry Hi-Tech, 22, 16–19.

Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D., & Robards, K. (2005). Investigation of Australian olive mill waste for recovery of biophenols. Journal of Agricultural and Food Chemistry, 53, 9911–9920.

Pérez-Jiménez, F. (2005). International conference on the healthy effect of virgin olive oil. European Journal of Clinical Investigation, 35, 421–424.

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández-Bolaños, J., et al. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. Journal of Agricultural and Food Chemistry, 53, 5212–5217.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. Journal of Agricultural and Food Chemistry, 60, 1175–1186.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. Journal of the Science of Food and Agriculture, 76, 270–276.

Singleton, V. L., & Rossi, J. A., Jr. (1965). Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144–158.

6.1.5. Capítulo 5.

Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E deficient rats.

Fátima Rubio-Senent, Baukje de Roos, Garry Duthie, Juan Fernández-Bolaños, Guillermo Rodríguez-Gutiérrez.

European Journal of Nutrition, DOI: 10.1007/s00394-014-0807-8.

La dieta mediterránea, que se caracteriza por un alto consumo de aceite de oliva, frutas, verduras, cereales y legumbres, se ha asociado con un menor riesgo de enfermedad cardiovasculares. Los beneficios para la salud de la dieta mediterránea se han atribuido a la alta concentración de polifenoles, que son antioxidantes naturales con propiedades anti-inflamatorias, anti-ateroscleróticas y afectan la función plaquetaria humana.

Se ha estudiado la actividad antiplaquetaría y la actividad de inhibición de la oxidación de microsomas provenientes de ratas con una dieta deficiente en vitamina E de distintos extractos y especies fenólicas obtenidas tras someter al alperujo a un tratamiento térmico. Dicho tratamiento favorece la liberación y fácil extracción de los compuestos fenólicos que permanecen remanentes en el subproducto generado durante el proceso de extracción del aceite de oliva mediante el sistema de dos fases.

Este trabajo ha sido realizado gracias a una estancia de tres meses llevada a cabo en el Rowett Institute of Nutrition & Health, de la Universidad de Aberdeen (Escocia, Reino Unido). Los resultados mostraron efecto positivo para los extractos sobre la inhibición de la agregación de plaquetas, obteniendo los mejores resultados cuado se probó el acetato de hidroxitirosilo y la mezcla de acetato de hidroxitirosilo más hidroxitirosol, observándose por primera vez este efecto sinérgico. El efecto positivo de estos fenoles presentes en el aceite de oliva en la inhibición de la agregación plaquetaría sugiere que estos extractos y compuestos podrían ser alternativas naturales para la prevención de enfermedades relacionadas con el trastorno cardiovascular. Por otro lado, el estudio realizado sobre la inhibición de la oxidación de los lípidos por parte de fenoles y extractos fenólicos aislados del alperujo tratado térmicamente mostraron efecto sinérgico cuando las mezclas hidroxitirosol más dihidroxifenilglicol fue estudiada, pudiéndose emplear para como alternativa natural para prevenir daños oxidativos.

Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E deficient rats.

Fátima Rubio-Senent, Baukje de Roos, Garry Duthie, Juan Fernández-Bolaños, Guillermo Rodríguez-Gutiérrez.

ABSTRACT

Purpose. This study explored the *in vitro* antioxidant and anti-platelet activities of hydroxytyrosol, hydroxytyrosol acetate, 3,4-dihydroxyphenylglycol and two phenolic olive extracts. These compounds and extracts were obtained from a new industrial process to hydrothermally treat the alperujo (160 °C/60 min), a by-product of olive oil extraction.

Methods. The extracts and the purified compounds were obtained chromatographically using both ionic and adsorbent resins. The antioxidant activity was determined by measuring inhibition of human platelet aggregation and inhibition of lipid peroxidation in liver microsomes of vitamin E-deficient rats.

Results. The positive effect of the extracts on the inhibition of platelet aggregation is showed, being higher in the case of hydroxytyrosol acetate up to 38 %, and for the first time, its synergist effect with hydroxytyrosol has been proved, obtaining more than double of inhibition. The phenolic extracts and the isolated phenols showed good results for inhibiting the lipid oxidation, up to 62 and 25 %, respectively. A synergistic effect occurred when the hydroxytyrosol acetate and the 3,4-dihydroxyphenylglycol were supplemented by hydroxytyrosol.

Conclusion. These results suggest the extract and these compounds obtained from a novel industrial process could be natural alternatives for the prevention of diseases related to cardiovascular disorder or oxidative damage.

KEYWORDS. Alperujo · Olive oil wastes · Phenols · Platelet function · Lipid peroxidation · Vitamin E

Introduction

Consumption of the Mediterranean diet, characterized by high consumption of olive oil, fruits, vegetables, grains and legumes, reduces the incidence of major cardiovascular events [1] and is associated with a lower risk of peripheral artery disease [2]. The health benefits of the Mediterranean diet have been attributed to high concentration of free radical-scavenging polyphenols such as flavonoids. Virgin olive oil is rich in unsaponifiable minor components such as sterols, tocopherols and polyphenols. The polyphenols are natural antioxidants that not only contribute to the stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties [3]. Dietary polyphenols have been shown to inhibit LDL oxidation, scavenge superoxide and other ROS, and increase plasma antioxidant capacity [4]. Furthermore, some dietary phenolic compounds, mainly polyphenols, have been shown to affect human platelet function *in vitro* and *in vivo* [5, 6]. Platelets play a central role in the formation of plaques within blood vessels, contributing to early inflammatory events [5]; so, the observed cardiovascular benefits attributed to olive oil may be linked to the anti-platelet activity of olive oil polyphenols and thus to the suppression of platelet activation.

The ability of many flavonoids and phenols to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats might indicate that these dietary compounds could have significant “vitamin E-like” antioxidant activity in biological systems [7]. The ability of dietary antioxidants to impair free radical-mediated oxidation of proteins, lipids and DNA, which are implicated in the pathogenesis of many chronic diseases [8], are believed to beneficially affect health.

After olive oil extraction, only a low percentage of the total phenolic compounds present in the olive fruits are found in the virgin olive oil. The remaining phenolics (98–99 %) end up in alperujo, a by-product from the modern two-phase processing technique used in the olive oil production [9].

Nowadays, the olive oil industry is starting to generate new by-products richer in phenols by thermal process applications such as hydroxytyrosol (HT), 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol acetate (HTA), or polymeric phenolic fractions (PPF) [10]. The industrial use of a patented steam treatment (ST) [11] allows the formation of liquid source that enables the extraction and the isolation of the most important phenols present in virgin olive oil. The ST leads to obtain a natural liquid source without suspended solid that is richer in phenols than the other liquid sources obtained from olive oil wastes. This treatment was designed in base of the effects found in the “steam explosion” system (SE) in which high temperatures and pressures (up to 240 °C and 40 kg/cm^2) are needed for a few minutes followed by an explosive depressurization. The main effects were the high solubilization of phenols and sugars and the easy separation of its phases. The new ST operates at lower temperatures up to 170 °C and

retention time of 1 h, avoiding the explosion and then, the technical complications and the high costs of operation, making easier its scale up that has been successfully done.

In previous work [6], the anti-platelet effects of alperujo extract obtained after an SE treatment were measured by comparing it with the effect of simple phenols such as hydroxytyrosol and 3,4-dihydroxyphenylglycol (DHPG), detecting for the first time the synergic effect of these two simple phenols. The effects on animal models of the alperujo extract treated by SE have been also studied [12]. Because the thermal conditions and the concentration of phenols of both treatments are different, together with the industrial use of the ST, the study of the *in vitro* activities of the phenols obtained from alperujo treated by ST are necessary. In the present work, the phenolic extract (PE) obtained from the alperujo treated industrially by ST has been tested, for the first time, to assess *in vitro* their antiplatelet in human platelet screening tool and their antioxidant effects in an animal microsomes model.

Materials and methods

Test compounds

Phenolic extracts (PE) were obtained from ST of alperujo at 160 °C for 60 min [10]. Polymeric phenolic fractions (PPF) were isolated from the PE by chromatography fractionation using Amberlite® XAD [13]. Hydroxytyrosol (HT) and 3,4-dihydroxyphenylglycol (DHPG) were purified by an ionic resin column [14, 15]. Hydroxytyrosol acetate (HTA) was isolated by an ionic resin column [16]. **Figure 1** shows the HPLC profile of each of the test compounds used.

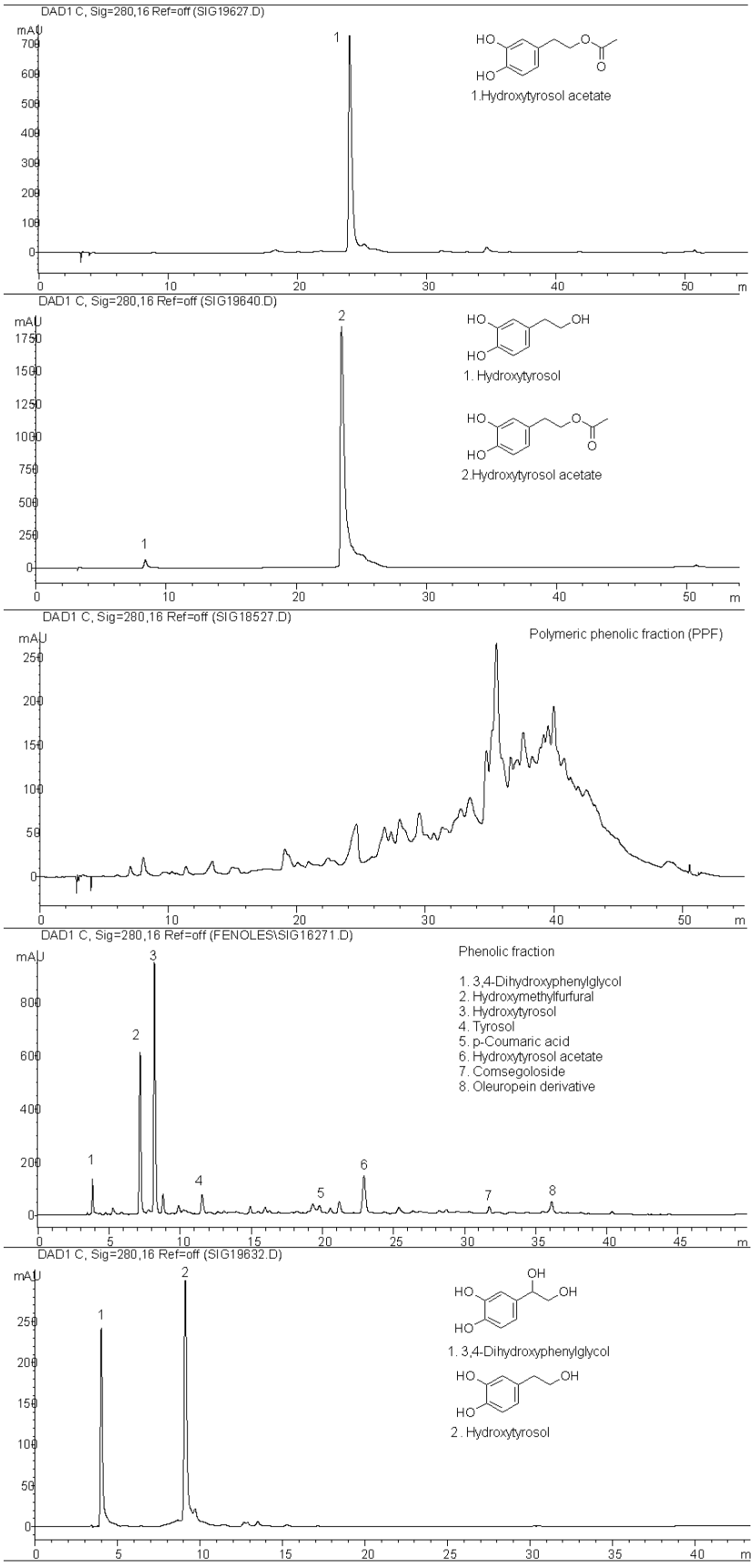


Fig. 1. HPLC profile of different samples used in this work.

Determination of the total phenolic content

To complete the analysis of phenols, the total phenolic content of the test compounds was measured according to the Folin-Ciocalteu method [17] and expressed as grams of gallic acid equivalents per kilogram of extract.

HPLC-DAD

The different phenols were quantified using a Hewlett-Packard 1100 liquid chromatography system with a C-18 column (Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm i.d., 5 μm). The system was equipped with a diode array detector (DAD; the wavelengths used for quantification were 254, 280 and 340 nm) and Rheodyne injection valves (20 μL loop). The mobile phases were 0.01 % trichloroacetic acid in water and acetonitrile utilizing the following gradient over a total run time of 55 min: 95 % A initially, 75 % A at 30 min, 50 % A at 45 min, 0 % A at 47 min, 75 % A at 50 min and 95 % A at 52 min until the run was completed. Quantification was completed by integration of peaks at different wavelengths with reference to calibrations made using external standards. The linearity of standard curves was expressed in terms of the determination coefficient plots of the integrated peak area versus concentration of the same standard. These equations were obtained over a wide concentration range in accordance with the levels of these compounds in the samples. The system was linear in all cases (r > 0.99). Three replicates were carried out on the same day.

Measurement of platelet aggregation

Blood sampling procedure

Blood samples were obtained from 23 healthy volunteers from European countries (9 males and 14 females and 25–60 years of age). Each volunteer signed consent form before donating blood. Volunteers had abstained from anti-inflammatory drugs and food supplements for at least 2 weeks prior to blood sampling and had a normal blood cell count. Blood was obtained using siliconized 21 gauge butterfly needles into 10-mL S-Monovette blood collection tubes containing 1 mL trisodium citrate as anticoagulant (Sarstedt Ltd, Beaumont Leys, UK).

In vitro platelet aggregation

Agonist-induced platelet aggregation was measured in platelet-rich plasma (PRP) upon incubation with the test compounds using a PACKS 4 machine (platelet aggregation chromogenic kinetic system) as described by us previously [6, 18]. Briefly, blood from healthy volunteers was collected into sodium citrate 3.8 % (9:1 v/v), and the PRP was obtained according to standardized procedures. The plasma poor in platelets (PPP) was used to adjust the PRP to a platelet count of 300 ± 20 platelets/μL using the Sysmex haematology analyser (KX-21N, Sysmex, Germany). Once adjusted, the PRP was left to rest at 37 °C. PRP was incubated at least ten times with different platelets with 100 and 500 mg/L of PE; 10, 50 and 100 mg/L of HTA; 10, 50 and 100 mg/L of PPF; 8 + 0.6, 43 + 3, 85 + 6 mg/L HTA + HT, respectively;

or PBS as a control, for 10 min at 37 °C. To induce the platelet aggregation, collagen (final concentration 3 and 5 µg/mL) or thrombin receptor analogue peptide (TRAP) (final concentration 25 µM) was added. The platelet aggregation measurement was started 90 min after blood sampling. After 10 min, the curve of platelets aggregation was obtained, and platelet aggregation was expressed as percentage of maximal aggregation.

Inhibition of lipid peroxidation in vitamin E-deficient microsomes

Microsomal lipid peroxidation was assessed by measuring the reaction of malonaldehyde, a product of lipid oxidation, with thiobarbituric acid to produce thiobarbituric acid-reactive substances (TBARS). These are quantified by high-pressure liquid chromatography (HPLC). Each extract was tested in triplicate.

For this study, liver microsomes from vitamin E-deficient male weanling rats of the Rowett Hooded Lister strain were used, as described by us previously [12]. Briefly, rats that had been on a diet containing less than 0.5 mg/kg vitamin E (−VE) for 13 weeks were anesthetized with ether and bled by cardiac puncture prior to removal of the liver. This protocol was approved by the Ethical Review Committee of Animal Studies at the Rowett Research Institute and was conducted in compliance with the Animals (Scientific Procedures) Act, 1986.

Microsomes were extracted from homogenized liver samples by washing with 0.154 M KCl and suspension in potassium phosphate buffer 0.05 M pH 7.4. The protein concentration was determined by the Biuret method and adjusted to 10 mg/mL with 0.05 M potassium phosphate buffer pH 7.4. Vitamin E deficiency in rats was confirmed by plasma, tissue and microsomal vitamin E concentrations, which were below the limit of detection by HPLC. The effect of the test compounds on *in vitro* microsomal lipid peroxidation in the microsomes was determined as described by the method of Duthie et al. [19]. Briefly, ethanol solutions (20 µL) with the test compounds in a concentration 25 times higher than required were incubated with microsomal preparations (0.5 mL) for 30 min at room temperature. Solutions with α-tocopherol were used as a control. For the control sample deficient in α-tocopherol, only 20 µL of EtOH was added. 0.1 mL of microsome solution with compounds was mixed with 0.5 mL of 0.05 mM ascorbic acid, 1 mL of a 2 mMADP/6 µM Fe^{2+} solution and 3.4 mL of 0.05 M phosphate buffer pH 7.4. The mixture was incubated at 30 °C and different aliquots were taken at 0, 5, 10 and 20 min for determination of thiobarbituric acid-reactive substances (TBARS) by HPLC. To this, 1 mL of 0.67 % thiobarbituric acid/acetic acid (1:1) and 2 mL of water were added and the solution was heated for 30 min at 100 °C in order to let the colour develop (measured at 535 nm).

The amount of generated TBARS was quantified by HPLC (Allience 2695) equipped with a fluorescence detector (Waters 2475). The column used was Phenomenex Luna 5u C18 (2) 100 A, 150 × 4.60 mm; the mobile phase was 60 % of KH_2PO_4 50 mM pH 7.0 and 40 % of MeOH at a flow of 0.8 mL/min for 12 min, in isocratic mode. Inhibition of lipid peroxidation was calculated as follows:

$$\%Inhibition = \left(\frac{AUC(-VE) - AUC(-VE + Comp)}{AUC(-VE)} \right) x\,100$$

where AUC (−VE) = area under curve in microsomes from rats deficient in vitamin E and AUC (−VE + Comp) = area under curve in microsomes from rats deficient in vitamin E plus test compound. The ability to inhibit microsomal lipid peroxidation by each extract or compound (mixture) was compared with a negative control (no incubation) and two positive controls (effect in microsomes from rats with adequate diet of vitamin E (on a vitamin E-adequate diet 100 mg dα-tocopherol/kg) and effect in microsomes from rats on a vitamin E-deficient diet supplemented with 100 mg/kg diet of dα-tocopherol.

Statistical analysis

Statgraphics® plus software was used for statistical analysis. Comparisons amongst samples were made using oneway analysis of variance (ANOVA, equal variance test) and the least significant difference (LSD) method. A *p* value <0.05 was considered significant.

Results

Inhibition of platelet aggregation

In this study, the anti-platelet properties of two natural extracts (PE and PPF) and a purified compound (HTA) obtained from a new industrial source were screened. Furthermore, the potential synergic effects between hydroxytyrosol acetate and hydroxytyrosol (HTA + HT) have been also studied for the first time.

The characteristics of the volunteers from whom we obtained blood samples are shown in **Table 1**. Incubation of platelet-rich plasma from healthy volunteers with 100 mg/L of hydroxytyrosol acetate, which equates to approximately 510 μM, resulted in a significant (p < 0.05) inhibition of 3 and 5 μg/L collagen-and 25 μM TRAP-induced platelet aggregation by 38, 27 and 37 %, respectively (**Fig. 2**). Incubation with 50 mg/L HTA also resulted in a significant inhibition of 5 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 7 and 22 %, respectively (**Fig. 2**).

Table 1: Subject characteristic.

	Male	Female	Total
Number	9	14	23
Platelet count ($x10^9$/L)	221.65±9.69 [a]	204.58±13.32	213.12±11.50
Mean platelet volume(fL)	8.14±0.26	8.61±0.56	8.37±0.15
Hematocrit (%)	38.72±1.81	35.05±0.78	36.89±0.52
Hemoglobin (g/dL)	13.07±0.89	11.74±0.14	12.40±0.38

[a]Values are mean±SD.

In this study, two natural phenolic extracts, obtained from alperujo hydrothermally treated at 160 °C/60 min (PE) and a polymeric phenolic fraction (PPF) isolated from phenolic extract were also screened. The activity of this new phenolic polymeric fraction (PPF) isolated and characterized has been tested for the first time. This PPF was composed mainly of phenolic compounds with small amounts of carbohydrates, proteins and ash, and it was formed during the ethyl acetate extraction process from the autohydrolysis liquids of steam-treated alperujo [13]. Incubation with 100 mg/L PPF resulted in significant ($p < 0.05$) inhibition of 3 and 5 µg/L collagen and 25 µM TRAP-induced platelet aggregation by 23, 13 and 22 %, respectively. For the assays with PE, a concentration of total phenols of 240 g phenols/kg extract was found by the Folin–Ciocalteu method. The HPLC results suggested that 1 g of PE contained 109.3 mg HT, 10.3 mg PPF, 9.9 mg DHFG and 8.5 mg HTA. Only the highest test concentration of PE (i.e. 500 mg/L) significantly inhibited 3 and 5 µg/L collagen and 25 µM TRAP-induced platelet aggregation by 52, 40 and 19 %, respectively (**Fig. 2**).

Inhibition of microsomal lipoxidation from vitamin E-deficient rats

The antioxidant properties of two natural extracts (PE and PPF) and three purified compounds (HT, HTA and DHPG) were screened. Furthermore, two mixtures of compounds (HT + DHPG) and (HTA + HT) were evaluated. Previous studies showed that a decrease in membrane concentration of α-tocopherol increased the rates of TBARS formation in all tissues, but the effect was especially pronounced in adrenal mitochondria and microsomes [20].

The PE, HTA and the HT + DHPG mixture showed most inhibition of lipid oxidation at the lowest test concentrations (**Tables 2** and **3**). 0.05 mM of HTA inhibited lipid peroxidation by 20.8 % (Table 3). Interestingly, this was very similar to the highest concentration (0.4 mM), but much more effective than the intermediate concentration (13.5 % of inhibition). HT and DHPG decreased lipid peroxidation of the microsomal preparation from vitamin E-deficient rats in a time-dependent manner, and the protection against peroxidation improved with increasing concentrations (**Fig. 3**).

Discussion

Inhibition of platelet aggregation

The anti-platelet property of the HTA and HT mixture was more effective than of each component separately. So when HT was tested alone, 6 mg/L of compound inhibited platelet aggregation by 4 % using collagen-induced platelet aggregation and no inhibitory effect was observed for 3 mg/L. Indeed, incubation with 43 + 3 mg/L (219 + 19.5 μM) HTA and HT resulted in a significant ($p < 0.05$) inhibition of 3 and 5 μg/L collagen and 25 μM TRAP-induced platelet aggregation by 30, 22 and 31 %, respectively (**Fig. 2**). A similar effect was observed for 85 + 6 mg/L HTA + HT (433 + 30.6 μM), inhibiting platelet aggregation by 85, 71 and 50 %, respectively (**Fig. 2**). González-Correa et al. [21] observed that 26 and 48 μM HTA inhibited collageninduced platelet aggregation by 50 %. This anti-platelet effect was stronger than that of HT and similar to that of acetylsalicylic acid (ASA), Hubbard et al. [22] considered different simple phenols and found that for p-coumaric, caffeic, ferulic and sinapic acid, a concentration of 478–816 μM was necessary to inhibit collagen-induced platelet aggregation by 50 %. Furthermore, concentrations between 10 and 100 μM of gallic acid inhibited TRAP-induced platelet aggregation by 10–50 % [23]. Ostertag et al. [18] found that 100 μM of catechol, resorcinol, pyrogallol and hippuric acid inhibited *in vitro* collagen-induced platelet aggregation. A previous study in our group using the same samples [6] showed that 40 mg/L of HT inhibited collagen induced platelet aggregation by 5 %, whereas a mixture of HT and DHPG (40 + 5 mg/L, respectively) inhibited collagen-induced platelet aggregation by approximately 12 % in a synergistic manner. In most of these studies, only relatively high concentrations of phenolic compounds caused significant anti-platelet effects in an *in vitro* screening model. Although these models can effectively measure the potential pharmacological anti-platelet effects of a large range of bioactive compounds, the effective concentrations may not necessarily be physiologically relevant from a dietary perspective [6].

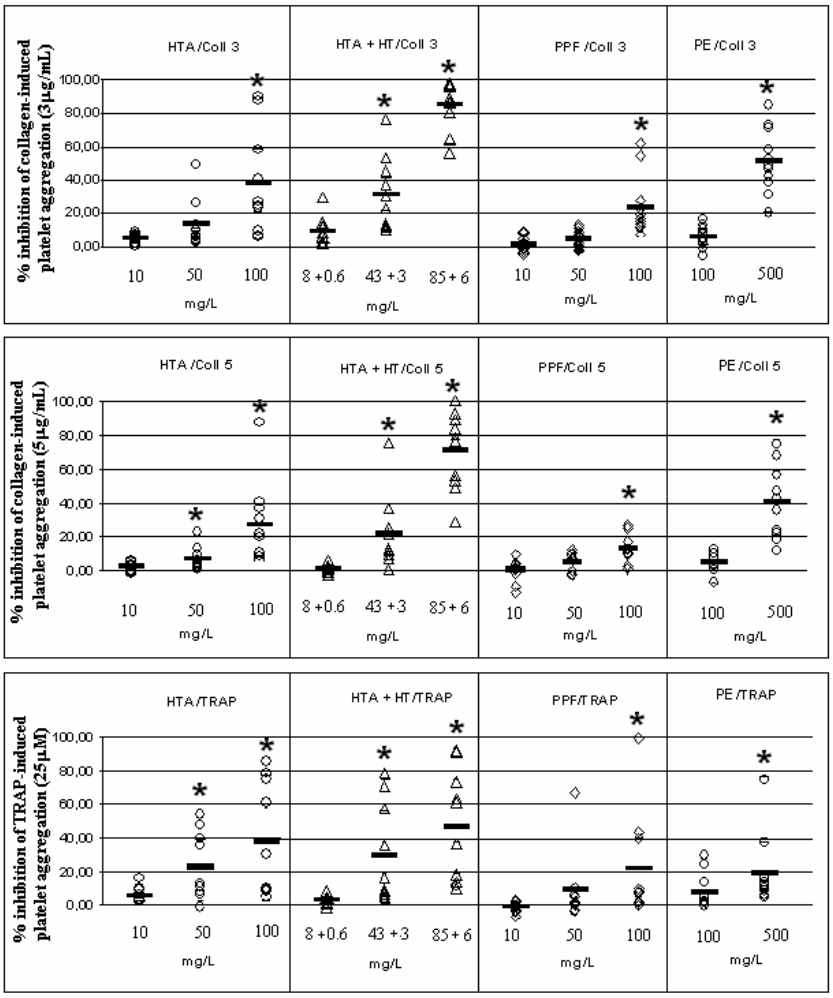


Fig. 2. Inhibition of platelet aggregation by HTA, HTA + HT, PPF and PE using 3 µg/L collagen (a), 5 µg/ collagen (b) or 25 µM TRAP (c) as an agonist. Platelet inhibition is expressed as the % decrease in the areas under the curve for platelet aggregation measured by light-transmission aggregometry when compared with control (PBS). *Significantly different from control ($p > 0.05$); (solid line) mean value

With regard to the PE, in a previous study [6] using an PE obtained by SE, a similar concentration of 500 mg/L alperujo extract significantly inhibited collagen-and TRAP-induced platelet aggregation by 25 and 16 %, respectively. The higher efficacy of PE in the current study could be due to a different phenol composition because the lower severity used to treat the alperujo by the industrial ST system.

Inhibition of microsomal lipoxidation from vitamin E-deficient rats

The HT + DHPG mixture produced a higher percentage inhibition (31 %) for lower test concentrations (0.25 + 0.25 mM), which was more powerful than the efficacy of individual compounds (**Table 2**). Also increasing concentrations of PPF showed improved inhibition of

lipid peroxidation, up to 58 % inhibition for the highest concentration (200 mg/L) (**Table 3**). The high efficacy of PE to act as an antioxidant may be explained by the fact that the extract contains different compounds that work in a synergistic fashion to inhibit lipid peroxidation, or it contains individual minor components that have high capacity of inhibition of lipid peroxidation.

Table 2. Abilities of HT and DHPG to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats

Compound	DHPG		HT	
	mg/L	% Inhibition	mg/L	% Inhibition
+VE		86.38		81.39
-VE+0.05mM	8.5	1.02	8	-2.42
-VE+0.1mM	17	4.89	15	4.84
-VE+0.25mM	43	10.74	39	5.53
-VE+0.5mM	85	11.8	77	8.92
-VE+1mM	170	13.83	154	12.29
-VE+2mM	340	24.61	308	25.45
Mixture	DHPG + HT			
	mg/L		% Inhibition	
-VE+(0.25+0.25)mM	(43+39)		31.26	
-VE+(0.50+0.50)mM	(85+77)		9.34	
-VE+(0.75+0.75)mM	(128+116)		18.07	

* Significant difference ($p < 0.05$) between DHPG + HT and the individual sum of DHPG and HT

Table 3. Abilities of different phenols and PE to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats

Compound	HTA		α-tocopherol	
	mg/L	% Inhibition	mg/L	% Inhibition
-VE+0.05mM	9.8	20.76±4.82	23.7	47.6±4.05
-VE+0.2 mM	39.2	13.45±1.27	94.6	69.8±2.36
-VE+0.4mM	78.4	21.06±0.79	189.2	72.9±1.65
Extracts	PPF		PE	
	mg/L	% Inhibition	mg/L	% Inhibition
-VE+Extract	10	15.4±3.73	39	62.6±1.51
-VE+Extract	100	16.5±4.69	58	45.4±0.66
-VE+Extract	200	57.9±4.30	77	50.7±0.33
Mixture	HTA+HT			
	mg/L		% Inhibition	
-VE+(0.1+0.01)mM	(19.6+1.5)		23.8±3.17	
-VE+(0.4+0.04)mM	(78.4+6.2)		23.72±0.70	
-VE+(0.8+0.08)mM	(157+12.4)		37.72±1.62	

In a detailed study, Duthie and Morrice [7] found that different flavonoids (i.e. quercetin, kaempferol, myricetin, galingin and fisetin) inhibited lipid peroxidation by 68–88 %, a similar range to the olive phenols studied in microsomes derived from livers of rats deficient in vitamin E. Mitchell et al. [24] observed similar results for kaempferol, which of all phytoestrogens had

the greatest ability to inhibit lipid peroxidation in vitamin E-deficient microsomes with an IC_{50} of 160 μM. In comparison, IC_{50} concentrations required to inhibit lipid peroxidation in liver microsomes was 31 μM for α-tocopherol and 124 μM for quercetin. The IC_{50} values for the isoflavones, chalcones and coumestan were approximately 35-, 22- and 16-fold higher than that of α-tocopherol, respectively.

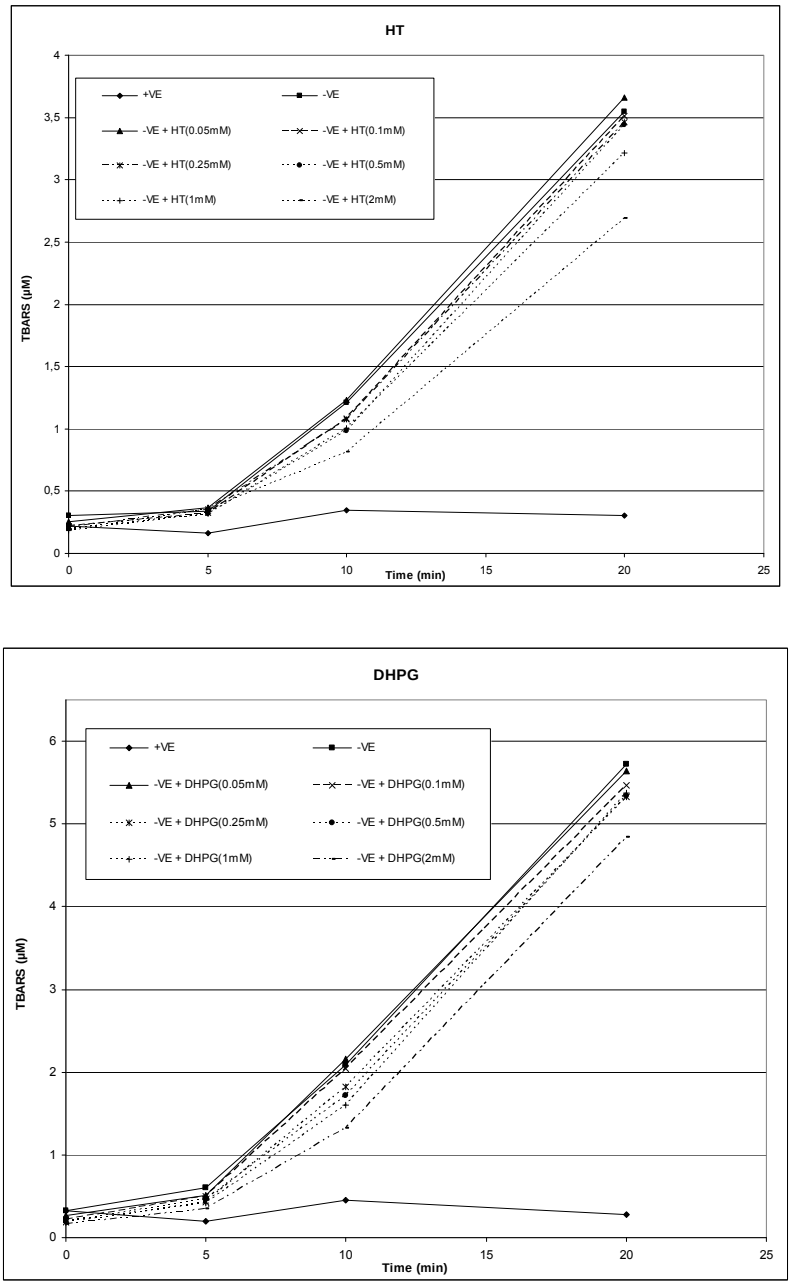


Fig. 3. Pre-incubation of hepatic microsomal preparations from vitamin E-deficient rats with a HT and DHPG on production of thiobarbituric reactive substances (TBARS) following initiation of peroxidation with Fe/ADP

Conclusion

The results of this study show that the olive extracts obtained from a new industrial liquid source produced by ST application to alperujo have higher activities that the PE previously reported using the SE as a treatment. Besides, a synergist effect of HTA and HT has been found for the first time. The PE and the isolated phenols obtained may protect against platelet activation and platelet adhesion and have antioxidant properties. The extraction of these compounds can be accomplished in a sustainable manner through effective use of the industrial source from the olive oil byproduct manufacturing process, offering a unique opportunity to produce functional ingredients that alone, or in effective combinations, could be added to foods to enhance their health properties.

Acknowledgments. The authors wish to express their gratitude to the Ministerio de Economía y Competitividad of Spain and co-funded by European Social Fund (ESF) (Project AGL2013-48291-R) for providing financial support. Dr. Rodríguez-Gutiérrez (RYC-2012-10456 contract) wishes to thank to the "Ramón y Cajal" Program from the Spanish Ministry of Economy and Competitiveness, and Ms RubioSenent (JAE-Pre104) to the Spanish National Research Council and co-funded by European Social Fund (CSIC-ESF) for providing financial support.

Conflict of interest. None.

References

1. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gómez-Gracia E, Ruíz-Gutiérrez V et al (2013) Prevention of cardiovascular disease with a Mediterranean diet. New Engl J Med 368:1279–1290

2. Ruiz-Canela M, Estruch R, Corella D, Salas-Salvado J, MartínezGonzález MA (2014) Association of Mediterranean diet with peripheral artery disease: the PREDIMED randomized trial. J Am Med Assoc 311:415–417

3. González-Santiago M, Martín-Bautista E, Carrero JJ, Fonolla J, Baro L, Bartolomé MV, Gil-Loyzaga P, López-Huertas E (2006) One-month administration of hidroxitirosol, a phenolic anti-oxidant present in olive oil, to hyperlipemic rabbits improve blood lipid profile, anti-oxidant status and reduces atherosclerosis development. Atherosclerosis 188:35–42

4. Visioli F, Poli A, Galli C (2002) Anti-oxidant and other biological activities of phenols from olives and olive oil. Med Res Rev 22:65–75

5. Ostertag LM, O'Kennedy N, Kroon PA, Duthie GG, de Roos B (2010) Impact of dietary polyphenols on human platelet function—a critical review of controlled dietary intervention studies. Mol Nutr Food Res 54:60–81

6. De Roos B, Zhang X, Rodríguez-Gutiérrez G, Wood S, Rucklidge GJ, Reid MD, Duncan GJ, Cantlay LL, Duthie GG, O'Kennedy N (2011) Anti-platelet effects of olive oil extract: *in vitro* functional and proteomic studies. Eur J Nutr 50:553–562

7. Duthie GG and Morrice P (2012) Anti-oxidant capacity of flavonoids in hepatic microsomes is not reflected by anti-oxidant effects *in vivo*. Oxid Med Cell Longev 165127

8. Aruoma OI (1998) Free radicals, oxidative stress, and antioxidants in human health and disease. J Am Oil Chem Soc 75:199–212

9. Fernández-Bolaños J, Rodríguez G, Gómez E, Guillén R, Jiménez A, Heredia A, Rodríguez R (2004) Total recovery of the waste of two-phase olive oil processing: isolation of added-value compounds. J Agric Food Chem 52:5849–5855

10. Rubio-Senent F, Rodríguez-Gutiérrez G, Lama-Munoz A, Fernández-Bolaños J (2012) New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. J Agric Food Chem 60:1175–1186

11. Fernández-Bolaños J, Rodríguez G, Lama-Muñoz A, Sánchez P (2011) Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de oliva. Patent Request No. PCT/ES2011/070583

12. Rodríguez-Gutiérrez G, Duthie GG, Wood S, Morrice P, Nicol F, Reid M, Cantlay LL, Kelder T, Horgan GW, Fernández-Bolaños J, de Roos B (2012) Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have anti-oxidant properties in vitamin E-deficient rats proteomics and network analysis approach. Mol Nutr Food Res 56:1137–1147

13. Rubio-Senent F, Rodríguez-Gutiérrez G, Lama-Muñoz A, Fernández-Bolaños J (2013) Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. Food Res Int 54:2122–2129

14. Fernández-Bolaños J, Rodríguez G, Rodríguez R, Heredia A, Guillén R, Jiménez A (2002) Production in large quantities of highly purified hidroxitirosol from liquid–solid waste of twophase olive oil processing or "Alperujo". J Agric Food Chem 50:6804–6811

15. Fernández-Bolaños J, Rodríguez G, Lama A, Rodríguez-Arcos R, Jiménez A, Guillén R (2008) Purification of 3,4-dihydroxyphenylglycol (DHPG) from vegetable products. Patent Request No 200803630

16. Fernández-Bolaños J, Rodríguez-Gutiérrez G, Lama-Muñoz A, Fernández-Bolaños JM, Maya-Castilla I, Rubio Senent F, López López O, Marset Castro A (2013) Method for obtaining hydroxytyrosol extract, mixture of hydroxytyrosol and 3,4-dihydroxyphenylglycol extract, and hydroxytyrosyl acetate extract from by-products of the olive tree and the purification of thereof. International Patent No: WO 2013/007850A1

17. Singleton VL, Rossi JA Jr (1965) Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. Am J Enol Vitic 16:144–158

18. Ostertag LM, O'Kennedy N, Horgan GW, Kroon PA, Duthie GG, de Roos B (2011) *In vitro* anti-platelet effects of simple plantderived phenolic compounds are only found at high, non-physiological concentrations. Mol Nutr Food Res 55:1–13

19. Duthie GG, González BM, Morrice PC, Arthur JR (1991) Inhibitory effects of isomers of tocopherol on lipid-peroxidation of microsomes from vitamin E-deficient rats. Free Radic Res Commun 15:35–40

20. Burczynski JM, Southard SJ, Hayes JR, Longhurst PA, Colby HD (2001) Changes in mitochondrial and microsomal lipid peroxidation and fatty acid profiles in adrenal glands, testes, and livers from α-tocopherol-deficient rats. Free Radic Biol Med 30:1029–1035

21. González-Correa JA, López-Villodres JA, Asensi R, Espartero JL, Rodríguez-Gutiérrez G, de la Cruz JP (2009) Virgen olive oil polyphenol hidroxitirosol acetate inhibits *in vitro* platelet aggregation in human whole blood: comparison with hidroxitirosol and acetylsalicylic acid. Br J Nutr 101:1157–1164

22. Hubbard GP, Wolffram S, Lovegrove JA, Gibbins JM (2003) The role of polyphenols compounds in the diet as inhibitors of platelet function. Proc Nutr Soc 62:469–478

23. Lill G, Voit S, Schror K, Weber AA (2003) Complex effect of different green tea catechins on human platelet. FEBS Lett 546:265–270

24. Mitchell JH, Gardner PT, McPhail DB, Morrice PC, Collins AR, Duthie GG (1998) Anti-oxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 360(1):142–148

6.1.6. Capítulo 6.

Isolation and characterization of a secoiridoid derivative from two-phase olive waste (alperujo).

Fátima Rubio-Senent, Sergio Martos, Aránzazu García, José G. Fernández-Bolaños, Guillermo Rodríguez-Gutiérrez, Juan Fernández-Bolaños.

J. Agric. Food Chem. DOI: 10.1021/jf5057716.

El pretratamiento térmico realizado sobre el alperujo para facilitar la extracción de los compuestos fenólicos presentes en el mismo provoca la solubilización de gran parte de estos compuestos, los cuales han sido analizados y cuantificados en capítulos previos. El análisis realizado sobre los extractos fenólicos obtenidos nos ha permitido identificar un derivado del ácido elenólico el cual se encuentra en elevadas concentraciones. En este capítulo nos centramos en identificar la estructura del derivado aislado. Estudios previos muestran que los compuestos derivados de la estructura del ácido elenólico, como compuestos con estructura glutaraldehídica presentan actividades antimicrobianas importantes.

En este trabajo se aisló un derivado del ácido elenólico y su estructura fue identificada correctamente por primera vez mediante datos espectroscópicos de RMN. Además, se ha estudiado como afecta el pretratamiento realizado sobre el alperujo, así como el proceso de extracción con acetato de etilo, tanto en frío como en caliente, sobre el proceso de formación/recuperación del compuesto de interés. Finalmente, como la estructura del compuesto deriva del glutaraldehído se realizaron unos ensayos de medida de actividad antimicrobiana, tanto del compuesto puro, como de dos extractos que contenían a dicho compuesto entre otras especies fenólicas.

Los ensayos realizados mostraron que la molécula era un derivado de ácido elenólico, concretamente el ácido (*E*)-3-(1-oxobut-2-en-2-il) glutárico. Así mismo se confirmó que para que tenga lugar su recuperación en elevadas concentraciones es necesario realizar un proceso de extracción con acetato de etilo, el cual acelera su formación y/o recuperación, ya que la especie también ha sido detectada en alperujos almacenados durante largos periodos. Además la actividad antimicrobiana del compuesto queda probada.

Isolation and characterization of a secoiridoid derivative from two-phase olive waste (alperujo).

Fátima Rubio-Senent, Sergio Martos, Aránzazu García, José G. Fernández-Bolaños, Guillermo Rodríguez-Gutiérrez, Juan Fernández-Bolaños.

ABSTRACT

A secoiridoid derivative was isolated from the ethyl acetate extract of two-phase olive waste (alperujo). The structure of this compound was fully characterized as *s-trans*-(*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid. The spectroscopic data including one-and two-dimensional NMR, mass spectrometry, infrared analysis and UV spectrum were showed. The origin of this compound has not been previously studied, although it most likely results from the breakdown of the oleuropein (or ligstroside) secoiridoid skeleton via oxidation and decarboxylation of dialdehydic form of elenolic acid, being this transformation enhanced by extraction of phenolics with ethyl acetate. In addition, the bactericidal activity of (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid and extracts containing it were evaluated against two phytopatoghenic microorganisms *Pseudomonas syringae* and *Agrobacterium tumefaciens*.

KEYWORDS: Alperujo; antimicrobial activity; ethyl acetate extract; elenolic acid; glutaric acid derivatives.

Introduction

Olive oil production is a very important and traditional industrial activity in the countries of the Mediterranean area, which account for 95% of worldwide olive oil production.[1] The olive oil manufacturing process by a two-phase centrifugation system generates huge quantities of a semi-solid by-product called alperujo, recognized as a valuable source of natural phenol antioxidants. After olive oil extraction, only a small percentage (1-2 %) of the total phenols present in the olive fruit remain in the oil, and 98-99 % of the phenol remain in the alperujo.[2]

The phenolic compounds of olives and olive products have been extensively studied due to their biological properties.[3-4] To increase the concentration of these phenols and facilitate their extraction from alperujo, an environmentally friendly process based on hydrothermal treatment was developed.[5] This process led to an easy separation of the solid and liquid phases of alperujo and allows the recovery of value-added compounds in the water-soluble fraction.

The alperujo is partially solubilised in water by the hydrothermal treatment, being the liquid fraction enriched in phenolic compounds that are easily extractable with ethyl acetate.[6]

The ethyl acetate extract was an interesting fraction composed of numerous phenolic compounds.[6-8] Many of the phenolic compounds present in the extract have been identified and their structures elucidated; however, some compounds remain unidentified including different derivatives of elenolic acid present in large amounts. In this work we have isolated and identified a compound whose structure, derived from elenolic acid, has been well-established.

It has been shown that compounds with elenolic acid-derivatives structures, such as glutaraldehyde-like compounds, exhibit antimicrobial activities important. Numerous works have studied the antimicrobial activity of olive mill wastewaters and alperujo[9-10] due to the high concentrations of phenolic compounds present in these by-products. For many years this activity was attributed to oleuropein and its hydrolysis products (oleuropein aglycon, elenolic acid and hydroxytyrosol), and the antimicrobial activity of these substances has been extensively studied.[11] Medina et al.[12] demonstrated that the main antimicrobials in olives are the dialdehydic form of decarboxymethyl elenolic acid, either free (EDA) or linked to hydroxytyrosol (HT-EDA), due to their content in glutaraldehyde-like compounds. Phytopathogenic bacteria such as *Pseudomonas syringae* and *Agrobacterium tumefaciens* can cause severe disease in a wide range of plants cultivated in the Mediterranean basin. *P. syringae* contributes to many diseases in tomato, peas, maple, kiwifruit, wheat, barley, and others, and *A. tumefaciens* produces tumors known as crown galls in a diverse group of dicotyledonous plants. The use antimicrobials from olive mill wastewaters against these bacteria would be of great interest.

In this work an elenolic acid derivative was isolated and its structure was elucidated by NMR spectroscopy. Moreover, the role of ethyl acetate in the formation of this compound was analyzed. Finally as the structure of the compound derives from a glutaraldehyde-like compound, an antimicrobial assay was realized to evaluate its bactericidal activity.

Materials and methods

Isolation of the compound of interest

Materials

Fresh alperujo samples (not stored) were obtained after virgin oil extraction from Picual olive fruits in an experimental mill at the Instituto de la Grasa in Seville, Spain. Samples of wet alperujo were taken from the output of the horizontal centrifuge of the two-phase process. The alperujo (a semisolid residue composed of olive peels, pulp, seeds, and ground stones) was processed without removal of the stones.

Standard compounds.

Hydroxymethylfurfural, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, 4-methylcatechol, catechol, 3,4-dihydroxyhydrocinnamic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma-Aldrich (Deisenhofer, Germany). Tyrosol was obtained from Fluka (Buchs, Switzerland). Oleuropein was obtained from Extrasynthese (Lyon Nord, Geney, France). Hydroxytyrosol was obtained by the method described by Fernández-Bolaños et al.[13] Hydroxytyrosol acetate was isolated following the process described by Fernández-Bolaños et al.[14]

Thermal Treatment.

The hydrothermal treatment used has been patented[5] and was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor has a stainless steel reservoir (100 L capacity) that can operate at temperatures between 50 and 190 °C and at a maximum pressure of 1.2 MPa. 10 kg of fresh alperujo were loaded into the reactor and treated for 60 min at 140 and 170 °C increasing the liquid by steam condensation. The wet material was centrifuged at 4700g (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, 10 L of the liquid phase was concentrated to 1 L by rotary evaporation under vacuum at 30 °C.

Phenol Extraction with refluxing ethyl acetate.

The liquid portions obtained after treatment were washed with hexane to remove the lipid fraction. 1 L of liquid was mixed with 500 mL of hexane, the mixture was shaken vigorously, and then the phases were separated by decantation and washed twice. Extraction of phenolic compounds was carried out with ethyl acetate. 200 mL of aqueous sample was extracted with 500 mL of ethyl acetate. The liquid−liquid extraction was performed with ethyl acetate (refluxed at 77 °C) in a continuous extractor of a heavier liquid (water) by lighter liquid (ethyl acetate) for 8 h.

Chromatographic Fractionation of the Ethyl Acetate Extract.

Approximately 12 g of extract were dissolved in 60 mL of H_2O/MeOH (80:20). The extracts were passed through a column 3.5 cm in diameter and 40 cm in height filled with Amberlite XAD16. The elution was performed with 1 L of H_2O, 30% EtOH (v/v), 50% EtOH (v/v), and 95% EtOH (v/v). Ten fractions of 100 mL each were collected. Fractions were analyzed by HPLC, and those containing the derivative of elenolic acid of interest were mixed and concentrated to 50 mL. The fractions 1 and 2 used in the antimicrobial test were obtained in this step.

HPLC-DAD.

The compounds were identified using a Hewlett-Packard 1100 liquid chromatography system with a C_{18} column (Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm i.d., 5 μm particle size). The system was equipped with a diode array detector (DAD; the wavelengths

used for quantification were 254, 280, and 340 nm) and Rheodyne injection valves (20 µL loop). The method used of quantification was described by Rubio-Senent et al.[6] Quantification was performed in triplicate using external standards; standard curves were linear in all cases (R^2 > 0.99).

Isolation.

The compound of interest was isolated using a semi-preparative system. A HPLC method similar to that described for HPLD-DAD analysis was used. Jasco HPLC (LC-Net II ADC, Kyoto, Japan) equipped with a DAD detector and a semipreparative Synergi 4 µ Hydro-RP80A reverse phase column (25 cm x 21.20 mm i.d., 4 µm particle size; Phenomenex, Macclesfield, Cheshire, UK) was used. The flow rate was maintained at 12 mL/min, and the injection volume was 600 µL. Before the DAD detector, the flow was divided with a T connection (0.25 mL/min to the DAD detector and 11.75 mL/min to the collection port).

Structural analysis.

^{1}H and ^{13}C NMR, COSY, HSQC, NOESY and HMBC experiments.

^{1}H (300.1 and 700.2 MHz) and ^{13}C (75.5 and 176.1 MHz) NMR spectra were recorded on Bruker Avance-300 III and Avance-700 III spectrometers using CD_3OD as solvent. The latter spectrometer was equipped with a cryogenically cooled 5 mm QCI gradient probe. Chemical shifts are reported in δ units (ppm) relative to the solvent peak (3.31 and 49.0 ppm for ^{1}H- and ^{13}C-NMR, respectively). ^{13}C NMR spectrum registered at 75.5 MHz was acquired with the following acquisition parameters: acquisition time 0.9 s, relaxation delay 1.5 s, spectral width of 239 ppm, data points 32k, number of scans 1,434, and line broadening of 1.0 Hz; whereas the ^{13}C spectrum registered at 176.1 MHz was acquired with an acquisition time of 0.8 s, relaxation delay of 3.0 s, spectral width of 220 ppm, data points 64k, number of scans 14,336, and line broadening of 1.0 Hz. The enhanced sensitivity of the NMR at 176.1 MHz with cryoprobe for ^{13}C NMR spectrum, allowed the signal for the carboxyl groups to be clearly observable, whereas that signal was missing at 75.5 MHz and a shorter relaxation delay (1.5 s).

The assignments of ^{1}H and ^{13}C signals were confirmed by homonuclear COSY, and heteronuclear HSQC spectra (Avance-300 III), respectively. The configuration of the double bond was confirmed by a 2D-NOESY experiment (Avance-300 III). The HMBC (Avance-700 III), experiment confirmed the connexion of the glutaric acid with the crotonaldehyde moiety through C-3 of the dicarboxylic acid.

Mass spectra.

Mass spectra (CI and HR-CI) were recorded on a Micromass AutoSpec-Q mass spectrometer with a resolution of 1000 or 10,000 (10% valley definition).

IR spectra.

IR spectra were recorded on Jasco FT/IR 4100 equipped with ATR accessory.

(*E*)-3-(1-Oxobut-2-en-2-yl)glutaric acid.

IR ν_{max} 3300-2400, 2727, 1684, 1440, 1405, 1310, 1234, 1160, 982, 932 (br), 844, 801 cm^{-1}. ^{1}H-NMR (700.2 MHz, CD_3OD): δ 9.28 (d, 1H, $J_{1',3}$ = 2.0 Hz, H-1'), 6.79 (q, 1H, $J_{3',4'}$ = 7.0 Hz, H-3'), 3.59 (m, 1H, H-3), 2.68 (dd, 2H, $J_{2a,2b}$ = 15.5 Hz, $J_{2a,3}$ = 9.1 Hz, H-2a, H-4a), 2.61 (dd, 2H, $J_{2b,3}$ = 6.2 Hz, H-2b, H-4b), 2.08 (d, 3H, $J_{4',3'}$ = 7.0 Hz, H-4'); ^{13}C-NMR (176.1 MHz, CD_3OD): δ 197.0 (C-1'), 176.3 (C-1, C-5), 155.9 (C-3'), 145.3 (C-2'), 38.4 (C-2, C-4), 31.9 (C-3), 15.4 (C-4'); CIMS *m/z* 201 ($[M + H]^+$, 22%), 183 ($[M - H_2O + H]^+$, 66%), 165 ($[M - 2H_2O + H]^+$, 100%); HR-CIMS *m/z* calcd for $C_9H_{13}O_5$ ($[M + H]^+$): 201.0763, found: 201.0769.

Assay of extraction.

Fresh alperujo was submitted to extractions with water, methanol-water and two hydrothermal treatments. Further phenol extractions from the aqueous fraction were performed with ethyl acetate at 77 ºC (hot) and room temperature (cold), in order to evaluate the optimal conditions for extraction of a high concentration of glutaric acid derivative.

Extraction with water at room temperature.

500 g of fresh alperujo was mixed with 500 mL of water at room temperature for 1 h with stirring. The mixture was filtered and the liquid extracted with cold and hot ethyl acetate.

Extraction with aqueous methanol at room temperature.

We used the method reported by Obied et al.,[15] for the extraction from 300 g of fresh alperujo with aqueous methanol (80% v/v; 15 mL/10 g of alperujo) at root temperature for 30 min. The extraction was repeated twice more using 10 mL/10 g of alperujo. The combined organic fractions were filtered, evaporated to dryness in a rotary evaporator (Buchi, Milan, Italy) at 40 ºC and then redissolved in water. The phenols were extracted with cold and hot ethyl acetate.

Thermal treatment 140 ºC/60 min.

20 kg fresh alperujo sample was hydrothermally treated at 140 ºC during 60 min with saturated steam, obtaining 35 L of aqueous fraction by steam condensation, which was filtered and extracted with cold and hot ethyl acetate.

Thermal treatment 170 ºC/60 min.

20 kg fresh alperujo sample was hydrothermally treated at 170 ºC during 60 min with saturated steam, obtaining 41 L of aqueous fraction by steam condensation, which was filtered and extracted with cold and hot ethyl acetate.

Liquid-liquid extraction with ethyl acetate at 77 ºC (hot).

We used the procedure as described in the paragraph *2.1.4*.

Liquid-liquid extraction with ethyl acetate at room temperature (cold).

The aqueous portions (200 mL) obtained after treatment were mixed with ethyl acetate (250 mL) in a separating funnel and vigorously shaken for 10 min at room temperature and then allowed to settle for 20 min. The phases were separated, and the extraction was repeated twice. The combined organic phases were evaporated to dryness at reduced pressure.

Bactericidal activity.

Pseudomonas syringae CECT 4429T and *Agrobacterium tumefaciens* CECT 4119T bacterial strains were purchased from the Spanish Type Culture Collection (CECT) at Burjasot, Valencia, (Spain). Bactericidal activity of the samples (extracts and pure compound) were tested using 150 µl of sample inoculated with 10 µl of an overnight broth culture of each microorganism, diluted with saline to obtain an initial population between 5×10^5 and 5.7×10^5 CFU/mL. The inoculated samples were incubated at room temperature for 5 minutes with occasional shaking and plated onto nutrient agar to count the number of survivors after incubation. Experiments were performed in duplicate.[16]

The pH of the extracts (2.8−2.6) and the pure compound (3.1) were adjusted to 5.6 with dilute NaOH and filtered through cellulose membranes 0.20 µm pore size before inoculation. Controls were run with saline.

Results and discussion.

Isolation and structural analysis of compound 1.

The phenolic extract obtained from hydrothermally treated alperujo was characterized as containing different phenolic compounds and various elenolic acid derivatives.[6] In a previous work these derivatives were called compounds A, B and C, with derivative A present in greater proportions, and one of the most abundant constituents in ethyl acetate extract, after hydroxytyrosol. This compound was purified by HPLC using a C_{18} semipreparative column (as described in Materials & Methods). HPLC/DAD analysis revealed the presence of a pure compound at 11 min with a UV absorption maximum at 230 nm (**Figure 1**).

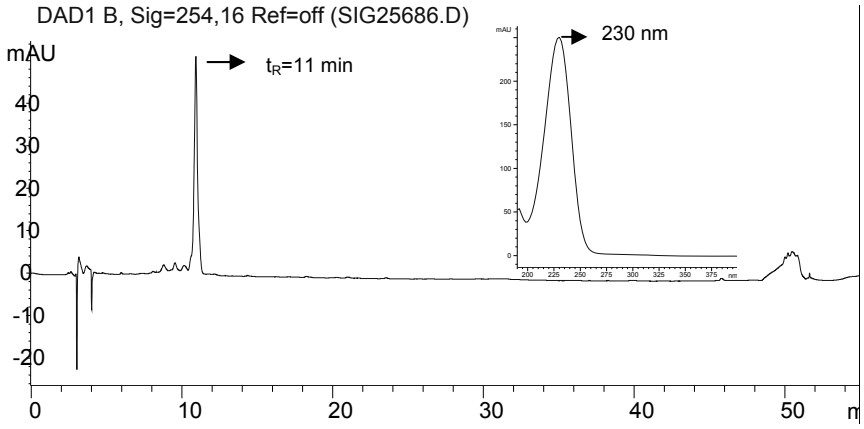
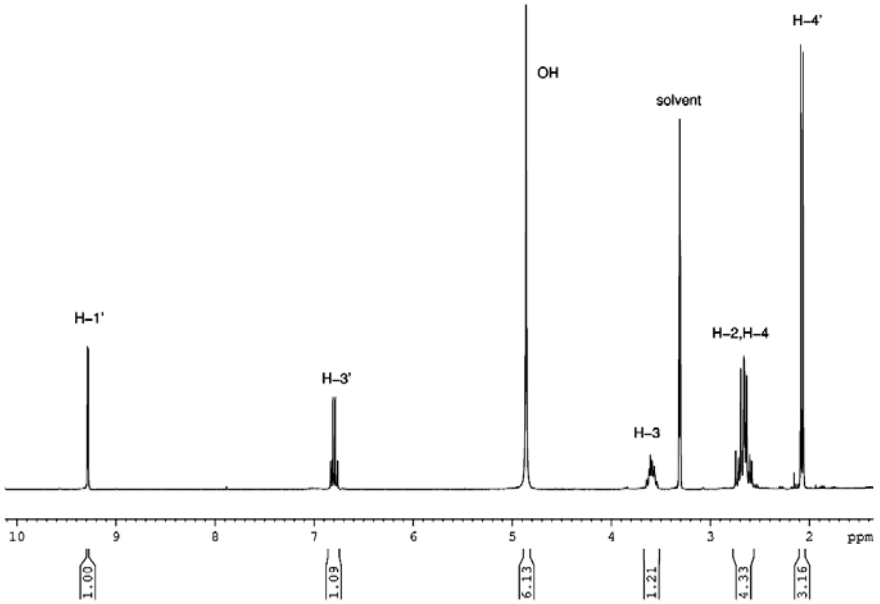


Figure 1: HPLC chromatogram and ultraviolet spectrum of **1**.

The structure of the compound was elucidated using 1D ^{1}H and ^{13}C NMR experiments (**Figures 2A and 2B**). Structural analysis was completed by using DEPT-45 (Supporting Information S1) and homonuclear and heteronuclear two-dimensional NMR techniques, such as COSY (Supporting Information S2), HSQC (Supporting Information S3), NOESY and HMBC experiments (**Figure 3A and B**, respectively). In addition, infrared analysis and mass spectrometry (low and high resolution chemical ionization) were also utilized.

A



B

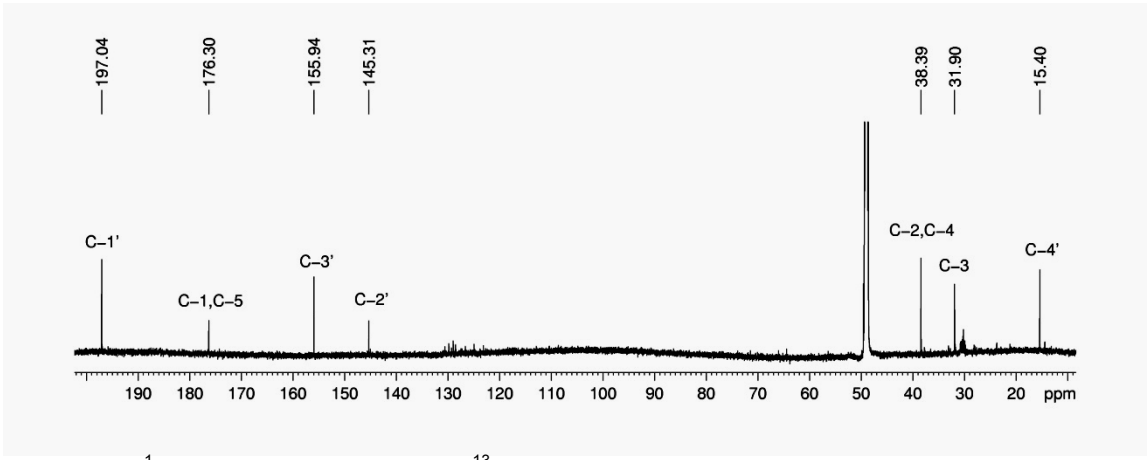
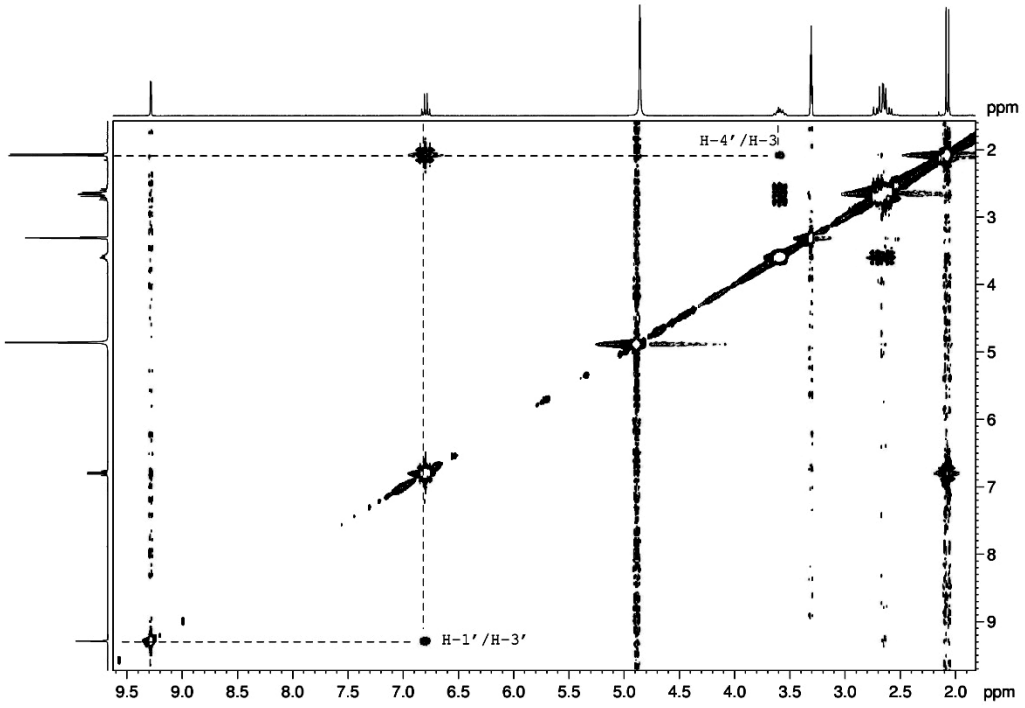


Figure 2: A, ^{1}H spectrum at 300.1 MHz. **B**, ^{13}C NMR spectra 176.1 MHz, for **1** in MeOH-d_4. The signals at 4.85 and 3.31 ppm in A and at 49.0 ppm in B correspond to the solvent.

A



B

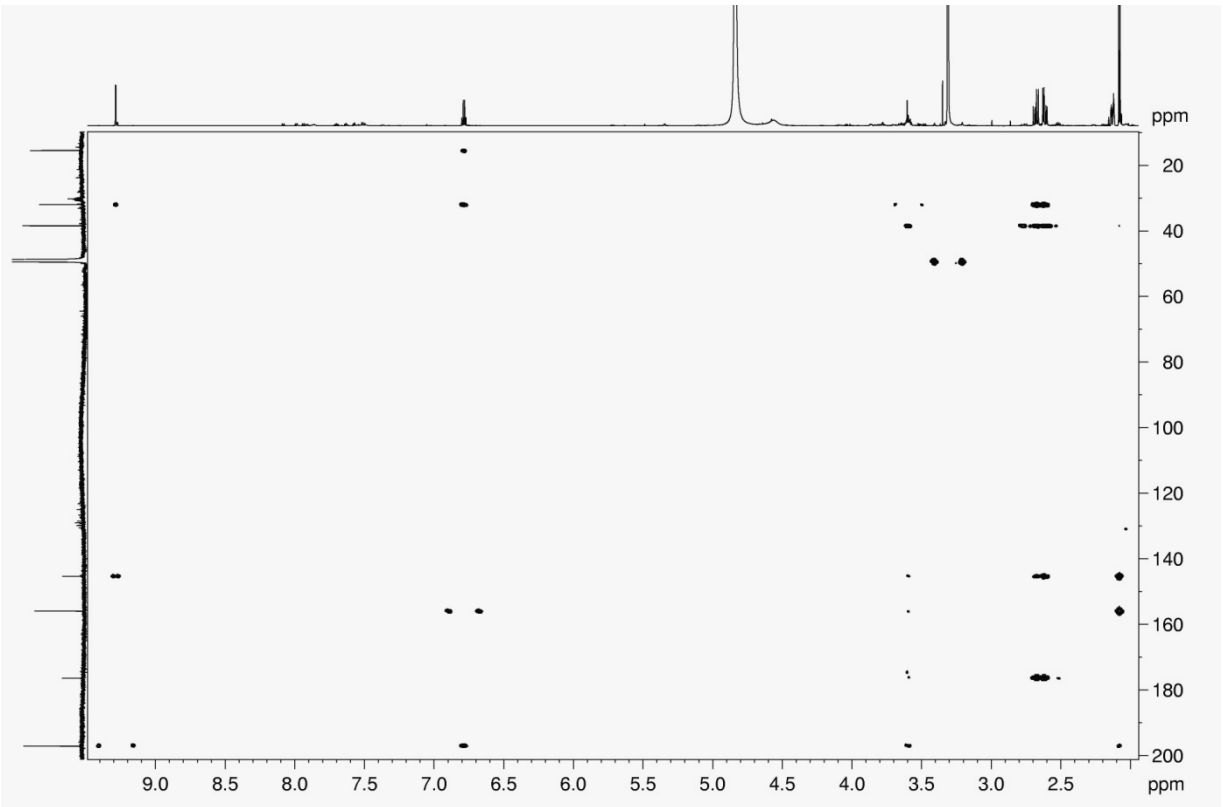


Figure 3: A, NOESY spectrum for **1** in MeOH-d_4 recorded at 300 MHz. **B**, HMBC spectrum for **1** in MeOH-d_4 recorded at 700 MHz.

The ^{1}H NMR data showed a doublet at δ 9.28 ppm (*J* =2.0 Hz), corresponding to an aldehyde group (H-1') coupled with H-3 (^{4}J) of glutaric acid moiety, as deduced from the COSY experiment. It is remarkable that the aldehyde proton of 2-alkylalk-2-enals, with either *E* or *Z* configuration, do not show any H,H coupling (Robert, Héritier, Quiquerez, Simian, & Blank, 2004). Furthermore, a quartet at δ 6.79 ppm (*J* = 7.0 Hz) indicated the presence of an olefinic proton (H-3') coupled (^{3}J) to a methyl (H-4') at δ 2.08 ppm, these data suggest that the compound contains a crotonaldehyde moiety substituted at the α-carbon. ^{1}H NMR spectrum also showed two symmetrical pairs of geminal protons as doublet of doublets at δ 2.61 (H-2a, H-4a) and δ 2.68 (H-2b, H-4b) ppm (2H each, $^{2}J_{2a,2b}$= 15.5 Hz) and a multiplet at δ 3.59 (1H), for protons of the CH_2CHCH_2 fragment, as confirmed by a COSY spectrum (Supporting Information S2). Furthermore, the HSQC experiment allowed complete assignments of the carbon signals in the spectrum corresponding to a symmetrical glutaric acid derivative, C-2/C-4 and C-3 for signals at 38.4 and 31.9 ppm, respectively, whereas the crotonaldehyde moiety had carbon signals at 197.0, 155.9 and 15.4 ppm, for C-1', C-3' and C-4', respectively (**Figure 2**). The signals observed at 176. 3 and 145.3 ppm in ^{13}C-NMR corresponded with the carboxylic carbons (C-1, C-5) and a quaternary olefinic carbon (C-2'), respectively; this information was corroborated, by DEPT-45 experiment in which quaternary carbon atoms do not appear (Supporting Information S1).

E-configuration of the C=C double bond of **1**, and the *s-trans* conformation for the C-1'−C-2' bond was confirmed by a 2D-NOESY experiment (**Figure 3A**) where the off-diagonal cross peaks, H-1'/H-3' and H-3/H-4', indicate through-space interaction and therefore suggesting close proximity of the involved hydrogen atoms (**Figure 4A**). The planar W pathway between formyl and H-3 protons, depicted in **Figure 4A**, also explains the long range coupling (^{4}J 2.0 Hz) observed in the ^{1}H NMR spectrum, and it is in agreement with a planar conformation of the crotonaldehyde moiety including in the plane the C-3−H-3 bond of the glutaric acid. No NOE interactions were observed between H-2/H-4 and crotonadehyde protons, also in agreement with the suggested conformation for 1.

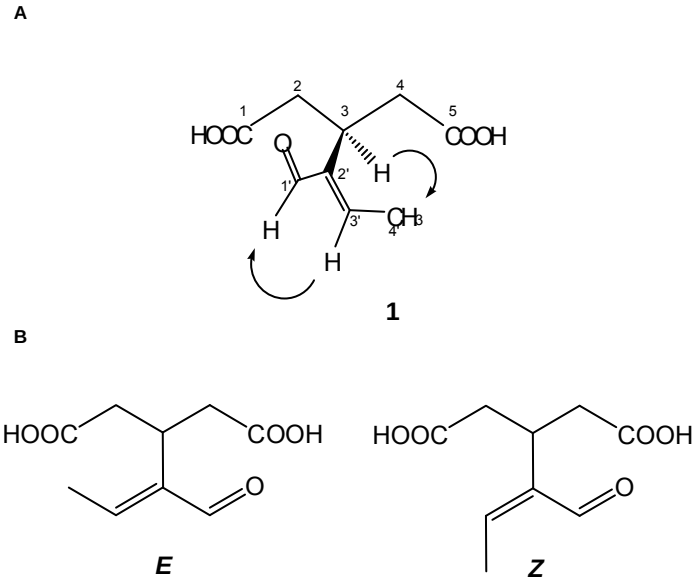


Figure 4: A, Significant NOE correlations for (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid (**1**) observed in MeOH-d_4. **B,** Possible *E* and *Z* configurations for the cronotonadehyde moiety of **1**.

The centrality of C3 was confirmed by the HMBC spectrum (**Figure 3B**), as it showed correlations between C-3 of glutaric acid with protons H-1', H-3' of the crotonaldehyde moiety, and strong correlation with (H-2, H-5) of glutaric acid. Three bond couplings were also observed between aldehydic carbon (C-1') and both H-3' of crotonaldehyde moiety and H-3 of the glutaric acid. Furthermore, the carboxylic carbons (C-1) were strongly coupled to H-2/H-4 and weakly coupled to H-3.

Next, we analysed the CI-MS and IR spectra (Supporting Information Figure S4 and S5, respectively) in order to complete the structural deductions for the isolated compound. The pseudomolecular ion $[M + H]^+$ obtained by high resolution CI-MS was in accordance with the assigned molecular formula $C_9H_{13}O_5$ (*m/z* 201); Additionally, the fragment obtained for loss of two water molecules successively, $[M + H - H_2O]^+$ and $[M + H - 2H_2O]^+$ with *m/z* 183 and 165 respectively, were identified. The IR spectrum of the compound revealed an absorption band at 3300−2400 cm^{-1} (vbr), which was attributed to the O−H stretch of hydrogen-bonded carboxylic acids and a band at 930 cm^{-1} associated with O−H bend. Absorption bands at 2727 and 1684 cm^{-1} can be attributed to C−H(O) and C=O of the conjugated aldehydic group, respectively. Finally, a signal at 1440 cm^{-1} was associated with the asymmetric bending vibrations of the methyl group.

Taking into account the structural descriptions as detailed above, the compound was identified as (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid (**1**). A similar compound has been described by Gil et al.[18] from olive oil residue, although in this case the compound was isolated in its methylated form previous treatment of the extract with diazomethane, and characterized as its dimethyl ester derivative. Also, Fotinos et al.[19] claimed to have isolated the same compound

from oil-press waste of a Greek olive crop variety, the Koroneiki olives, although in this case no structural chemical data were reported.

The configuration of this compound was reported to be *Z* (**Figure 4B**) in Chemical Abstract Service (CAS registry number 220007-88-5), the opposite to the one described herein, as noted above. Curiously enough, Gil et al.[18] and Fotinos et al.[19] named their isolated compound as the *E* estereoisomer, although they both formulated the compound as *Z*.

Assay of extraction.

The molecule identified in this paper was obtained and quantified from the ethyl acetate extract resulting from the hydrothermal treatment of olive oil waste in a previous work.[6] In order to identify the origin of this molecule, four solid-liquid extractions were realized for fresh alperujo from an olive mill plant. The first was realized with water at room temperature, the second with methanol/water (80:20) followed by methanol elimination, and the other two extractions were performed using a hydrothermal treatment. Subsequent extractions with ethyl acetate were realized in cold and hot conditions for the four aqueous fractions obtained.

The amount of **1** measured in the four initial aqueous samples from alperujo, and in each of the eight organic fractions is shown in **Table 1**. To our surprise, the data show that in the aqueous initial samples obtained after solid-liquid extraction, prior to extraction with ethyl acetate, the compound **1** was not detected. However, the compound was identified after extraction with ethyl acetate (in hot and cold) following extractions with H_2O, MeOH/H_2O (80:20) and hydrothermal treatment of 140 °C/60 min. Higher values were obtained in extractions with refluxing ethyl acetate in all cases. These data are in agreement with those obtained by Gil et al.[18] and Fotinos et al.[19], who in both cases isolated their glutaric acid derivative from olive products after extraction with ethyl acetate. The fact that before the extraction with ethyl acetate, the compound **1** was not identified, and after extraction is identified in high concentrations makes us think that the compound **1** is formed during the extraction process, favouring its formation when the process is done at temperature of 77 ºC. However, in samples of the wastewater from stored alperujo (not ethyl acetate extracted) from a pomace processing mill a compound named as decarboxymethyl elenolic acid (EDA)-like derivative, with a maximum absorbance at 230 nm and MS spectrum with a signal at *m/z* 199 in the negative mode was identified,[10] which is present in significant amounts (0.9-1.6 g/Kg alperujo) and matches the compound that we have isolated and identified, although in this case after the extraction with ethyl acetate in higher amounts. Thus, these results suggest that the compound could be produced chemically by a hypothetical hydrolytic reaction during the storage and/or processing of the alperujo, in similar conditions that occur when dealing with fresh alperujo and ethyl acetate extraction, as described herein. In both cases, the compound likely originates from

elenolic acid, the terpenoid skeleton of oleuropein (or ligstroside). Three alternative routes (**Figure 5**) can be devised for the formation of **1** from carboxymethyl elenolic acid **3**, involving ester hydrolysis of **3** or **6**, decarboxylation of β-ketoacid **4** or malonic acid derivative **7**, and oxidation of the formyl group of **3**, **4**, or **5**. Significativaly, glutaraldehyde **5** is the only intermediate that has been previously described; for example in stored alperujo[10] and in extra virgin olive oil.[20] Therefore, the pathway through **4** and **5** is the most likely. Nevertheless, two compounds named as "oxidised 3,4-DHPEA-EDA" (oxidised dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol) with *m/z* 335 and 393 (by MS in negative mode), have been recently detected among oxidation products in stored virgin olive oil,[21] might, in fact, be hydroxytyrosyl esters of **1** and **6** respectively. These authors have also detected peaks with *m/z* 183 and 199 which match with the structures **5** and **1**, respectively.[21]

Table 1: Amount of Compounds Measured in the Initial Aqueous Samples and in Each of the Organic Fractions Isolated After Extraction With Hot and Cold Ethyl Acetate.

	g compound/Kg fresh alperujo		
	Initial aqueous samples	Hot extraction	Cold extraction
H_2O	n.d.[a]	2.85±0.05	1.77±0.05
(80:20) MeOH/H_2O	n.d.	4.06±0.25	2.36±0.18
140ºC/60 min[b]	n.d.	1.30±0.07	0.82±0.19
170ºC/60 min[b]	n.d.	n.d.	n.d.

[a]n.d: not detected. [b]Hydrothermal treatment

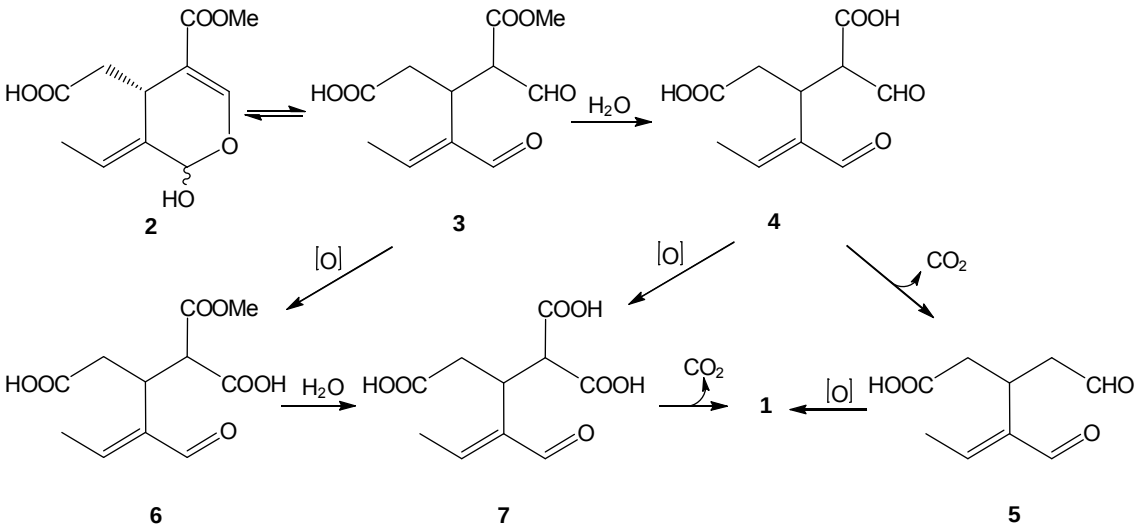


Figure 5: Alternative routes for the formation of glutaric acid derivative **1** from elenolic acids **2** and **3** in which demethylation, decarboxylation and oxidation steps are involved.

Table 1 also shows that the highest yield of the compound is obtained by extraction with MeOH/H_2O (80:20), followed by extraction with water, and the yield decreasing with the increasing severity of the solid-liquid extraction process, such that the molecule was not identified in the process of 170 °C / 1 h. These results are consistent with the results previously obtained[6], in which the concentration of the compound herein described as **1** decreases at 160 ºC with increasing treatment time.

A logical explanation for the absence of **1** in aqueous extractions of fresh alperujo and the detection of important amounts of it after extraction with ethyl acetate might be that one or several enzymatic processes (methyl ester hydrolysis, oxidation or decarboxylation) take place in a faster fashion in ethyl acetate than in aqueous media. Enzymatic oxidation of VOO secoiridoids by enzymes such as polyphenol oxidase (PPO) and peroxidase (PPP), activated during the oil mechanical extraction process, has been previously described.[22] Furthermore, the no detection of **1** after the thermal treatment under harsh conditions (170 ºC/60 min) might be related to enzyme denaturalization. These results showed that the formation of compound **1** can take place over long storage processes, or be accelerated by the extraction with ethyl acetate. In future studies we will address the origin of the molecule 1 and will study whether the activity of PPO and PPP is improved in ethyl acetate.

Antimicrobial activity.

The phenolic ethyl acetate extract obtained from hydrothermally treated alperujo (160 ºC/60 min) was fractionated using Amberlite XAD16. This method led to the isolation of two fractions (Fraction 1 and 2) that contain the glutaric acid derivative **1**, together with other phenolic compounds in different ratios (**Table 2**). Antimicrobial activity against *Pseudomonas syringae* and *Agrobacterium tumefaciens* of Fractions 1, 2 and the purified compound were screened. In this work the antimicrobial ability was determined after only 5 minutes of contact,[10] as such a bactericidal effect would be of more interest for the application of active compounds for field trials on plants than other methods. The method employed to evaluate the antimicrobial activity of the extracts and the compound is characterized by a contact time of five minutes. The method evaluates the antimicrobial activity considering that the application is done on the plant and there may be external agents (rain and / or wind) that delimit the most prolonged time of contact. This method is employed by numerous authors to evaluate the antimicrobial activity of different compounds in plants.[10, 23]

Table 2: Phenolic Composition of Fraction 1 and 2 Obtained After the Fractionation of Hydrothermally Treated Alperujo Using Amberlite XAD16.

	g compound/100 g dry weight	
Fraction	1	2
3.4-Dihydroxyphenylglycol	0.25±0.02	n.d.[a]
Hydroxymethylfurfural	0.97±0.06	n.d.
Hydroxytyrosol	33.68±0.77	4.53±0.01
Protocatechuic acid	0.83±0.02	0.59±0.06
(*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid	**38.90±0.57**	**8.13±0.47**
Catechol	0.43±0.05	1.25±0.04
Tyrosol	2.81±0.03	4.46±0.14
p-Hydroxybenzoic acid	n.d.	0.59±0.01
3.4-dihydroxyhidrocinamic acid	0.45±0.01	1.24±0.04
Vanillic acid	n.d.	0.71±0.01
4-Methylcatechol	1.39±0.02	9.85±0.08
Oleuropein	0.02±0.01	7.67±0.15
Hydroxytyrosyl acetate	n.d.	4.77±0.05

[a]n.d: not detected

The bactericidal effect of Fraction 1 (**Figure 6A**) was assessed at concentrations of 50 mg/mL for *P. syringae* reducing the initial population by 4 log UFC/mL (10,000-fold), and for *A. tumefaciens* reduced by only 2 log units (100-fold). Fraction 2 (**Figure 6B**) had the same bactericidal effect on *P. syringae* that Fraction 1 at 50 mg/mL, however 75 mg/mL of this fraction were required to elicit a bactericidal effect against *A. tumefaciens*. The purified glutaric derivative, (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid, dramatically decreased the *P. syringae* population by more than 4 log units at the concentration of 18 mg/mL, however no bactericidal effect was observed at the same concentration for *A. tumefaciens* (**Figure 6C**).

The concentrations of phenolic compounds in the Fractions 1 and 2 are shown in **Table 2.** Observing the results obtained for fraction 1, the bactericidal effect on *P. syringae* at 50 mg/mL could be attributed to its high glutaric derivative content (38.9%), corresponding to 19 mg/mL, a concentration that coincides with the 18 mg/mL of purified compound that showed a strong bactericidal effect. Nevertheless, Fraction 1 also contained other known antimicrobial compounds, such as hydroxytyrosol (33.7%), representing 16.8 mg/mL of the 50 mg/mL that provoked inhibition of microbial growth. We determined that the hydroxytyrosol concentration required to cause an antimicrobial effect on *P. syringae* and *A. tumefaciens* was less than 16.8 mg/mL (data not shown). The effect of purified hydroxytyrosol from aqueous alperujo solution was higher than in the presence of the other substances comprising fraction 1, which would suggest an antagonistic interaction.

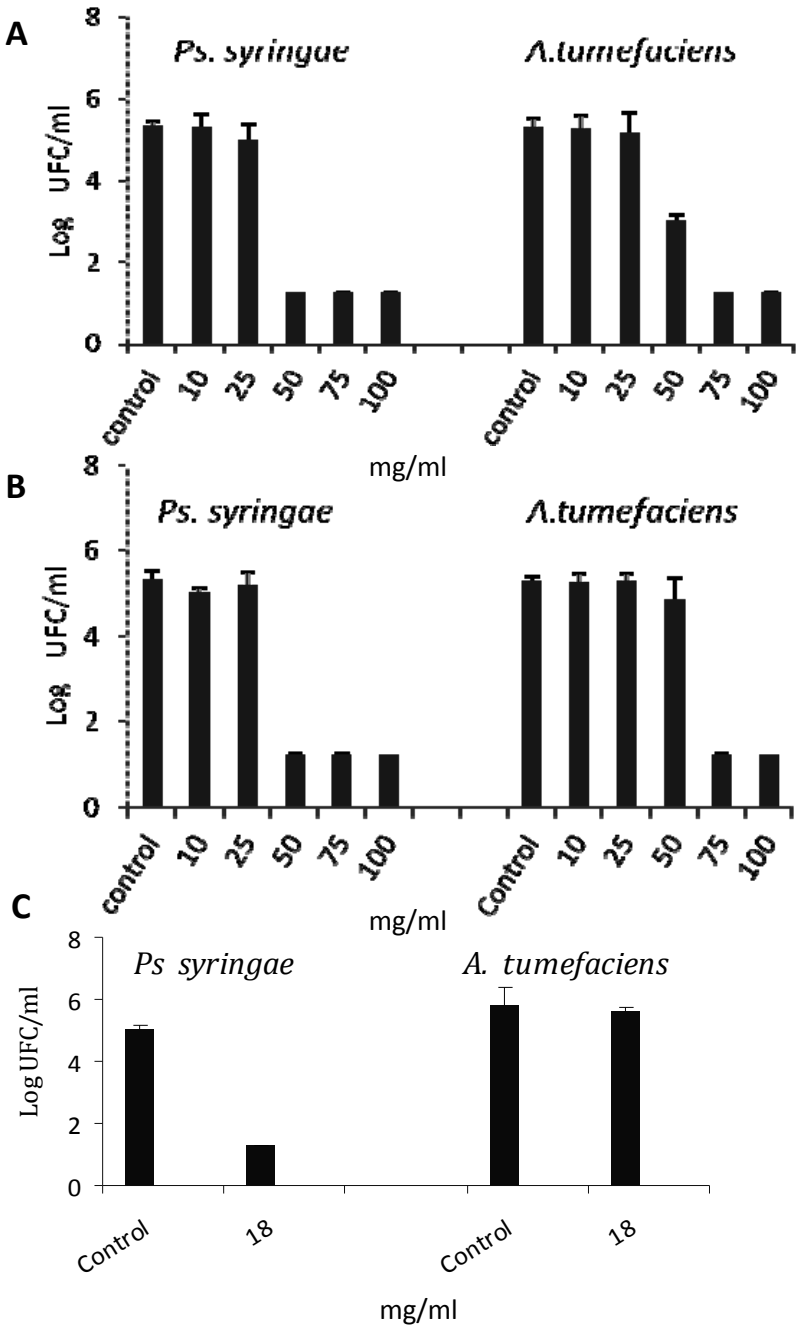


Figure 6: Bactericidal activity of phenolic fraction 1 (**A**), fraction 2 (**B**) and isolated (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid (**C**), against *Pseudomonas syringae* CECT 4429T and *Agrobacterium tumefaciens* CECT4119T. Bars indicate the standard deviation of duplicates. Line indicates the detection limit.

Fraction 2 exerted bactericidal activity against *P. syringae* at a concentration of 50 mg/mL, containing 8% of glutaric acid derivative (4 mg/mL) and 4.5% of hydroxytyrosol (2.2 mg/mL), while 75 mg/mL of this fraction were needed to act on *A. tumefaciens*, representing 6 mg/mL of of glutaric acid derivatives and 3.3 mg/mL of hydroxytyrosol. In this case the bactericidal effect of Fraction 2 can be explained by the concentration of only hydroxytyrosol, since the effect of purified hydroxytyrosol was detected at a similar concentration (data not

shown). However, it could also be explained by the combined effects of hydroxytyrosol, oleuropein and 4-methylcathecol, being the last one the most toxic.[24]

These preliminary studies of antimicrobial activity of compound **1** show that the isolated compound has good antimicrobial activity when compared to hydroxytyrosol. The hydroxytyrosol is a polyphenol found in high concentrations in the alperujo, which present an important antimicrobial activity, its activity has been studied against many bacterial and fungi, and many studies have been realized evaluated this compound.[23, 25] Particularly, the hydroxytyrosol at 1 mg/mL has bactericidal effect for the two microorganisms evaluated in this article.[25] Therefore, this first screening opens a new way for to study the bactericidal effect of this compound on other pathogens for plants and humans. Furthermore, compound **1** could be used not only as an antimicrobial agent, but also a key source of a lactone with significant cytoprotective properties against DNA damage induced by oxidative stress.[19]

General Comments

In the present investigation, chemical studies of a phenolic extract obtained from by aqueous, methanolic aqueous, or hydrothermically treated fresh alperujo led to the isolation and identification of an elenolic acid derivative, (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid (**1**). To the best of our knowledge, this is the first time that the structure has been well established and their spectroscopy date are showed. The *s-trans* conformation for the Cα−CHO bond is agreement with the NOESY experiment. The compound was quantified in large amounts and synthetic routes to explain its formation involving ester hydrolysis, decarboxylation and oxidation have been suggested. The role of ethyl acetate as extracting solvent seems to be key in the process. From a practical point of view, (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid could be used as an important source of a lactone with significant cytoprotective properties,[19] and as an antimicrobial agent against bacteria responsible for plant diseases.

Funding

Funding was received from the Spanish Ministry of Economy and Competitiveness and co-funded by European Social Fund (ESF) (Project AGL2013-48291-R). Dr. Rodríguez-Gutiérrez (RYC-2012-10456 contract) wish to thank to the “Ramón y Cajal” Program from, and Ms Rubio-Senent (JAE-Pre104) to the Spanish National Research Council (CSIC) program for providing financial support, both co-funded by ESF.

References.

(1) Aragón, J. M.; Palancar, M. C. Present and Future of Alpeorujo. In (2001). *Improlive 2000*, Ed. Complutense: Madrid, Spain, 2001; 242−300.

(2) Lesage-Meessen, L.; Navarro, D.; Maunier, S.; Sigoillot, J-C.; Lorquin, J.; Delattre, M.; Simon, J-L.; Asther, M.; Labat, M. Simple phenolic content in olive oil residues as a function of extraction systems. *Food Chem.*, **2001**, *75*, 501−507.

(3) López-Miranda, J. (2010). Olive oil and health: Summary of the II International conference on olive oil and health consensus report. Jaen and Córdoba (Spain). *Nutr., Metab. & Cardiovascular Dis.,* **2010***, 20*, 284−294.

(4) Ghanbari, R.; Anwar, F.; Alkharfy, K.M., Gilani, A.-H.; Saari, N. Valuable Nutrients and Functional Bioactives in Different Parts of Olive (Olea europaea L.)—A Review. *Int.l Journal of Mol. Sci.,* **2012***, 13*, 3291−3340.

(5) Fernández-Bolaños, J.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Sánchez, P. Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de olive. ES2374675. **2010.**

(6) Rubio-Senent, F.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. New phenolic compounds hydrothermally extracted from the olive oil byproduct alperujo and their antioxidative activities. *J. Agric. and Food Chem.,* **2012***, 60*, 1175−1186.

(7) Rubio-Senent, F.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity. *LWT-Food Sci. Technol.,* **2013a***, 54,* 114−124.

(8) Rubio-Senent, F.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Fernández-Bolaños, J. Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. *Food Res. Int.*, **2013b,** *54*, 2122–2129.

(9) Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M. A.; Cristinzo, G. Antibacterial polyphenols from olive oil mill waste waters. *J. Appl. Bact.*, **1995**, *79*, 393-398.

(10) Medina, E.; Romero, C.; de los Santos, B.; de Castro, A.; García, A.; Romero, F.; Brenes, M. Antimicrobial activity of olive solutions from stored alperorujo against plant pathogenic microorganisms. *J. Agric. Food Chem.,* **2011***, 59,* 6927−6932.

(11) Zampa, A.; Silvi, S.; Servili, M.; Montedoro, G. F.; Orpianese, C.; Cresci, A. In vitro modulatory effects of colonia microflora by olive oil iridoids*. Microbial Ecol. Health Dis.,* **2006***, 18*, 147−1532.

(12) Medina, E.; de Castro, A.; Romero, C.; Brenes, M. Comparison of concentration of phenolic compounds in olive oils and other plant oils: correlation with antimicrobial activity. *J. Agric. and Food Chem.,* **2006***, 54*, 4954−4961.

(13) Fernández-Bolaños Guzmán, J.; Heredia Moreno, A.; Rodríguez Gutiérrez, G.; Rodríguez Arcos, R.; Jiménez Araujo, A.; Guillén Bejarano, R. Method for obtaining purified hydroxytyrosol from products and by-products derived from the olive tree. U.S. Patent 6,849,770 B2. **2005**.

(14) Fernández-Bolaños, J.; Rodríguez-Gutiérrez, G.; Lama-Muñoz, A.; Rubio-Senent, F.; Fernández-Bolaños, J.M.; Maya Castilla, I.; López-López, O. Procedimientos para la extracción de extracto de hidroxitirosol, extracto mezcla de hidroxitirosol y 3,4-dihidroxifenilglicol, y extracto de acetato de hidroxitirosol a partir de subproductos del olivo y su purificación. WO2013/007850A1. **2011**.

(15) Obied, H. K.; Bedgood, D. R. Jr.; Prenzler, P. D.; Robards, K. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Analytical Chim. Acta*, **2007**, *603*, 176−189.

(16) National Committee for Clinical Laboratory Standards. Methods for determining bactericidal activity of antimicrobial agents. Approved guideline M26-A. National Committee for Clinical Laboratory Standards, Wayne, PA. 1999.

(17) Robert, F.; Héritier, J.; Quiquerez. J.; Simian, H.; Blank, I. Synthesis and sensorial properties of 2-alkylalk-2-enals and 3-(acetylthio)-2-alkyl alkanals. *J. Agric. Food Chem.,* **2004**, *52*, 3525−3529.

(18) Gil, M.; Haïdour A.; Ramos, J. L. Two glutaric acid derivatives from olives. 1998. *Phytochemistry,* **1997***, 49*, 1311−1315.

(19) Fortinos, S.; Panaitescu, L.; Skaltsounis, A.-L.; Mitakou, S.; Magiatis, P.; Aligiannis, N.; Galaris, D. Compounds and compositions derived from olives and methods of use thereof. US2003/0185921. **2003**.

(20) Pérez-Trujillo, M.; Gómez-Caravaca, A.M.; Segura-Carretero, A.; Fernández-Gutiérrez, A.; Parella, T. Separation and identification of phenolic compounds of extra virgin olive oil from *Olea europaea L*. by HPLC-DAD-SPE-NMR/MS. Identification of a new diastereoisomer of the aldehydic form of oleuropein aglycone. *J. Agric. Food Chem.,* **2010**, *58*, 9129−9136.

(21) Di Maio, I.; Esposto, S.; Taticchi, A.; Selvaggini, R.; Veneziani, G.; Urbani, S.; Servili, M. Characterization of 3,4-DHPEA-EDA oxidation products in virgin olive oil by high performance liquid chromatography coupled with mass spectrometry. *Food Chem.*, **2013**, *138*, 1381−1391.

(22) Servili, M.; Taticchi, A.; Esposto, S.; Urbani, S.; Selvaggini, R.; Montedoro, G. F. Effect of olive stoning on the volatile and phenolic composition of virgin olive oil. *J. Agric. Food Chem.*, **2007**, *55*, 7028–7035.

(23) Brenes, M.; García, A.; de los Santos, B.; Medina, E.; Romero, C.; de Castro, A.; Romero, F. Olive glutaraldehyde-like compounds against plant pathogenic bacteria and fungi. *Food Chem.,* **2011***, 125*, 1262–1266

(24) Capasso, R.; Evidente, A.; Schivo, L.; Orru, G.; Marcialis, M.; Cristinzo, G. Antibacterial polyphenols from olive oil mill waste water. *J. Appl. Bacteriology,* **1995***, 79,* 393−398.

(25) Medina, E.; Brenes, M.; Romero, C.; García, A.; de Castro, A. Main Antimicrobial Compounds in Table Olives. *J. Agric. Food Chem.,* **2007***, 55*, 9817–9823.

Supporting Information

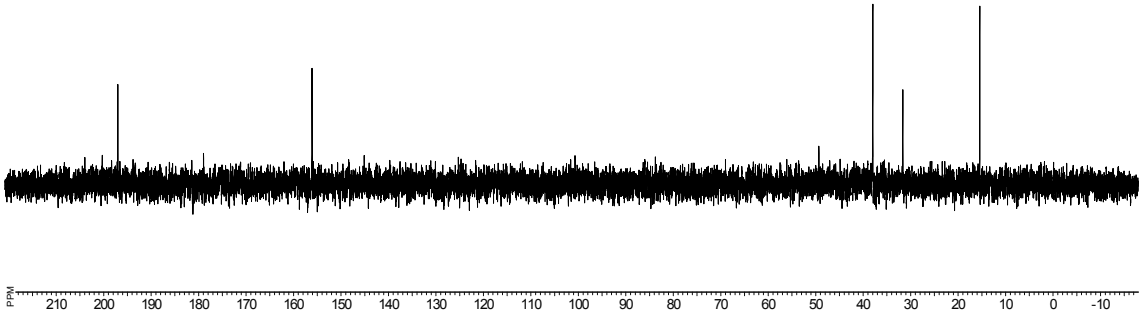


Figure S1: DEPT-45 experiment for **1.**

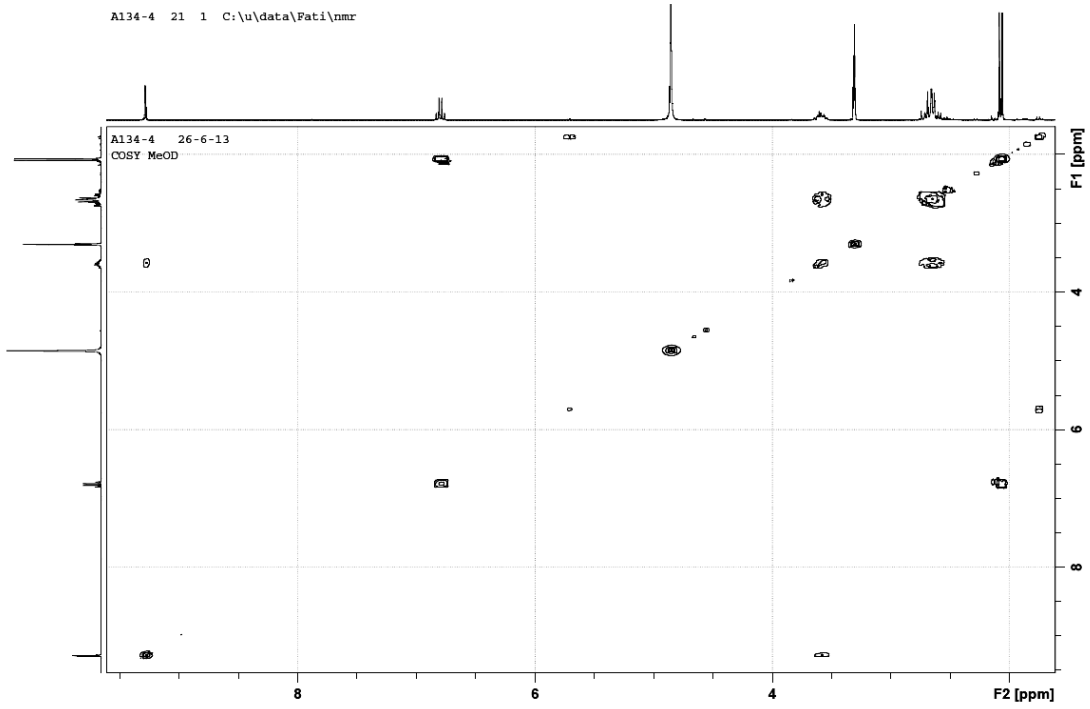


Figure S2: COSY bidimensional spectrum analysis for **1**.

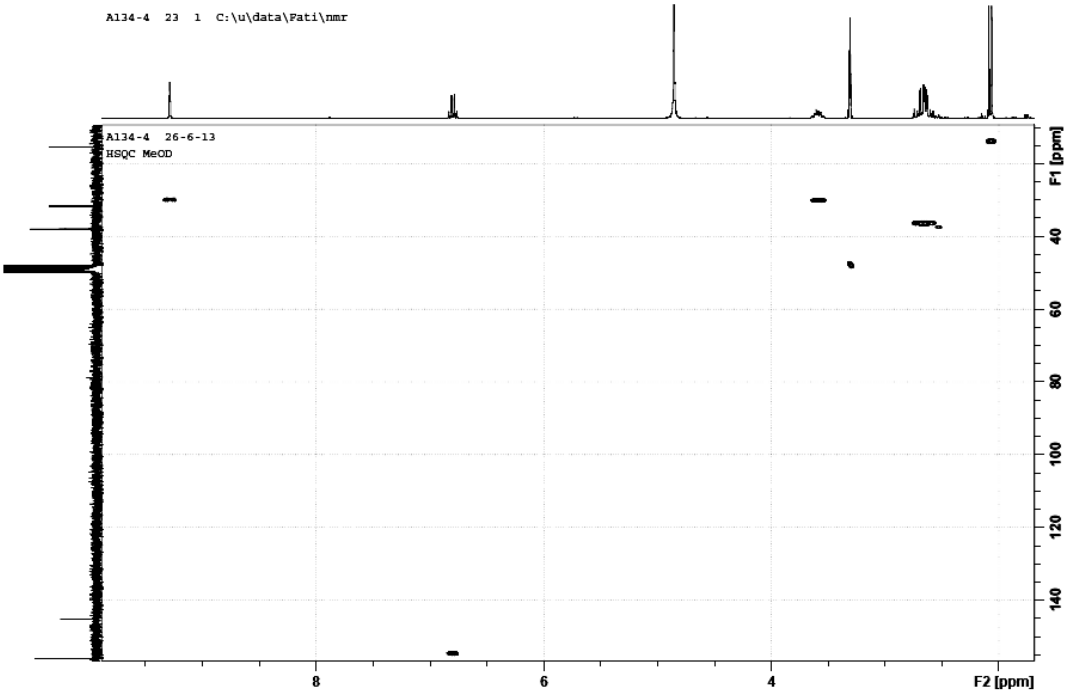
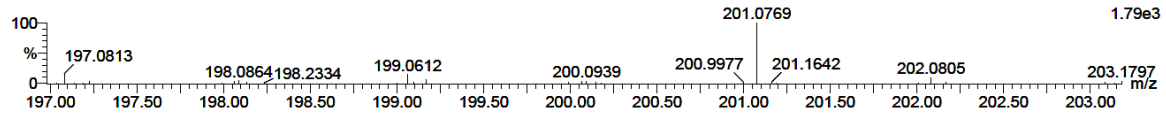


Figure S3: HSQC bidimensional spectrum analysis for **1**.



Minimum:	80.00				-100.0		
Maximum:	100.00		200.0	10.0	100.0		
Mass	RA	Calc. Mass	mDa	PPM	DBE	Score	Formula
201.0769	100.00	201.0763	0.6	3.0	3.5	1	C9 H13 O5

Figure S4: Spectrum of high resolution CI-MS for compounds **1.**

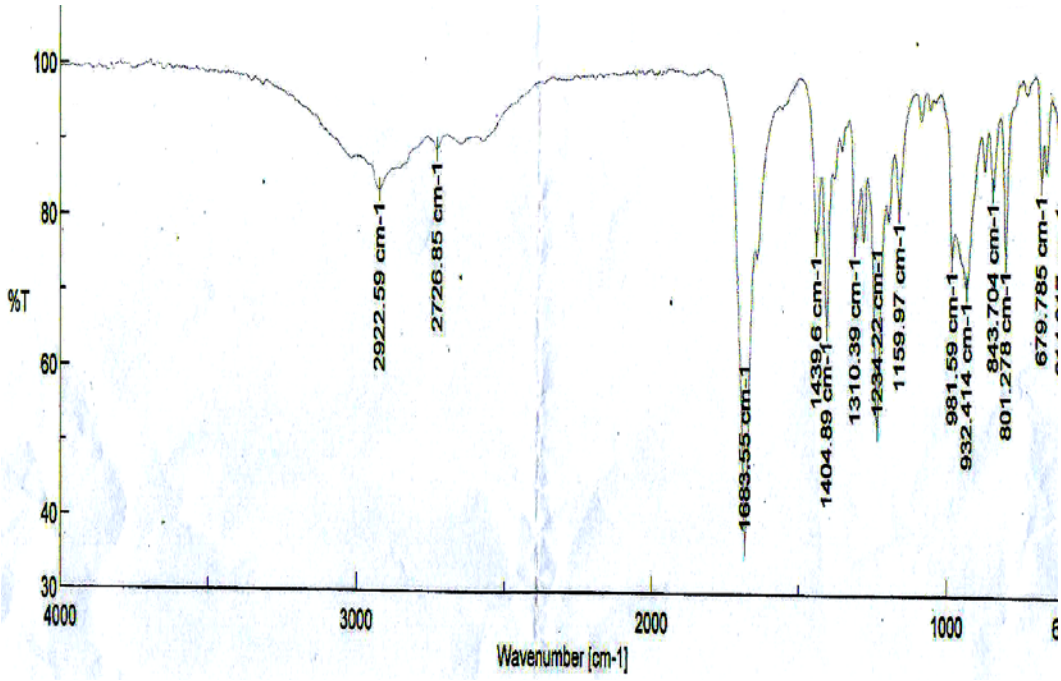


Figure S5: IR spectra for compound **1**

6.1.7. Capítulo 7.

Isolation and identification of minor secoiridoids and phenolic components from thermally treated olive oil by-products.

Fátima Rubio-Senent, Sergio Martos, Antonio Lama-Muñoz, José G. Fernández-Bolaños, Guillermo Rodríguez-Gutíerrez, and Juan Fernández-Bolaños.

La aplicación de un nuevo procedimiento industrial basado en el tratamiento hidrotérmico a 160 ° C / 60 min del alperujo, un subproducto de la extracción de aceite de oliva, permite la recuperación de una fase líquida que contiene una alta concentración de compuestos fenólicos y secoiridoides. Estos compuestos son fácilmente extraíbles mediante el empleo de acetato de etilo, lo que permite la obtención de un extracto fenólico.

Los compuestos fenólicos mayoritarios presentes en este extracto ya han sido identificados en capítulos anteriores y sus propiedades antioxidantes probadas. Este capítulo se centra en aquellos compuestos fenólicos minoritarios que se encuentran en la fracción de acetato de etilo y que por lo tanto también contribuyen a las características del extracto. Además, la extracción con acetato de etilo proporciona una fracción acuosa remanente en la cual también se detectan fenoles, principalmente aquellos que presentan una elevada polaridad. El estudio de los fenoles minoritarios del extracto fenólico, así como los presentes en la fracción acuosa, nos ha permitido detectar compuestos conocidos tales como diferentes derivados secoiridoires (oleuropeína, ligustrósido y derivados), acteósidos (verbascósido e hidroxiverbascosido) y flavonoides.

El estudio detallado de los fenoles presentes en la fracción acuosa que permanece remanente tras la extracción con acetato de etilo de la fracción líquida obtenida a partir de alperujo tratado térmicamente nos ha permitido identificar una nueva molécula. Esta molécula desconocida es la que se encuentra en una mayor concentración en la fracción acuosa y presenta un peso molecular de 408. Su estructura se ha caracterizado por RMN de ^{1}H y ^{13}C, espectrometría de masas de ionización química y análisis infrarrojo. Todos estos resultados nos han permitido identificar la molécula como el ácido 1-glucosil aciclodihidroelenólico.

Isolation and identification of minor secoiridoids and phenolic components from thermally treated olive oil by-products.

Fátima Rubio-Senent, Sergio Martos, Antonio Lama-Muñoz, José G. Fernández-Bolaños, Guillermo Rodríguez-Gutíerrez, and Juan Fernández-Bolaños

ABSTRACT

The application of a novel industrial process based on the hydrothermal treatment of 160 ºC/60 min of alperujo, a by-product of olive oil extraction, allows the formation of a liquid phase containing a high concentration of phenolic and secoiridoid compounds. Ethyl acetate was used to extract these phenolic compounds from the aqueous matrix. The phenols present in high concentrations in the ethyl acetate extract have been studied in previous work. In this study, we confirmed the presence of several phenolic compounds existing in minor concentrations in the steam-treated alperujo extract, but which contribute to the characteristics of this organic extract. The polar compounds that remain in the aqueous fraction after extraction with ethyl acetate were also studied. We report the presence of known compounds and we also detected an unknown molecule with a molecular weight of 408 whose structure was characterized. This new secoiridoid glucoside was identified as 1-glucosyl acyclodihydroelenolic acid.

KEYWORDS: pomace olive waste (Alperujo); steam-treatment; ethyl acetate extract; secoiridoid derivatives;.acteosides; flavonoids; phenolic compounds.

1. Introduction.

A modern two-phase centrifugation system to obtain extra virgin olive oil is now widely used in Spain (accounting for 90% of the total production). Besides olive oil, the process releases a semisolid by-product called two-phase pomace or “alperujo”, with over 4 million of tonnes generated annually in Spain. Olive fruits and derived products represent a recognized valuable source of phenolic compounds with important beneficial effects for human health including antioxidant, anti-inflammatory, antimicrobial, and anticarcinogen activities (Estruch et al., 3013; Kalogerakis, Polito, Foteinis, Chatzisymeon & Mantzavinos, 2013; Ruiz-Canela, Estruch, Corella, Salas-Salvador, & Martínez-González, 2014). The possible recovery of bioactive compounds from this by-product, containing some 98% of the total phenolic content of the olive fruit in comparison to only 2% in the oil, is an attractive way of valorising it. In addition, to improve the productivity of olive oil processing, using the waste material is being promoted.

One interesting approach toward the valorisation of alperujo is the use of a hydrothermal treatment at high temperature (150-170 ºC) and pressure (0.6-1.2 MPa), which allows an easy separation of the solid and liquid phases and the recovery of the bioactive compounds from each phase (Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, & Sánchez, 2010), and whose methodology is already implemented on an industrial scale. Recent studies have shown that a substantial part of the phenolic compounds, the pectins and hemicelluloses present in alperujo could also be recovered from the water soluble fraction after the steam treatment (Rubio-Senent, Lama-Muñoz, Rodríguez-Gutiérrez, & Fernández-Bolaños, 2013; Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012; Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, García, & Fernández-Bolaños 2015). Many of these phenols were isolated and identified from the ethyl acetate extract obtained from the aqueous phase of steam-treated alperujo. Moreover, many interesting antioxidant activities have been attributed to this class of compounds (Rubio-Senent et al., 2012; Rubio-Senent et al., 2013). However, the complete characterization of the ethyl acetate extract has yet to be achieved. In this work we considered the minor phenols present in the organic fraction, which could contribute to the antioxidant activity of the total extract. In fact, Obied, Prenzler, Konczak, Rehman, & Robards (2009) showed that a mixture of olive biophenols in ethyl acetate extacts of olive mill waste were more effective in protecting DNA from oxidative damage and inhibiting the growth of cancer cells than individual biophenols.

The more polar components that remain in the aqueous fraction after the ethyl acetate extraction were also studied in previous work. After ultra-filtration through a 3000 Da molecular weight cut-off membrane, a fraction over 3000 Da was found to be enriched in pectin. The fraction below 3000 Da was mainly composed of neutral oligosaccharides with a degree of polymerisation (DP) between 4−10, together with several secoiridoid glycosides esterified to phenolic compounds, their aglycones, and mono- and disaccharides linked to phenolic compounds (Fernández-Bolaños, Rubio-Senent, Lama-Muñoz, García, & Rodríguez-Gutiérrez, 2014). The occurrence of these interesting compounds prompted us to continue our investigation on the same aqueous fraction. In this work, we identified another series of known phenol and secoiridoid derivatives by the analysis of their spectral mass data and comparison with literature values. In addition, an unknown compound with a molecular weight of 408 was identified for the first time based on nuclear magnetic resonance (NMR) spectra of both the unknown compound and its acetylated derivative.

2. Materials and methods.

2.1. Materials.

The sample of alperujo (a semisolid residue composed of olive peels, pulp, seeds, and ground stones) was obtained in March 2009, from Picual olives processed at a Spanish oil mill (Almazara Experimental, Instituto de la Grasa, Seville). The alperujo was processed in the pilot reactor without removal of the stones.

2.2. Thermal Treatment.

The hydrothermal treatment used has been patented (Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, & Sánchez, 2010) and was performed using a prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor has a stainless steel reservoir (100 L capacity) that can operate at temperatures between 50 and 190 °C and at a maximum pressure of 1.2 MPa.

20 kg of fresh alperujo was loaded into the reactor and treated for 60 min at 160 °C. Then the wet material was centrifuged at 4,700 g (Comteifa, S.L., Barcelona, Spain) to separate the solids and liquids. After centrifugation, 10 L of the liquid phase was concentrated to 1 L by rotary evaporation at 30 °C under reduce pressure.

2.3. Phenol Extraction.

The aqueous portion obtained after thermal treatment was washed with hexane to remove the lipid fraction: 1 L of liquid was mixed with 500 mL of hexane; the mixture was shaken vigorously, and then the phases were separated by decantation and washed twice. The liquid−liquid extraction of phenolic compounds was carried out with ethyl acetate (500 mL per 200 mL of sample; refluxed at 77 °C) in a continuous extractor of a heavier liquid (water) by a lighter liquid (ethyl acetate) for 8 h.

2.4. Concentration of minor phenols in phenolic extract.

Approximately 3 g of extract was dissolved in 50 mL of H_2O/MeOH (80:20). First, the extracts were passed through a polyamide column 3.5 cm in diameter and 30 cm in height. The elution was performed stepwise with 500 mL of H_2O, 25% MeOH (v/v), 50% MeOH (v/v), 75% MeOH (v/v) and 100% MeOH (v/v), and each fraction was collected. The fractions were analyzed by HPLC, and those that contained a greater number of unknown phenols were passed through a second column of Amberlite XAD16, 3.5 cm in diameter and 15 cm in height. The elution was performed with 250 mL of H_2O, 25% MeOH (v/v), 50% MeOH (v/v), 75% MeOH (v/v), and 100% MeOH (v/v), and each collected fraction was monitored by HPLC. The phenols were purified and concentrated using a cartridge of Sep-Pak C_{18}, with elution by H_2O, MeOH 20 % (v/v), MeOH 40 % (v/v),, MeOH 80 % (v/v) and MeOH 100 % (v/v). This process allowed the

collection of three main fractions: Polyamide water/XAD 50/SPE 20, Polyamide water/XAD 75/SPE 20 and Polyamide water/XAD 75/SPE 40, referred to as PXS50/20, PXS75/20 and PXS75/40 respectively. These three fractions were enriched in minor compounds, which were later identified by HPLC-MS.

2.5. Isolation of phenolic compounds in aqueous fraction after extraction with ethyl acetate.

In order to increase the proportion of low molecular weight oligosaccharides in the initial liquid, a mild chemical hydrolysis with 0.5 N HCl was carried out (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández-Bolaños, 2012). A protocol for the isolation of these oligosaccharides, including separation by ultrafiltration, adsorption chromatography and size exclusion, has been developed according to a previously published procedure (Rubio-Senent et al., 2013). Briefly, hydrolyzed fractions were ultra-filtered at room temperature using a Prep/Scale®-TFF Cartridge of 3000 Da regenerated cellulose (Millipore Corp., Bedford, MA, USA). Fractions with a size smaller than 3000 Da were fractionated and purified by adsorption on Amberlite XAD-16 resin and eluted with water, methanol/water 20% (v/v) and 50% (v/v), successively. After the separation of the monosaccharides and fractionation of the oligosaccharides by adsorption chromatography, the fractions were fractionated by size exclusion chromatography on two Superdex Peptide HR 10/30 (30 x 1 cm) columns (Pharmacia Biotech, Uppsala, Sweden) connected in line to a Jasco LC-Net II/ADC (Easton, MD, USA) HPLC system.

Samples (100 µL) were eluted with a flow rate of 0.5 mL/min. The peaks were monitored by a Jasco MD-1550 Diode Array detector (DAD) and a Jasco RI-1530 Refraction Index detector. Column calibration was performed with a variety of standards, using compounds of a series of cellobiose (RT= 66.5 min), cellotriose (RT= 63.5 min), cellotetraose (RT= 61.1 min), cellopentaose (RT= 59.0 min) and cellohexaose (RT= 56.2 min) (purchased from Megazyme).

2.6. HPLC-DAD.

The different phenols were analyzed using a Hewlett-Packard 1100 liquid chromatography system with a C_{18} column (Teknokroma Tracer Extrasil ODS-2, 250 mm × 4.6 mm i.d., 5 µm). The system was equipped with a diode array detector and Rheodyne injection valves (20 µL loop). The mobile phases were 0.01% trichloroacetic acid in water (A) and acetonitrile (B), utilizing the following gradient over a total run time of 55 min: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, and 95% A at 52 min, until the run was completed.

2.7. HPLC-MS.

The phenolic compounds present in the different fractions were identified by electron impact mass spectra collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters Inc., Manchester, UK). Electron spray ionization (ESI) mass spectra were obtained at ionization energies of 50 and 100 eV in negative mode and of 50 eV in positive mode; the capillary voltage was 3 kV, the desolvation temperature was 200 °C, the source temperature was 100 °C, and the extractor voltage was 12 V. The flow was maintained at 1 mL min^{-1} in split mode (UV detector MS) for each analysis. A Tracer Extrasil ODS-2 column (250 mm × 4.6 mm i.d., 5 μm) (Teknokroma, Barcelona, Spain) was used. The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile, utilizing the gradient used in HPLC-DAD.

2.8. Structural analysis.

2.8.1. ^{1}H and ^{13}C-NMR, COSY, HSQC and NOESY spectra.

^{1}H (500.1 MHz) and ^{13}C (125.8 MHz) NMR spectra were recorded on Bruker Avance-500 spectrometer using D_2O or CD_3OD as solvent. Chemical shifts are reported in δ units (ppm) relative to the solvent peak (D_2O set at 4.79 for ^{1}H-NMR, CD_3OD set at 33.31 and 49.0 ppm for ^{1}H- and ^{13}C-NMR, respectively). The assignments of ^{1}H and ^{13}C signals were confirmed by homonuclear COSY, and heteronuclear HSQC spectra, respectively. The configuration of the double bond was confirmed by a 2D-NOESY experiment.

2.8.2. Mass spectra.

High Resolution Electrospray Ionization (HR-ESI) was performed using a Qexactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific) with a resolution of 70,000. The spray needle voltage was +3.5 kV in positive ionization, or -2.5 kV in negative ionization, as indicated for each compound. Samples were solved in MeOH:H_2O (50%) with 0.1% formic acid as additive and automatically introduced using a UHPLC Ultimate 3000 (Dionex).

2.8.3. IR spectra.

IR spectra were recorded on Jasco FT/IR 4100 equipped with an ATR accessory.

4-[(β-D-Glucopyranosyloxy)methyl]-3-[3-hydroxy-2-(methoxycarbonyl)propyl)] hex-4-enoate 1 (Trivial name: **1-glucosyl acyclodihydroelenolic acid**)

IR ν_{max} 3650-2500 (br), 1717, 1438, 1366, 1268, 1233, 1163, 1068 (sh), 1019 (str) cm^{-1}. ^{1}H-NMR (500.1 MHz, D_2O): δ 5.97 (q, 1H, $J_{8,10}$ = 7.0 Hz, H-8), 4.53 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1'), 4.43 (d, 1H, $J_{1a,1b}$ = 11.7 Hz, H-1a), 4.19 (d, 1H, $J_{1b,1a}$ = 11.7 Hz, H-1b), 3.99 (dd, 1H, $J_{6a',5'}$ = 2.1 Hz, $J_{6a',6b'}$ = 12.3 Hz, H-6a'), 3.83 (s, 3H, OCH_3), 3.81-3.76 (m, 3H, H-6b', H-3a, H-3b), 3.55 (t,

1H, $J_{3',2'}$ = $J_{3',4'}$ = 9.1 Hz, H-3'), 3.53-3.42 (m, 3H, H-4', H-5', H-5), 3.34 (dd, 1H, $J_{2',1'}$ = 8.0 Hz, $J_{2',3'}$ = 9.1 Hz, H-2'), 3.04 (ddd, 1H, $J_{4,3a}$ = 4.8 Hz, $J_{4,5}$ = 8.9 Hz, $J_{4,3b}$ = 11.3 Hz, H-4), 2.67 (dd, 1H, $J_{6a,5}$ = 9.6 Hz, $J_{6a,6b}$ = 15.4 Hz, H-6a), 2.56 (dd, 1H, $J_{6b,5}$ = 5.4 Hz, $J_{6b,6a}$ = 15.4 Hz, H-6b), 1.77 (d, 3H, $J_{10,8}$ = 7.0 Hz, H-10); ^{13}C-NMR (125.8 MHz, D_2O): δ 177.0 (C-7), 176.6 (*C*OOMe), 132.3 (C-9), 131.7 (C-8), 101.7 (C-1'), 2 x 75.9 (C-3', C-5'), 73.2 (C-2'), 71.5 (C-1), 69.7 (C-4'), 61.8 (C-3), 60.9 (C-6'), 52.5 (O*C*H_3), 50.9 (C-4), 36.8 (C-6), 34.7 (C-5), 13.0 (C-10); ESI-MS *m/z* 407 ([M−H] $^-$, 29%); HR-ESI-MS *m/z* calculated for $C_{17}H_{27}O_{11}$ ([M−H] $^-$): 407.1559, found: 407.1562.

3-[3-Hydroxy-2-(methoxycarbonyl)propyl]-4-[2',3',4',6'-tetra-O-acetyl-β-D-glucopyranosyl-oxy)methyl]hexenoate 2.

IR v_{max} 2929, 2861, 1739 (str), 1570, 1424, 1372, 1219 (str), 1038 (str), 903 (weak), 837 (weak) cm^{-1}. ^{1}H-NMR (500.1 MHz, CD_3OD): δ 5.78 (q, 1H, $J_{8,10}$ = 6.9 Hz, H-8), 5.24 (t, 1H, $J_{3',2'}$ = $J_{3',4'}$ = 9.4 Hz, H-3'), 5.03 (dd, 1H, $J_{4',3'}$ = 9.4 Hz, $J_{4',5'}$ = 10.0 Hz, H-4'), 4.87 (dd, 1H, $J_{2',1'}$ = 8.0 Hz, $J_{2',3'}$ = 9.4 Hz, H-2'), 4.76 (d, 1H, $J_{1',2'}$ = 8.0, H-1'), 4.28 (dd, 1H, $J_{6a',5'}$ = 4.6 Hz, $J_{6a',6b'}$ = 12.3 Hz, H-6a'), 4.27 (d, 1H, $J_{1a,1b}$ = 11.0 Hz, H-1a), 4.24 (d, 1H, $J_{1a,1b}$ = 11.0 Hz, H-1b), 4.24 (dd, 1H, $J_{3a,4}$ = 4.0 Hz, $J_{3a,3b}$ = 10.5 Hz, H-3a), 4.15 (dd, 1H, $J_{6b',5'}$ = 2.3 Hz, $J_{6a',6b'}$ = 12.3 Hz, H-6b'), 4.02 (dd, 1H, $J_{3b,4}$ = 9.8 Hz, $J_{3a,3b}$ = 10.5 Hz, H-3b), 3.88 (ddd, 1H, $J_{5',6b'}$ = 2.4 Hz, $J_{5',6a'}$ = 4.6 Hz, $J_{5',4'}$ = 10.0 Hz, H-5'), 3.71 (s, 3H, OCH_3), 3.49 (ddd, 1H, $J_{5,6b}$ = 5.1 Hz, $J_{5,6a}$ = 10.2 Hz, $J_{5,4}$ = 11.4 Hz, H-5), 2.86 (ddd, 1H, $J_{4,3a}$ = 4.0 Hz, $J_{4,3b}$ = 9.8 Hz, $J_{4,5}$ = 11.4 Hz, H-4), 2.37 (dd, 1H, $J_{6a,5}$ = 10.2 Hz, $J_{6a,6b}$ = 14.2 Hz, H-6a), 2.31 (dd, 1H, $J_{6b,5}$ = 5.1 Hz, $J_{6a,6b}$ = 14.2 Hz, H-6b), 2.08, 2.07, 2.01, 2.00, 1.96 (5s, 3H each, CH_3CO), 1.73 (d, 3H, $J_{10,8}$ = 6.9 Hz, H-10). ^{13}C-NMR (125.8 MHz, CD_3OD): δ 178.7 (C-7), 175.5 (*C*OOMe), 172.5, 172.4, 171.6, 171.3, 171.2 (5 CH_3OH), 134.9 (C-9), 131.5 (C-8), 101.1 (C-1'), 74.8 (C-3'), 73.7 (C-1), 73.0 (C-2'), 72.9 (C-5'), 69.9 (C-4'), 65.7 (C-3), 63.2 (C-6'), 52.3 (O*C*H_3), 50.0 (C-4), 40.2 (C-6), 37.0 (C-5), 20.9, 20.7, 20.6, 2 x 20.5 (5 *C*H_3CO), 14.0 (C-10). ESI-MS *m/z* 641 ([M+Na]$^+$, 17%), 214 (100%); HR-ESI-MS *m/z* calculated for $C_{27}H_{38}O_{16}Na$ ([M+Na]$^+$): 641.2052, found: 641.2051.

2.9. Acetylation of 1-glucosyl acyclodihydroelenolic acid.

A solution of 1-glucosyl acyclodihydroelenolic acid (42 mg, 0.103 mmol) in Ac_2O/Py (1:1, 4 mL) was stirred at room temperature for 24 h. Then, the mixture was concentrated to dryness under high vacuum and the residue was purified by column chromatography (AcOEt-cyclohexane 1:1 → AcOEt-MeOH 4:1). Yield: 45 mg, 70%.

3. Results and discussion.

3.1 Minor phenolic compounds present in the organic fraction.

We have previously characterized the phenolic extracts recovered with ethyl acetate from the liquid phase obtained from a new hydrothermal process of alperujo (Rubio-Senent et al., 2012). Among the compounds detected, hydroxytyrosol was observed in the highest concentration, followed by elenolic acid derivatives and a polymeric phenolic fraction. In addition, almost 25 other compounds were identified using HPLC-MS, which demonstrated the great complexity of the steam-treated alperujo phenolic fraction. In this work, the organic ethyl acetate extract was further separated in order to recover and characterise other minor phenolic compounds from steam-treated alperujo, which have not been previously identified. The extract was subjected to fractionation by means of an atmospheric-pressure chromatographic system through a series of adsorbent polyamide resins followed by Amberlite XAD resin, and further desorption with water and methanol in order to separate the minor compounds from those present in greater proportions. Finally, to facilitate the identification of the minor compounds, they were concentrated and purified by solid phase extraction (SPE) using a reversed-phase C18 cartridge. This process allowed the collection of three main fractions PXS50/20, PXS75/20 and PXS75/40, enriched in minor compounds that were identified by HPLC-MS (**Figure 1; Table 1**).

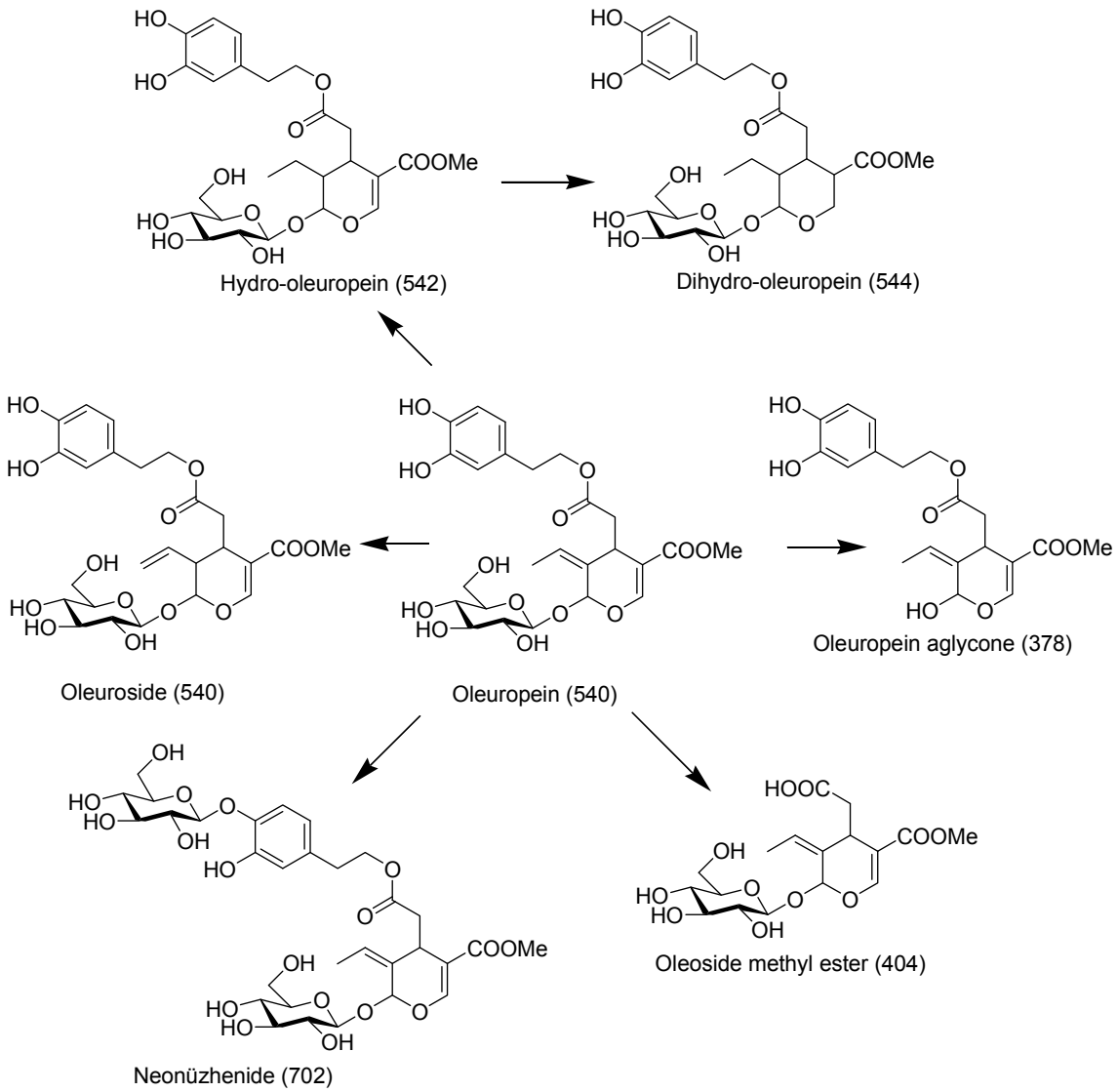


Figure 1: Different derivatives from oleuropein. Molecular weight indicated in brackets.

Table 1: Minor phenolic compounds identified in phenolic fractions. Rt, retention time in HPLC-MS. MW, molecular weight. *m/z*, major fragments detected by HPLC-MS.

Fraction	R_t (min)	Compound	MW	*m/z*$^-$	Reference
PXS50/20	19.9	Oleoside methyl ester	404	403	Cardoso et al., 2005
PXS75/20	30.1	Dihydro oleuropein	544	543, 525 and 151	Obied et al., 2008
PXS75/40	30.1	Dihydro oleuropein	544	543, 525 and 151	Obied et al., 2008
	31.3	Neonüzhenide	702	701 and 539	Cardoso et al., 2005
	34.3	Oleuropein derivative	540	539, 361, 225 and 121	-
	36.6	Hydro oleuropein	542	541, 405, 137 and 123	-

The mass spectrum of the PXS50/20 fraction identified an oleoside methyl ester, characterized by a quasi-molecular ion in negative mode $[M-H]^-$ with *m/z* 403. In the PXS75/20 fraction, the dihydro-oleuropein was identified; its mass spectrum presented signals at *m/z* 543,

525 and 151, which were associated with $[M-H]^-$, $[M-H_2O]^-$, and with the remaining elenolic acid core (De Nino, Mazzotti, Morrone, Perri, Raffaelli, & Sindona, 1999), respectively. The PXS75/40 fraction was particularly enriched in minor phenolic compounds. Furthermore, in this fraction, dihydro oleuropein was also identified. A molecule with a quasi-molecular ion at *m/z* 701 was detected. This signal could be attributed to neo-nüzhenide or oleuropein diglucose since both species produced the fragment corresponding to the loss of a glucose molecule, *m/z* 539. Nüzhenide was previously found to be the major phenolic compound in olive seeds (Servili, Baldioli, Selvaggini, Macchioni, & Montedoro, 1999). The alperujo sample, from which the phenolic extract was obtained, contains fragments of stone, and the molecule nüzhenide has been previously detected and quantified in alperujo extracts following hydrothermal treatment (Rubio-Senent et al., 2012). Therefore, it is possible that lower concentrations of neo-nüzhenide were present in minor concentrations. The species with *m/z* 540 was identified as a derivative of oleuropein, due to similarities in the absorbance profile and molecular weight, but the fragments obtained by MS are different from those described for oleuropein. Finally, another species with a molecular weight of 542 was detected; this molecule is identified as hydro oleuropein. The hydro oleuropein had a quasi-molecular ion at 541 and a fragment was observed at *m/z* 405, which could be attributed to $[M-HT-H]^{-;}$ this species has not been detected to date, but the presence of oleuropein and dihydro oleuropein in the extract led us to consider it as an intermediate compound.

3.2 Secoiridoids and phenolic glycosides present in the aqueous fraction.

The aqueous fraction obtained after steam treatment of alperujo and subsequent ethyl acetate extraction was treated by a mild acid hydrolysis to increase the proportion of oligosaccharides of low molecular weight released from the cell wall material of olive pulp. Fractions eluted from adsorption chromatography were fractionated by size exclusion chromatography using Superdex-Peptide resin, purified, and characterized, in order to contribute to the knowledge of the more polar phenolic and secoiridoids compounds in steam-treated olive pomace. The identification of the compounds present was based on the search for quasi-molecular ions $[M-H]^-$ ions using electrospray ionization mass spectrometry (ESI-MS) together with the interpretation of the different fragments formed (**Table 2**).

Table 2: Phenolic compounds identified in aqueous fraction. Rt, retention time in HPLC-MS. MW, molecular weight. *m/z*, major fragments detected by HPLC-MS.

R_t (min)	Compound	MW	*m/z*$^-$	Reference
11.7	Molecule 1	408	407	-
11.8	Molecule 1 glycosylated	570	569	-
18.6	β-hydroxyverbascoside	640	639, 621, 179 and 161	Mulinacci et al., 2005
23.1	Verbascoside	624	623, 461 and 161	Savarese et al., 2007
26.6	Luteolin-7-*O*-Glucoside	478	477 and 285	Obied et al., 2008
30.3	Oleuropein aglycon derivative	360	359, 153 and 123	De Nino et al., 2000
30.5	Oleuropein	540	539, 377, 307, 275 and 223	Obied et al., 2007
42.2	Quercitin Arabinose	434	433 and 301	Owen et al., 2003
45.1	Ligstroside aglycon isomer	362	361, 329, 291, 259, 241,223 and 139	Fu et al., 2009

Spectroscopy data showed the presence of a predominant ion at *m/z* 407 which likely corresponds to the depronated molecule $[M-H]^-$, and the corresponding sodium $[M+Na]^+$adduct at *m/z* 431 was observed in the positive mode for a sample with R_t of 11.7 min. Also, the appearance of the peak at *m/z* 569, for another fraction from Superdex-Peptide, is consistent with the existence of second hexose unit, as suggested by the loss of 162 u from *m/z* 569. Although this ion, *m/z* 407, has previously been found in olive fruit (Cardoso, Guyot, Marnet, Lopes-Da-Silva, Renard, & Coimbra, 2005; Menéndez et al., 2008), the structure of the compound was unknown, although it appears consistently in the Superdex-Peptide fractions studied (Fernández-Bolaños et al., 2014). We named this molecule, with a molecular weight at 408, as Molecule **1.** Molecule 1 was purified exhaustibly, and we determined the structure for the first time, as detailed in the next section.

Analysis of the others fractions gave an ion $[M-H]^-$ at *m/z* 639 and another at *m/z* 623, which corresponded with the molecular ions of β-hydroxyverbascoside and verbascoside, respectively. In both cases, the characteristic fragments ions due to the loss of caffeic acid were also present, and the peak at *m/z* 161 would result from a proton transfer and the formation of an anionic ketene (Ryan, Antolovich, Herlt, Prenzler, Lavee, & Robards, 2002). Both acteosides are complex biophenols associated with beneficial health properties, such as an important antioxidant and antibacterial activity, a protection in the concentration of free radicals, and inhibition of the lipid perroxidation (Liu, & Wyman, 2003). The β-hydroxyverbascoside has been described in previous work as a precursor of 3,4-dihydroxyphenylglicol (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, Palacios-Díaz, & Fernández-Bolaños, 2013).

The flavonoids luteolin-7-*O*-glucoside and quercetin-arabinose were also identified. While the first, a flavone, has previously been detected in the ethyl acetate extract of steam-treated alperujo (Rubio-Senent et al., 2012), the flavonol, quercetin-arabinose was detected for the first time in olive fruit or their by-products in this study. The precursor of this last compound is probably the predominant flavonoid in olive pomace, the quercetin-3-arabinose-glucoside

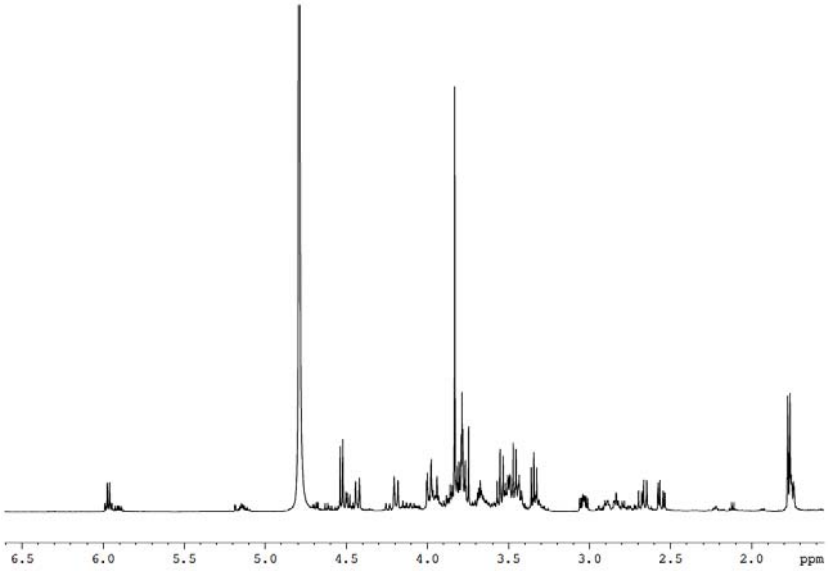
(Bouaziz, Chamkha, & Sayadi, 2004; Yahyaoui et al. 2014), since the loss of glucose during the steam-treatment would result in the quercitin-arabinose detected. Its mass spectrum showed an ion at *m/z* 433 in negative mode and the corresponding signal at *m/z* 301 due to the loss of the pentose unit.

In addition, oleuropein, the major phenol in olive fruit, and other derivatives from phenolic secoiridoids, which are closely correlated to oleuropein and ligstroside, were also isolated by Superdex-Peptide chromatography from the aqueous fraction and identified by spectral characterization. These last molecules were identified as oleuropein aglycone derivatives, also identified in olive pulp (De Nino, Mazzotti, Perri, Procopio, Raffaelli, & Sindona, 2000), and as an isomer of ligstroside aglycone identified in olive oil (Fu, Segura-Carretero, Arráez-Román, Menéndez, De la Torre, & Fernández-Gutiérrez, 2009); both were not detected in the ethyl acetate extract.

3.3. Characterization of Molecule 1

Molecule **1,** with a quasi-molecular ion at *m/z* 407 in negative mode, is not apparent from the HPLC chromatogram generated at 280 nm due to the relatively weak absorption of the compound at this wavelength. Molecule **1** was purified by HPLC using a C_{18} semi-preparative column and subsequent detection by electrospray ionization mass spectrometry (ESI-MS) (Supplementary data 1) (as described in Materials & Methods). The structure was elucidated using ^{1}H and ^{13}C NMR techniques (**Figure 2A** and Supplementary data 2, respectively), and chemical ionization mass spectrometry and infrared analysis. Structural analysis was confirmed using bi-dimensional NMR techniques, such as the COSY (^{1}H-^{1}H correlation) (Supplementary data 3), HSQC (^{1}H-^{13}C correlation) (Supplementary data 4), DEPT-45 and NOESY (through-space coupling) (data not show). In addition, because the signal overlapping in the ^{1}H-NMR spectrum, the sample was peracetylated to confirm the structure of Molecule **1** through the structural analysis of its peracetylated derivative, Molecule **2** (**Figure 3**).

A



B

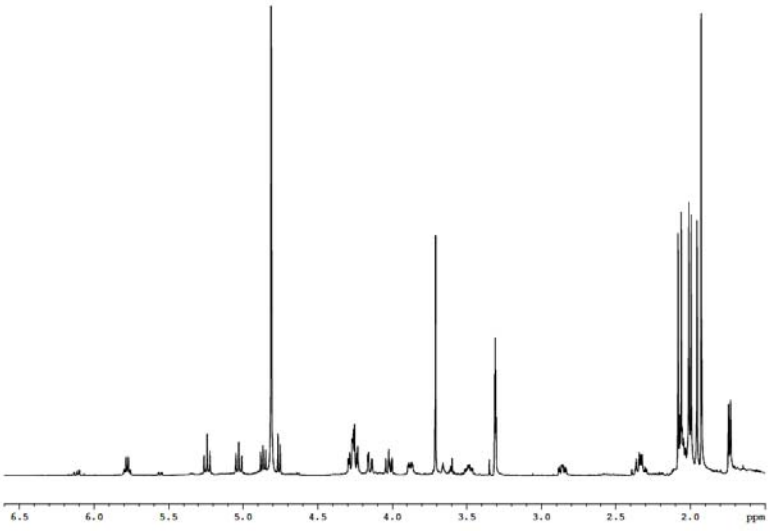


Figure 2: **A**,[1]H-RMN Molecule 1. **B**, [1]H-RMN Molecule **2**.

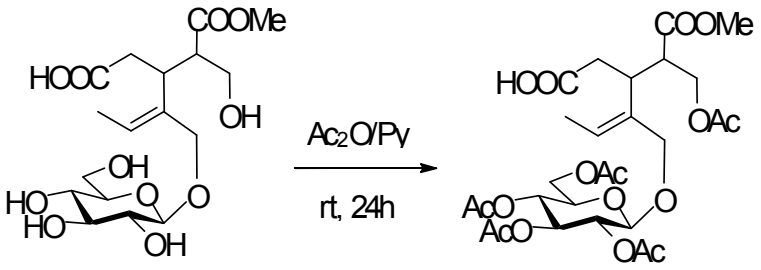


Figure 3: Acetylation of dihydroelenolic acid glucoside using acetic anhydride and pyridine at room temperature (rt).

The IR spectrum of the compound (Supplementary data 5) revealed a broad absorption band, 3650-3100 cm^{-1}, corresponding to O-H stretching vibrations of hydroxyl groups and the carboxylic group. The absorption band at 1717 cm^{-1} was attributed to the C=O stretching of the carboxylic and ester groups. Finally, the strong signal at 1019 cm^{-1} was associated with C-O tension of the ester. The ^{1}H-NMR data **(Figure 2A)** showed a quartet at δ 5.78 (*J*=6.9 Hz) coupled with a signal of methyl at δ 1.73, suggesting that the compound contains a vinyl moiety characteristic of the elenolic acid. ^{1}H-NMR also showed two geminal protons at δ 4.43 and 4.19 (2 doblets, $J_{1a,1b}$ = 11.7 Hz, H-1a, H-1b) that confirm the reduction of the C-1 of elenolic acid glucoside. COSY (Supplementary data 3) gave information about the correlation between H-3a/H3b/H-4, H-4/H-5, H-5/H-6a/H-6b. The HSQC (Supplementary data 4) correlation of H-3a and H-3b with the carbon signal at 61.8 ppm, confirms the presence of the hydroxyl in position 3. Furthermore, the HSQC correlation of H-1a and H-1b with the carbon at 71.5 ppm confirms that presence of a glycosyloxy group at C-1. In fact, glycosylation of the hydroxyl group at C-1 induces a strong deshielding in the signal of the carbon attached to it (71.5 ppm for C-1), compared with C-3 (61.8 ppm) (Jiménez & Riguera, 1994). The *E* configuration for the olefin moiety was confirmed by 2D-NOESY, where cross peaks H-1a/=CH, H-1b/=CH and H-5/CH_3 indicate through-space coupling, and therefore spatial proximity (**Figure 4A**). Finally, we proved the molecular formula $C_{17}H_{28}O_{11}$ by High Resolution Electrospray Ionization Mass Spectrometry (HR-ESI-MS) on negative mode (Supplementary data 1).

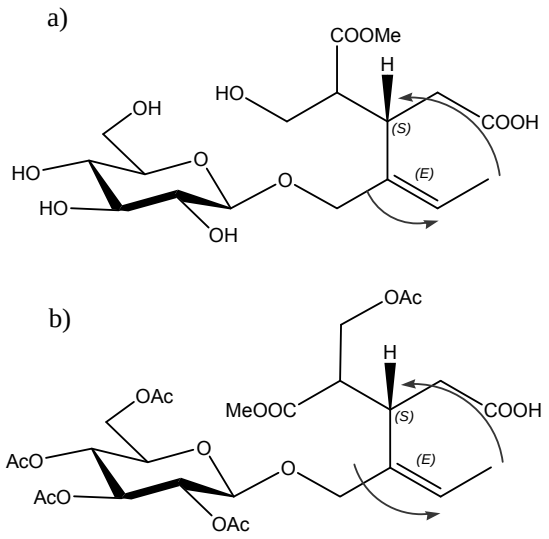


Figure 4: Through-space correlation found in 2D-NOESY experiment for Molecule 1 (**a**) and Molecule 2 (**b**).

Acetylation of 1-glucosyl acyclodihydroelenolic acid was carried out in a mixture of acetic anhydride and pyridine (1:1) at room temperature and the compound (Molecule **2**) was purified by column chromatography using silica gel as stationary phase. The presence of five acetyl groups in the final compound was confirmed by ^{1}H-NMR (δ 2.08, 2.07, 2.01, 2.00, 1.96 ppm)

(**Figure 2B**) and ^{13}C-NMR (δ 20.9, 20.7, 20.6, 20.5, 20.5 ppm) (Supplementary data 6). The deshielding of protons H-3a (4.24 ppm) and H-3b (4.02 ppm) for Molecule **2** compared with those protons for Molecule **1** (3.81-3.76 ppm) confirms the presence of a free hydroxyl group at C-3 of **1.** The *E* configuration was again confirmed by 2D-NOESY (**Figure 4B**, Supplementary data 7). On the basis of configuration of C-5 of oleuropein (Inouye, H., Yoshida, T., Tobita, S., Tanaka, K., & Nishioka, T,), and assuming a similar biosynthetic pathway from 7-ketologanin (Gutierrez-Rosales, Romero, Casanovas, Motilva, & Mínguez-Mosquera, 2010), the configuration of C-5 of the acyclodihydroelenolic acid **1** should be *R;* however, The configuration of C-4 could not be determined. A planar-zigzag conformation for the 5-acetoxypentanoic acid moiety of **2** is in agreement with vicinal H,H coupling constants ($J_{3a,4}$ = 4.0 Hz $J_{3b,4}$ = 9.8 Hz, $J_{4,5}$ = 11.4 Hz, $J_{5,6a}$ = 10.2 Hz, $J_{6b,5,6b}$ = 5.1 Hz).

The IR spectrum (Supplementary data 8) showed a weak absorption band at 2929 cm^{-1} that was attributed to the presence of the carboxylic group. The strong band at 1739 cm^{-1} corresponds to the C=O stretching of the carboxylic and ester groups. Finally, the strong bands at 1220 and 1038 cm^{-1} were associated with C-O stretching vibrations of the acetyl and the methyl ester groups, respectively. The molecular formula $C_{27}H_{38}O_{16}$ was also corroborated by HR-ESI mass spectrometry (Supplementary data 9). The major fragment *m/z* 214 coincides with the loss of sugar, acetyl and methyl moieties.

Taking into account the structural descriptions as detailed above, the compound was identified as 1-glucosyl acyclodihydroelenolic acid. This compound has not previously been identified. However, another non-aldehydic open cycle secoiridoid had been reported by Obied, Bedgood, Prenzler, & Robards (2007) hydroxytyrosyl acyclodihydroelenolate. In this case, the hydroxytyrosol was linked to a non-glycosylated secoiridoid nucleus. In both cases, the compounds with diol groups likely originate from reduction reactions of elenolic acid, the terpenoid skeleton of oleuropein. It is likely that the formation of **1** involves a glycosylation step after the reduction at C-1 has taken place.

4. Conclusions.

The results of this study show the presence of diverse compounds in the alperujo extract (organic and aqueous) obtained from a new industrial liquid source by the steam treatment of alperujo and subsequent extraction with ethyl acetate. In a first step, we considered the phenolic compounds present in low concentrations in a phenolic extract obtained with ethyl acetate following purification procedures, which due to their minor concentration have not been previously studied. In a second step, the polar compounds present after the extraction with ethyl acetate were studied. Both steps led to the identification of numerous known compounds, including secoiridoids derivatives such as oleuropein and ligstroside derivatives, their hydrolytic

derivatives (oleuropein and ligstroside aglycones), acteosides (verbascoside and hydroxyverbascoside) and flavonoids. In addition, we isolated, purified, and elucidated the structure of a new secoiridoid derivative with a molecular weight of 408. The study of spectroscopy allowed the identification of the molecule 1-glucosyl acyclodihydroelenolic acid for the first time.

Acknowledgements.

Funding was received from the Spanish Ministry of Economy and Competitiveness and co-funded by European Social Fund (ESF) (Project AGL2013-48291-R). Dr. Rodríguez-Gutiérrez (RYC-2012-10456 contract) wishes to thank the "Ramón y Cajal" Program, and Ms Rubio-Senent (JAE-Pre104) the Spanish National Research Council (CSIC) program, for providing financial support, both co-funded by ESF.

References

Bouaziz, M., Chamkha, M., & Sayadi, S. (2004) Comparative Study on Phenolic Content and Antioxidant Activity during Maturation of the Olive Cultivar Chemlali from Tunisia. *Journal Agricultural and Food Chemistry. 52*, 5476−5481.

Cardoso, S. M., Guyot, S., Marnet, N., Lopes-Da-Silva, J. A., Renard, C. M., & Coimbra, M. A. (2005). Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *Journal of the Science of Food and Agriculture, 85*, 21–32.

Carlos Jiménez, C. & Riguera, R.(1994). Phenylethanoid Glycosides in Plants*:* Structure and Biological Activity*. Natural Products Reports, 11*, 591-606.

De Nino, A., Mazzotti, F., Morrone, S. P., Perri, E., Raffaelli, A., & Sindona, G. (1999). Characterization of Cassanese olive cultivar through the identification of new trace components by ionspray tandem mass spectrometry. *Journal Mass Spectrometry, 34*, 10−16.

De Nino, A., Mazzotti, F., Perri, E., Procopio, A., Raffaelli, A., & Sindona, G. (2000). Virtual freezing of the hemiacetal-aldehyde equilibrium of the aglycones of oleuropein and ligstroside present in olive oils from Carolea and Coratina cultivars by ionspray ionization tandem mass spectrometry. *Journal Mass Spectrometry*, *35,* 461−467.

Estruch, R., Ros, E., Salas-Salvado, J., Covas, M.I., Corella, D., Aros, F., Gomez-Gracia, E., Ruiz-Gutierrez, V., Fiol, M., Lapetra, J., Lamuela-Raventos, R.M., Serra-Majem, L., Pinto, X., Basora, J., Munoz, M.A., Sorli, J.V., Martinez, J.A., & Martinez-Gonzalez, M.A. (2013). Primary Prevention of Cardiovascular Disease with a Mediterranean Diet. *New England Journal of Medicine, 368*, 1279-1290.

Fernández-Bolaños, J. Rodríguez-Gutiérrez, G. Lama-Muñoz, A. & Sánchez, P. (2010). *Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de oliva*. (Spanish Patent request No. P201031236).

Fernández-Bolaños, J., Rubio-Senent, F., Lama-Muñoz, A., García, A., & Rodríguez-Gutiérrez, G. (2014). Production of oligosaccharides with low molecular weights, secoiridoids and phenolic glycosides from thermally treated olive by-products. In: Oligosaccharides: Food Sources, Biological Roles and Health Implications. ISBN: 978-1-62948-328-3.

Fu, S., Segura-Carretero, A., Arráez-Román, D., Menéndez, J. A., De la Torre, A., & Fernández-Gutiérrez, A. (2009). Tentative characterization of novel phenolic compounds in extra virgin olive oils by rapid-resolution liquid chromatography coupled with mass spectrometry. *Journal of Agricultural and Food Chemistry, 57*, 11140-11147.

Gutíerrez-Rosales, F., Romero M. P., Casanovas, M., Motilva, M. J., & Mínguez-Mosquera, M. I. (2010). Metabolites involved in oleuropein accumulation and degradation in fruits of *Olea europaea* L.: Hojiblanca and Arbequina varieties. *Journal of Agricultural and Food Chemistry*, *58*, 12924-1233.

Carlos Jiménez, C. & Riguera, R. (1994). Phenylethanoid glycosides in plants: structure and biological activity. Natural products Reports, 11, 591-606.

Kalogerakis, N., Polito, M., Foteinis, S., Chatzisymeon, E., & Mantzavinos, D. (2013). Recovery of antioxidants from olive mill wastewaters: A viable solution that promotes their overall sustainable management. *Journal of Environmental Management, 128*, 749-758.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids, 28*, 1-13.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., Palacios-Díaz, R., & Fernández-Bolaños, J. (2013). A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste. *Food Chemistry, 140*, 154-160.

Liu, C., & Wyman, C.E. 2003. The effect of flow rate of compressed hot water on xylan, lignin and total mass removal from corn stover. *Industrial & Engineering Chemistry Research, 42*, 5409– 5416.

Menéndez, J. A., Vázquez-Martín, A., García-Villalba, R., Carrasco-Pancorbo, A., Oliveras-Ferraros, C., Fernández-Gutiérrez, A., & Segura-Carretero, A. tabAnti-HER2 (erbB-2) oncogene effects of phenolic compounds directly isolated from commercial extra-virgin olive oil (EVOO). *BCM Cancer, 2008*, 8, 377.

Mulinacci, N., Innocenti, M., La Marca, G., Mercalli, E., Giaccherini, C., Romani, A., et al. (2005). Solid olive residues: Insight into their phenolic composition*. Journal of Agricultural and Food Chemistry, 53*, 8963–8969.

Obied, H. K., Bedgood, D. R. Jr., Prenzler, P. D., & Robards, K. (2007). Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Analytical Chemical Acta, 603*, 176−189.

Obied, H. K., Prenzler, P. D., Ryan, D., Servili, M., Taticchi, A., Esposto, S., & Robards, K. (2008). Biosynthesis and biotransformations of phenol-conjugated oleosidic secoiridoids from Olea europaea L. *Natural Product Reports, 25*, 1167−1179.

Obied, H. K., Prenzler, P. D., Konczak, I, Rehman, A. U., & Robards, K. (2009). Chemistry and bioactivity of olive biophenols in some antioxidant and antiproliferative in vitro bioassays. *Chemistry and Research Toxicology, 22*, 227-234.

Owen, RW., Haubner, R., Mier, W., Giacosa, A., Hull, W. E ., Spiegelhalder, B., & Bartsch, H. (2003). Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food and Chemical Toxicology, 41*, 703–717.

Rubio-Senent, F., Lama-Muñoz, A., Rodríguez-Gutiérrez, G. & Fernández-Bolaños, J. (2013). Isolation and identification of phenolic glucosides from thermally treated olive oil byproducts. *Journal of Agricultural and Food Chemistry, 61*, 1235-1248.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil by-product alperujo and their antioxidative activities. *Journal of Agricultural and Food Chemistry, 60*, 1175–1186.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Munoz, A., García, A., & Fernández-Bolaños, J. (2015). Pectin extracted from thermally treated olive oil by-products: characterization physico-chemical properties, in vitro bile acid and glucose binding, *Food Hydrocolloids*, DOI: 10.1016/j.foodhyd.2014.06.001.

Ruiz-Canela, M., Estruch, R., Corella, D., Salas-Salvado, J., & Martinez-Gonzalez, MA. (2014). Association of Mediterranean diet with peripheral artery disease: The PREDIMED randomized trial. *Journal of the American Medical Association*, 311-415.

Ryan, D., Antolovich, M., Herlt, T., Prenzler, P.D., Lavee, S., & Robard, K. (2002). Identification of phenolic compounds in tissues of the novel olive cultivar Hardy's Mammoth. *Journal Agricultural and Food Chemistry*, 50, 6716-6724.

Savarese, M., De Marco, E., & Sacchi, R. (2007). Characterization of phenolic extracts from olives (Olea europaea cv. Pisciottana) by electrospray ionization mass spectrometry. *Food Chemistry*, 105, 761−770.

Servili, M., Baldioli, M., Selvaggini, R., Macchioni, A., & Montedoro, G. (1999). Phenolic compounds of olive fruit: One- and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. *Journal Agricultural and Food Chemistry*, 47, 12–18.

Supplementary Data

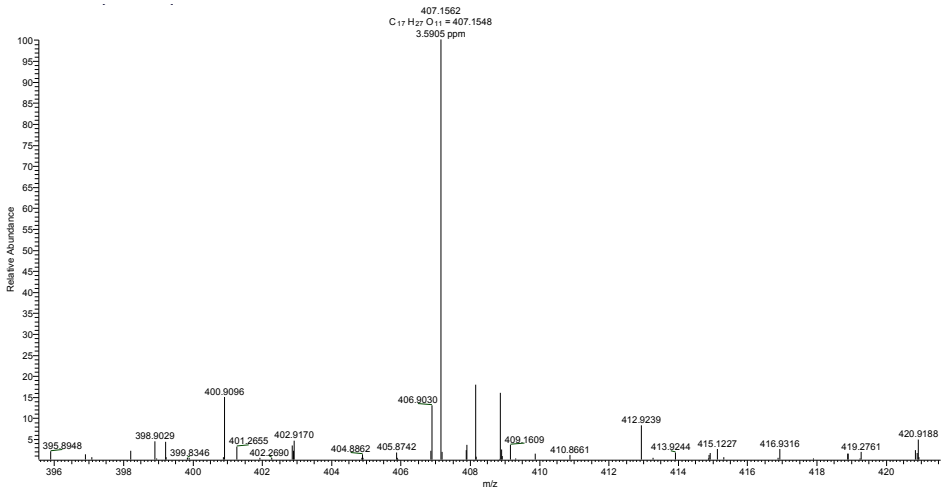


Figure 1: MS Molecule 1.

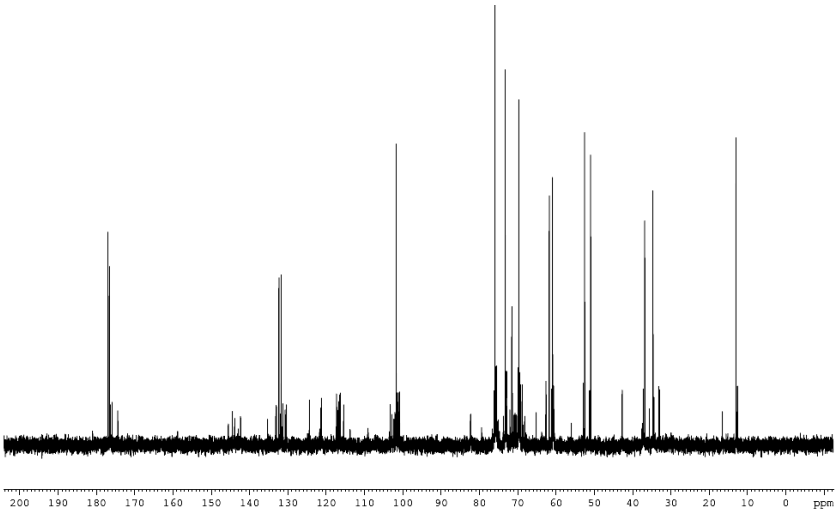


Figure 2: ^{13}C-RMN Molecule 1.

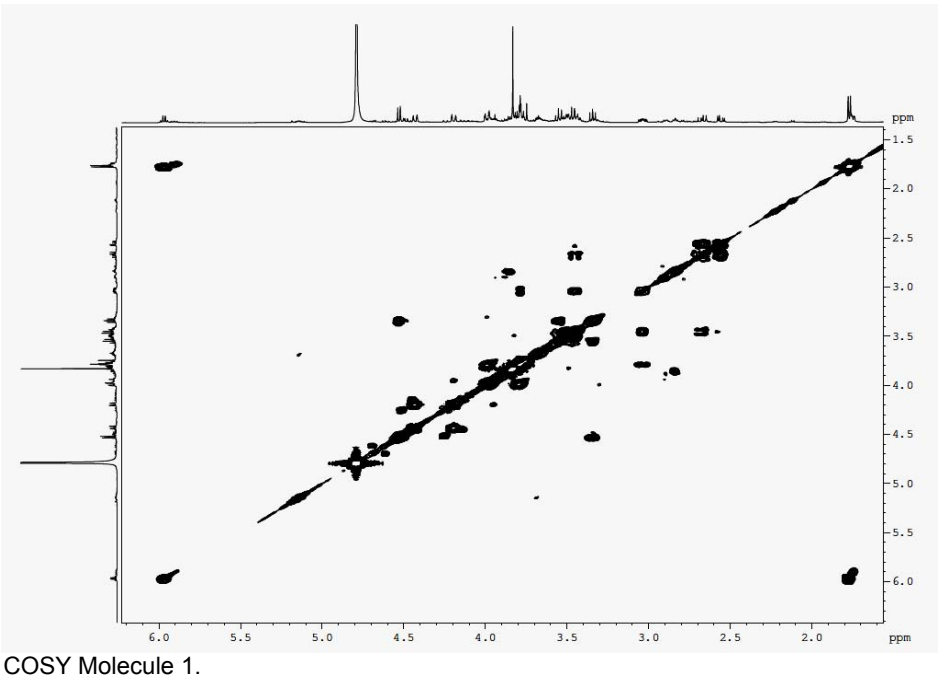


Figure 3: COSY Molecule 1.

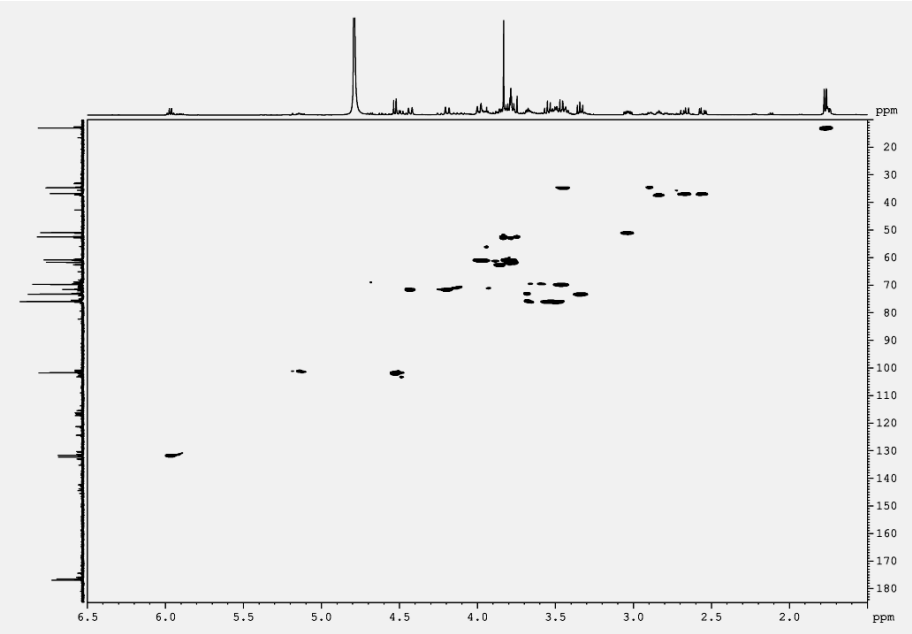


Figure 4: HSQC Molecule 1.

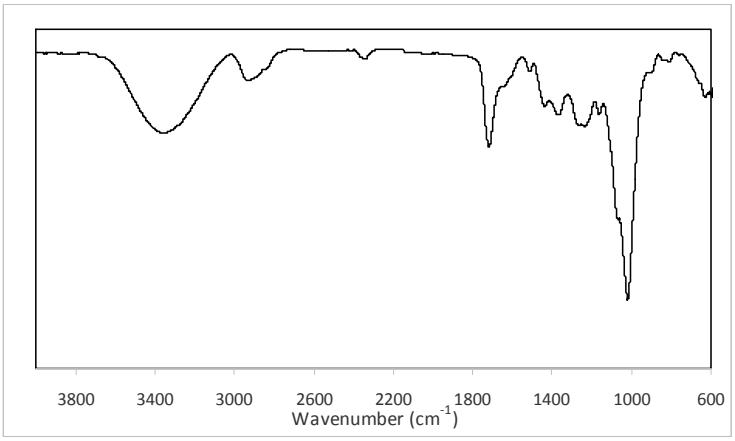


Figure 5: IR Molecule 1.

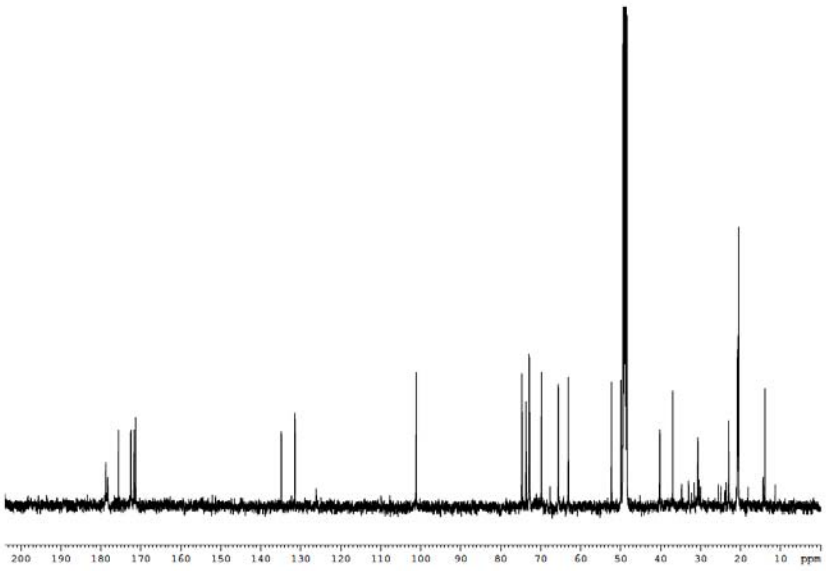


Figure 6: ^{13}C-RMN Molecule 2.

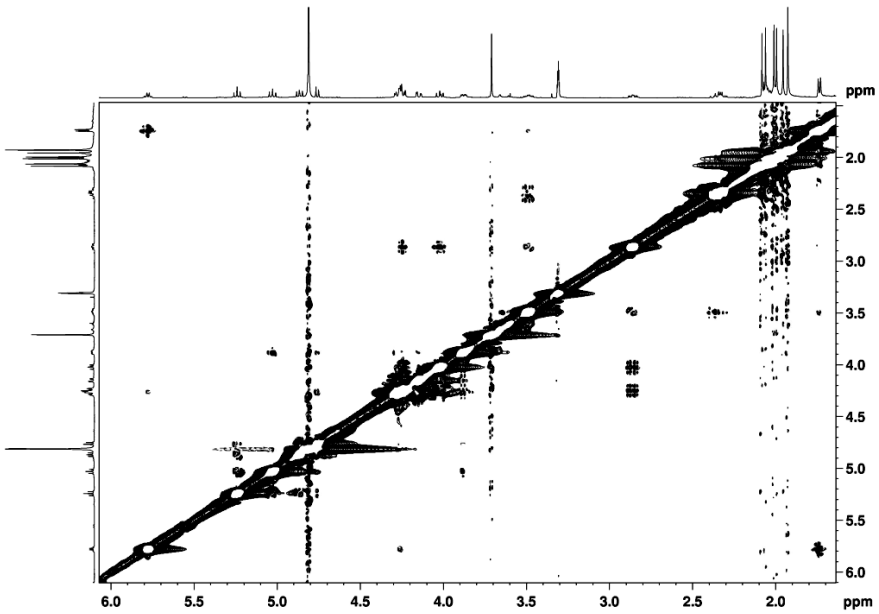


Figure 7: NOESY Molecule 2.

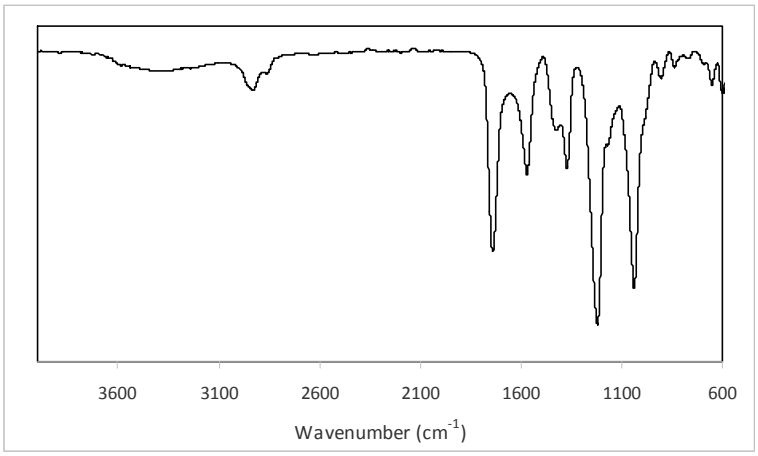


Figure 8: IR Molecule 2.

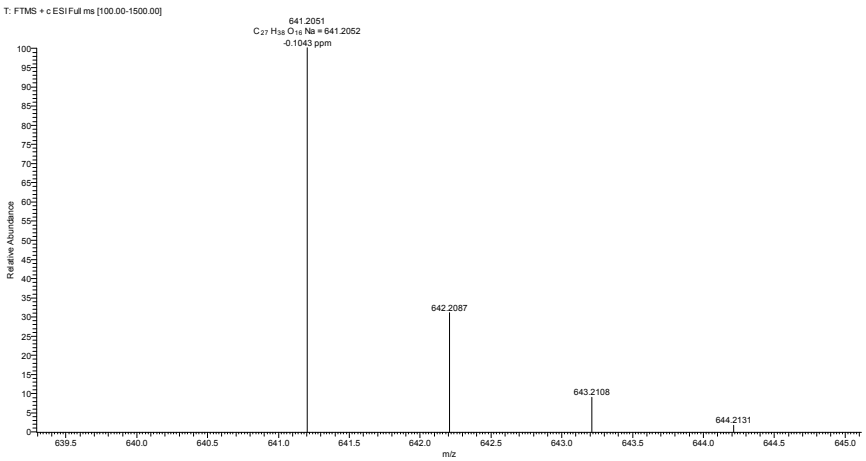


Figure 9: MS Molecule 2.

6.1.8. Capítulo 8.

A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste.

Lama-Muñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Palacios-Díaz. R., Fernández-Bolaños, J.

Food Chemistry, 2013, 140, 154-160.

El fenol 3,4-dihidroxifenilglicol (DHFG) se encuentra en cantidades importantes en el agua de vegetación de la aceituna y en las aceitunas de mesa, además de encontrarse en pequeñas cantidades en el aceite de oliva. También ha sido recuperado en el alperujo tratado térmicamente. El sistema de purificación DHFG a partir de alperujo mediante el uso de resinas de intercambio iónico y polímeros absorbentes ha sido patentado por nuestro grupo de investigación. Estudios previos realizados muestran que el DHFG tiene mayor capacidad antioxidante, antirradical y poder reductor que el hidroxitirosol (HT) y evita la peroxidación lipídica en un grado que es comparable con la vitamina E.

Aunque el DHFG es un potente antioxidante, no está disponible comercialmente en grandes cantidades. En consecuencia, la recuperación de DHFG a partir del alperujo podría proporcionar una fuente de un compuesto de alto valor añadido. Estudios previos han mostrado que tanto el tratamiento térmico, como calentamientos suaves del alperujo provocan un aumento en la concentración de DHFG, estos datos indican la presencia de compuestos que contienen DHFG como parte de su estructura o actúan como sustratos para la generación de DHFG. El objetivo de este capítulo es estudiar el origen de la liberación DHFG e identificar los posibles precursores que aumentan la cantidad de DHFG libre en los extractos.

Los resultados obtenidos nos permitieron aislar e identificar dos compuestos que se caracterizan por poseer en sus estructuras una unidad de DHFG, actuando como claros precursores de dicha especie.

A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste

Antonio Lama-Muñoz, Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Rafael Palacios-Díaz, Juan Fernández-Bolaños

ABSTRACT

3,4-Dihydroxyphenylglycol (DHPG) is a potent antioxidant recently found in the free form in olive oil and table olives. DHPG can be recovered from olive oil solid waste by a hydrothermal treatment. It was observed that an increase in the concentration of DHPG occurred when alperujo aqueous extracts were subjected to mild thermal conditions (post-treatment). This fact indicates that certain solubilized compounds or precursors containing DHPG which is released with the post-treatment. In the present study, the precursors of DHPG were identified and characterized after extraction from alperujo using thermal treatment and purification by fractionation on Amberlite® XAD16 polyamide and semi-preparative reverse-phase HPLC columns. Their structures were elucidated using HPLC coupled to diode array detector (DAD) and electrospray ionization mass spectrometry (ESI-MS). The results identified three compounds as precursors, and their structures can be attributed to the diastereoisomeric forms of the two β-hydroxy derivatives of verbascoside and isoverbascoside (β-hydroxyacteoside and β-hydroxyisoacteoside), and 2”-hydroxyoleuropein, all of which contain a DHPG moiety, potentially explaining the increases in the concentration of this phenolic compound in olive oil waste.

KEYWORDS: Alperujo; Antioxidants; 3,4-Dihydroxyphenylglycol; Hydroxytyrosol; Phenols; ESI mass spectrometry; Oleuropein; Verbascoside.

1. Introduction

In Spain, olive oil is commonly extracted using a two-phase centrifugation system, a process that generates a semisolid waste that is called two-phase pomace or ‘‘alperujo’’. It has been estimated that the generation of alperujo as olive processing by-product in Spain is approximately 4-6 million tonnes every year. However, alperujo presents a serious environmental problem for Mediterranean countries due to its highly polluting organic load that includes lipids, pectins, polyalcohols, sugars, tannins and phenolic compounds, which limit its biodegradation because of their high toxicity (Roig, Cayuela, & Sánchez-Monedero, 2006). However, alperujo can provide a large, inexpensive, and unexploited source of chemicals: alperujo is rich in phenolic compounds (it contains 98-99% of the available phenols in olive fruit)

with strong antioxidant properties and well-recognized health-beneficial activities (Cicerale, Lucas, & Keast, 2010).

The phenolic compounds present in alperujo can be recovered by a novel process based on hydrothermal treatment (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012). This treatment is conducted in a discontinuous reactor using steam at high pressures and temperatures (170 ºC, 8 kg/cm^2) and allows the separation of alperujo into two phases (liquid and solid), an operation that is practically impossible without pretreatment. The result is a significant solubilization of the phenolic compounds in the liquid phase. The simple phenol 3,4-dihydroxyphenylglycol (DHPG) is structurally similar to hydroxytyrosol (HT) (the major phenolic in olive fruit) and has an additional hydroxyl group in the β-position. DHPG is found in vegetation water of the olive fruit (Limiroli, Consonni, Ranalli, Bianchi, & Zetta, 1996), table olives (Rodríguez, Lama, Jaramillo et al., 2009) and olive oil (Medina, de Castro, Romero, & Brenes, 2006) and has been recovered from alperujo using the above treatment (Rodríguez, Lama, Trujillo et al., 2009). A patented system for purifying DHPG (> 90% dry weight) from this natural source has been developed by our group and utilizes ion-exchange resins and adsorbent polymers (Fernández-Bolaños et al., 2008). Despite the many studies that show the health-beneficial biological activities of HT (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010; Killeen, Pontoniere, & Crea, 2011), only a few studies have been conducted on the functional properties of DHPG. DHPG has higher antioxidant and antiradical capacities and reducing power than hydroxytyrosol (HT) and prevents lipid peroxidation to a degree that is comparable with vitamin E (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007). Furthermore, DHPG is bioavailable and has antioxidant properties in vitamin E-deficient rats (Rodríguez-Gutiérrez et al., 2012); it may also protect against platelet activation and adhesion and possibly has anti-inflammatory properties (de Roos et al., 2011).

Although DHPG is a strong antioxidant, it is not commercially available in large quantities. Consequently, the recovery of DHPG from alperujo could provide a source of a high added-value compound that may be used as a natural antioxidant ingredient with biological activity to enhance food properties, for nutritional purposes and for preservation. However, DHPG is present in alperujo at a low concentration (approximately 10-fold less than HT). In a previous study (Rodríguez, Lama, Trujillo et al., 2009), the possibility of applying a hydrothermal treatment for obtaining a good recovery of DHPG from alperujo, focusing on the optimal conditions of the thermal treatment for DHPG solubilization was investigated. A long-term stability study of DHPG was also conducted using olive oil mill wastewaters (obtained by a mild heating treatment at the industrial level of the alperujo obtained through olive oil extraction) stored under different temperature and pH conditions. It was found that the concentration of DHPG was increased by 35% under all of the conditions, indicating the presence of compounds that contain DHPG as part of their structure or act as substrates for DHPG generation. The presence of DHPG precursors was also confirmed during the thermal treatment of alperujo

aqueous extracts, resulting in the release of additional DHPG. This finding prompted us to hypothesize the existence of certain compounds, previously reported by some authors, which could be good candidates for explaining the observed increases in DHPG concentrations upon the separation of aqueous extracts of alperujo as the primary source of DHPG. Dellagreca, Previtera, Temussi, and Zarrelli (2004) isolated and identified two compounds from the reverse osmosis fraction of olive mill waste waters as C-6-C-2 dimers of tyrosol (Ty) and HT with 3,4-dihydroxyphenylglycol as the possible precursors. Mulinacci et al. (2005) identified two diastereoisomers of β-hydroxyacteoside with the presence of DHPG in their structures, and Di Donna et al. (2007) provided mass spectrometric evidence for the presence of 2”-hydroxyoleuropein in olive drupe tissues, with DHPG constituting the phenolic moiety.

The aim of the present work was to study the origin of DHPG release and to identify the possible precursors that increase the amount of free DHPG in the aqueous extracts of alperujo subjected to a thermal post-treatment. For this purpose, the aqueous extract of alperujo were extracted with ethyl acetate and fractionated by adsorption columns to obtain pure precursors. The quantitative determination of DHPG and the qualitative determination of the precursors were performed by HPLC-DAD. The fractions containing DHPG precursors were then structurally characterized by electrospray ionization mass spectrometry (ESI-MS).

2. Materials and methods

2.1. Plant materials

Four alperujo samples from different olive cultivars and seasons were obtained from an experimental olive oil mill located at the Instituto de la Grasa (CSIC) in Seville (Spain) and were stored at -20ºC until use and analysis.

2.2. Chemicals

The pure standard of 3,4-dihydroxyphenylglycol was purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany), and ultrapure water was obtained using a Milli-Q water system (Millipore, Milford, MA, USA). The extraction solvents ethyl acetate and methanol were obtained from Romil Ltd. (Waterbeach, UK). The hexane used for the oil extraction was of analytical grade.

2.3. Solubilization of DHPG precursors by hydrothermal treatment

The alperujo (250 g) was mixed with water using a solid/liquid ratio of 1/5 (w/v) and heated in a water bath Unitronic 320 OR (J.P. Selecta, Barcelona, Spain) with slow agitation and the temperature controlled at 50 ºC for 1 h. This first thermal treatment of the alperujo was

aimed to solubilize DHPG and, chiefly, its potential precursors. According to a previous work (Rodríguez, Lama, Trujillo et al., 2009), the highest percent of solubilized precursors in the aqueous extract is obtained using these optimal conditions. The solid and liquid phases were separated by centrifugation and filtration.

2.4. Analysis of free and linked DHPG by HPLC–DAD

Aliquots from the water-soluble fraction obtained after the hydrothermal treatment (50 ºC for 1 h) were filtered through 0.45 µm membranes and used for the direct HPLC determination of DHPG. The HPLC analyses were performed using a Hewlett Packard Series 1100 liquid chromatography system equipped with a diode array detector and a Rheodyne injection valve (20 µL loop). A Spherisorb ODS-2 column (250 x 4.6 mm i.d.; particle size, 5 µm) (Teknokroma, Barcelona, Spain) was used at room temperature. A linear gradient elution was performed at a flow rate of 1.0 mL/min using ultrapure water (pH 2.5 adjusted with trifluoroacetic acid) and acetonitrile as the mobile phase, from 5% to 25% acetonitrile over 30 min. The system was equilibrated between the runs for 5 min using the starting mobile-phase composition. The chromatograms were recorded at 280 nm. The quantitative evaluation of free DHPG was based on a comparison of the retention times with that of the reference compound and recording the UV spectra in the range of 200-360 nm. The quantification was performed by means of a five-point regression curve in triplicate of individual stock solutions of DHPG ($r^2 \geq 0.99$).

The liquid phase (or aqueous extract) was subjected to a second thermal treatment or post-treatment at 90 ºC for 1-5 h to release DHPG from its precursors. The released DHPG from the precursors in the extracts, i.e., increased DHPG concentrations, were calculated as the difference between the DHPG content determined before and after post-treatment at 90 ºC. The time selected was that for which the highest conversion of precursors into DHPG was achieved.

2.5. Isolation of precursors

First, the liquid phase or aqueous extract of the alperujo was defatted using *n*-hexane to remove all possible traces of oil, and the DHPG precursors were extracted from the aqueous extract of alperujo using ethyl acetate in a separatory funnel at room temperature. The mixture, aqueous extract/solvent (1:10, v/v), was vigorously shaken for 10 min to achieve equilibrium and then allowed to settle for 20 min. The phases were separated, and the extraction was repeated twice. The ethyl acetate fractions were then combined and evaporated to dryness under vacuum at 40 ºC in a rotator evaporator (Buchi, Milan, Italy). The resulting ethyl acetate fraction was dissolved in water, and the DHPG precursors in the ethyl acetate extract were purified by fractionation using adsorbent resins. Two resins were consecutively used in the present work: Amberlite® XAD16 (Rohm and Haas Ltd., Copenhagen, Denmark) and polyamide (50-160 µm; Sigma-Aldrich, St. Louis, MO, USA). These adsorbents have been used previously in the selective recovery of natural phenols from olive mill wastewater and olive fruits (Bertin, Ferri, Scoma, Marchetti, & Fava, 2011; He, Xia, & Chen, 2008). Prior to their use, the resins

were soaked with methanol and washed with water and then were placed in 3.5 cm internal diameter columns in an amount sufficient to achieve a height of 40 cm. The elution of the Amberlite® XAD16 column was performed using 600 mL of water and 100 mL of 20% MeOH (v/v), 40% MeOH (v/v), 60% MeOH (v/v), 80% MeOH (v/v) and 100% MeOH; six fractions of 100 mL each were collected for water, and two fractions of 50 mL each were collected for each methanol solution. The elution of the polyamide column was performed with 30 mL of water and 10 mL of solutions of water and methanol (from 5% to 100%); twenty-three fractions of 10 mL each were collected. All of the fractions were monitored and analyzed before and after of the post-treatment at 90 ºC for 4 h by HPLC-DAD. Those fractions showing the highest increases in DHPG were selected. Lastly, the DHPG precursors were isolated using a Hewlett Packard Series 1100 HPLC system fitted with a Synergi 4 µm Hydro-RP 80A semi-preparative reverse-phase column (250 x 21.20 mm internal diameter and 4 lm particle size) (Phenomenex, Torrance, CA, USA). The separation was performed at a flow rate of 20 mL/min using a linear gradient elution of ultrapure water (pH 2.5 adjusted with trifluoroacetic acid) and acetonitrile as the mobile phase, from 5% to 25% acetonitrile over 30 min; the compounds were detected at 280 nm.

2.6. Identification of DHPG precursors by HPLC–ESI-MS

The analysis of the purified precursors from the aqueous extract of alperujo was performed in the selected fractions by mass spectrometry. The data were collected using a Micromass ZMD4 quadrupole mass spectrometer (Waters Inc., Manchester, UK) equipped with an electrospray ionization (ESI) source. The mass spectra were obtained at an ionization energy of 50 and 100 eV in the negative mode. The capillary voltage was set to 3 kV, the desolvation temperature was set to 200 ºC, the source temperature was set to 100 ºC, and the extractor voltage was set to 12 V. The flow rate was maintained at 1 mL/min, and the split mode (DAD-MS) was used for each analysis. A Tracer Extrasil ODS-2 column was used (250 x 4.6 mm i.d.) (Teknokroma, Barcelona, Spain). The mobile phase was 0.01% trichloroacetic acid in water (solvent A) and acetonitrile, for a total running time of 55 min, using the following gradient or proportions of solvent A for the elution: 0 min, 95% A; 30 min, 75% A; 45 min, 50% A; 47 min, 0%; 50 min, 75% A; and 52-55 min 95% A. The UV spectra were recorded from 200 to 400 nm during the analysis.

3. Results and discussion

The effect of the post-treatment time on the DHPG release in the recovered aqueous extracts of two different alperujo samples was examined. The post-treatment was performed for 5 h; aliquots were collected at five time points, and the released DHPG levels were measured. As shown in **Fig. 1**, in both cases, the results obtained after the post-treatment at 90 ºC

indicated that DHPG was most efficiently released from the precursors after 4 h, a time at which the highest DHPG concentration was achieved. The DHPG concentration did not differ significantly after this time. The differences between the alperujo samples are explainable because the concentration of phenolic compounds depends on the olive cultivar used to produce the oil.

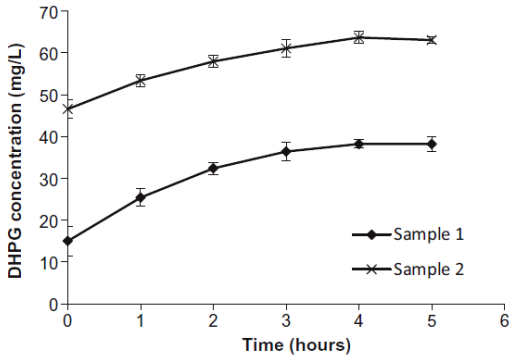


Fig. 1. Release of DHPG from precursors during post-treatment at 90 ºC for different times of the aqueous extracts of two alperujo samples.

Fig. 2a and **b** shows the comparison of two chromatograms of the aqueous extract of alperujo, demonstrating the effect of the post-treatment, which causes the release of DHPG from the solubilized precursors; the major phenolic compounds are indicated. The DHPG concentration was measured before and after the post-treatment for 4 h at 90 ºC. The observed increase in the concentration of DHPG (the concentration changed from 0.15 to 0.27 g/L, which represents an increase of 80%) is not related to variations in other well-known phenolic compounds, such as HT, Ty and their glucosides, as no changes in their peak areas were observed. This fact allows us to discard the dimers of HT-DHPG and Ty-DHPG as possible precursors because hydrolytic cleavage would involve an increase in the concentration of the olive phenolics HT and Ty. Furthermore, and for the same reason, the oxidation of HT to o-quinone and further conversion to DHPG via the addition of an H_2O molecule (Roche, Dufour, Mora, & Dangles, 2005) was also rejected because that would also imply a decrease in the concentration of HT, which did not occur in this case.

The aqueous extract of alperujo was partitioned with ethyl acetate to obtain a more concentrated extract to evaluate the differences in the chromatographic profile and to facilitate the identification of the DHPG precursors. The extraction of the precursors with ethyl acetate at room temperature was found to be effective, and the distribution of precursors between the aqueous extract and ethyl acetate fraction was determined by the increases in the DHPG concentration in each phase. The solubility of the DHPG precursors in the ethyl acetate phase is much higher than in the aqueous phase, with the DHPG concentration showing a significant increase after the post-treatment at 90 ºC (360% compared with 14% for the opposite aqueous phase). Moreover, approximately 80% of all of the free DHPG is found in the aqueous extract, as indicative of its polar nature.

The ethyl acetate fraction was applied to a 3.5 x 40 cm Amberlite® XAD16 column and eluted first with water and then with aqueous methanol (from 20 to 100%) to collect six fractions (F1-F6). F1, eluted with water, contained free DHPG and other simple phenolic compounds, whereas the bulk of the DHPG precursors (81%) were eluted from the column in fractions F3 to F5, from 40% MeOH (v/v) to 80% MeOH (v/v). An aliquot of each fraction was monitored by HPLC-DAD after the post-treatment. The F3-F5 fractions were pooled for further fractionated using a 3.5 x 40 cm polyamide column. The ten precursor-containing fractions eluted with aqueous methanol (from 10% to 55%) were combined and subjected to a subsequent purification step using a semi-preparative reverse-phase HPLC system. Two individual fractions (3 and 10) were successfully isolated and monitored by HPLC-DAD using a reverse- phase analytical column after the post-treatment, presumably responsible for the DHPG release. To structurally identify the precursors, the fractions were further characterized by ESI-MS.

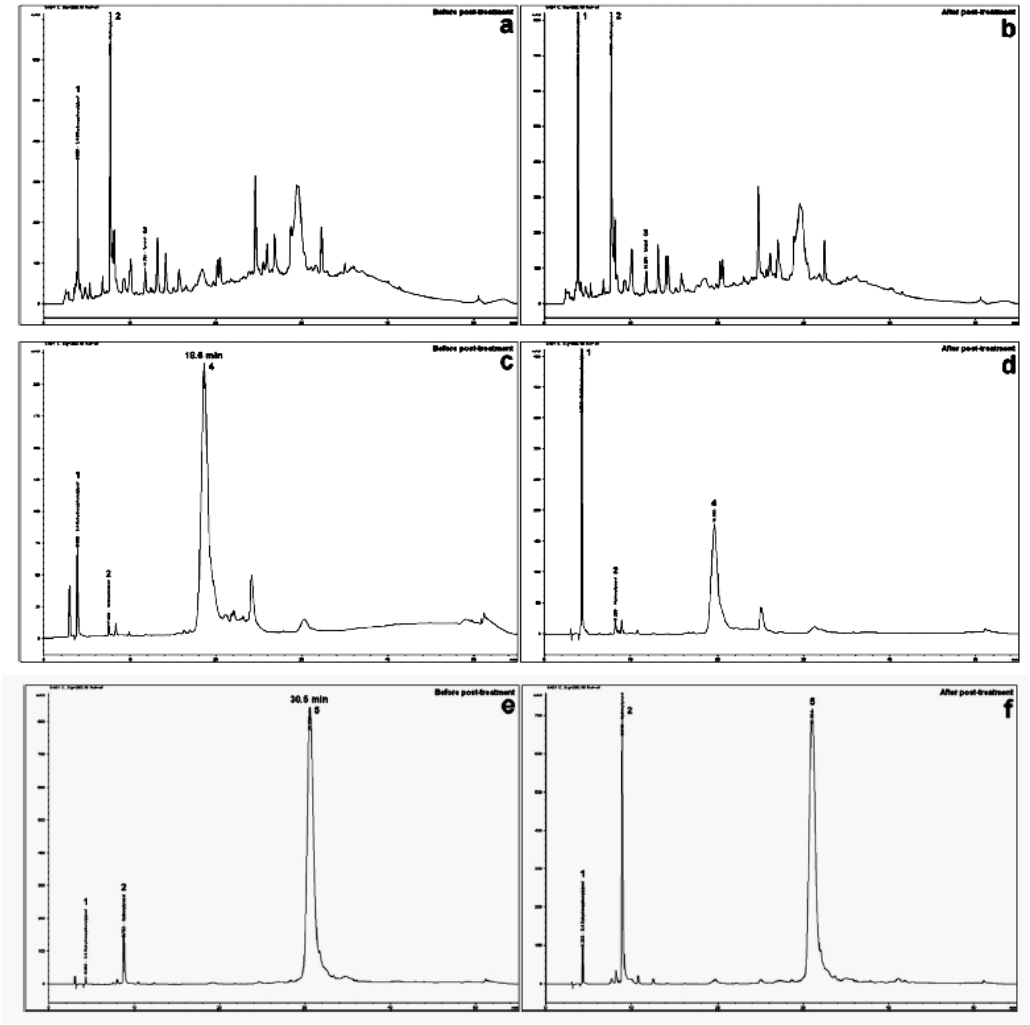


Fig. 2. HPLC-DAD profiles at 280 nm of the aqueous extract of alperujo before (**a**) and after (**b**) the post-treatment at 90 ºC for 4 h. Peak (1) 3,4-dihydroxyphenylglycol, (2) hydroxytyrosol and (3) tyrosol, respectively. Only 3,4-dihydroxyphenylglycol increased significantly. The profiles of the isolated fractions 3 (in the middle) and 10 (below) by semi-preparative reversed-phase chromatography before (**c, e**) and after (**d, f**) the post-treatment, respectively, also are shown. Peak numbers 4 and 5 correspond to the compounds identified as precursors of DHPG.

The HPLC-DAD analysis of fraction 3 at 280 nm showed a peak with a retention time of 18.6 min; after the post-treatment (90 ºC/4 h), the peak area decreased slightly, whereas the peak of DHPG increased significantly (**Fig. 2c** and **d**). The mass spectrum in negative mode and absorbance profile of fraction 3 (**Fig. 3a**) provides useful structural information to identify the precursors of DHPG. The abundant peak at *m/z* 639 is due to the molecular ion $[M-H]^-$ of two diastereoisomers of the molecule β-hydroxyacteoside or β-hydroxyverbascoside (MW 640) (Innocenti et al., 2006). The presence of a hydroxyl group in the β-position of the hydroxytyrosol moiety is the only difference with respect to verbascoside, as previously reported by Mulinacci et al. (2005). However, the separation of these two isomers was not possible by reverse-phase chromatography due to their almost identical structures. The ion at *m/z* 621 is produced by the loss of a water molecule $[M-H-H_2O]^-$. According to the findings of Ryan, Robards, and Lavee (1999), the two fragment ions at *m/z* 179 and 161 are attributable to the caffeic acid ion [caffeic acid-H]$^-$ and a dehydrated ion [caffeic acid-H_2O]$^-$, respectively. The isolated compounds exhibited the UV spectral characteristics of verbascoside isomers, with absorption maxima (λ_{max}) at 224, 281 and 332 nm (Obied, Allen, Bedgood, Prenzler, & Robards, 2005; Owen et al., 2003). However, there is another possibility, as this mass spectrum is also consistent with the two diastereoisomeric structures of the β-hydroxyl derivative of isoverbascoside; indeed, these two structures have been recently identified in olive mill wastewater by Cardinali et al. (2012). All of these stereoisomers have the same fragmentation scheme and, therefore, are not distinguishable by mass spectrometry. Their structures are shown in **Fig. 4**. These compounds identified as precursor of DHPG would explain the hydrolytic release of the DHPG moiety during the thermal post-treatment supported by the acidic pH (4-5) of the aqueous extracts of alperujo. It has been found that acidity cause a significant increase in the concentration of major phenolic compounds due to the rupture of more complex molecules as much from in alperujo as in olive oils and brines.

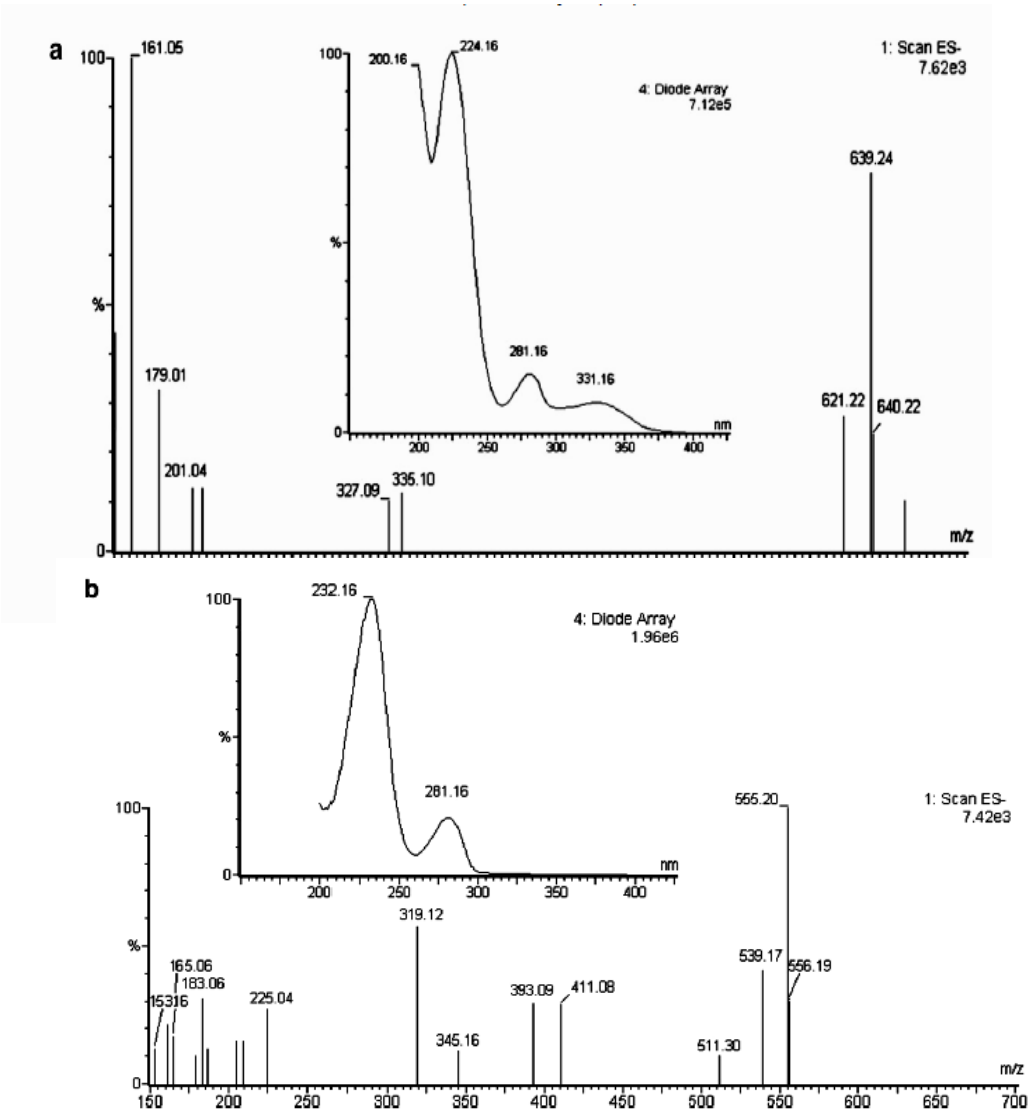


Fig. 3. Mass spectra in negative ion mode and absorbance profiles of precursors of DHPG identified in the fractions 3 (a) and 10 (b).

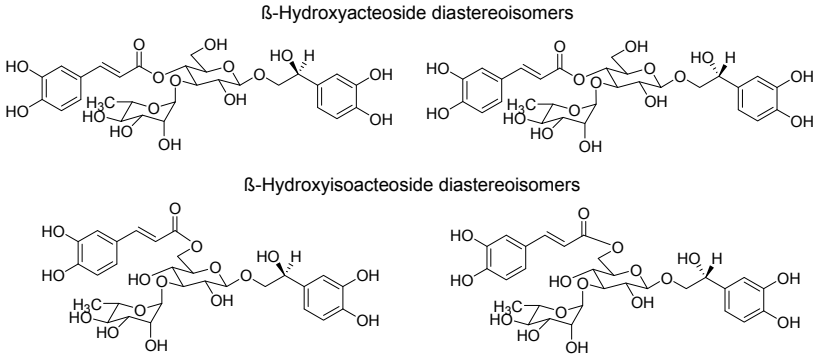


Fig. 4. Chemical structures of DHPG precursors identified in the fraction 3 by ESI-MS.

The HPLC-DAD analysis of fraction 10 at 280 nm indicated that it contained one main peak with a retention time of 30.5 min; as for peak 3, the area of this peak decreased slightly, whereas, both DHPG and HT increased considerably (**Fig. 2e** and **f**). The negative ESI-MS mass spectrum of fraction 10 (**Fig. 3b**) showed a molecular ion at *m/z* 555 of a phenol-conjugated oleosidic secoiridoid named 2”-hydroxyoleuropein (MW 556), a recently identified compound (Di Donna et al., 2007) in which the hydroxyl group is located at the phenylethanolic moiety. The peak observed at *m/z* 153 indicates that the proposed structure contains DHPG and confirms the identity of this molecule as a precursor. However, the negative ion mass spectrum also showed a molecular ion of oleuropein at *m/z* 539, though this is most likely a fragment as a result of the loss of the hydroxyl group from 2”-hydroxyoleuropein because other characteristic ions of oleuropein, such as *m/z* 377 and 361, were not observed. **Fig. 5** represents a possible fragmentation scheme of 2”-hydroxyoleuropein, a scheme that is very similar to that proposed by Di Donna et al. (2007). Fragment at *m/z* 393 suggest the loss of 162 Da that correspond to one glucose moiety. A fragment at *m/z* 225 attributed to elenolic acid was also observed and may arise from a McLafferty-type rearrangement of the ester function. The observed fragment ion at *m/z* 345 is likely a result of the loss of the methoxyl and hydroxyl groups from *m/z* 393. Lastly, there is a correlation between the structure of 2”-hydroxyoleuropein and UV spectrum with a λ_{max} at 232 nm characteristic of a secoiridoid core (Cardoso et al., 2005) and 281 nm (**Fig. 3b**).

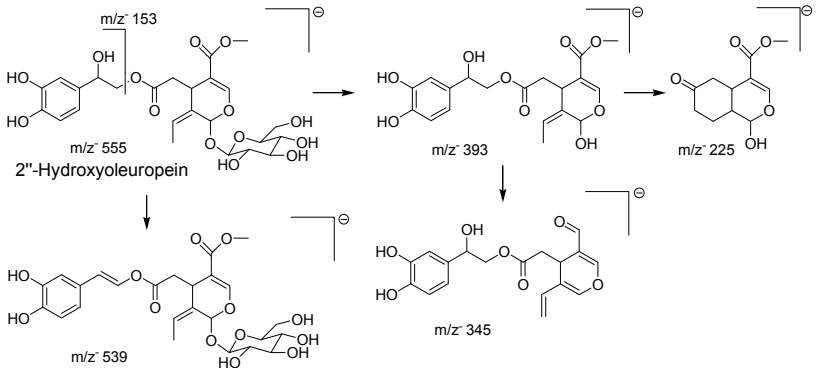


Fig. 5. Fragmentation scheme of 2”-hydroxyoleuropein identified in the fraction 10 as precursor of DHPG.

The HPLC analysis of this fraction after post-treatment at 90 ºC revealed a significant increase in the HT concentration (**Fig. 2e** and **f**), suggesting that there is a second molecule present in peak number 5 that appears as a mixture of compounds/isomers that were not successfully resolved by HPLC and that by the mass spectrum can also be attributed to deacetoxy-oleuropein aglycon. The peak recorded at *m/z* 319 corresponds to the molecular ion and fragment ions at *m/z* 183 and 165 caused by the loss of the 3,4-dihydroxyphenylethyl moiety (Romani et al., 2001) and subsequent loss of a water molecule, respectively. Some authors found that the concentration of hydroxytyrosol increased notably during storage as a result of the hydrolysis of deacetoxy-oleuropein aglycon (Cinquanta, Esti, & La Notte, 1997).

Hydrolytic reactions are involved in the degradation of the secoiridoid derivative during the prolonged storage of oil (Brenes, García, García, & Garrido, 2001; Gómez-Alonso, Mancebo-Campos, Salvador, & Fregapane, 2007; Lavelli, Fregapane, & Salvador, 2006) and are one of the main effects observed in the phenolic fraction. Due to enzymatic or chemical reactions, this mechanism leads to a breaking of the ester bonds into simple phenolic forms, such as elenolic acid, hydroxytyrosol and tyrosol. The hydrolysis of secoiridoid derivatives is associated with a decrease in bitterness and pungency. In fact, this same reasoning can be applied to the hydrolysis of 2”-hydroxyoleuropein in the aqueous extracts of alperujo, as supported by the loss of this compound and the corresponding increase of DHPG under the forced conditions of the post-treatment.

4. Conclusions

In this work, the structures of two phenylpropanoidic compounds (β-hydroxyacteoside and β-hydroxyisoverbascoside) and one hydroxylated derivative at the elenolic moiety of oleuropein (2”-hydroxyoleuropein) were isolated, partially purified and identified by HPLC-DAD-ESI-MS as precursors of DHPG. These findings support our previous hypothesis on the identity of the probable precursors. DHPG is a potent antioxidant biophenol that might have interesting applications in cosmetics, nutraceuticals, and functional foods. The identification of the precursors responsible for increasing the DHPG concentration is important to improve the use of alperujo and to extend the knowledge of new olive phenolic compounds with biological activities. Further studies about the biological activities of the identified precursors are necessary to determine their potential uses.

Acknowledgments

This research was funded by Ministerio de Ciencia e Innovación (AGL2009-12352) of the Spanish Government. Dr. Guillermo Rodríguez is grateful to the JAE-Doc Programme (CSIC) co-funded by European Social Fund (ESF). We also wish to thank Junta de Andalucía (Consejería de Innovación, Ciencia y Empresa) (P08-AGR-03751) for financial support.

References

Bertin, L., Ferri, F., Scoma, A., Marchetti, L., & Fava, F. (2011). Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction. Chemical Engineering Journal, 171, 1287–1293.

Brenes, M., García, A., García, P., & Garrido, A. (2001). Acid hydrolysis of secoiridoid aglycons during storage of virgin olive oil. Food Chemistry, 49, 5609–5614.

Cardinali, A., Pati, S., Minervini, F., D'Antuono, I., Linsalata, V., & Lattanzio, V. (2012). Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants. Journal of Agricultural and Food Chemistry, 60, 1822–1829.

Cardoso, S. M., Guyot, S., Marnet, M., Lopes-da-Silva, J. A., Renard, C. M. G. C., & Coimbra, M. A. (2005). Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. Journal of the Science of Food and Agriculture, 85, 21–32.

Cicerale, S., Lucas, L., & Keast, R. (2010). Biological activities of phenolic compounds present in virgin olive oil. International Journal of Molecular Sciences, 11, 458–479.

Cinquanta, L., Esti, M., & La Notte, E. (1997). Evolution of phenolic compounds in virgin olive oil during storage. Journal of the American Oil Chemists' Society, 74, 1259–1264.

Dellagreca, M., Previtera, L., Temussi, F., & Zarrelli, A. (2004). Low-molecular-weight components of olive oil mill waste-waters. Phytochemical Analysis, 15, 184–188.

de Roos, B., Zhang, X., Rodríguez Gutiérrez, G., Wood, S., Rucklidge, G. J., Reid, M. D., et al. (2011). Anti-platelet effects of olive oil extract: in vitro functional and proteomic studies. European Journal of Nutrition, 50, 553–562.

Di Donna, L., Mazzotti, F., Napoli, A., Salerno, R., Sajjad, A., & Sindona, G. (2007). Secondary metabolism of olive secoiridoids. New microcomponents detected in drupes by electrospray ionization and high-resolution tandem mass spectrometry. Rapid Communications in Mass Spectrometry, 21, 273–278.

Fernández-Bolaños, J., Guillén Bejarano, R., Jiménez Araujo, A., Rodríguez Arcos, R., Rodríguez Gutiérrez, G., & Lama-Muñoz, A. (2008). Method for purifying 3,4-dihydroxyphenylglycol (DHPG) from plant product. WO/2010/070168/A1. PCT/ ES2009/070541.

Gómez-Alonso, S., Mancebo-Campos, V., Salvador, M. D., & Fregapane, G. (2007). Evolution of major and minor components and oxidation indices of virgin olive oil during 21 months storage at room temperature. Food Chemistry, 100, 36–42.

Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C. L., Sanchez-Rovira, P., & Ramirez-Tortosa, M. C. (2010). Hydroxytyrosol: From laboratory investigations to future clinical trials. Nutrition Reviews, 68, 191–206.

He, Z., Xia, W., & Chen, J. (2008). Isolation and structure elucidation of phenolic compounds in Chinese olive (Canarium album L.) fruit. European Food Research and Technology, 226, 1191–1196.

Innocenti, M., la Marca, G., Malvagia, S., Giaccherini, C., Vincieri, F. F., & Mulinacci, N. (2006). Electrospray ionisation tandem mass spectrometric investigation of phenylpropanoids

and secoiridoids from solid olive residue. Rapid Communications in Mass Spectrometry, 20, 2013–2022.

Killeen, M. J., Pontoniere, P., & Crea, R. (2011). Hydroxytyrosol An examination of its potential role in cardiovascular disease, inflammation, and longevity. Agro Food Industry Hi-tech, 22, 16–19.

Lavelli, V., Fregapane, G., & Salvador, M. D. (2006). Effect of storage on secoiridoid and tocopherol contents and antioxidant activity of monovarietal extra virgin olive oils. Journal of Agricultural and Food Chemistry, 54, 3002–3007.

Limiroli, R., Consonni, R., Ranalli, A., Bianchi, G., & Zetta, L. (1996). 1H NMR study of phenolics in the vegetation water of three cultivars of Olea europaea: Similarities and differences. Journal of Agricultural and Food Chemistry, 44, 2040–2048.

Medina, E., de Castro, A., Romero, C., & Brenes, M. (2006). Comparison of the concentrations of phenolics compounds in olive oils and other plant oils: Correlation with antimicrobial activity. Journal of Agricultural and Food Chemistry, 54, 4954–4961.

Mulinacci, N., Innocenti, M., La Marca, G., Mercalli, E., Giaccherini, C., Romani, A., et al. (2005). Solid olive residues: Insight into their phenolic composition. Journal of Agricultural and Food Chemistry, 53, 8963–8969.

Obied, H. K., Allen, M. S., Bedgood, D. R., Jr., Prenzler, P. D., & Robards, K. (2005). Investigation of Australian olive mill waste for recovery of biophenols. Journal of Agricultural and Food Chemistry, 53, 9911–9920.

Owen, R. W., Haubner, R., Mier, W., Giacosa, A., Hull, W. E., Spiegelhalder, B., et al. (2003). Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. Food and Chemical Toxicology, 41, 703–717.

Roche, M., Dufour, C., Mora, N., & Dangles, O. (2005). Antioxidant activity of olive phenols: Mechanistic investigation and characterization of oxidation products by mass spectrometry. Organic and Biomolecular Chemistry, 3, 423–430.

Rodríguez, G., Lama, A., Jaramillo, S., Fuentes-Alventosa, J. M., Guillén, R., Jiménez-Araujo, A., et al. (2009). 3,4-Dihydroxyphenylglycol (DHPG): An important phenolic compound present in natural table olives. Journal of Agricultural and Food Chemistry, 57, 6298–6304.

Rodríguez, G., Lama, A., Trujillo, M., Espartero, J. L., & Fernández-Bolaños, J. (2009b). Isolation of a powerful antioxidant from Olea europaea fruit-mill waste: 3,4-Dihydroxyphenylglycol. LWT – Food Science and Technology, 42, 483–490.

Rodríguez, G., Rodríguez, R., Fernández-Bolaños, J., Guillén, R., & Jiménez, A. (2007). Antioxidant activity of effluents during the purification of hydroxytyrosol and 3,4-dihydroxyphenylglycol from olive oil waste. European Food Research and Technology, 224, 733–741.

Rodríguez-Gutiérrez, G., Duthie, G. G., Wood, S., Morrice, P., Nicol, F., Reid, M., et al. (2012). Alperujo extract, hydroxytyrosol, and 3,4-dihydroxyphenylglycol are bioavailable and have antioxidant properties in vitamin E-deficient rats – A proteomics and network analysis approach. Molecular Nutrition and Food Research, 00, 1–17.

Roig, A., Cayuela, M. L., & Sánchez-Monedero, M. A. (2006). An overview on olive mill wastes and their valorisation methods. Waste Management, 26, 960–969.

Romani, A., Pinelli, P., Mulinacci, N., Galardi, C., Vincieri, F. F., Liberatore, L., et al. (2001). HPLC and HRGC analyses of polyphenols and secoiridoid in olive oil. Chromatographia, 53, 279–284.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil byproduct alperujo and their antioxidative activities. Journal of Agricultural and Food Chemistry, 60, 1175–1186.

Ryan, D., Robards, K., & Lavee, S. (1999). Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. Journal of Chromatography, A, 832, 87–96.

6.2. Oligosacáridos y polisacáridos

6.2. Polisacáridos y oligosacáridos.

Como ya se ha comentado con anterioridad, el tratamiento térmico realizado sobre el alperujo facilita la solubilización de una gran cantidad de compuestos atrapados en el entramado de la matriz de la pared celular vegetal, así como la hidrólisis de moléculas complejas, provocando el aumento de la concentración de los compuestos fenólicos, oligosacáridos y polisacáridos en la fracción acuosa. Los fenoles solubilizados son separados de los oligos y polisacáridos mediante una extracción con acetato de etilo, tras la cual se obtiene una fracción acuosa enriquecida en estos compuestos.

La fracción acuosa obtenida tras los diferentes tratamientos térmicos y posterior extracción con acetato de etilo es sometida a una hidrólisis suave con HCl, con el objetivo de hidrolizar los polisacáridos neutros a oligómeros permitiendo la posterior separación entre oligosacáridos neutros y polisacáridos ácidos (pectinas) mediante la ultrafiltración a través de membrana de 3000 Da. Esta separación nos ha permitido por un lado estudiar como afectan las diferentes condiciones del tratamiento térmico a la composición de las fracciones enriquecidas en oligosacáridos neutros (Capítulo 9), en las cuales se han identificado oligómeros y compuestos formados por oligómeros y fenoles. Por otro lado las fracciones pécticas obtenidas tras la separación de los oligosacáridos neutros tras los diferentes tratamientos térmicos realizados sobre el alperujo también han sido estudiadas (Capítulo 10). Para ello los extractos de pectinas han sido purificados, su composición ha sido estudiada y diferentes propiedades como el grado de esterificación, la capacidad de captación de agua/aceite, la capacidad de emulsificación y su estabilidad, el índice de retardo de la glucosa y la capacidad de retención de ácidos biliares han sido evaluadas.

También se ha realizado un estudio del material péptico recuperado a partir de un alperujo que ha sufrido un tratamiento térmico suave industrial (50-80 ºC durante 1-2 horas). En la actualidad dicho subproducto se está empleando como fuente de hidroxitirosol, esta explotación está siendo realizada por la empresa de base tecnológica (EBT) Subproductos Vegetales del Mediterráneo S.L (SVM S.L.). El estudio de recuperación, análisis y evaluación de las propiedades de las pectinas recuperadas a partir de este nuevo subproducto ha sido considerado (Capítulo 11).

Finalmente y para completar el aprovechamiento de los subproductos generaros en la industria de la aceituna se realiza un estudio sobre los diferentes componentes de la pared celular (hemicellulosa, celulosa y lignina) que son recuperadas mediante un pretratamiento “steam explosion” sobre un material puramente lignocelulósico como es el hueso de la aceituna y sus diferentes partes. Los diferentes componentes son aislados mediante un proceso de fraccionamiento basado en solubilizaciones selectivas (Capítulo 12). A las fracciones así obtenidas se les estudian diferentes propiedades.

6.2.1. Capítulo 9.

Production of oligosaccharides with low molecular weights, secoiridoids and phenolic glycosides from thermally treated olive by-products.

Fernández-Bolaños, J., Rubio-Senent, F., Lama-Muñoz, A., García, A., Rodríguez-Gutiérrez, G.

Oligosaccharides: Food Sources, Biological Roles and Health Implications, Cap 6, pag 173-208. Editors: Lori S. Schweizer and Stanley J. Krebs. Enero, 2014. ISBN: 978-1-62948-328-3.

El tratamiento hidrotérmico aplicado sobre el alperujo permite la recuperación de compuestos de alto valor añadido en la fracción soluble en agua, entre los que cabe descartar los fenoles y los azúcares. Estudios previos han mostrado que una parte sustancial de las hemicelulosas presentes en el alperujo son recuperadas en forma de oligosacáridos tras tratamiento con vapor. El tratamiento térmico puede proporcionar un gran número de compuestos bioactivos, incluyendo oligosacáridos y compuestos fenólicos asociados con polisacáridos.

En este capítulo se realiza un estudio de los oligosacáridos neutros y ácidos procedentes de las hemicelulosas, y de los compuestos secoiridoides, mono- y disacáridos vinculados a los compuestos fenólicos, liberados del alperujo tratado térmicamente seguido de una hidrólisis ácida suave. Además en aquellas fracciones en la cuales una importante proporción de fenoles fue detectada, se realizaron ensayos de actividad antioxidante.

Los resultados obtenidos para las actividades antioxidantes demostraron que algunas fracciones pueden ser consideradas como nuevos compuestos bioactivos. Además de los oligosacáridos identificados con un DP de 3 a 10, se detecto un grupo de fracciones con estructuras secoiridoides conjugadas con compuestos fenólicos y se confirmo la presencia de una fracción significativa de di-y trisacáridos unidos a compuestos fenólicos (hidroxitirosol y tirosol) que podría ser considerado fibra soluble y fibra soluble antioxidante.

Production of oligosaccharides with low molecular weights, secoiridoids and phenolic glycosides from thermally treated olive by-products.

Juan Fernández-Bolaños, Fátima Rubio-Senent, Antonio Lama-Muñoz, Aránzazu García, Guillermo Rodríguez-Gutiérrez

ABSTRACT

Alperujo was hydrothermally pre-treated to solubilize the oligosaccharides from the cell wall material of olive pulp. A protocol for the isolation of oligosaccharides of low molecular weight has been developed, which involves thermal treatment, mild acid hydrolysis, separation by ultra-filtration, adsorption chromatography and size exclusion chromatography using Superdex Peptide HR. Three groups of fractions with common characteristics were obtained and studied in two samples of alperujo from different seasons, origin and variety of olives. In the first group, neutral and acidic oligosaccharides with a low DP (4-10) bound or not to phenol-containing compounds and with antioxidant activity were identified. In the second group, several secoiridoid glycosides esterified to phenolic compounds and their aglycones were also detected. In the third group, a significant fraction of di- and trisaccharides of glucose or glucose-xylose bound to phenol residues (hydroxytyrosol and tyrosol) that could be considered antioxidant-soluble fiber with bioactive properties was also isolated. All of them were identified by HPLC, CG, and ESI-MS and in certain cases by ^{1}H and ^{13}C MNR. The similar behavior obtained for both samples confirms the protocol followed as a useful tool for bioactive compound isolation.

KEYWORDS: Alperujo, steam treatment, secoiridoids, xylo-oligosaccharides, xyloglucan, arabinans, antioxidants, phenolic compounds.

1. Introduction

Alperujo is a semisolid, residual by-product of virgin olive oil produced by a continuous, two-phase extraction system. In Spain, the annual production of this by-product is approximately 2.5-6 million tons, depending on the season. From an environmental point of view, alperujo represents a serious problem due to its highly polluting organic load which limits its biodegradation because of its high toxicity. The combustion of alperujo as a fuel is a commonly used procedure to eliminate its harmful effects on the environment. In recent years, many management options have been proposed for the treatment and valorization of alperujo. One of the most interesting is a

hydrothermal treatment developed and patented by our research group [1] already implemented at industrial scale. This treatment is conducted in a continuous reactor using steam at high pressures and temperatures (150-170ºC, 5-8 kg/cm^2) and allows for easy separation of the solid and liquid phases and for recovery of the bioactive compounds and valuable fractions from each phase. In this steam treatment, an auto-hydrolytic process occurs and solubilizes part of the alperujo. It allows for the recovery of added-value compounds in the water-soluble fraction to create a fraction which is rich in valuable phenols (hydroxytyrosol, 3,4-dihydroxyphenylglycol and others) and sugars leaving a solid residue with oil which has been enriched with minor components [2, 3].

Recent studies have shown that a substantial part of the hemicelluloses present in alperujo could be also recovered as oligosaccharides by steam treatment or auto-hydrolysis of the water-soluble fraction [4]. Cell wall material for olive pulp comprises a number of molecular components, with considerable quantities of cellulose (30%), pectic polysaccharides (39%) and hemicellulosic polymers rich in xylans and glucuronoxylans (14%), xyloglucans (15%) and mannans (2%) [5, 6, 7], which could provide a wide range of functional components.

The phenolic compounds hydroxytyrosol, tyrosol or secoiridoid derivatives are abundant in olive pulp but were not identified in the olive cell wall [8]. However, it has been reported that the interaction between polysaccharides and the hydrophilic compounds present in the olive paste may be involved in the apparent loss in the phenolic compounds from the oil during crushing and malaxation [9]. Thermal treatment can provide a large number of bioactive compounds, including oligosaccharides and phenolics associated with polysaccharides, with potential applications in the food or nutraceutical industry. The optimization and integration of the process for revaluing alperujo, which in this case can also be used for the production of this type of oligosaccharides for intermediate pharmaceutical or food use, could be an important economical improvement.

There is increasing interest in generating non-digestible oligosaccharides that would promote the growth of beneficial bacteria in the large intestine, providing protection from infection and maintaining the normal function of the gastro-intestinal system [10, 11]. Small oligomers of galacturonic acid (with degrees of polymerization [DP] from 2 to 7) were also found responsible for inhibiting the adherence of bacteria to epithelial cells, the initial and crucial stage of infection [12]. Xylo-oligosaccharides act as pre-biotics, maintaining gastrointestinal health and reducing the risk of colon cancer [13]. These pre-biotics reduce cholesterol levels, improve the biological availability of calcium and have beneficial effects on type 2 diabetes mellitus; in addition, they cause the apoptosis of human leukemia cells [14]. The acidic xylo-oligosaccharides show antibacterial activity against Gram-positive bacteria, especially against *Helicobacter pylori* [15] (Christakopoulus et al., 2003) and direct mitogenic activity, indicating a potential inmunostimulatory [16] (Nabarlatz, Montané, Kardosova, Bekesová, Hribalová, & Ebringerová, 2007).

The acidic and neutral oligosaccharides, together as secoiridoid compounds and mono- and disaccharides linked to phenolic compounds, released from alperujo that was steam-treated in a bath pilot reactor (capacity of 100 L), following mild acid hydrolysis has been reported in a previous paper [17, 18]. Process definition and results obtained on different raw material and on a larger scale for the ultra-filtration steps are presented here and compared with previous results.

2. Materials and Methods

Raw material

Two samples of olive pomace or "alperujo" (a semisolid residue composed of skin, pulp, ground stone and olive seeds) from different olive cultivars and consecutive seasons were directly collected from the two-phase centrifugal system decanter before recovery of the olive-pomace oil. One of them (Sample1) was supplied by the local pomace-processing mill (Oleícola el Tejar, Córdoba, Spain), in January, at the halfway mark of the olive oil production season (2009-2010), and the other (Sample2) was taken in an experimental olive oil mill plant located at the Instituto de la Grasa (CSIC) in Seville (Spain), in December, also in the half-way mark of the olive oil production season (2010-2011) in the province of Seville.

Thermal treatments or steam processing of Alperujo

The hydrothermal treatment has been patented [1], and it was performed using a steam treatment reactor prototype designed by our research group at the Instituto de la Grasa (Seville, Spain). The reactor has 100 L capacity and a stainless steel reservoir that can operate at temperatures between 50 and 190°C and at a maximum pressure of 1.2 MPa.

Twenty kilogram samples of alperujo were treated with saturated steam for 15 min at 170°C (Sample 1) and 60 min at 170ºC (Sample 2). Heating of the alperujo was performed by direct injection of pressure and temperature steam enhancing the contact between the steam and the alperujo. After treatment the steam inlet valve was closed and the pressure reduced to atmospheric pressure at a controlled rate. The wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain) to separate the solid and liquid phases.

Phenol extraction

At the end of the treatment, an aliquot of the liquid phase (10 L) was concentrated to 1 L by rotary evaporation a vacuum at 37ºC; five portions of 200 mL were extracted with 500 mL volumes of ethyl acetate (refluxed at 77ºC) for 5-6 h in a continuous extraction from a heavier liquid (water) to a lighter one (ethyl acetate) in order to separate the phenolic compounds from the carbohydrate components. The aqueous solution was concentrated and lyophilized.

Acid Hydrolysis and Ultra-Filtration through a Membrane of 3000 Da

In order to increase the proportion of oligosaccharide of low molecular weight in the initial lyophilized liquid, a mild chemical hydrolysis with 0.5 N HCl was carried out [17].

In Sample 1 a small volume (100 mL) of hydrolyzed fraction was ultra-filtered at room temperature using an Amicon 8400 stirred cell (Millipore Corp., Bedford, MA. USA) through 3000 Da molecular weight cut off membranes (cellulose regenerated). The solutions were washed with water at 40ºC until 500 mL of permeate were collected.

In Sample 2, hydrolyzed fractions (1000 mL) were ultra-filtered at room temperature using a Prep/Scale®-TFF Cartridge holder XX42PS001 with Prep/Scale®-TFF Cartridge 3000 Da regenerated cellulose (Millipore Corp., Bedford, MA, USA). The solutions were washed with 5000 mL of water at 40ºC and concentrated under vacuum at 40ºC until obtaining a final volume of 150 mL.

Adsorption XAD Chromatography

Fractionation and purification of the neutral and pectic oligosaccharides were performed by adsorption on Amberlite XAD-16 resin (**Figure 1**). 25 mL of hydrolyzed sample (Sample 1) were injected onto a column (35 x 3.0 cm) of Amberlite XAD-16 and eluted at 1.5 mL/min with water (200 mL) and 20% (v/v) methanol/water (200 mL), successively. In Sample 2 the fraction obtained after ultra-filtration with a membrane of 3000 Da and concentrated to 700 mL was injected onto a column (74.5 x 8.0 cm) of Amberlite XAD-16 and eluted with water (10 L) and methanol/water 20% (v/v) (7 L) and 50% (v/v) (5 L), successively.

The carbohydrates and uronic acids in each eluted fraction were assayed with the anthrone-sulphuric acid colorimetric assay [19] and the m-hydroxybiphenyl method [20], respectively.

Quantification of Individual Neutral Sugars by Gas Chromatography (GC) after Converting Them into Alditol Acetates

Individual neutral sugars were analyzed from duplicate samples with and without initial TFA hydrolysis (2N TFA at 121ºC for 1h) prior to reduction, acetylation and analysis by GC [21]. Inositol was used as an internal standard. A Hewlett-Packard 5890 series II chromatograph, fitted with a 30 m x 0.25 mm fused silica capillary column (SP-2330 from Supelco, Bellefonte, PA), was employed. The oven temperature program used was as follows: 180ºC for 7 min and then increased to 220ºC at 3ºC/min (15 min). The carrier gas was helium, which had a flow rate of 1 mL/min. The injector temperature was 250ºC, and the FID detector temperature was 300ºC. The split ratio was 1/100.

Because oligosaccharides were converted into monosaccharides during the post-hydrolysis treatment, the increase in the concentration of monosaccharides caused by post-hydrolysis provided a measurement of the concentration of oligosaccharides. All determinations were made by triplicate.

Ultra-Filtration through a Membrane of 1000 Da

The fractions eluted from Amberlite XAD-16 resin with water and methanol/water were subjected to ultra-filtration through a 1000 Da molecular weight cut-off membrane on a fiber unit similar to the one described above for Samples 1 and 2, respectively. In Sample 1 four fractions were obtained: Fraction A (<1000 Da), Fraction B (between 1000-3000 Da), eluted with water; Fraction C (<1000 Da) and Fraction D (between 1000-3000 Da), eluted with 20% methanol. In Sample 2 six fractions were obtained: Fraction A (<1000 Da), Fraction B (between 1000-3000 Da), eluted with water; Fraction C (<1000 Da) and Fraction D (between 1000-3000 Da), eluted with methanol 20%; Fraction E (<1000 Da) and Fraction F (between 1000-3000 Da), eluted with methanol 50%.

Determination of the Total Phenolic Content and Antioxidant Activity

Total phenols

The phenolic content was measured by the Folin-Ciocalteu method [22], and expressed as grams of gallic acid equivalents per kilogram of fresh alperujo.

Antiradical activity, DPPH (2,2-diphenyl-1-picrylhydrazyl)

Free-radical scavenging capacity was measured using the DPPH method described in a previous work [23]. The method is based on the measurement of the free-radical scavenging capacity of the antioxidant against the stable radical $DPPH^{\cdot}$. An iMark^{TM} microplate absorbance reader model 550 (Bio- Rad, Hercules, CA, USA) was used for the absorbance measurements. $DPPH^{\cdot}$ has an absorption band at 515 nm which disappears upon reduction by antiradical compounds. For each fraction and standard, the decrease in absorbance (expressed as a percentage of the initial absorbance) was plotted against the concentration of the antioxidant in the reaction mixture. The radical-scavenging capacity of each fraction was expressed as EC_{50} (effective concentration, mg/mL), which is the amount of antioxidant necessary to decrease the initial absorbance by 50%.

Reducing power

The reducing power assay was carried out according to the procedure described in a previous work [23]. The antioxidative potentials of the fractions were estimated for their ability to reduce $FeCl_3$. All of the analyses were made using a microplate reader and the absorbance was measured at 490 nm. The assay was calibrated using Trolox. To express the results, a calibration curve was established by plotting A_{490} against known concentrations of Trolox (0.059 to 0.56 mg/mL). The reducing power (P_R) was expressed as Trolox equivalents (TE) in mg/mL from the equation as determined from linear regression.

Size Exclusion Chromatography on Superdex Peptide HR

After the separation of the monosaccharides and fractionation of the oligosaccharides by adsorption chromatography, the fractions A, B, C and D of Sample 1 and the fractions A, B, C, D, E and F of Sample 2 were fractionated by size exclusion chromatography on two Superdex Peptide HR 10/30 (30 x 1 cm) columns (Pharmacia Biotech, Uppsala, Sweden) connected in line to a Jasco LC-Net II/ADC (Easton, MD, USA) HPLC system. Samples (100 µL) containing 10 mg of oligosaccharide/mL were applied several times to the column and eluted with 100 mM ammonium acetate buffer (pH 5) and water in Samples 1 and 2, respectively, at a flow rate of 0.5 mL/min in both cases. The peaks were monitored by a Jasco MD-1550 Diode Array detector (DAD) and a Jasco RI-1530 Refraction Index detector. Column calibration was performed with a variety of standards, including galacturonic acids (RT= 69.6 min) (purchased from Fluka) and maltose (RT= 66.5 min), maltotriose (RT= 64.5 min), maltotetraose (RT= 63.2 min), maltopentaose (RT= 60.5 min), maltohexaose (RT= 59.1 min) and maltoheptose (RT= 57.0 min) (purchased from Sigma); glucose (RT= 69.5 min), and compounds of a series of cellobiose (RT= 66.5 min), cellotriose (RT= 63.5 min), cellotetraose (RT= 61.1 min), cellopentaose (RT= 59.0 min) and cellohexaose (RT= 56.2 min) (purchased from Megazyme) for neutral oligosaccharides.

Electrospray Ionization Mass Spectrometry

Neutral oligosaccharides collected from the Superdex Peptide column fractions were identified by their electron impact mass data, which were collected on a quadrupole mass analyzer (ZMD4, Micromass, Waters Inc; Manchester, U.K.). Electrospray ionization (ESI) mass spectra were obtained at an ionization energy of 50 and 100 eV in negative mode and at 50 eV in positive mode; the capillary voltage was 3 kV, the desolvation temperature was 200°C, the source temperature, was 100°C and the extractor voltage was 12V. The flow was maintained at 0.3 mL/min in splitless mode (UV detector MS) for each analysis.

A Teknokroma Tracer Extrasil ODS-2 column (250 mm x 4.6 mm, i.d. of 5 µm) was used. The mobile phase was 0.01% trichloroacetic acid in water and acetonitrile using the following gradient over a total run time of 55 min: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, and 95% at 52 min until the run was completed.

NMR Spectroscopy

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX300 spectrometer operating at 300 and 75 MHz, respectively. Samples were collected from Superdex Peptide column fractions at 62-68 min, which coincided with the standard oligosaccharides with DP from 4-2 in the calibration sample (first group), and fraction eluted with the monosaccharide standard, after solvent (negative peak) and another eluted after solvent (second group) was examined in acetylated form in CD_3OD and $CDCl_3$ respectively. Chemical shifts (δ) are expressed in parts per million (ppm) values relative to the central solvent peak TMS, tetramethylsilane, $(CH_3)_4Si$ (δ = 0,00 ppm). The experiments were

performed at 25ºC. *Acetylation*: Fractions were evaporated to dryness and 20 mg were dissolved in pyridine (2 mL) and cooled in an ice bath. Acetic anhydride (2 mL) was added to this solution and the mixture was left over night at 4º C. The resulting solution was evaporated to dryness and co-evaporated four times with water (2 mL) and toluene (2 mL) to remove the pyridine. The solid was dissolved in 0.5 mL of water. *Sample preparation*: The acetylated samples (0.5 mL) were lyophilized and dissolved in isotopically enriched $2D_2O$ and lyophilized to replace exchangeable protons with deuterons. The residue was dissolved in methanol-d and chloroform-d and transferred to a 5 mm of NMR tube.

3. Results and Discussion

Extraction and Isolation of Oligosaccharides and Sugars Linked to Phenolics from Alperujo and their Antioxidant Activity

A steam treatment was applied to two different samples of alperujo collected from two different seasons. One of them was treated with saturated steam for 15 min at 170ºC and other for 60 min at 170ºC. In both samples the hydrothermal treatment of moist alperujo samples was followed by ethyl acetate extraction of the liquid phase in order to separate the phenolic compounds from carbohydrate components. A subsequent mild acid hydrolysis with 0.5 N HCl at 70ºC for 2 h was carried out to increase the proportion of oligosaccharides of low molecular weight. The overall fractionation procedure described in a previous paper [17] is presented in Scheme 1 (**Figure 1**). The mild hydrolysis with HCl did not hydrolyze most of the pectic polysaccharides and neutral oligosaccharides were isolated after an ultra-filtration through a 3000 Da membrane. The production of oligosaccharides was evaluated by the measurement of oligomers in the fraction below 3000 Da, calculated for the difference between those sugars which are completely free and those that are present as oligosaccharides. The monosaccharide compositions of the permeate and retentate of the ultrafiltration were determined by the total hydrolysis of the oligosaccharides with 2 N of TFA at 120ºC for 2 hours [24].

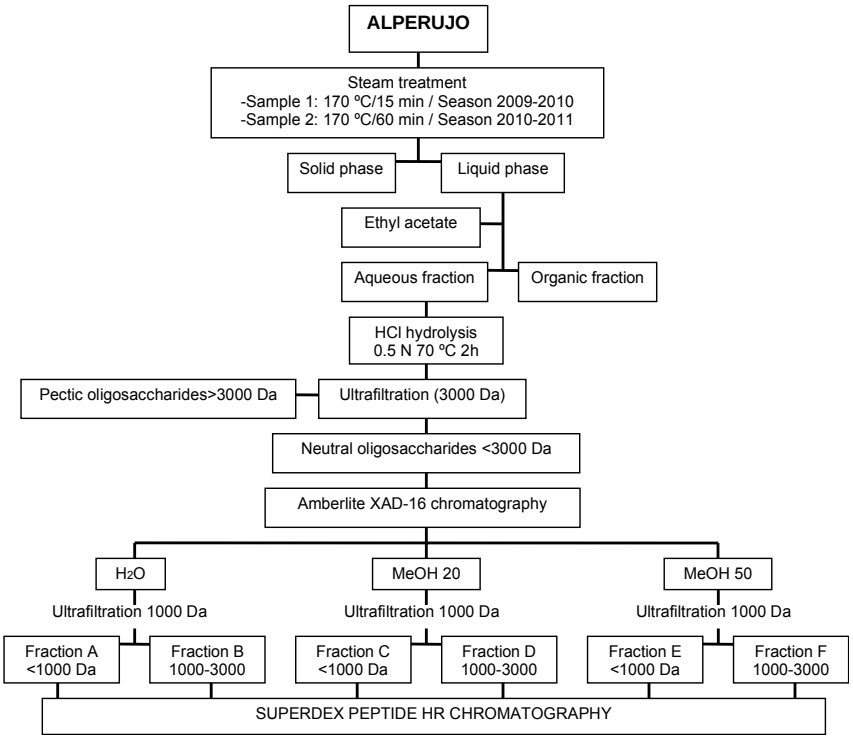


Figure 1. Scheme of the fractionation process performed to obtain oligosaccharides with a low degree of polymerization.

Analysis of the carbohydrates (**Table 1**) in fraction > 3000 Da revealed the presence mainly of pectic polysaccharides composed of galacturonic acid (46%) with galactose (28%) and rhamnose (9%). The fraction between < 3000 Da showed that they contained monosaccharides and partial hydrolysis products from xylan, xyloglucan, arabinan, arabinogalactan, glucomannan or mannan from the cell wall of the olive pulp.

Table 1. Composition of oligosaccharides (neutral sugars and uronic acids) in the fractions obtained after ultrafiltration through a 3000 Da membrane. The results are expressed in percentage with respect to the total content

	Sample 1		**Sample 2**	
	>3000 Da	**< 3000 Da**	**>3000 Da**	**< 3000 Da**
Uronic acids	45.96[a]±5.49[b]	n.d	18.92±1.61	8.02±0.23
Rhamnose	8.91[c]±0.85	1.42±0.08	2.96±1.28	6.32±0.11
Fucose	0.90±0.08	n.d	0.11±0.04	0.25±0.07
Arabinose	10.45±0.04	4.77±0.40	29.59±1.53	27.60±0.11
Xylose	2.74±0.22	0.79±0.08	27.91±1.21	17.42±1.62
Mannose	n.d [d]	27.90±2.70	1.89±0.09	1.80±0.08
Galactose	28.07±2.47	1.83±0.16	11.03±0.83	5.93±0.70
Glucose	2.97±0.22	63.59±6.20	7.60±0.39	32.67±1.57

[a] Uronic acids were determined following the method described by Blumenkrantz and Asboe-Hansen (1973).
[b] Standard deviation (n=3).
[c] Neutral sugars were quantified as alditol acetates by GC.
[d] n.d., not detected.

However, in this work we concentrated on the study of this last fraction < 3000 Da that resulted from mild acid hydrolysis, when the oligosaccharides were efficiently separated from an important proportion of the monosaccharides by a simple chromatography step on XAD-16 resin and eluted with water.

In Sample1 four fractions (A, B, C, and D) were obtained after chromatography on Amberlite XAD-16 resin and after ultrafiltration through a 1000 Da molecular weight cut off membrane (**Table 2**). The fraction obtained by elution with water fraction A (< 1000 Da) and B (between 1000 and 3000 Da) were rich in glucose and mannose, with substantial proportion of arabinose in fraction A and arabinose and galactose in fraction B; the fractions obtained by elution with 20% methanol/water (C and D) contained predominantly glucose but also mannose and xylose.

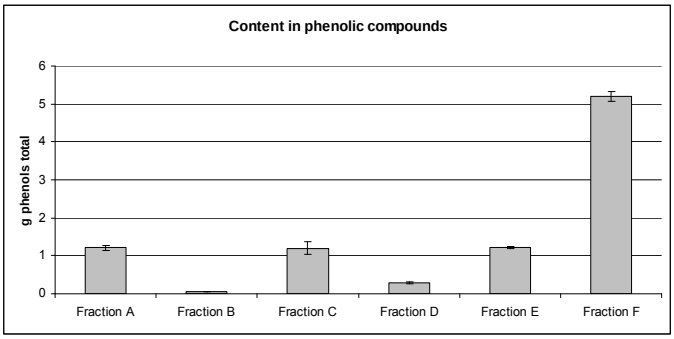
In Sample 2 six fractions (A-F) were obtained since it was necessary to increase the proportion of MeOH to 50% for the complete elution of carbohydrates from Amberlite XAD-16 (**Table 2**) probably due to certain interactions of phenolic compounds with the resin. However, each fraction presented a certain content in phenolic compounds (**Figure 2a**) and they were tested *in vitro* assays to evaluate their radical scavenging activity and reducing power capacity using hydroxytyrosol (HT), α-tocopherol and Trolox as a positive control (**Figure 2b, c**). The results show that fractions eluted with 50% MeOH were the fraction with a higher antiradical activity with an EC_{50} of 2.2 mg/mL for fraction F (between 1000 and 3000 Da) and 4.5 mg/mL for fraction E (< 1000 Da) with an EC_{50} for HT and α-tocopherol of 0.05 and 0.15 mg/mL, respectively. The antiradical capacity of all fractions had a high correlation with the phenolic content (r = 0.9579), following a lineal model (data not shown). The reducing power was low for all fractions and the statistical analysis confirmed that fractions A, C, D and E were not significantly different with α-tocopherol, and the fraction F had a higher reducing capacity than α-tocopherol. A high correlation with the phenolic content was also obtained (r = 09646), not following a lineal model in this case but an “inverse of x” (data not shown).

Table 2. Sugar composition of each fraction obtained after elution with water and methanol 20 and 50 % from XAD-16 chromatography and ultrafiltration through a 1000 Da membrane for each sample analyzed[a]

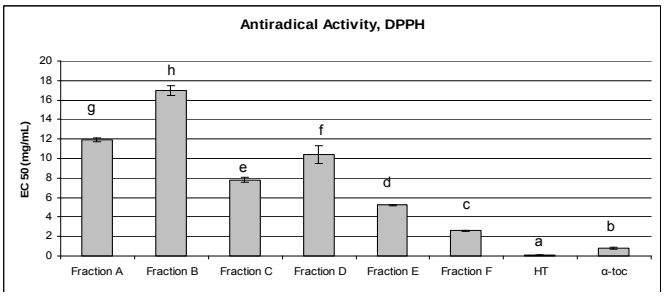
	Sample 1			
	A	**B**	**C**	**D**
Rhamnose	1.7±0.2[b]	3.8±0.1	0.9±0.1	3.6±0.3
Fucose	0.5±0.1	n.d [c]	n.d	n.d
Arabinose	13.4±0.3	8.3±0.5	2.0±0.2	6.1±0.5
Xylose	3.5±0.1	4.5±0.2	3.4±0.1	9.8±0.8
Mannose	19.9±1.4	17.2±1.5	8.5±0.8	9.9±0.9
Galactose	3.0±0.3	10.7±0.7	1.0±0.1	6.6±0.6
Glucose	58.1±2.7	55.5±1.7	84.3±8.3	64.0±6.3
% oligosaccharide	10.5	6.8	12	10.7

	Sample 2					
	A	**B**	**C**	**D**	**E**	**F**
Rhamnose	3.1±0.2	5.5±0.4	3.3±0.2	2.7±0.5	5.2±0.4	5.5±0.4
Fucose	--	1.4±0.1	0.2±.0.1	0.5±.1	0.4±.1	--
Arabinose	12.8±0.4	53.4±0.8	8.1±0.5	52.9±2.5	4.0±0.4	17.6±0.9
Xylose	3.5±0.1	15.1±0.2	0.9±0.1	25.3±1.4	8.0±0.8	9.7±0.5
Mannose	29.9±0.4	--	5.7±0.5	1.8±0.2	4.4±0.4	3.0±0.2
Galactose	1.4±0.1	5.5±0.3	0.5±0.1	2.7±0.1	0.4±0.1	18.2±1.8
Glucose	49.7±0.2	19.2±0.7	81.3±1.9	14.0±0.8	77.7±1.6	46.1±3.6
% oligosaccharide	26.6	42.3	33.1	60.5	19.1	40.9

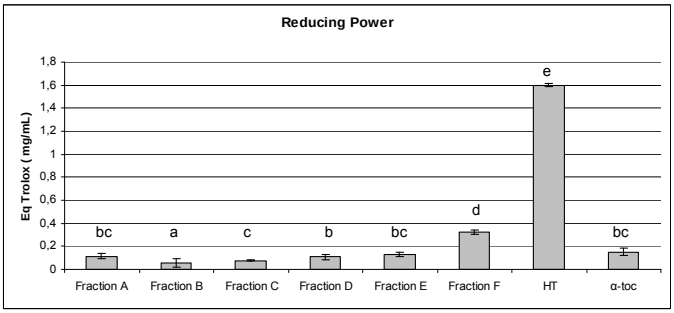
[a] The composition is determined by gas chromatography as alditol acetate. The results are expressed in percentage with respect to the total content.
[b] Standard deviation (n=3).
[c] n.d., not detected.



(a)



(b)



(c)

Figure 2. Content of total phenols (grams) presents in the different fractions obtained by elution with water and methanol 20 and 50% from XAD-16 chromatography and ultrafiltration for Sample 2. Radical scavenging capacity on DPPH (**b**) and reducing power (**c**) of each sample and two standards (hydroxytyrosol and α-tocopherol), data are presented as the means ± SDs. Different letters indicate significantly different results ($p < 0.05$).

Isolation and Separation of Three Groups of Compounds by Size Exclusion Chromatography

Four and six new fractions eluted from Amberlite XAD resin for Sample 1 and Sample 2, respectively were further separated and purified by gel filtration using a Superdex Peptide HR 10/30 resin column based on their molecular weight, although this resin also separates molecules on the basis of their interaction with phenolics. The refractive index elution profile obtained from the gel filtration column for Sample 2 is shown in **Figure 3**. In fact, we have confirmed that peaks eluted after the standard monosaccharide sample and solvent. Fractions corresponding to the peaks of refractive index were pooled and analyzed for their sugar content. In the case of faction F

(between 1000 and 3000 Da), with higher antioxidant activity, the peaks were collected and the total phenolic content together with DPPH radical-scavenging ability and reducing power were determined (**Table 3, Figure 4**) for evaluating the contribution of each peak to antioxidant activity. These results imply a synergistic effect between fractions since any individual fraction has less activity than the former. Both antioxidant assays were correlated with phenolic content, with r = 0.8781 for antiradical activity, by lineal model, and a better correlation for reducing power (r = 0.8918), described by an "inverse of x" regression model. Therefore it appears that peak 4, with higher contents in phenolic compounds had greater activity while the peak of larger DP with lower phenolic contents and higher in oligosaccharides showed the lowest ability.

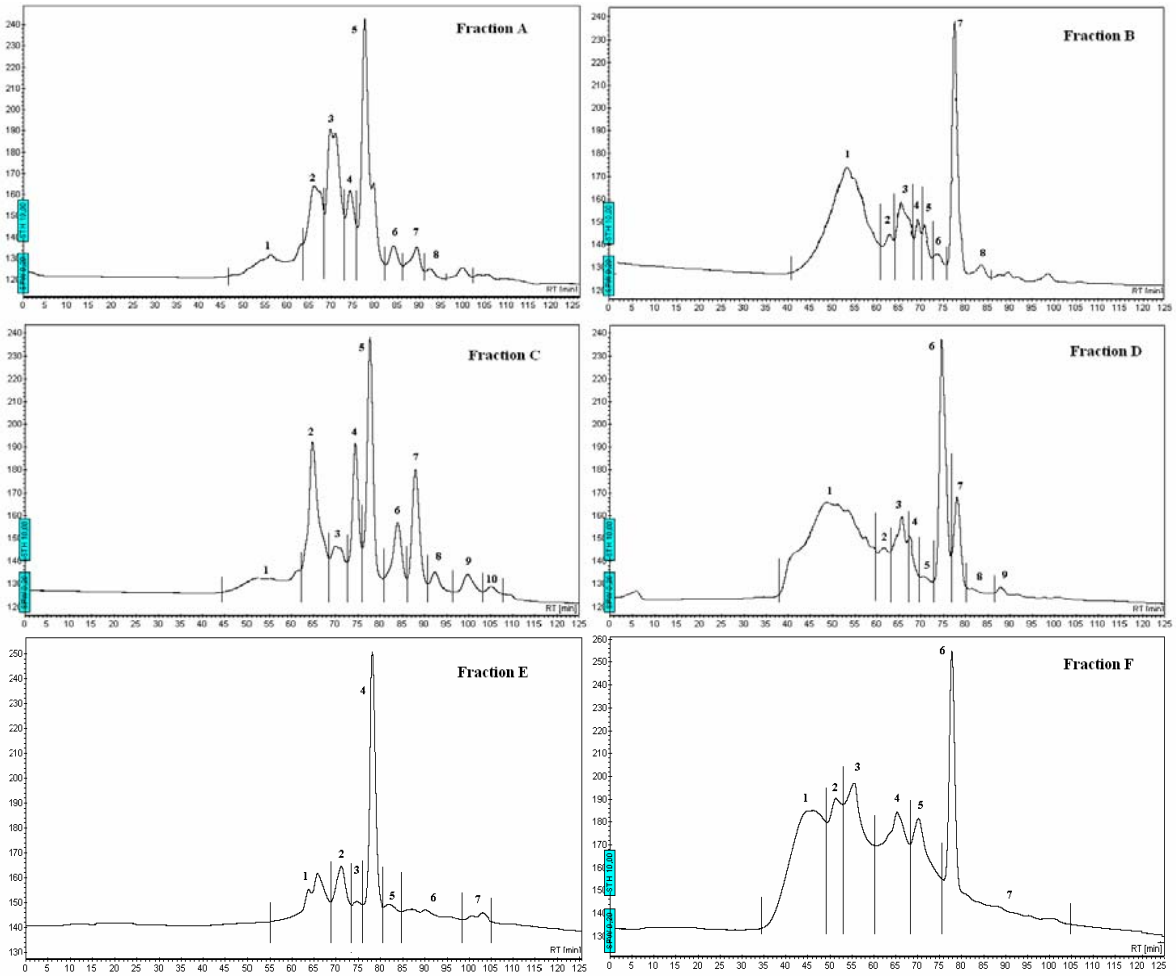


Figure 3. Refractive index profiles by size exclusion chromatography of the oligosaccharides obtained in fractions A, B, C, D, E and F from Sample 2.

Table 3. Composition of different subfractions separated by size exclusion chromatography from fraction F of Sample 2

	% (g/100 g extract dry) Sample 2-Fraction F			
	1+2	**3**	**4**	**5**
% phenols	12.1	12.26	29.68	18.66
% uronic acids	7.62	n.d.[a]	n.d.	n.d.
% neutral sugasr	45.24	12.08	24.68	21.05
	% molar neutral sugar			
Rhamnose	10.93[b]	13.27	9.18	10.46
Fucose	0.48	1.92	11.14	1.55
Arabinose	30.34	25.81	9.23	6.36
Xylose	43.13	16.43	6.01	2.95
Mannose	1.19	n.d.	5.62	10.73
Galactose	4.77	4.63	1.36	n.d.
Glucose	9.17	37.94	67.46	66.26

[a] n.d., not detected.
[b] The composition is determined by gas chromatography as alditol acetate. The results are expressed in percentage with respect to the total content.

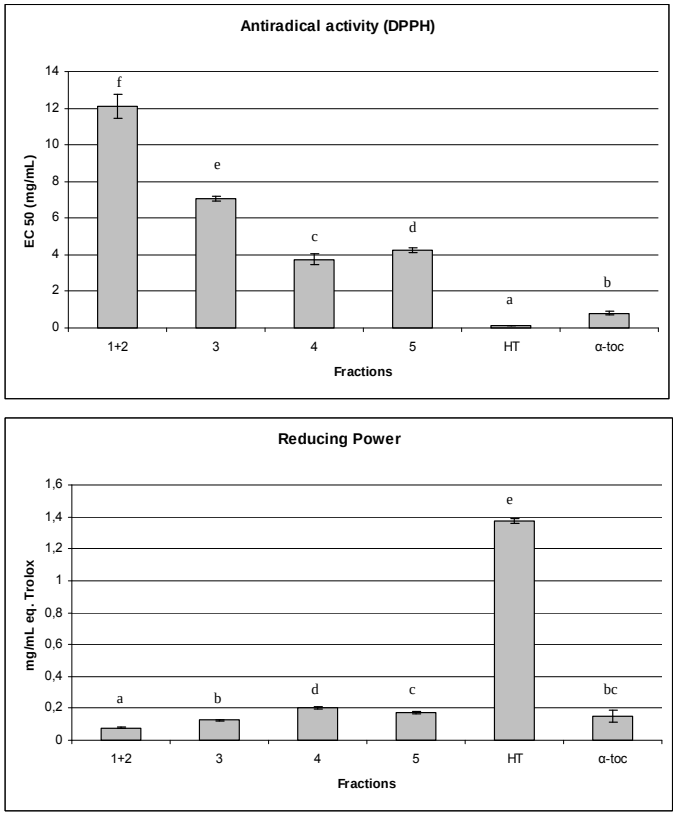


Figure 4. Radical scavenging capacity on DPPH and reducing power of each fraction isolated from Sample 2-Fraction F (F1+2, F3, F4 and F5) and two standards (hydroxytyrosol and α-tocopherol), date are presented as the means ± SDs. Different letters indicate significantly different result ($p < 0.05$).

In Sample 1 the results of its elution profile were similar to Sample 2 as were described in a previous paper [17]. In both cases, despite being of different samples and different treatment, we

have found three groups of fractions with common characteristics. One of them which included a fraction rich in neutral and acidic oligosaccharides and a low UV absorbance or no absorbance corresponds to molecules with a degree of polymerization (DP) from 10 to 3. The second group of fractions with theoretically smaller DP, next to standard oligosaccharides with DP from 4 to 2, showed similar absorption spectra with a maximum absorbance near 240 nm, characteristic of secoiridoid core [25], and a slight shoulder at 268-274 nm, characteristic of phenols (**Figure 5a**). This fact suggests that secoiridod glycosides esterified to phenolic compounds were detected. The third group of fractions included fractions with a retention time from Superdex Peptide that coincide with the monosaccharide standard in the calibration sample, or with the solvent (Rt = 78 min) and those fractions that eluted after the solvent because of interactions with the gel. Their absorption spectra differed from the previous group because they showed maximum absorbencies near 280 nm and 228 nm (**Figure 5b**), indicative of the presence of carbohydrates bound to phenolic compounds.

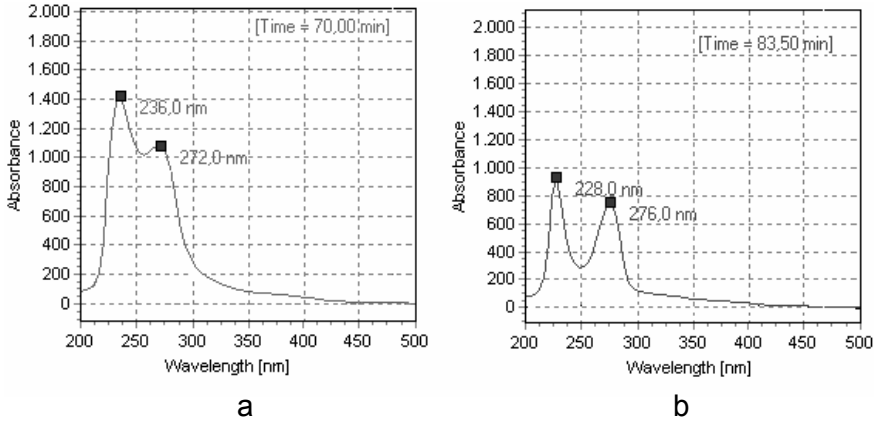


Figure 5. Ultraviolet absorption spectra for (**a**) fractions C5 and D4 from the Sample 1 (representative of the second group) have an absorption maximum near 240 nm and shoulder at 268-274 nm and (**b**) fractions C10 and D8 from the Sample 1 (representative of the third group) have two absorption maxima, one near 280 nm and a second at 228 nm.

Characterization of Three Groups of Fractions

The structural analysis of neutral oligosaccharides of the first group using electrospray ionization mass spectrometry (ESI-MS) was conducted. The mass spectrum of these fractions showed a variety of oligosaccharides which correspond mainly to a series that coincides for Samples 1 and 2. One of them with $[M+Na]^+$ for a neutral pentose-oligosaccharide composed of 3, 4, 5, 7 or 10 pentose residues and resulting in ions for the larger series at *m/z* 569, 701, 833, 965, 1097, 1229 and 1361. The loss of 132 Da was attributed to the successive loss of a pentose residue. The ESI mass spectra of neutral oligosaccharides (Pentose $_n$) for n = 7 and 5 are shown in **Figure 6**.

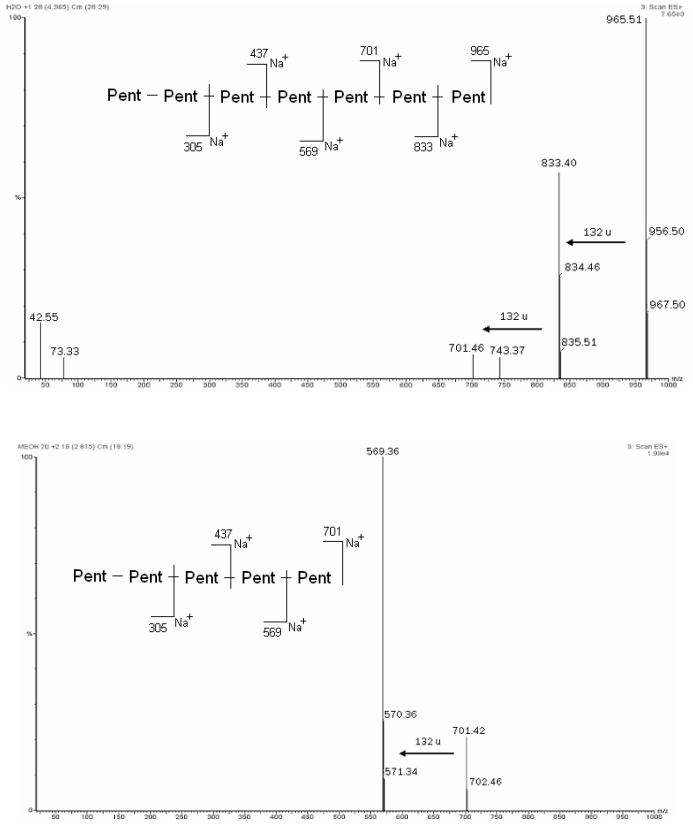


Figure 6. ESI mass spectra obtained for fractions A1, B1 and D1 of Sample 2 in positive mode, identified as neutral pento-oligosaccharides ($Pent_7$) (upper) and mass spectra obtained for fractions A2, B2 and D2 of Sample 2 in positive mode, identified as neutral pento-oligosaccharides ($Pent_5$) (lower).

A second series of abundant ions in both positive and negative modes was also observed in both samples. In the case of the Sample 1 was observed in mode positive ions at *m/z* 759 and 1023, which correspond to an increase of 58 Da on $pentose_5$ (*m/z* 701*)* and $pentose_7$ (*m/z* 965) and is attributed to the $[M\text{-}H_2O+Na]^+$ of the -methyl-D-glucuronic acid residue (-MeGlcA) of the $Pent_4MeGlcA$ and $Pent_6MeGlcA$. In Sample 2 was observed in mode negative ions at *m/z* 983, 851, 719, 587, 455, 323 and 191 which correspond to $Pent_6MeGlcA$. Ions that correspond to a series of $Pent_5MeGlcA$ (**Figure 7**) and $Pent_4MeGlcA$ were also found. In addition, only in the Sample 1 were ions at *m/z* 657 and 789 found, which revealed the presence of pentose-oligosaccharides that contain two GlcA residues ($Pent_2GlcA_2$ and $Pent_3GlcA_2$) and the most abundant ions at *m/z* 957, which corresponded to the $[M\text{-}H+2Na]^+$ ion with the presence of both GlcA and MeGlcA as substitute residues in the same pentose-oligosaccharide ($Pent_4GlcAMeGlcA$) [17].

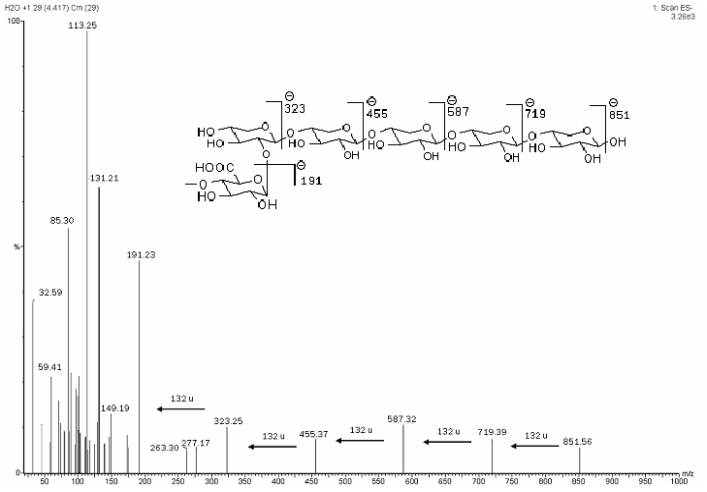


Figure 7. ESI mass spectra obtained in fraction D1 of Sample 1 and fraction C1 of Sample 2 in negative mode. Structure proposed for possible acidic pentose-oligosaccharide ($Pent_5MeGlcA$).

The analysis of the carbohydrates in these fractions (**Table 4**) revealed that the fractions with a neutral pentose-oligosaccharides series contained mainly arabinose, although some of them contained mainly xylose, while the fraction rich in acidic pentose-oligosaccharide contained mainly xylose. This fact suggests the presence of neutral arabinose- and xylose- oligosaccharide and acidic xylo-oligosaccharides, respectively, which is in accordance with the results previously reported in the alkali extract of olive pulp [26] (Reis et al. 2003).

Table 4. Total neutral sugars in ultrafiltration fractions A, B, C and D, and sugar composition expressed as mol % of the oligomers of subfractions separated by size-exclusion or gel filtration chromatography for Sample 1 (upper) and Sample 2 (lower). Retention time (RT) and oligosaccharides identified in pools of each of subfractions

	Sample 1					
Fraction	**A**	**B**	**C**	**D**		
	Total sugars (mg/L)[a]					
1	4	29	33	119		
2	--	--	--	50		
3	--	--	--	22		
Fraction	**A1**	**B1**	**C1**	**D1**	**D2**	**D3**
RT (min)	49-61	49-59	50-60	46-55	55-59	59-62
Sugars	Oligosaccharides (% molar)[b]					
Rhamnose	12.8	11.6	12	3.9	8	5.7
Fucose	3.5	--	6.6	--	1.5	--
Arabinose	15.8	16.8	17.5	13.9	12.2	9.5
Xylose	10.4	11.3	18.4	20.9	21.2	17.9
Mannose	10.8	10	8	3.6	2.4	6.3
Galactose	19	24.1	5.9	22.4	13	6.6
Glucose	27.8	26.2	31.5	35.2	41.6	54
Oligosaccharides identified	Hex_7	Hex_6	Hex_6Pent_3	Hex_6Pent_3 $Pent_{4\text{-}10(c)}$	Hex_6Pent_3 Hex_5Pent_4	Hex_6Pent_3

	Sample 2									
Fraction	**A**		**B**			**C**	**D**			
	Total sugars (mg/L)[a]									
1	48		36			47	104			
2	105		15			--	43			
3	--		52			--	66			
4	--		--			--	105			
Fraction	**A1**	**A2**	**B1**	**B2**	**B3**	**C1**	**D1**	**D2**	**D3**	**D4**
RT (min)	**45-62**	**62-68**	**41-61**	**61-64**	**64-68**	**44-62**	**38-60**	**60-63**	**63-66**	**66-69**
Sugars	Oligosaccharides (% molar)[b]									
Rhamnose	4.2	5	4.3	3.3	2.6	8.2	4.3	2.4	1.7	0.3
Fucose	0.1	0.2	0.2	--	--	0.7	0.2	0.3	--	0.2
Arabinose	60.9	15	58.4	36.5	19.9	72	67.2	20.9	6	1.8
Xylose	19	0.9	17.5	13.2	5.1	1.6	21.1	50.2	57.1	20.3
Mannose	2.9	7.9	0.4	11.5	9.2	14.3	0.4	3.6	0.6	0.3
Galactose	4	0.3	7.7	2.7	1.8	3.2	3.6	2.8	0.6	0.4
Glucose	8.8	63.5	11.5	32.9	67.4	--	3.2	19.9	33.1	46.7
Oligosaccharides identified	$Pent_7$ $Pent_5MeGlcA$	$Pent_5$	$Pent_7$ $Pent_5MeGlcA$	$Pent_5$	Hex_3	$Pent_6MeGlcA$	$Pent_7$ $Pent_5MeGlcA$	$Pent_5$ $Pent_4MeGlcA$	$Pent_4$	Hex_3 $Pent_3$

[a] Colorimetric determination by the Anthrone method.
[b] Quantification by GC as alditol acetates prior to hydrolysis with 2 N TFA at 120 °C for 2 hours.
[c] Also in this fraction D1 was detected: $Pent_6MeGlcA$, $Pent_4MeGlcA$, $Pent_2MeGlcA_2$, $Pent_3MeGlcA_2$ and $Pent_4GlcAMeGlcA$

In addition, the examination of the mass spectra shows an ion at *m/z* 1409, which is attributed to the $[M+Na]^+$; the predominant ions at *m/z* 1247, 1115, 1085, 953 and 629 (**Figure 8**) were together the sugar composition in accordance with the results obtained in olive pulp xyloglucan with gross formation of $(Hexose)_6(Pentose)_3$ [27]. In another fraction, the mass spectra revealed the presence of an ion pseudomolecular $[M+Na]^+$ at *m/z* 1380 that could correspond to a oligosaccharide with a structure like $(Hexose)_5(Pentose)_4$, which, based on the sugar composition (data not shown), could be a xyloglucan oligosaccharide found in the cell walls of olives [27]. Also, the spectra of fractions A and B of Sample 1 showed the ions at *m/z* 1175, 1013, 851 and 689 and 1013, 851 and 689, respectively, that corresponded at $(Hex)_7$ and $(Hex)_6$ with the loss of hexose residue of 162 Da in each series in positive mode (data not shown). Regarding the sugar composition, the oligosaccharides could be attributed to glucan, mannan or galactan chains. In Sample 2, an ion at *m/z* 527 in positive mode was observed, which corresponds to $(Hexose)_3$. The presence of 67.4% of glucose in this fraction confirmed the presence of $(Glucose)_3$, which was also proposed by Vierhuis et al. [27] .The MS spectrum of the de-protonated molecule $[M-H]^-$ with ion *m/z* 369 confirmed the presence of the dimer galacturonic acid- methyl-D-galacturonic acid in fraction A4, A5 and A6 of Sample 2.

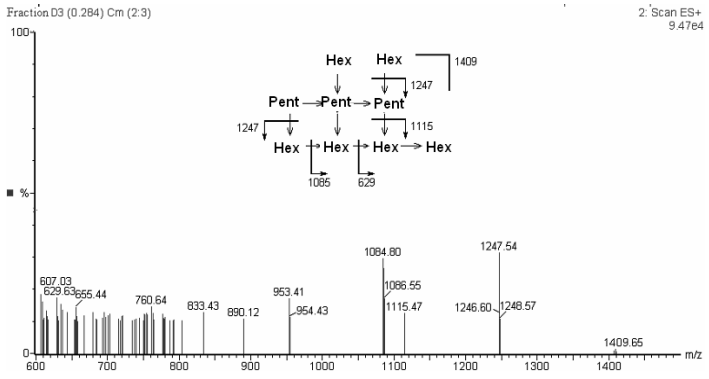
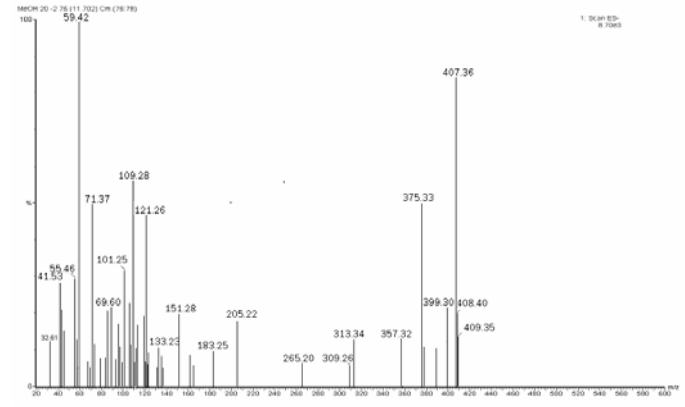


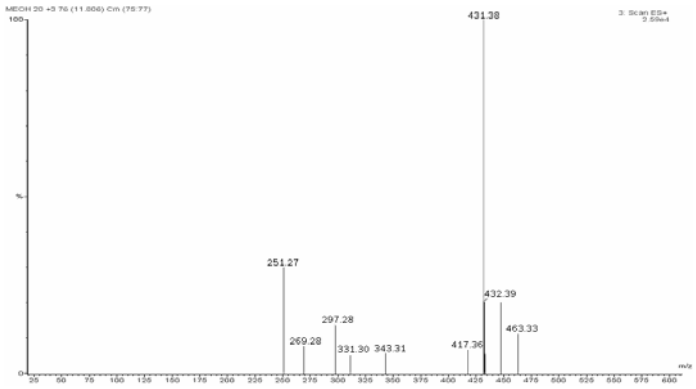
Figure 8. ESI mass spectra obtained for fractions C1, D1, D2 and D3 of the Sample 1 in positive mode and fragmentation scheme for the structure proposed of Hex_6Pent_3.

The analysis of the fractions in the second group of Samples 1 and 2, gave a similar pattern of fragmentation of mass in both positive and negative modes, which suggest they all have a common structure. The mass spectra (**Figure 9**) show a predominant ion at *m/z* 407 which likely corresponds to a deprotonated molecule $[M-H]^-$, and the corresponding sodium $[M+Na]^+$ adduct at *m/z* 431 was observed in the positive mode. The appearance of the peak at *m/z* 569 was found only in some fractions of Sample 1 and Sample 2 and is consistent with the existence of one hexose unit, as suggested by the loss of 162 u from *m/z* 569, and an aglycone unit, as proven by the ions observed at *m/z* 407. These data together with the absorbance maximum at 240 nm are characteristic of a secoiridoid core, which suggests that the molecule under investigation might correspond to a glucosyl secoiridoid bearing a phenol unit. Its structure was recently proposed by

our own research group and coincides with oleuropeinic acid [18]. The fragments in negative and positive mode have been proposed in **Figure 10**.



(a)



(b)

Figure 9. Mass spectra in full scan negative (**a**) and positive (**b**) identified as oleuropeinic acid in some of the fractions for the second group of Sample 1 and Sample 2.

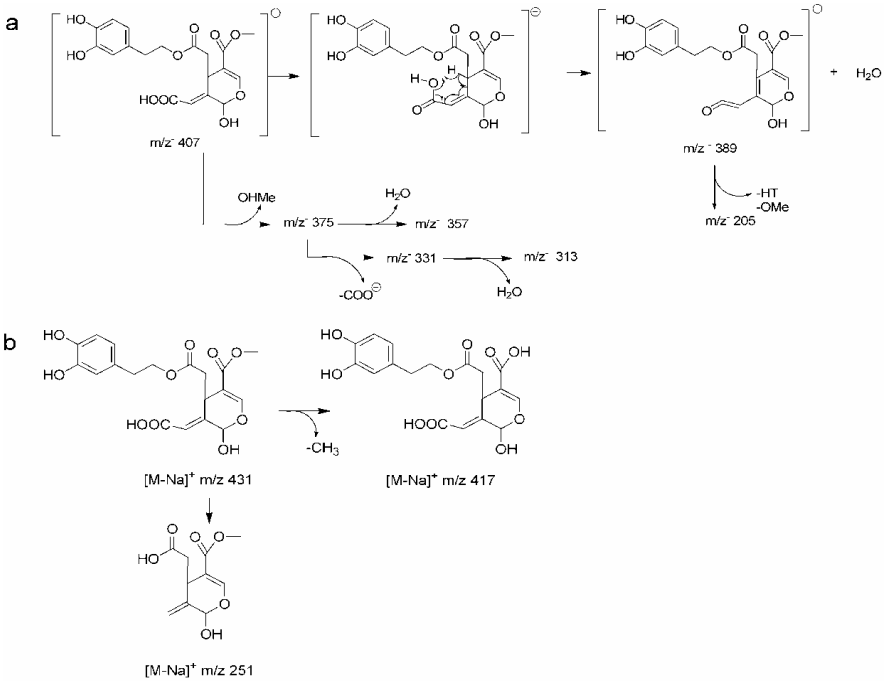


Figure 10. Scheme of fragmentation proposed for the oleuropeinic acid, in negative mode (**a**) and in positive mode (**b**).

In addition, in Sample 2 several other derivatives from phenolic secoiridoids, which are closely correlated to oleuropein were isolated and identified by UV absorbance and spectral characterization. The mass spectra revealed the presence of the pseudo-molecular ion $[M-H]^-$ at *m/z* 555 and the ions at *m/z* 537 that appear as a result of the loss of water $[M-H_2O-H]^-$. The loss of glucose yield in the ions at *m/z* 393 which are characteristic of 10-hydroxyoleuropein has been previously described by Cardoso et al. [28]. This molecule could be the precursor of the oleuropeinic acid since it could be formed by the oxidation of hydroxyoleuropein.

Another component isolated showed an pseudomolecular ion $[M-H]^-$ at *m/z* 393 and the corresponding sodium $[M+Na]^+$ adduct at *m/z* 417 in positive mode (data not shown) which were consistent with a listroside derivative, identified in olive pulp by de la Torres-Carbot et al. [29]. An isomer of the oleuropein aglycon with ions at *m/z* 377, 197 and 153 and secologanoside with ions at *m/z* 389, 209 and 121 were also identified.

The analysis of the fractions in the third group showed the presence of glucosides and di- and trisaccharides linked to a hydroxytyrosol or tyrosol moiety. In Sample 1 a fraction containing an $[M+Na]^+$ ion at *m/z* 663 was isolated by Superdex Peptide chromatography. The molecule's proposed structure and fragmentation are shown in **Figure 11**. The resonance signals from the ^1H and ^{13}C NMR of the acetylated molecule are in accordance with the proposed structure (**Figure 12**) of this compound. After the consecutive loss of glucose (fraction contains 99.5% glucose), it was

possible to observe the formation of the sodium adduct species at *m/z* 501 and 339. The analysis of the other fractions, by mass spectrometry, gave an $[M+Na]^+$ ion at *m/z* 501 and the corresponding deprotonated molecule, $[M-H]^-$ at *m/z* 477, in negative mode, and the groups of ions at *m/z* 315, 153 and 123, also present in the above molecule, which was consistent with the presence of a diglucosyl structure linked to hydroxytyrosol. The characteristic fragments of ions corresponding to a glucoside attached to hydroxytyrosol and tyrosol were also observed (data not shown).

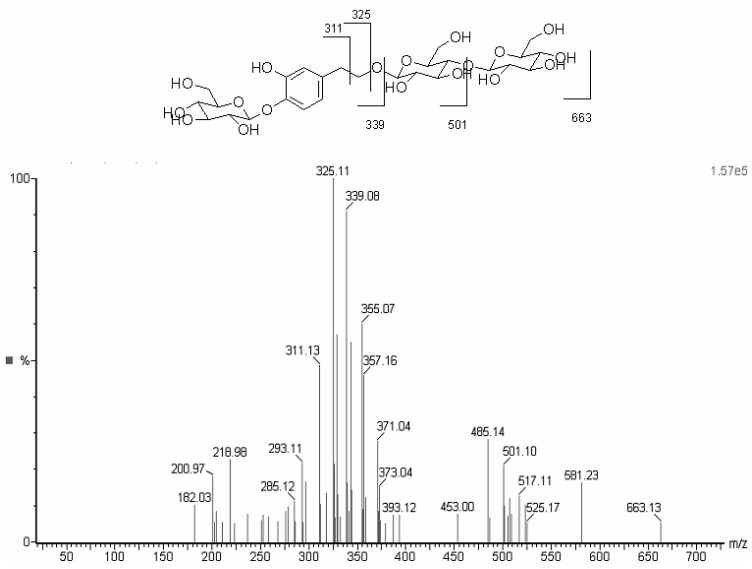


Figure 11. Mass spectra in positive mode obtained for the fraction D8 in Sample 1 and structure proposed according to the profile of fragmentation observed (all ions represented are sodium adducts).

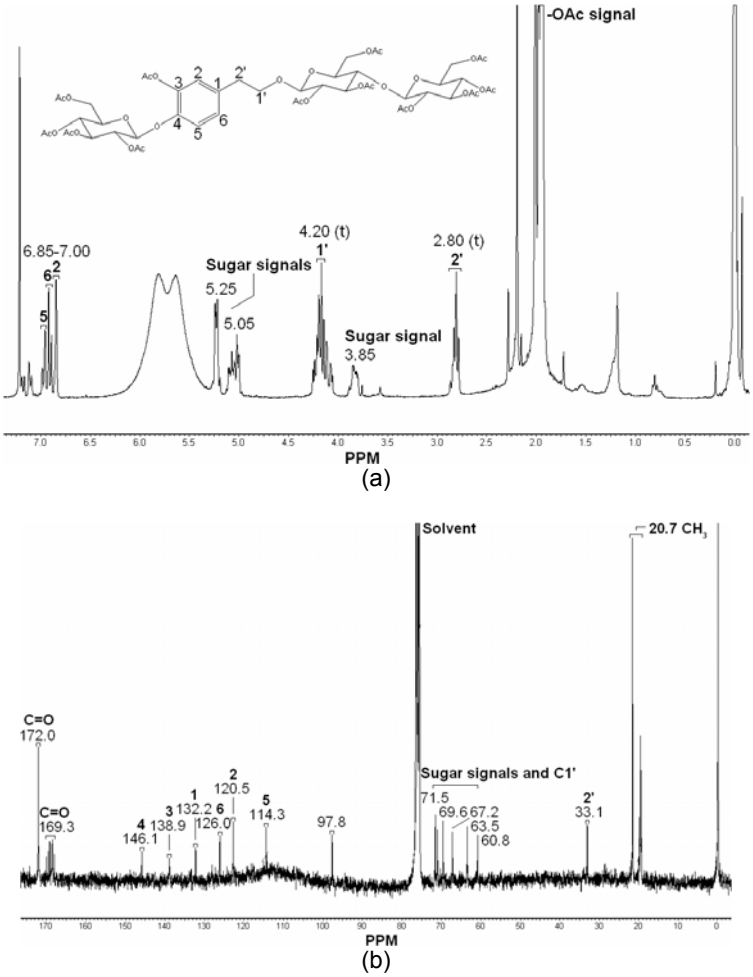


Figure 12. ^{1}H (**a**) and ^{13}C (**b**) NMR spectra (registered at 300 and 75 MHz, respectively) obtained for the acetylated simple. The chemical shifts are relative to TMS in $CDCl_3$.

Only in Sample 2 only was a molecule identified with a pseudo-molecular ion $[M-H]^-$ at *m/z* 477 and the species *m/z* 153 and 123 that could correspond to the molecule [Hydroxytyrosol-Hex-Pent]. The presence of 70% glucose and 15% xylose in this fraction led to the identification of [Hydroxytyrosol-Gluc-Xyl].

Conclusion

The protocol for the isolation of oligosaccharides of low molecular weight developed for two samples of olive by-products or alperujo from different seasons, origin and variety of olives results in a reproducible and useful tool for the recovery of bioactive compounds. Hydrothermally treated alperujo represents a good source of not only bioactive phenols but also poly- and oligosaccharides with potential biological properties. Chemical hydrolysis has been performed to increase the neutral and acidic oligosaccharides with low molecular weight obtaining di-

galacturonic acids, and different structures of neutral and acidic xylo-oligosaccharides, together with xyloglucan, glucans and arabinans.

The results obtained from the antioxidant test demonstrate that some fractions may be considered as new potentially useful bioactive compounds, especially those with the highest content in phenolic compounds, which are mainly responsible for the antioxidant capacity of the soluble fiber.

In addition, a group of fractions with oleosidic secoiridoid structures conjugated to phenolic compounds were identified as oleuropeinic acid, 10-hydroxyoleuropein, a ligstroside derivative, an isomer of oleuropein aglycon and secologonaside.

The presence of a significant fraction of di- and trisaccharides bound to phenolic compounds (hydroxytyrosol and tyrosol) could be considered soluble fiber and antioxidant soluble fibers with bioactive properties assigned to a phenolic moiety, respectively.

The fraction of neutral oligosaccharides or those bound to a phenol-containing compound can be fermented by colonic bacteria and may act as prebiotic compounds. The phenolic compound would be released and might exert the effect of scavenging free radicals and binding metal or to be absorbed, thus increasing their nutritional value.

Acknowledgments

We thank the Ministerio de Ciencia e Innovación of Spain for financial support (grant no. AGL2009-12352) and the Consejería de Innovación, Ciencia y Empresa de la Junta de Andalucía (Proyecto de Excelencia P08-AGR-03751).

REFERENCE

[1] Fernández-Bolaños, J. Rodríguez-Gutiérrez, G. Lama-Muñoz, A. & Sánchez, P. (2010). *Dispositivo y procedimiento para el tratamiento de los subproductos de la obtención del aceite de oliva*. (Spanish Patent request No. P201031236).

[2] Rodríguez, G., Lama, A., Trujillo, M., Espartero, J. L. & Fernández-Bolaños, J. (2009). Isolation of a powerful antioxidant from Olea europaea fruit-mill waste: 3, 4-Dihydroxyphenylglycol. LWT- *Food Science and Technology*, *42*, 483- 490.

[3] Lama-Muñoz, A. Rodríguez-Gutiérrez, G. Rubio-Senent, F. Gómez-Carretero, A. & Fernández-Bolaños, J. (2011). A New Hydrothermal Treatment of Alperujo Enhances the

Content on Bioactive Minor Components in Crude Pomace Olive Oil. *Journal of Agricultural and Food Chemistry*, *59*, 1115-1123.

[4] Rodríguez, G., Rodríguez, R., Guillén, R., Jiménez, A. & Fernández-Bolaños, J. (2007). Effect of steam treatment of alperujo on the enzymatic saccharification and in vitro digestibility. *Journal of Agricultural and Food Chemistry*, *55*, 136-142.

[5] Jiménez, A., Guillén, R., Fernández-Bolaños, J. & Heredia, (1994). A. Cell wall composition of olives. *Journal of Food Science*, *59*, 1192-1201.

[6] Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños, J. & Heredia, A. (2001). Olive fruit cell wall degradation of cellulosic and hemicellulosic polysaccharides during ripening. *Journal of Agricultural and Food Chemistry*, *49*, 2008-2013.

[7] Vierhuis, E. Schols, H. A. Beldman, G. & Voragen, A. G. J. (2001). Structural characterization of xyloglucan and xylans present in olive fruit (Olea europeae, cv Koroneiki). *Carbohydrates Polymers*, *44*, 51-62.

[8] Mafra, I. Barros, A. S. & Coimbra, M. A. (2006). Effect of black oxidising table olive process on the cell wall polysaccharides of olive pulp (Olea oleuopaea L. var. Negrinha do Douro). *Carbohydrates Polymers*, *65*, 1-8.

[9] Chiacchierini, E., Mele, G., Restuccia, D. & Vinci, G. (2007). Impact evaluation of innovative and sustainable extraction technologies on olive oil quality. *Trends in Food Science and Technology*, *18*, 299–305.

[10] Mussatto, S. I. & Mancilha, I. M. (2006). Non-digestible oligosaccharides: A review. *Carbohydrate Polymers*, *68*, 587-597.

[11] Gibson, G. R. & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota-introducing the concept of prebiotics. *Journal of Nutrition*, *125*, 1401-1412.

[12] Guggenbichler, J. P., Jurenitsch, J. & de Bettignies-Dutz, A. (1997). *Blocking the accumulation of germs on human cells*. (PCT/EP94/ 03006).

[13] Hsu, C. K., Liao, J. W., Chung, Y. C. & Chan, Y. C. (2004). Xylooligosaccharides and fructoligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats. *Journal of Nutrition*, *134*, 1523-1528.

[14] Broekaert, W. F., Courtin, C. M., Verbeke, K., van de Wiedele, T., Verstraete, W. & Delcour, J. A. (2011). Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides. *Critical Reviews in Food Science and Nutrition*, *51*, 178-194.

[15] Christakopoulos, P., Katapodis, P., Kalogeris, D., Kekos, D. Macris, B. J., Stamatis, H. & Skaltsa, H. (2003). Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases. *International Journal of Biological Macromolecules*, *31*, 171-175.

[16] Nabarlatz, D., Montané, D., Kardosova, A., Bekesová, S., Hribalová, V. & Ebringerová, A. (2007). Almond shell xylo-oligosaccharides exhibiting immunostimulatory activity. *Carbohydrate Research*, *342*, 1122-1128.

[17] Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F. & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids*, *28*, 1-13.

[18] Rubio-Senent, F., Lama-Muñoz, A., Rodríguez-Gutiérrez, G. & Fernández-Bolaños, J. (2013). Isolation and identification of phenoic glucosides from thermally treated olive oil byproducts *Journal of Agricultural and Food Chemistry*, *61*, 1235-1248.

[19] Dische, Z. (1962). Color reactions of carbohydrates. In Whistler, R. L., Wolfram, M. L., (Eds.), *Methods in Carbohydrates Chemistry* (477-512). New York: Academic Press.

[20] Blumenkrantz, N. & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484-489.

[21] Englyst, H. N. & Cummings, J. H. (1984). Simplied method for the measurement of total nonstarch polysaccharides by gas-chromatography of constituent sugars as alditol acetates. *Analyst*, *109*, 937-942.

[22] Singleton, V. & Rossi, J. A. (1965). Colorimetric of total phenolics with phosphomolybdic-Phosphotungstic acid reagents. *American Journal of Enolology and Viticulture*, *16*, 144-158.
Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A. & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil byproduct alperujo and their antioxidative activities. *Journal of Agricultural and Food Chemistry*, *60*, 1175-1186.

[23] Fernández-Bolaños, J., Rodríguez, G., Gómez, E., Guillen, R., Jiménez, A., Heredia, A. & Rodríguez, R. (2004). Total recovery of the waste of two-phase olive oil processing: Isolation of added-value compounds. *Journal of Agricultural and Food Chemistry*, *52*, 5849-5855.

[24] Obied, H. K., Allen, M. S., Bedgood, D. R., Prenzler, P. D. & Robards, K. (2005). Investigation of Australian olive mill waste for recovery of biophenols. *Journal of Agricultural and Food Chemistry*, *53*, 9911-9920.

[25] Reis, A., Domingues, R. M., Domingues, P., Ferrer-Correia, A. J., & Coimbra, M. A. (2003). Positive and negative electrospray ionisation tandem mass spectrometry as a tool for structural characterization of acid released oligosaccharides from olive pulp glucuronoxylans. *Carbohydrate Research*, *338*, 1497-1505.

[26] Vierhuis, E., Servili, M., Baldioli, M., Schols, H. A., Voragen, A. G. J. & Montedoro, G. (2001). Effect of enzyme treatment during mechanical extraction of olive oil on phenolic compounds and polysaccharides. *Journal of Agricultural and Food Chemistry*, *49*, 1218-1223.

[27] Cardoso, S. M., Guyot, S., Marnet, N., Lopes-da-Silva, J. A., Renard, C. & Coimbra, M. A. (2005). Characterisation of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *Journal of Science. Food Agriculture*, *85*, 21-22.

[28] De la Torre-Carbot, K., Jauregui, O., Gimeno, E., Castellote, A. I. Lamuela-Raventós, R. M. & López-Sabater, M. C. (2005). Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC-DAD, and HPLC-MS/MS. *Journal of Agricultural and Food Chemistry*, *53*, 4331-4340.

6.2.2. Capítulo 10.

Pectin extracted from thermally treated olive oil by-products: characterization, physico-chemical properties, *in vitro* bile acid and glucose binding.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J.

Food Hydrocolloids, 2015, 43, 311-321.

Con el objetivo de mejorar la utilización del alperujo se ha desarrollado un pretratamiento térmico que ha sido patentado y que facilita la separación de las fases sólida y líquida que lo componen, obteniendo en la fracción líquida una elevada recuperación de compuestos de alto valor añadido. Entre los compuestos solubilizados se detectan considerables cantidades de polisacáridos pécticos, ya que un tercio de la pared celular de la aceituna está compuesta por polisacáridos pécticos ricos en arabinosa. Las pectinas han sido ampliamente utilizadas como agentes gelificantes, estabilizantes y emulsionantes en la industria alimentaría. En el organismo actúan como prebióticos, anti-inflamatorios, anti-diarreicos, y puede prevenir el estreñimiento, además actúan de forma beneficiosa para controlar la diabetes, la prevención de la obesidad, el cáncer y son capaces de captar ácidos biliares.

En este trabajo, se estudia el material péctico solubilizado en la fracción acuosa obtenida tras el tratamiento térmico del alperujo, como resultado de la ruptura de la pared celular. El material obtenido a partir del alperujo tratado con diferentes tratamientos (para una misma temperatura, 160 ºC, y tres tiempo diferentes, 30, 45 y 60 minutos) es purificado, caracterizado y se realizó una investigación detallada de una serie de propiedades que fueron comparadas con los resultados obtenidos para pectina de limón y manzana comercial (peso molecular, grado de esterificación, capacidad de retención de agua/aceite, capacidad de emulsificación y estabilidad, capacidad de captación de ácidos biliares e índice de retardo de la glucosa).

Los resultados obtenidos nos han mostrado que las pectinas aisladas a partir de alperujo tratado térmicamente se caracterizan por estar enriquecidas en azúcares neutros, poseer un elevado grado de esterificación y un tamaño medio de 6 kDa. Estas pectinas de bajo peso molecular pueden ser consideradas como pectinas modificadas, las cuales son fácilmente absorbidas y se caracterizan porque se unen y bloquean a la proteína pro-metastásica galectina-3 (Gal-3). Así mismo presentan una elevada capacidad de captación de aceite y se caracterizan por ser capaces de captar ácidos biliares y retardar el índice de diálisis de la glucosa.

Pectin extracted from thermally treated olive oil by-products: Characterization, physico-chemical properties, *in vitro* bile acid and glucose binding

Fátima Rubio-Senent, Guillermo Rodríguez-Gutíerrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños

ABSTRACT

The pectin fraction released from steam-treated alperujo at 160 ºC for 30, 45, and 60 min was purified and characterized. Differences were observed in the composition, and physical and biological activities of the pectin extracts, depending on the length of the thermal treatment. The extracts presented a low molecular weight in the range of 2-40 KDa, a high content of neutral sugars, and a high percentage of acetylation. In comparison with commercial pectins, the extracts had low water and high oil holding capacities, a normal emulsifying activity and a similar emulsion stability to that of apple pectin. For the three pectin extracts studied, the *in vitro* analyses showed considerable bile-acid binding activity and a glucose retardation index similar to the values obtained for citrus pectins. Thus, the pectins isolated from olive oil waste have suitable physical and biological properties for commercial use. Pectin extraction from alperujo, a sizeable by-product of the olive oil manufacturing process, would facilitate the use and subsequent reduction of this environmentally damaging organic waste.

KEYWORDS: Alperujo; Steam treatment; Pectin; Glucose retardation index; Water/oil holding capacity; Bile acid binding

1. Introduction

The olive oil manufacturing process, by the continuous biphasic extraction system, generates oil and a by-product known as alperujo. Alperujo is a combination of liquid (olive vegetative water) and solid (skin, seed, pulp and pieces of stones) olive-pomace mill waste. Approximately 4-6 million tonnes of alperujo are produced by the olive oil extraction process every year in Spain. The production of such high quantities of alperujo has important environmental consequences, due to its high organic content and the presence of phytotoxic components, which makes it difficult to use in further bioprocesses.

The combustion of alperujo as a fuel is commonly used as a procedure to eliminate its harmful effect on the environment. However, several studies have investigated ways to exploit

this polluting by-product in order to obtain high value products, besides a waste reduction strategy. With the aim of improving the utilization of alperujo, we have recently developed an innovative steam treatment (Patent no. ES2374675; Fernández-Bolaños, Rodríguez- Gutiérrez, Lama-Muñoz, & Sánchez, 2010), which allows for an easy separation of the solid and liquid phases. In this process, an auto-hydrolysis of material occurs and facilitates the recovery of added-value compounds in the water-soluble fraction. Recoverable compounds include natural antioxidants (biophenols such as hydroxytyrosol and 3,4-dihydroxyphenylglycol), neutral and acid oligosaccharides, and phenolic glucosides (Rubio-Senent, Lama- Muñoz, Rodríguez-Gutiérrez, & Fernández-Bolaños, 2013). In addition, considerable amounts of pectic polysaccharides can be recovered by this system, because one third of the olive-pulp cell wall is made up of arabinose-rich pectic polysaccharides (Coimbra, Cardoso, & Lopes-da-Silva, 2010).

Pectins are natural hydrocolloids that are found in higher plants as principal structural elements of cell walls, and have been widely used as gelling agents, stabilizers, and emulsifiers in the food industry. Pectin is also a component of soluble dietary fibers, which is poorly digested in the small intestine but ferments in the colon, and plays a significant role in many physiological processes. It acts as a prebiotic, anti-inflammatory, anti-diarrheal, and can prevent constipation; it is also beneficial to the control of diabetes, and in the prevention of obesity and cancer (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Rodríguez, Jiménez, Ferneńdez-Bolaños, Guillén, & Heredia, 2006). In addition, interest has recently been raised in the possible application of pectin in tissue engineering, cancer treatment and gene delivery (Morris, Belshaw, Waldron, & Maxwell, 2013; Munarin, Tanzi, & Petrini, 2012). Eastwood and Hamilton (1968) demonstrated that pectin could bind bile acids, thereby causing their excretion in the faeces. The continual depletion of bile in this manner is thought to reduce serum LD cholesterol levels, by diverting cholesterol for the manufacture of bile acids, and hence reduces the risk of cardiovascular disease. The binding of pectin to bile salts has also led to an interest in its use to reduce the excessive level of bile in the colon, which lowers the risks of bowel cancer (Camire & Dougherty, 2003).

Essentially, pectins are complex polysaccharides containing 1,4-linked-α-D galacturonic acid residues that are occasionally interrupted by (1→2)-α-L-rhamnose residues carrying sugar side chains, typically galactose and arabinose. Apple pomace and citrus peel, and to a lesser extent sugar beet roots and sunflowers, are source of commercial pectins. However, there is a requirement for a novel source of pectin in order to manufacture pectins with tailored structures and beneficial properties, particularly adaptable for biomedical application (Munarin et al., 2012). Some studies have reported the extraction of pectins from olive by-products such as olive mill wastewater (Galanakis, Tornberg, & Gekas, 2010) or two-phase olive pomace (Cardoso, Coimbra, & Lopes-da-Silva, 2003). In the latter case, pectin was extracted from an ethanolic insoluble residue of alperujo using acid extraction at 80 ºC for 1.5 h, and despite the high neutral sugar content present, a potential source of gelling pectic material with distinct rheological properties from those available commercially was obtained (Cardoso et al., 2003).

Industrial extraction is usually carried out by acid treatment at high temperatures for long periods of heating, and posterior concentration of the pectin for precipitation by alcohol. The chemical structure of pectin can undergo important structural changes during the extraction procedure, and the length of heat treatment has been recently reduced by the use of hot water extraction with microwave pre-treatment (Fishman, Chau, Hoagland, & Hotchkiss, 2006).

In this work, we study the pectic material solubilized in the aqueous fraction of alperujo, as a result of the breakdown of the cell wall. We purified and characterized the pectin fraction released from steam-treated alperujo at 160 ºC for three different reaction times (30, 45, and 60 min). As hydrothermal conditions can modify the pectin structure and its molecular weight, and thus its functional properties, a detailed investigation of these properties are reported in this study. We also tested some of the important beneficial properties (water and oil holding capacities, emulsifying properties, bile-acid binding, glucose retardation index) of the pectic material obtained using *in vitro* assays and discuss the suitability of alperujo as a source of pectin for commercial or biomedical applications.

2. Materials and methods

2.1. Raw material

Olive pomace or “alperujo” (a semi-solid residue composed of olive peels, pulp, seeds, and ground stones) was collected directly after the two-phase centrifugal system used in the local pomace processing mill (Oleícola El Tejar, Córdoba, Spain) for the extraction of olive oil. The moisture level was determined.

2.2. Chemicals

Pectin from citrus peel and with different degrees of esterification (89%, 55-70% and 22%); cholestyramine; three bile acids (cholic, deoxycholic, and chenodeoxycholic acid) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Apple pectin was purchased from F.E.R.O.S.A. (Barcelona, Spain). The standards of Dextran from *Leuconostoc* ssp 250,110, 70, 40 and 6 kDa, were obtained from Fluka Bio Chemika (Switzerland). The extraction solvents ethyl acetate and ethanol were obtained from Romil Ltd. (Waterbeach, UK) and VWR International (Ph. Eur.) (Radnor, Pennsylvania, USA), respectively.

2.3. Thermal treatments or steam processing of alperujo

The hydrothermal treatment of the alperujo (Patent no. ES2374675) was performed using a steam treatment reactor prototype, designed by our research group at the Instituto de la Grasa (Seville, Spain).

Fresh alperujo samples (10 Kg) were treated with saturated steam for 30, 45 and 60 min at a temperature of 160 ºC. Heating of the alperujo was performed by direct injection of steam, enhancing the contact between the steam and the alperujo. After closing the steam inlet valve, the pressure was blended down to atmospheric pressure at a controlled rate. The wet material was centrifuged at 4700 g (Comteifa, S.L., Barcelona, Spain) to separate the solid and liquid phases.

2.4. Phenol extraction

At the end of the thermal treatment, an aliquot of the liquid phase (10 L) was concentrated to 1 L by rotary evaporation at 37 ºC in a vacuum, and extracted with ethyl acetate (refluxed at 77 ºC) for 5-6 h. The aqueous solutions obtained in each treatment after the extraction of phenolic compounds are rich in neutral and acid oligosaccharides.

2.5. Acid hydrolysis and ultrafiltration through a membrane of 3 KDa

In order to increase the proportion of low molecular weight oligosaccharides in the aqueous phase obtained after extraction with ethyl acetate, a mild chemical hydrolysis at 70 ºC with 0.5 M HCl for 2 h was carried out (Rubio-Senent et al., 2013).

The hydrolyzed fractions (1000 mL) of the three treatments were ultra-filtered at room temperature using a Prep/Scale®-TFF Cartridge holder XX42PS001 with Prep/Scale®-TFF Cartridge 3000 Da regenerated cellulose (Millipore Corp., Bedford, MA, USA). The solutions were washed with 5000 mL of water at 40 ºC and concentrated by vacuum at 40 ºC until a final volume of 150 mL was obtained. The fractions recovered with a size over 3 KDa were rich in pectic material.

2.6. Isolation and purification of raw pectin extract and fractional precipitation with ethanol (EtOH)

The fraction obtained after ultrafiltration with a molecular weight greater than 3 KDa was submitted to precipitation with 85% ethanol and further purified by EDTA complexation, followed by percolation through a strong resin ionic exchanger, according to the procedure described by Cardoso et al. (2003). The purified extracts of the three steam-treated samples are referred to as Pectoliv30, Pectoliv45 and Pectoliv60.

The fraction obtained from treatment at 160 ºC/60 min was also fractionally precipitated with ethanol, in order to compare it with the previous procedure of purification, following the scheme in **Fig. 1**. This sample was chosen to be fractionally precipitated because the lowest percentage of purified extract was obtained by purification following the procedure of Cardoso et al. (2003) (see Results and Discussion section). The first precipitation was carried out with EtOH 60% (v/v) causing the precipitation of pectins with high molecular weight between 0 and 60% of EtOH. The resulting precipitate, named PEt60, was removed by centrifugation at 15000 g for 20 min at 4 ºC. EtOH was added to the new supernatant to obtain an 80% ethanol solution

(v/v). An insoluble material was obtained between 60 and 80% EtOH, facilitating the precipitation of lower molecular weight pectins, and the resulting precipitate was named PEt80. Each precipitate was dissolved in water and concentrated by rotary evaporation at 40 ºC to completely remove the ethanol, and then lyophilized.

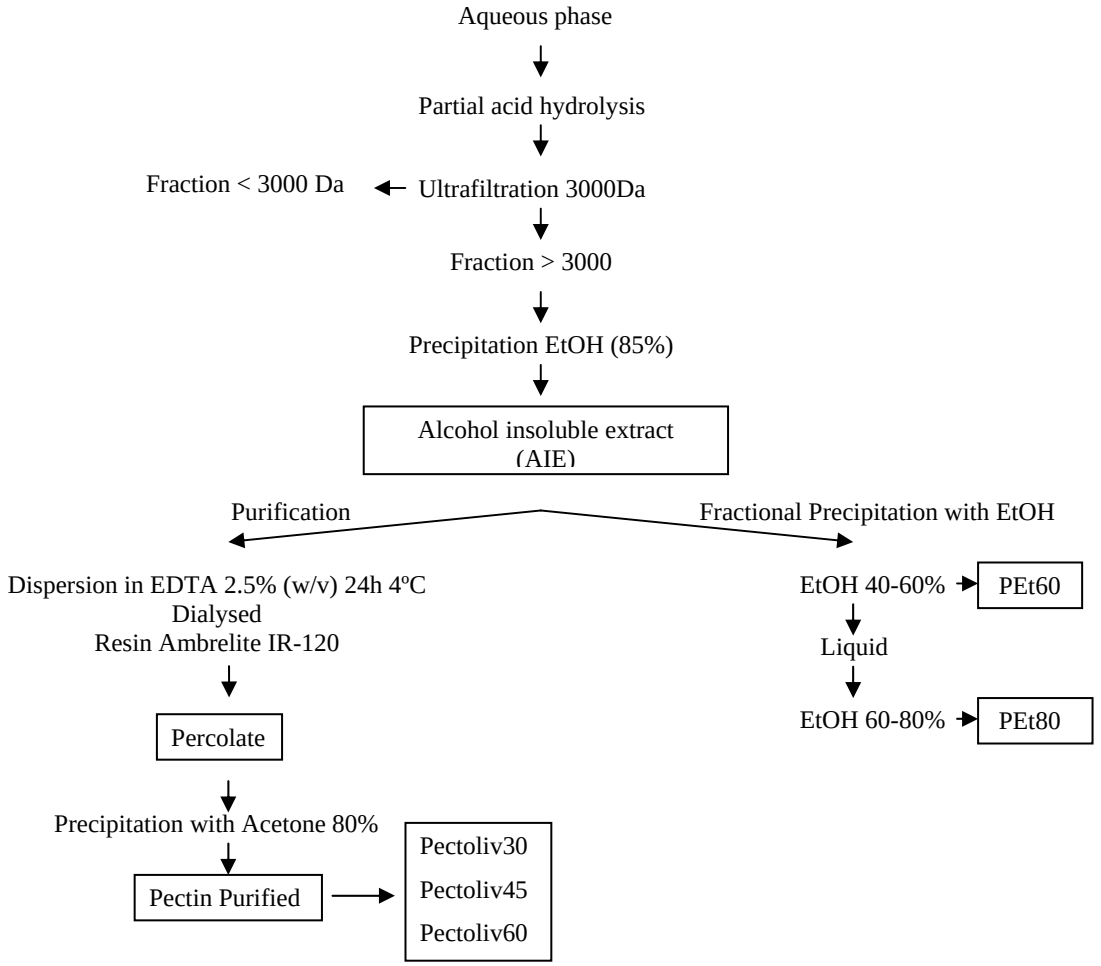


Fig. 1. Scheme of recovery and purification to obtain purified pectin from the aqueous phase of thermally treated alperujo.

2.7. Polysaccharide analysis

Galacturonan (anhydrogalacturonic acid, GalA) was estimated according to the m-hydroxydiphenyl method, described by Blumenkrantz and Asboe-Hansen (1973) for uronic acids. It is worth noting the very high proportion of galacturonic acid with respect to the glucuronic acid found in the aqueous phase of the thermal treatment, which has been described in previous work (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, & Fernández- Bolaños, 2012).

Glycosyl compositions of the different samples were determined by gas chromatography (GC) after their conversion and quantification to alditol acetates. Individual neutral sugars were analyzed from duplicate samples with initial TFA hydrolysis (2 N TFA at 121 ºC for 1 h) prior to

reduction, acetylation and analysis by GC (Englyst & Cummings, 1984). Inositol was used as an internal standard. Calibrationwas performed with a series of standard sugar solutions of L-rhamnose (Rha), L-fucose (Fuc), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man) and D-xylose (Xyl). Chromatographic conditions utilized were described by Lama-Muñoz et al. (2012).

The total neutral sugars were determined colorimetrically according to the anthrone-sulfuric acid assay of Dische (1962), using glucose as a standard and in triplicate.

2.8. Determination of the total phenolic, protein and ash contents

Total phenolic content was determined by the Folin-Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents (Singleton & Rossi, 1965). Protein was determined by the micro Kjeldahl method (Kjeldahl,1983) using the multiplication of the total nitrogen content by a conversion factor of 6.25. The analysis of ash was performed according to the AOAC procedure (AOAC, 1990, pp. 915-919).

2.9. High-Performance Size Exclusion Chromatography (HPSEC) and Molecular Weight (MW) distribution of the pectins

HPSEC was performed as described by Dos-Santos, Jiménez- Araujo, Rodríguez-Arcos, and Fernández-Trujillo (2011). The MW was measured using Jasco equipment (LC-Net II ADC, Kyoto, Japan) with a refractive index detector (Jasco RI-1530) and two different columns (300 x 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA) in sequence TSKgel GMPWXL (dextran MW < 50000 KDa) and TSKgel G3000PWXL (dextran MW < 60 KDa). The system was calibrated with dextrans of 252, 110, 70, 40, 6 kDa and glucose. Fractions of 250 µL were collected using a Redifrac fraction collector (Pharmacia Biotech, Uppsala, Sweden). The uronic acid and total sugar content of the fractions were determined by measuring absorbance at 520 and 620 nm respectively, in a iMark™ microplate absorbance reader (Bio-Rad, Hercules, CA, USA).

2.10. Ion exchange chromatography

The method used was described by Jiménez et al. (2001) with some modification. 5 mL Hi-Trap Columns (anionic exchanger: Q-Sepharose) from Pharmacia (Uppsala, Sweden) were used. The column was equilibrated in a 0.01 M imidazole-HCl buffer, pH 7. The flow during injection was 1 mL/min. Two steps in the fractionation were made with increasing buffer concentration of imidazole-HCl from 0.01 to 2.0 M, pH 7. In the first step, a flow of 1 mL/min was employed and neutral oligosaccharides were collected in fractions of 2 mL. In the second step, a flow of 5 mL/min was employed and acid oligosaccharides were collected in fractions of 5 mL. All fractions were assayed for uronic acids and neutral sugar according to the colorimetric method.

2.11. Saponification, degree of methylesterification (DM) and acetylation (DAc)

The determination of the DM and DAc of the pectic polysaccharides was based on the estimated methanol and acetic acid contents released by saponification with 2 M NaOH at 20 ºC for 60 min (Waldron & Seveldran, 1990). The amount of methanol released was measured enzymatically using alcohol oxidase and a spectrophotometric method, as described previously by Galanakis et al. (2010), and expressed as % (mol MeOH/100 mol GalA). The amount of acetic acid released after saponification was determined by HPLC (Hewlett Packard Serie 1100) on an AminexHPX-87H (300 x 7.8mm, Bio-Rad), according to Cardoso et al. (2003), and expressed as % (mol AcOH/ 100 mol GalA). The samples were analyzed in duplicate for both methods, and compared to the values obtained for three commercial pectins with different degrees of esterification. For pectin from citrus with degrees of esterification of 89, 55-70 and 22% the results obtained were 86, 53 and 28%, respectively.

2.12. Isolation of galacturonan: hydrolysis of neutral sugar

The method used for hydrolyzing the neutral sugar attached to the pectin chain was described by Markov et al. (2011) with modifications. Pectin (1 g) was exposed to partial acid hydrolysis with 2 M TFA (200 mL) for 5 h at 100 ºC to obtain galacturonan. The resulting solution was ultra-filtrated through a membrane of 1 KDa. The fraction with a size greater than 1 KDa was lyophilized to produce D-galacturonan, eliminating approximately 95% of the neutral sugar chains.

2.13. Functional properties

2.13.1. Water/Oil-holding capacity (WHC/OHC)

These properties were determined by centrifugation using the method described by Fuentes-Alventosa et al. (2009). The oil holding capacity (OHC) was determined under the same conditions as WHC using soybean oil (0.92 g/mL density), and was expressed as g oil retained/g sample.

2.13.2. Emulsification activities (EA) and stability of the emulsion (ES)

Emulsification activities and emulsion stability were evaluated in duplicate using the method of Betancur-Ancona, Peraza-Mercado, Moguel-Ordoñez, and Fuertes-Blanco (2004). EA was expressed as percentage of the volume of the emulsified layer respect to the volume of the entire layer in the centrifuge tube. ES was determined using the prepared emulsions and heated at 80 ºC for 30 min. The emulsion was cooled to room temperature and centrifuged at 1200 g for 5 min in a Sorvall RT 6000D (DuPont, Nemours, Mechelen, Belgium). ES was expressed as the percentage of the remaining emulsified layer volume of the original emulsion volume. Also, the stability of the emulsions were determined by letting them stand for 36 h at 4 ºC, as described by Dea and Madden (1986).

2.13.3. Binding of bile acid (BA)

The capacity of pectins to bind BA *in vitro* was evaluated following the method described by Camire and Dougherty (2003) with some modifications. The change in the BA concentration upon exposure to the pectin solution during an *in vitro* digestion process, which included an acidic digestion of the sample at pH 2, followed by pancreatine digestion, was used to estimate the binding of BA. Individual BAs such as cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) (Sigma, St. Louis, MO) were selected for analysis. The results obtained for each sample of isolated pectin were compared with the results obtained for Cholestyramine resin (Sigma C 4650), cellulose (Macherey Nagel, GmbH & Co. KG) and commercial pectin from citrus peel (Sigma P 9135).

In vitro digestion procedure. Each BA was dissolved in 0.1 M $NaHCO_3$ to make a 31.25 mM BA solution, which in the final mixture would have a BA in the range of that found in the human gut (1.5-7 mM). 100 mg of sample (pectin, cholestyramine and cellulose) were added to each tube in triplicate. The individual sample without BA was used as a blank, and the reagent mixture including BA but without sample, was considered as 100% of BA concentration. The samples were digested in 1 mL of 0.01 M HCl for 1 h at 37 ºC in a shaking water bath (P-Selecta Unitronic 320 OC), to simulate gastric digestion. The sample was neutralized with 0.1 M NaOH to a pH of 7.0, and 4 mL of BA solution added to each test sample, except for the sample blank, to which 4mL of $NaHCO_3$ were added without BA. 5 mL of porcine pancreatin (activity equivalent of 8 x USP) with a concentration of 10 mg/mL in 0.01 M buffer phosphate at pH 7.0 was added and the tubes were incubated for 1 h at 37 ºC in a shaking water bath. Supernatants were recovered after centrifugation for 10 min at 1120 g in a Sorvall RT 6000D (DuPont, Nemours, Mechelen, Belgium). An additional 5 mL of phosphate buffer were used to rinse out the incubation tubes, which were vortexed and centrifuged as before, and the resulting supernatant combined with the relevant extract.

Bile acid analysis. BA were analyzed by a the modified Pettenkofer colorimetric method, described by Boyd, Eastwood, and MacLean (1966). 100 µL of supernatant (samples, blank and standards of individual BA) with 5 mL sulfuric acid (70%) were heated for 1 h at 137 ºC. After 5 min incubation, 1 mL of a freshly prepared furfural (0.25%) solution was added and the solutions were thoroughly mixed. A pink colour develops, reaching maximum intensity after 60 min. Readings were made at the absorption maximum of 490 nm in iMark™ microplate absorbance reader (Bio-Rad, Hercules, CA, USA) and the values were determined from the obtained standard curve. The ranges for the different standards were 0-30 µg for CA, 0-750 µg for DCA and 0-750 µg for CDCA. Binding capacity was expressed as mM bile acid/100 mg of pectin and was calculated from the decrease in BA concentration (mM) in the test solution after exposure to 100 mg of added pectin.

2.13.4. Glucose dialysis retardation index (GDRI)

GDRI was determined as described by Fuentes-Alventosa et al. (2009). The glucose concentration was determined spectrophotometrically according to the anthrone method (Dische, 1962).

2.14. Statistical analysis

Results were expressed as mean values ± standard deviations. Sample comparison, by multivariate analysis of variance (ANOVA), followed by Duncan's comparison test (Statgraphics Plus program version 2.1), was used to assess the differences in the composition and functional characteristics among the different isolated pectins. The level of significance used was $p < 0.05$.

3. Results and discussion

3.1. Purification and chemical characterization of the isolated pectin extracts

The aqueous phase from hydrothermally treated alperujo, including carbohydrates, phenols and polyphenols, was treated with ethyl acetate, a mild acid hydrolysis and a subsequent ultra-filtration through a membrane of 3 KDa, to isolate pectic polysaccharides as previously reported (Lama-Muñoz et al., 2012; Rubio-Senent et al., 2013). This isolation was carried out with three samples obtained from hydrothermal treatments at 160 ºC for 30, 45 and 60 min. **Table 1** shows the concentration of uronic acid (in grams per kilograms of fresh alperujo) for each treatment for the initial solution and following the isolation of pectic polysaccharides. During the process of hydrolysis and ultra-filtration no loss in pectic material was observed. These results show that the 45 min treatment liberated the maximum concentration of uronic acid from the initial sample (1.72 g uronic acid/Kg fresh alperujo; 0.6% dry weight), and from the fraction with a size less than 3 KDa, whereas, the 60 min hydrothermal treatment gave the lowest concentration of uronic acid. Therefore, these results suggest that the length of hydrothermal treatment did not affect pectins with a size >3 KDa, but resulted in the degradation of pectins with a size <3 KDa.

Table 1: Concentration of uronic acid in the initial solution and after the ultrafiltration of 3 KDa, of the three heat treatment conditions.

	g Uronic acid/Kg fresh alperujo		
	Initial	Fraction <3000	Fraction >3000
160 ºC/30	1.48±0.13 (0.5%)[a]	0.78±0.02	0.96±0.05
160 ºC/45	1.72±0.01 (0.6%)	1.12±0.09	0.60±0.03
160 ºC/60	0.78±0.09 (0.3%)	0.19±0.02	0.58±0.01

[a] Yield (% dry weight) of the pectin recovered from steam-treated alperujo.

The three samples containing pectic material with a size greater than 3 KDa were precipitated with 85% EtOH to obtain an alcohol insoluble extract (AIE), and then purification by two procedures were compared; the procedure described by Cardoso et al. (2003), and sequential precipitation with ethanol (60 and 80% ethanol (v/v)) (**Fig. 1**). Extracts purified by the

first procedure are referred to as Pectoliv30, Pectoliv45 and Pectoliv60, and the extracts obtained by fractional precipitation of the sample treated at 160 ºC/60 min are referred to as PEt60 and PEt80.

The composition of the initial alcohol insoluble extracts (AIE) of the fractions > 3 KDa and the purified extracts (Pectoliv30, Pectoliv45, Pectoliv60, PEt60 and PEt80) were analyzed and the results are shown in **Table 2**. The total sugar contents (the sum of the percentage of uronic acid and neutral sugar) were increased from 30-50% to 80-90% by the first purification process, whereas the phenolic compounds present (4-9%), responsible for the brown colour of the pectin extract, did not change significantly (**Table 2**). The purified pectic material obtained for the shortest treatment time (Pectoliv30) was mostly composed of GalA (48%) and Ara (45%), with minor proportions of Rha (1%), Xyl (2%) and Man (2%). The composition of this pectic extract was similar to the pectic polysaccharide rich in Ara, a galacturan backbone linked to Ara residue, previously reported by Cardoso et al. (2003) for olive pomace. The steam treatment promotes the cleavage of the galacturonan backbone and the arabinan side chain, and indeed a more prolonged steam treatment successively reduced the proportion of GalA and Ara, and increased the sugars Rha, Xyl, Gal, Glc and Man. The composition of the neutral sugar content would suggest the presence of other polysaccharides (arabinoxylans, glucomannans, xyloglucans), which have been previously described as constituents of the olive pulp (Jiménez, Guillén, Fernández-Bolaños, & Heredia, 1994) and have also been described in pectin with high contents of Glc (15-17%) and Gal (7-9%) obtained from olive mill wastewater (Galanakis et al., 2010). Prolonged heat treatment leads to certain degradation of GalA, as polyuronide polymers are susceptible to degradation by β-elimination by heating (Fishman et al., 2006), and also produce a more heterogeneous pectic material with differences in the sugar composition.

Table 2
Chemical composition of alcohol insoluble extract (AIE) (initial sample precipitated with EtOH 85%) and the purified extracts of steam-treated olive by-product (alperujo). Composition of the pectic material sequentially precipitated with EtOH of 60 and 80% from the initial sample (AIE) obtained by treatment at 160ºC/60 min.

	Treatment		Treatment		Treatment		Treatment	
	160 ºC/30 min		160 ºC/45 min		160 ºC/60 min		160 ºC/60 min	
	AIE	Pectoliv30	AIE	Pectoliv45	AIE	Pectoliv60	Pectin EtOH 60%	Pectin EtOH 80%
g/ Kg fresh alperujo	3.13		3.16		3.08		0.77	1.04
% purified extract[a]		38.94		33.97		26.61		
Composition (g/100g extract)								
Uronic acid	19.65±0.48	42.32±2.52	14.43±0.33	38.38±1.84	13.49±1.41	22.22±2.12	17.88±0.55	10.99±1.02
Neutral sugar	29.25±0.60	46.01±0.45	31.37±2.46	51.78±5.62	18.83±0.20	57.89±1.81	36.04±0.82	36.86±2.11
Phenol	7.09±0.45	5.91±0.77	4.55±0.62	4.01±0.54	9.74±0.96	8.53±0.81	13.2±0.28	9.98±0.81
Protein	-[c]	1.24±0.02	-	1.08±0.01	-	1.00±0.04	-	-
Ash	-	1.42±0.38	-	0.12±0.05	-	1.24±0.19	-	-
Metoxyl+O-acetyl	-	6.85±0.85	-	9.01±1.03	-	5.99±0.62	-	-
Total	53.99	103.75	50.35	104.38	42.06	96.88	67.12	57.83
Total sugars[b]	48.9	88.33	45.8	90.16	32.32	80.11	53.92	47.85
% molar								
Galacturonic acid	-	47.91±2.87	-	42.57±2.04	-	27.74±2.64	33.16±0.20	22.98±2.13
Rhamnose	-	0.96±0.01	-	2.82±0.60	-	6.61±0.61	9.08±0.01	7.79±0.72
Fucose	-	0.55±0.04	-	4.16±0.25	-	0.67±0.02	0.36±0.05	0.21±0.01
Arabinose	-	45.37±1.77	-	28.38±3.50	-	19.81±0.45	15.71±0.31	19.40±1.23
Xylose	-	2.20±0.89	-	10.26±0.57	-	16.26±0.23	10.15±0.10	22.17±1.32
Mannose	-	2.48±0.58	-	3.48±0.31	-	6.48±0.39	4.24±0.41	2.89±0.21
Galactose	-	0.55±0.03	-	3.42±0.07	-	13.52±0.03	14.29±0.31	12.00±0.98
Glucose	-	n.d.	-	4.95±0.49	-	8.97±0.07	13.11±0.31	12.38±1.02

n.d. not detected.
[a] Percentage of purified pectin obtained from AIE.
[b] Sum of the uronic acids and neutral sugars.
[c] Not realized.

For the second purification procedure, the pectic material obtained from the 60 min treatment at 160 ºC was separated into two fractions according to their solubility in aqueous ethanol solutions. The fraction precipitated in 60 and 80% EtOH had a percentage of total sugar of 54 and 48%, respectively, with a substantial increase in both cases with respect to the initial material (AIE, with 32% total sugar; **Table 2**). Although the precipitated material increased in purity (total of known compounds) from 42% to 67 and 58%, this purity is below that of the extracts obtained using the procedure of Cardoso et al. (2003) with 97% purity, indicating the presence of numerous unknown compounds in the precipitated fraction of the material that remained soluble between 60 and 80% of ethanol.

The three purified extracts (Pectoliv30, Pectoliv45 and Pectoliv60) that contain considerable quantities of neutral sugars, 46, 52 and 58%, respectively, were fractionated previously saponified (Pectoliv30Sap, Pectoliv45Sap and Pectoliv60Sap) by anion exchange chromatography on Q-Sepharose in order to determine whether these neutral sugar fractions were unbound fractions, present merely as co-extracts, or were covalently linked with

galacturonan backbone. An analysis of these fractions is shown in **Table 3**. The non-retained material (neutral polysaccharides), eluted with 0.01 M imidazole buffer (Fraction I), represented 28, 38 and 48% of the total sugars for each sample. The retained fractions (acidic polysaccharides), eluted with 2.0 M imidazole buffer (Fraction II), represented 72, 62 and 52% of the total sugars for each of the samples. In both elutions, mixtures of neutral and acidic polysaccharides were obtained. For the non-retained fraction (Fraction I), the % of acidic sugars increased (6, 10 and 21%) whereas the % of neutral sugars decreased with longer hydrothermal treatment. The opposite pattern was observed for the retained fraction (Fraction II): the % of acidic sugars decreased (33, 29 and 17%) whilst the % neutral sugars increased (67, 71, 83%) with longer treatment. Therefore, it would appear that an important proportion of neutral sugar moieties were linked to the acidic pectin backbone. The neutral sugar composition of Fraction I from the three purified extracts indicated the presence of pectic material similar to typical pectin polymers from the olive fruit (Cardoso et al., 2003), with Ara and Gal the major neutral sugars, although a large proportion of Xyl and a minor proportion of Glc were also present and therefore could be considered a co-extract. The neutral sugar compositions of Fraction II were similar for the pectin extracts, mainly because of the presence of Ara and Man. However, the relative proportion of Man increased with longer treatment while that of Ara decreased. Therefore the polysaccharides recovered in eluates of 2.0 M imidazole buffer would appear to contain neutral sugars which are covalently linked to the acidic sugar chain, probably to give a new or a-typical pectic polysaccharide structure. Hydrothermal treatment might have resulted in solubilized pectic material with structural features that can only be explained if, during the thermal processing, there is a random hydrolysis of polymeric material of the cell wall of alperujo.

Table 3
Distribution of the total sugars and glycosil composition of both fractions eluted by ion exchange chromatography (Neutral - Fraction I, and acidic - Fraction II) on Q-Sepharose from the three purified pectin extracts.

	Pectoliv30Sap		Pectoliv45Sap		Pectoliv60Sap	
	Fraction I[a]	Fraction II[b]	Fraction I	Fraction II	Fraction I	Fraction II
% Distribution of the total sugar applied						
Total sugars	28.2±1.7	71.8±6.1	38.2±2.0	61.8±6.7	48.5±3.3	51.5±11.0
% Distribution of composition in the elution						
Neutral sugar	94.2±2.1	67.2±1.6	90.2±3.0	71.1±5.8	79.4±5.1	83.0±10.1
Uronic acid	5.8±0.5	32.8±0.4	9.8±0.4	28.9±1.0	20.6±6.6	17.0±0.9
% molar						
Rhamnose	1.8±0.1	3.9±0.7	2.3±0.3	2.9±0.1	7.5±0.3	2.9±0.3
Fucose	0.2±0.1	n.d.	0.1±0.1	n.d.	0.3±0.1	n.d.
Arabinose	31.4±0.2	42.7±3.3	20.5±0.8	29.5±1.0	29.7±0.5	10.5±0.4
Xylose	22.6±0.4	7.0±2.4	40.4±1.6	14.7±1.6	24.1±0.3	3.8±0.1
Mannose	7.5±0.4	30.5±5.4	4.7±0.3	39.6±0.9	4.1±4.8	75.2±8.9
Galactose	25.5±0.7	12.3±2.3	25.0±0.1	10.8±3.9	22.1±0.3	3.0±1.5
Glucose	11.1±0.4	3.6±1.8	7.0±0.2	2.6±0.4	12.1±0.3	4.7±0.5

[a] Neutral fraction eluted with 0.01 M imidazole buffer and collected as Fraction I.
[b] Acidic fraction eluted with 2.0 M imidazole buffer and collected as Fraction II.

3.2. Determination of molecular size distribution

The three purified pectin extracts obtained for each treatment were subjected to High-Performance Size exclusion Chromatography (HPSC). The profiles of refractive index (RI), and the distribution of uronic acids (Abs 520 nm) and neutral sugar (Abs 620 nm), are displayed in **Fig. 2**.

The results for the three samples Pectoliv30, Pectoliv45 and Pectoliv60 identified three zones, which correspond approximately to the retention times of 30-35, 36-40 and 41-47 min and to estimated molecular weights of 250-110 KDa, 110-40 KDa and 40-2 KDa, respectively. The extract obtained after 60 min steam treatment (Pectoliv60), revealed a decrease in the intensity of zones I and II corresponding to high molecular weight eluates, indicating that longer treatment time resulted in the breakage of the pectin chain into other smaller ones. In accordance, the highest proportion of compounds eluted in zone III for the three samples, indicating that the pectic material had an average size of 2-40 KDa, with a maximum peak at 6 KDa. Furthermore, these polymers appear to be composed of varying amounts of this identificable macromolecular population, which match in amounts of uronic acid and neutral sugar and profile of refraction index (RI). Thus the molecular distribution of the pectin extracts supports the previous observation that the majority of neutral sugars are linked to the acidic pectin backbone.

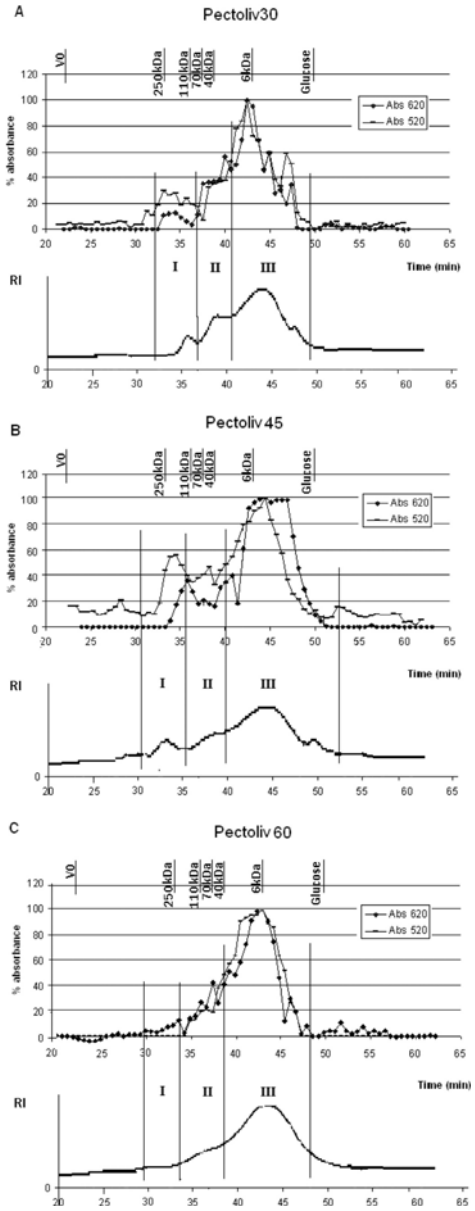


Fig. 2. Neutral sugar (absorbance at 620 nm), uronic acid (absorbance at 520 nm) and refractive index (RI) elution profiles obtained by HPSEC from the purified pectic material obtained from steam treated olive by-products (alperujo). A, Pectoliv30, B, Pectoliv45 and C, Pectoliv60. The three zones (I, II and III) were assigned according to the characteristic peak observed.

The low average molecular weight of the pectins may affect the formation of gels; in fact, we do not obtain a gel for any of the pectin samples. However it has recently been shown that small and short chain molecules of citrus pectin, known as “modified pectin”, can be more easily absorbed in the intestinal tract and have a potential role in the prevention and reduction of carcinogenesis (Morris et al., 2013). Currently, ultrasound treatment as a tool to obtain this modified pectin is being tested for the prevention of cancer metastasis, replacing chemical or enzymatic de-polymerization processes (Zhang et al., 2013). Modified pectin includes pectin with differing molecular weights, degrees of acetylation and sugar composition. Therefore, the low molecular weight pectins, Pectoliv30, Pectoliv45 and Pectoliv60, extracted from alperujo

could be good candidates for potential inhibitors of the pro-metastatic protein galectin-3 (Morris et al., 2013).

3.3. Determination of the degrees of methylation (DM) and acetylation (DAc)

The degree of methylation (DM) corresponds to the percentage of carboxyl groups esterified with methanol at the C-6. The degree of acetylation (DAc) is defined as the percentage of galacturonic acid units esterified with acetic acid, assuming that only the hydroxyl groups at the C-2 and/or C-3 of the carbohydrate ring are acetylated. Methyl-esterification is widespread in native pectins, in contrast, acetyl-esterification is usually low or absent in commercial citrus pectin, with some exceptions such as highly acetylated sugar beet pectin (DAc ≈ 30) (Dea & Madden, 1986).

The DM and DAc results obtained for the three samples of purified pectic material are shown in **Table 4**. Pectoliv30 had a DM of 5% and increased with longer treatment time to 15% and 19% for Pectoliv45 and Pectoliv60, respectively. As such, the pectin extracts can be characterized as low methoxylated pectin (LM). According to the literature, the DM obtained are lower than the DM of the soluble pectic material recovered from olive mill wastewater (59%; Galanakis et al., 2010), and the pectic material extracted from the residue of olive pomace (with a DM between 31 and 43%; Cardoso et al., 2003). The lower DM observed in the extract from steam treated olive pomace is probably due to the high heat treatment, although when the length of treatment was extended certain esterification reactions took place.

The pectin extracts obtained had a high level of acetylation (DAc from 48 to 84%), especially when compared to a previously reported DAc value of 11% for pectin extracted from olive pomace residue (Cardoso et al., 2003), and assuming that the O-acetyl groups are located in the galacturonyl residue. However, the sum of DM and DAc was greater than 100% for Pectoliv60; this could be explained by the possible acetylation of the monosaccharide of neutral or acidic chains (Gille et al., 2011; Hazendonk, Reinerink, de Waard, & Dam, 1996). To study the repartition of the acetyl groups between both chains, an extensive acid hydrolysis of the neutral side chains was carried out, following the method of Markov et al. (2011). This procedure allowed us to hydrolyze approximately 95% of the neutral sugar chains. The hydrolysis was followed by an ultra-filtration through a membrane of 1 KDa, which separated the neutral chains of smaller sizes (<1 KDa) of acidic oligosaccharides from the larger ones (>1 KDa). The DAc of the fraction >1 KDa in the three samples Pectoliv30, Pectoliv45 and Pectoliv60, was reduced to 15, 23 and 54%, respectively, which represents an important decrease (69, 63 and 35%) and is consistent with an initial distribution of the acetyl group between the neutral and acidic chains. However, the considerable quantity of acetyl groups is an unusual aspect of pectin from olive pulp. The O-acetyl content (g/100 g extract) of the three

extracts (6-8%) are close to that reported for the acetylated pectin of sugar beet, which had a high level of acetylation in the range of 2-9% (Dea & Madden, 1986). The high level of acetylation obtained could possibly be a result of the extraction with hot ethyl acetate in the initial steps, used to extract phenol compounds, which together with the hydrothermal treatment, also promotes esterification (data not shown). Nevertheless, the presence of these substituents could inhibit the gelation of the pectin, possibly due to steric hindrance or the hindrance of pectin-pectin interactions, although they should enhance the hydrophobic nature of the molecule (Dea & Madden, 1986). This latter property would give a surface active character with potential as an interfacial agent in oil/water with the ability to lower the surface tension in water, and will be studied further in the next section.

3.4. Functional properties

3.4.1. Water holding capacity (WHC) and oil holding capacity (OHC). Emulsification activity (EA) and stability of the emulsion (ES)

The results obtained for WHC, OHC, EA and ES of the purified pectin extracts are presented in **Table 4**. The WHC and the OHC are the abilities of a material to retain water and oil, respectively, after centrifugation (Fuentes-Alventosa et al., 2009). WHC is the ability of dietary fiber, related to soluble fiber, to absorb and retain water to form a viscous solution. WHC is an important property from both a physiological and technological point of view, since it can increase the bulk volume of food, and modify the viscosity and texture of a formulated product in addition to reducing calories (Rodríguez et al., 2006). Commercial citrus pectin significantly showed the highest WHC (10.3 g water/g sample). The low WHC of the different samples of low molecular weight pectic material obtained could be attributed to changes in the composition, with low amounts of galacturonic acid, and the structure of polymers occurring during heating. The values experimented a certain increase with longer treatment time, from 0.3 to 1.9 g water/g sample; this last value is comparable to the value obtained for commercial apple pectin. Important differences can be found between values for commercial pectin from citrus and apple, when a high WHC appears to be linked to a greater pectin content, which has a high capacity to absorb water (Sila et al., 2009); however, the contents in pectin in commercial citrus and apple pectin are similar. So the main explanation may be a difference in the structure of these pectins.

Table 4
Degree of acetylation (DAc) and methylation (DM) and functional properties of the purified pectin material obtained from steam-treated olive by-product (alperujo) and two commercial pectins (citrus and apple pectin): water holding capacity (WHC), oil holding capacity (OHC), emulsification activity (EA) and stability of the emulsion (ES).

	Dac %	DM %	DAc + DM %	WHC g water holding/g sample	OHC g oil holding/g sample	EA Emulsion volume/Total volume	ES %
Pectoliv30	47.89±0.94[a] (7.08±0.14)[b]	4.76±0.39[c]	52.65±1.33	0.34±0.01	2.99±0.02	54.19±3.03	19.09±1.29
Pectoliv45	61.20±6.39 (8.05±0.84)	14.77±1.35	75.97±7.74	0.43±0.06	4.43±0.07	51.89±0.86	14.58±2.95
Pectoliv60	84.33±2.55 (6.13±0.19)	18.72±1.16	103.05±3.71	1.87±0.05	6.17±0.05	50.33±1.89	n.d.
Citrus pectin	n.d.	34.68±1.41	34.68±1.41	10.35±0.27	2.59±0.19	64.58±2.95	100±0.01
Apple pectin	n.d.	6.08±0.87	6.08±0.87	2.00±0.07	2.22±0.05	54.31±6.10	21.14±3.38

n.d. not detected.
[a] Results expressed in (mol AcOH/mol GalA)·100.
[b] Results expressed in (mg AcOH/mg total sugar)·100.
[c] Results expressed in (mol MeOH/mol GalA)·100.

OHC is another important functional property of some ingredients used in formulated food. The more prolonged hydrothermal treatment resulted in an increase in this property, from 3.0 to 6.2 g oil/g sample. The mechanism of oil adsorption is not well understood and high OHC could be due to high pectin content; however it appears that the OHC values obtained in this study for commercial pectin (citrus and apple) are lower than those for the three pectin samples. The higher OHC values could be explained by the increased degree of acetylation and methylation of the pectin samples, due to an increased hydrophobicity. Ingredients with a high OHC allow for the stabilization of high fat food products and emulsions, facilitating the solubilization or dispersion of two immiscible liquids (Grigelmo-Miguel, Gorinstein, & Martín-Belloso, 1999). Subsequently, we studied the emulsification activity (EA) and the stability of the emulsion (ES) in order to evaluate the functionality of the pectin samples extracted from alperujo.

Citrus pectin was found to have the highest EA, 64.6% (**Table 4**). The isolated pectins had values similar to apple pectin, of 50-54%, despite the fact that the OHC of commercial pectin was lower. These results show that the pectins extracted in this study from olive oil by-products can be considered as good emulsifiers, as their EA values were higher than 50% (Abdul-Hamid & Luan, 2000). However, only citrus pectin showed good emulsification stability (100%) after heating the emulsion to 80 ºC for 30 min. Nevertheless, the stability of the emulsions was also determined by standing for 24, 36 and 48 h at 4 ºC (Dea & Madden, 1986). Any separated oil layer was visible in the emulsions containing the pectin extracts for at least 48 h, and therefore can be considered as 100% stable.

Taken together, we demonstrate that the pectic material can act as an emulsifier in the oil-in-water emulsions, in spite of the low stability indices after heating the emulsion, and has the emulsifying properties similar to the acetylated pectin of sugar beet. As such, Pectoliv30,

Pectoliv45 and Pectoliv60 could be utilized to stabilize foods with a high percentage of fat and emulsion.

3.4.2. In vitro bile acid (BA) binding

Human bile contains three major BAs, which were tested in these binding studies. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary BAs secreted by the liver and Deoxycolic acid (DCA) is one of the secondary BAs. Secondary acids, formed through the transformation of primary acid by intestinal bacteria, are thought to be involved in the etiology and development of colorectal cancer (Nair, 1988).

The BA binding of pectin extracted from olive by-products and of these pectin extracts de-esterified (saponified) on a dry matter, was compared to the BA binding of cholestyramine, cellulose and commercial citrus pectin (**Fig. 3**). Cholestyramine, a well known BA binding and cholesterol reducing agent, was used as a positive control and bound 6.7, 12.7 and 6.4 mM of CA, DCA and CDCA per 100 mg of sample, respectively, which is equal to 81.8, 96.6 and 91.8% of the total added BA. The negative control, commercial cellulose, bound only 0.08, 1.12 and 0.59 mM of CA, DCA and CDCA per 100 mg of sample or 1.0, 9.6 and 7.6% of the total added BA. These results are similar to the previously published results for cholestyramine and cellulose (Kim & White, 2010). The BA binding values of the pectic material resulting from the different steam treatments of olive by-products were in the range of 0.7-5.6 mM/100 mg of pectin, depending on the type of BA measured. No differences were found when we compared the BA binding of the Pectoliv30, Pectoliv45 and Pectoliv60 pectin extracts with commercial citrus pectin for two of the primary bile acids considered (CA and CDCA). However, for the secondary bile acid (DCA), Pectoliv30 and Pectoliv60 bound lower amounts of BA than citrus pectin, although there were no significant differences between the three samples of pectin. The cholestyramine, citrus pectin and the three samples of pectin obtained showed higher percentages of BA binding with DCA (secondary bile acid) than for CA (primary bile acid). This result was similar to those obtained by other groups who showed that BA adsorption decreases with a hydroxyl group increase in steroid nuclei (Drzikova, Dongowski, Gebhardt, & Habel, 2005). A comparison of these results with those from previous studies on pectin and another dietary fiber (Camire & Dougherty, 2003; Kim & White, 2010) show an adequate BA binding capacity, especially to secondary acid DCA. Together, these BA binding results point to a possible health-promoting potential for the pectins extracted from alperujo.

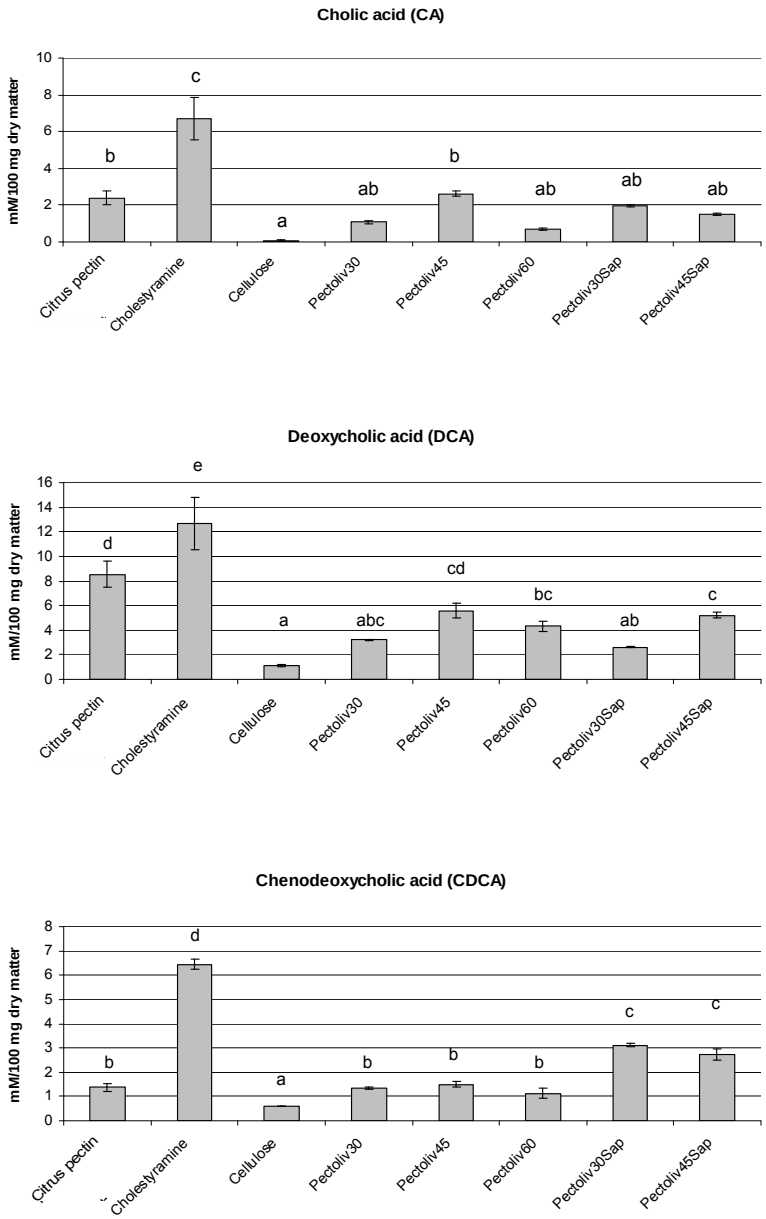


Fig. 3. *In vitro* bile acid binding ability of purified pectin obtained from three different treatments, Pectoliv30, Pectoliv45 and Pectoliv60, non-esterified (saponified) pectin Pectoliv30Sap and Pectoliv45Sap, cellulose, cholestyramine and citrus pectin. Data are presented as the mean ± SD of three replicates. Letters indicate significant differences ($p < 0.05$).

Pectin is one of the dietary fiber components that interact with BA, although its mechanism of interaction with BA is currently not fully understood. The differences in BA binding among the samples tested may be related to their structure or to the presence of other compounds besides pectin substances, such as polyphenols. However, there is no obvious correlation between BA adsorption and the polysaccharide composition, or with phenolic compounds (**Table 2; Fig. 3**). In this work, the influence of methoxyl content (at C6 carboxyl group) or the presence of an acetyl group (at -OH group on the C2 and C3 of every galacturonic acid unit or -OH of other sugars) were also determined. About 3.12 and 2.73 mM/100 mg dry

matter of CDCA (**Fig. 3**) is adsorbed by non-esterified (saponified) Pectoliv30Sap and Pectoliv45Sap, respectively, surpassing even commercial citrus pectin (1.37 mM/100 mg dry matter), which has a low degree of methyl esterification The pectins Pectoliv30 and Pectoliv45 possess a degree of acetylation (DAc) of 48 and 61% (expressed as mol AcOH by mol GalA) or a DAc of 7 and 8% (expressed as g/100 g extract; **Table 4**), that lead to a decrease in their interaction with CDCA of 56.4 and 45.1%, respectively, with respect to non-esterified pectins. These results were similar to those observed by Dongowski (1995), who showed that the acetylation of pectin led to a decreased interaction with bile acids. However a drastic reduction in DM and DAc by saponification did not seem to improve the degree of interaction with CA or DCA (**Fig. 3**), since they were not statistically different.

3.4.3. Glucose dialysis retardation index (GDRI)

For the study of GDRI, the retardation curves of the pectin samples were adjusted by the "square root x" model ($y = a + bx^{-2}$) with $R^2 \geq 0.9626$, except for the control with a lineal model. Using the regression equation for each sample, the different GDRI's at 60 min were calculated and the results are presented in **Fig. 4**. The GDRI is a useful *in vitro* index to predict the effect that fiber has on the delay of glucose absorption in the gastrointestinal tract (Larrauri, Goñi, Martín-Carrón, Rupérez, & Saura-Calixto,1996). The GDRI phenomenon seems to be related to the soluble dietary fiber and uronic acid contents of insoluble fiber, although other authors have pointed to a relationship between the internal structure and surface properties of fiber and glucose diffusion (López et al., 1996).

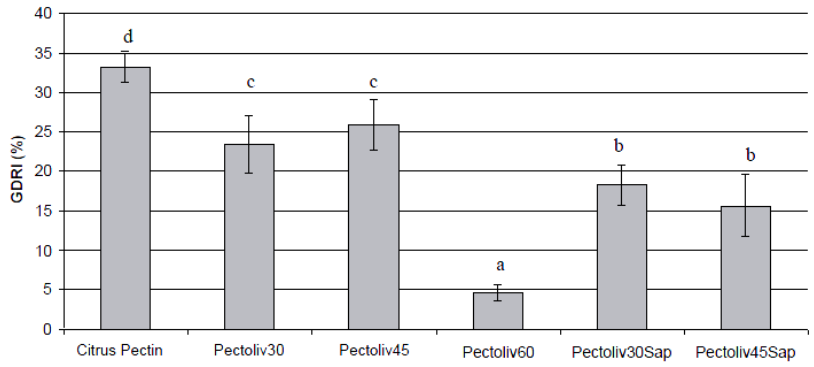


Fig. 4. Glucose dialysis retardation index (GDRI %) of the different samples of pectin. Data are presented as the mean ± SD of two replicates. Letters indicate significantly different results ($p < 0.05$).

The citrus pectin presented the highest value of GDRI (34%), in agreement with the values reported by Larrauri et al. (1996) (34.9%) and Adiotomre, Eastwood, Edwards, and Brydon (1990) (34.0%) for apple pectin. The pectins Pectoliv30 and Pectoliv45 have similar values (~24%), whereas Pectoliv60 shows a low GDRI (~5%). The uronic acid contents of the

first two samples (42.3% and 38.4%) are almost double that of Pectoliv60 (22.2%), and may explain their higher retardation indices. The GDRI of the pectin samples Pectoliv30 and Pectoliv45 after saponification were also determined and found to be even lower. The degrees of acetylation and methylation seem to have some influence on GDRI, because the saponified samples presented a slightly shorter delay in glucose dialysis.

4. Conclusions

Pectins obtained from thermally treated alperujo (160 ºC) and followed by extraction with ethyl acetate, present important physical and biological properties depending on the time of reaction. The low degree of polymerization of all the extracts affects gel formation, however it also makes alperujo a natural source of low molecular weight pectins easily absorbed by the intestinal tract and with potentially beneficial biological activities, such as for the prevention of cancer metastasis.

In comparison with commercial pectins, the alperujo extracted pectins showed low water and high oil holding capacities, a normal emulsifying activity and similar emulsion stability to apple pectin. These characteristics make these pectin extracts suitable for possible use in the food industry, as emulsifiers or stabilizers.

The *in vitro* studies showed an important activity of bile-acid binding of the three pectin extracts, with a higher percentage of binding with DCA than CA and CDCA, and at similar levels to the values obtained for citrus pectins when primary bile acid (CA and CDCA) were assayed. The studies of glucose retardation index for the three pectin extracts showed a high capacity of retention, similar to that observed for citrus pectin. These pectins could therefore have a biomedical application to contribute to reducing serum cholesterol levels and decreasing the risk of bowel cancer. Alternatively, they could be incorporated as low-calorie bulk ingredients into high-fiber foods to lower postprandial serum glucose levels.

Finally, exploiting olive-pomace mill waste (alperujo) for the extraction of pectins for commercial or biomedical use would result in the obtention of high value products and aid the reduction of a highly polluting organic waste.

Acknowledgments

Funding was received from the Ministerio de Economía y Competitividad of Spain and co-funded by European Social Fund (ESF) (project AGL2013-48291-R). F.R.S. received funding from the Spanish JAE-PRE program (CSIC-ESF).

References

Abdul-Hamid, A., & Luan, Y. S. (2000). Functional properties of dietary fibre prepared from defatted rice bran. Food Chemistry, 68, 15-19.

Adiotomre, J., Eastwood, M. A., Edwards, C. A., & Brydon,W. G. (1990). Dietary fibre: *in vitro* methods that anticipate nutrition and metabolic activity in human. American Journal of Clinical Nutrition, 52, 128-134.

AOAC (Official Methods of Analysis). (1990). Food composition (15th ed.). Vol. 1. Arlington, Virginia: AOAC International.

Betancur-Ancona, D., Peraza-Mercado, G., Moguel-Ordoñez, Y., & Fuertes-Blanco, S. (2004). Physicochemical characterization of lima bean (Phaseolus lunatus) and Jack bean (Canavalia ensiformis) fibrous residues. Food Chemistry, 84, 287-295.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. Analytical Biochemistry, 54, 484-489.

Boyd, G. S., Eastwood, M. A., & MacLean, N. (1966). Bile acids in the rat: studies in experimental occlusion of the bile duct. Journal of Lipid Research, 7, 83-93.

Camire, M. E., & Dougherty, M. P. (2003). Raisin dietary fiber composition and *in vitro* bile acid binding. Journal of Agricultural and Food Chemistry, 51, 834-837.

Cardoso, S. M., Coimbra, M. A., & Lopes-da-Silva, J. A. (2003). Calcium-mediated gelation of an olive pomace pectic extract. Carbohydrate Polymers, 52, 125-133.

Coimbra, M. A., Cardoso, S. M., & Lopes-da-Silva, J. A. (2010). Olive pomace, a source for valuable arabinan-rich pectic polysaccharides. Chemistry and Materials Science, 294, 129-141.

Dea, I. C. M., & Madden, J. K. (1986). Acetylated pectic polysaccharides of sugar beet. Food Hydrocolloids, 1, 71-88.

Dische, Z. (1962). Color reactions of carbohydrates. In R. L. Whistler, & M. L.Wolfram (Eds.), Methods in carbohydrates chemistry (pp. 477e512). New York: Academic Press.

Dongowski, G. (1995). Influence of pectin structure on the interaction with bile acids under *in vitro* conditions. Zeitschrift für Lebensmittel-Untersuchung und e Forschung, 201, 390-398.

Dos-Santos, N., Jiménez-Araujo, A., Rodríguez-Arcos, R., & Fernández-Trujillo, J. P. (2011). Cell wall polysaccharides of near-isogenic lines of melon (Cucumis melo L.) and their inbred parental which show differential flesh firmness or physiological behaviour. Journal of Agricultural and Food Chemistry, 59, 7773-7784.

Drzikova, B., Dongowski, G., Gebhardt, E., & Habel, A. (2005). The composition of dietary fibre-rich extrudates from oat affects bile acids binding and fermentation *in vitro*. Food Chemistry, 90, 181-192.

Eastwood, M. A., & Hamilton, D. (1968). Studies of the adsorption of bile salts to non-absorbed components of diet. Biochemical et Biophysica Acta, 152, 165-173.

Englyst, H. N., & Cummings, J. H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as aditol acetates. Analyst, 109, 937-942.

Fernández-Bolaños, J., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Sánchez, P. (2010). Dispositivo y procedimiento para el tratamiento de los subproductos de las obtención del aceite de oliva (Publication Number no. ES2374675).

Fishman, M. L., Chau, H. K., Hoagland, P. D., & Hotchkiss, A. T. (2006). Microwaveassisted extraction of lime pectin. Food Hydrocolloids, 20, 1170-1177.

Fuentes-Alventosa, J. M., Rodríguez-Gutiérrez, G., Jaramillo-Carmona, S., Espejo- Calvo, J. A., Rodríguez-Arcos, R., Fernández-Bolaños, J., et al. (2009). Effect of extraction method on chemical composition and functional characteristics of high dietary fibre powders obtained from asparagus by-products. Food Chemistry, 119, 665-671.

Galanakis, C. A., Tornberg, E., & Gekas, V. (2010). A study of the recovery of the dietary fibres from olive mill wastewater and the gelling ability of the soluble fibre fraction. LWT-Food Science and Technology, 43, 1009-1017.

Gille, S., de Souza, A., Xiong, G., Benz, M., Cheng, K., Scgultink, A., et al. (2011). OAcetylation of Arabidopsis hemicellulose xyloglucan requires AXY4 or AXY4L, proteins with a TBL and DUF231 domain. The Plant Cell, 23, 4041-4053.

Grigelmo-Miguel, N., Gorinstein, S., & Martín-Belloso, O. (1999). Characterization of peach dietary fibre concentrate as a food ingredient. Food Chemistry, 65, 175-181.

Hazendonk, J. M., Reinerink, E. J. M., de Waard, P., & Dam, J. E. G. (1996). Structural analysis of acetylated hemicellulose polysaccharides from fibre flax (*Linun usitatissimum L*). Carbohydrate Research, 291, 141-154.

Jiménez, A., Guillén, R., Fernñandez-Bolaños, J., & Heredia, A. (1994). Cell wall composition of olives. Journal Food Science, 59, 1192-1201.

Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños, J., & Heredia, A. (2001). Olive fruit cell wall degradation of pectic polysaccharides during ripening. Journal of Agricultural and Food Chemistry, 49, 409-415.

Kjeldahl, J. (1983). Neue methode zùr bestimmung des stickstoffs in organichen kòrpen. Zeitschrift für Analytische Chemie, 22, 366-382.

Kim, H. J., & White, P. J. (2010). *In vitro* bile-acid binding and fermentation of high, medium, and low molecular weight β-glucan. Journal of Agricultural and Food Chemistry, 58, 628-634.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. Food Hydrocolloids, 28, 92-104.

Larrauri, J. A., Goñi, I., Martín-Carrón, N., Rupérez, P., & Saura-Calixto, F. (1996). Measurement of health-promoting properties in fruit dietary fibres: antioxidant capacity,

fermentability and glucose retardation index. Journal of Science and Food Agricultural, 71, 515-519.

López, G., Ros, G., Rincón, F., Periago, M. J., Martínez, M. C., & Ortuño, J. (1996). Relationship between physical and hydration properties of soluble and insoluble fiber of artichoke. Journal of Agricultural Food Chemistry, 44, 2773-2778.

Markov, P. A., Popov, S. V., Nikitina, I. R., Ovodova, R. G., & Ovodov, Y. S. (2011). Anti-Inflammatory activity of pectins and their galacturonan backbone. Russian Journal of Bioorganic Chemistry, 37, 817-821.

Morris, V. J., Belshaw, N. J., Waldron, K. W., & Maxwell, E. G. (2013). The bioactivity of modified pectin fragments. Bioactive Carbohydrates and Dietary Fibre, 1, 21-37.

Munarin, F., Tanzi, M. C., & Petrini, P. (2012). Advances in biomedical applications of pectin gels. International Journal of Biological Macromolecules, 51, 681-689.

Nair, P. P. (1988). Role of bile acids and neutral sterols in carcinogenesis. The American Journal of Clinical Nutrition, 48, 768-774.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. Trends in Food Science and Technology, 17, 3-15.

Rubio-Senent, F., Lama-Muñoz, A., Rodríguez-Gutiérrez, G., & Fernández Bolaños, J. (2013). Isolation and identification of phenolic glucosides from thermally treated olive oil byproducts. Journal of Agricultural and Food Chemistry, 61, 1235-1248.

Sila, D. N., Van Buggenhout, S., Duvetter, T., Fraeye, I., De Roeck, A., Van Loey, A., et al. (2009). Pectins in processed fruits and vegetables: Part II-Structurefunction relationships. Comprehensive Reviews in Food Science and Food Safety, 8, 86-104.

Singleton, V. L., & Rossi, J. A., Jr. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 16, 144-158.

Waldron, K. W., & Selvendran, R. R. (1990). Composition of the cell walls of different asparagus (Asparagus officinalis) tissues. Physiologia Plantarum, 80, 568-575.

Zhang, L., Ye, X., Ding, T., Sun, X., Xu, Y., & Liu, D. (2013). Ultrasound effects on the degradation kinetic, structure and rheological properties of apple pectin. Ultrasonics Sonochemistry, 20, 222-231.

6.2.3. Capítulo 11.

Novel pectin present in new olive mill wastewater with similar emulsifiers and better biological properties than citrus pectin.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., García, A., Fernández-Bolaños, J.

La producción de aceite de oliva es una actividad muy importante y tradicional, sobre todo en los países de la zona del Mediterráneo, que representan el 95% de la producción de aceite de oliva de todo el mundo. Los avances tecnológicos en molinos de aceite de oliva han introducido métodos más eficientes de extracción del aceite de oliva, tales como el sistema de centrifugación de dos fases, que permite la extracción del aceite de oliva virgen sin adición de agua, liberándose un subproducto semisólido llamado orujo de dos fases o "alperujo". El alperujo se encuentra enriquecido en compuestos bioactivos de interés, como el hidroxitirosol, un potente antioxidante fenólico natural fácilmente recuperable mediante un proceso industrial patentado por nuestro grupo de investigación.

En este capítulo se estudian los polisacáridos de la pared celular que se solubilizan en la fracción acuosa del alperujo como resultado de la hidrólisis enzimática de la pared celular por enzimas degradantes (pectinasa, hemicelulasa, celulasa) durante periodos de almacenamiento, o por la descomposición química favorecida durante el tratamiento térmico suave sufrido en la orujera (50-80 ºC durante 1-2 horas). Para ello el material péctico disponible es aislado mediante precipitación fraccionada con etanol, y a continuación purificado, pasándose a evaluar una serie de propiedades características de las pectinas. Entre estas propiedades cabe destacar la capacidad de retención de agua y aceite, capacidad de emulsificación, de retención de ácidos biliares, del índice de retardo de la glucosa y actividad antioxidante, obteniéndose resultados diferentes a los obtenidos en el capítulo anterior, donde el alperujo es sometido a un tratamiento térmico fuerte.

Los resultados obtenidos mostraron que en comparación con las pectinas comerciales, presentan alta capacidad de retención de aceite, así como de retención de los ácidos biliares e índice de retardo de la glucosa. Además de una importante actividad antioxidante como consecuencia de su vinculación a compuestos fenólicos, pudiendo actuar como fibra dietética antioxidante, la cual combina los efectos de la fibra y de los antioxidantes, provocando efectos beneficiosos en el colón y previniendo de enfermedades crónicas.

Novel pectin present in new olive mill wastewater with similar emulsifiers and better biological properties than citrus pectin.

Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Aranzazu García, Juan Fernández-Bolaños.

ABSTRACT

Today alperujo from the olive oil industry was subjected a new treatment industrial that consisting in a gentle heating at 50-80 °C for 1-2 hours, following by a new three-phase centrifugation system that gives alperujo oil, olive pomace and aqueous by-products. This process is a prerequisite to reduce humidity of solid to allow its use as biomass, and allows recovering a new olive mill wastewater, which was used in this work for isolate water-soluble polysaccharides. Two polysaccharide-enriched extracts were obtained by ethyl alcohol precipitation with 40 and 80% (v/v) EtOH from the new by-product. These extracts were characterized and the polysaccharides were purified. The pectin material presented high molecular size and low percentage of methyl esterification and acetylation. In comparison with commercial pectins, the extracts had better oil holding capacity and similar emulsifying activity that of citrus pectin. For first time bile-acid binding and glucose retardation activity were considered for pectin from oive by-product, obtaining better results than those observed for commercials pectins. In addition, antioxidant activities of these extracts were investigated using various *in vitro* assay systems. The two raw polysaccharides extracts rich in associated polyphenol compounds exhibited the strongest scavenging activity against DPPH and ABTS radicals and reducing power. Thus, polysaccharide-enriched extracts have suitable emulsifiers properties for commercial uses and important biological properties, better than that observed for citrus pectin and apple pectin

KEYWORDS: Alperujo; Pectin; Emulsifier; Glucose retardation index; Bile acid binding; dietary fiber antioxidant.

1. Introduction.

Olive oil production is a very important and traditional activity, particularly in the countries of the Mediterranean area, which account for 95% of worldwide olive oil production. Technological advances in olive oil mills have introduced more efficient methods of olive oil extraction, such as the two-phase centrifugation system, which allows the extraction of virgin

olive oil without a water addition. This extraction system is widespread in Spain (accounting for 90% of production) and many other olive oil producing countries. Besides olive oil, the process releases a semisolid by-product called two-phase pomace or “alperujo”, with over 4 millions of tonnes generated annually in Spain. This paste is usually used for the extraction of olive pomace oil with hexane after drying, or by centrifugation without drying. The change from the three phase extraction system to a two phase one results in a very wet pomace (with 60-70% of moisture) due to retention of water from the fruit in the residue.

Currently, the alperujo by-product is treated to recover the maximum amount of oil, using the final solid as biomass. Due to the considerable energy required for the drying process, an alternative process that helps to reduce the moisture is being implemented at an industrial level. This process consist in a gentle heating at 50-80 ° C for 1-2 hours, following by a new three-phase centrifugation system that gives alperujo oil, olive pomace and aqueous by-products. This process is a prerequisite to reduce humidity of solid to allow its use as biomass, and allows recovering a new olive mill wastewater, enriched in bioactive components of interest, with a nature and varied composition, which can be extracted and used for industrial purposes. The treatment realized in this work is considered as gentle heating in comparison with other hydrothermal treatment applied over alperujo in previous work, which used temperatures of 160 ºC during different times (30-60 min) studied (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2015). Therefore the by-product used in this work is a new raw material.

In this study we considered the cell wall polysaccharides and associated phenolic compounds that are solubilized in the aqueous fraction after gentle heating, which come from the degradation of cell wall material during alperujo storage due to enzymatic activities (as a result of degrading enzymes like pectinase, hemicellulase, cellulase) before to the treatment, or are solubilized due that this thermal treatment enhances the enzymatic activity and/or the chemical breakdown. The presence of phenolic compounds in the olive cell wall is scant (Mafra, Barros, & Coimbra, 2006) although the interaction between polysaccharides and the hydrophilic compounds (phenols, proteins, etc) during crushing and malaxation of the olive paste before oil extraction, with oxidation, condensation or polymerization reaction, provide an important non-carbohydrate polymeric material associated with the cell wall polysaccharides (Obied, Allen, Bedgood, Prenzler, & Robards, 2005)..

Some studies have reported the extraction of pectins from olive by-products such as olive mill wastewater from traditional three-phase system without mild heating (Galanakis, Tornberg, & Gekas, 2010) or two phase olive pomace (Cardoso, Coimbra, & Lopes-da-Silva, 2003). In other hand, a previous work realized in our lab (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2015) considered the pectin material isolated from alperujo hydrothermally treated (160 ºC during 30, 45 or 60 min), this treatment favors the

recovering of pectins with low molecular weigh and high degree of esterification, these properties causes that the materials studied present different and characteristic properties.

Pectins are natural hydrocolloids that are found in higher plants as principal structural elements of cell walls, and have been widely used as gelling agents, stabilizers, and emulsifiers in the food industry. One of the most important properties of pectin material is its emulsificaction activity due to their important industrial applications. This property measured their potential to produce fine oil-in-water droplets and their capacity to keep the emulsion droplets small for a substantial period of time. Emulsification and emulsion stabilization, should involve the interfacial absorption of all or of a part of the components making up the extracts, as to provide a stabilizing layer which would protect against droplet coalescense via electrostatic and/or steric interactions (Ritzoulis, et al., 2014). For study this property the emulsification activity (EA) of material is measured, considering that a material can be considered as good emulsifiers when their EA values are higher than 50% (Abdul-Hamid & Luan, 2000).

The polysaccharides are the main component of dietary fiber and play a significant role in many physiological processes and in the prevention of several diseases (Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006). In addition, the antioxidant capacity of associated phenolic compounds can produce a variety of beneficial effects (Perez-Jiménez et al., 2008). Saura-Calixto (1998) defined antioxidant dietary fiber as a natural product that combines the beneficial effects of dietary fiber and natural antioxidants, and as such, polyphenolic compounds can be considered to fit these criteria. Polysaccharides from food plants are reported to possess important bioactive functions, including hypoglycemic and hypolipidic activities. Water-soluble dietary fibers hamper the diffusion and absorption of glucose, thus resulting in lower postprandial blood glucose (Ou, Kwok, Li, & Fu, 2001). Also, dietary fiber can bind bile acids, thereby aiding their excretion in the feces hence reduces the risk of cardiovascular disease and therefore lower the risk of bowel cancer (Eastwood & Hamilton, 1968; Camire & Dougherty, 2003).

In this work the functional properties of the new olive by-product, such as water and oil holding capacities and emulsion properties were evaluated and the result were compared with commercial pectin from apple and citrus. Furthermore *in vitro* assays like bile-acid binding, glucose retardation capacity and antioxidant activity of the polysaccharidic material obtained were evaluated for first time.

2. Materials and methods.

2.1. Olive material.

The aqueous effluent for the polysaccharide purification was supplied by Subproductos Vegetales del Mediterráneo (SVM) S. L., Seville (Spain). The SVM factory receives aqueous by-product from alperujo oil extraction from several local pomace processing mills for the purification of hydroxytyrosol. The wastewater is a dark liquid with a high hydroxytyrosol content, which is obtained from stored alperujo and kneaded at 50-80º C for 1-2 h using a three-phase centrifugation system.

2.2. Chemicals.

Pectin from citrus; cholestyramine and three bile acids (cholic, deoxycholic, and chenodeoxycholic acid) were purchased from Sigma-Aldrich (St Louis, MO, USA). Apple pectin was purchased from F.E.R.O.S.A. (Barcelona, Spain). The standards of Dextran from Leuconostoc ssp 250, 110, 70, 40 and 6 kDa, were obtained from Fluka Bio Chemika (Switzerland).

2.3. Isolation of alcohol insoluble extracts enriched in polysaccharides.

100 L of percolate from the recovery of hydroxytyrosol was ultra-filtered at room temperature, using a semi-industrial system with a cartridge of 3 kDa ceramic membrane HUF-L1-500 mm. The filtration was conducted using a pump. The permeate was removed at at constant cross-flow velocity and the concentrate was recycled back into the feed tank until a volume of 13.8 L was recovered. This fraction, with a size greater than 3 kDa, was submitted to precipitation with 40% (v/v) ethanol: water, causing the precipitation of polysaccharide-enriched extracts with a high molecular weight between 0 and 40% of EtOH. The resulting precipitate named alcohol insoluble extract at EtOH 40 % (AIE40) was removed by centrifugation at 4700 g (Comteifa, S.L., Barcelona, Spain). EtOH was added to the remaining liquid to obtain an 80% (v/v) ethanol solution. An insoluble material was obtaining between 40 and 80% EtOH, facilitating the precipitation of lower molecular weight polysaccharide-enriched extracts, named alcohol insoluble extract at EtOH 80 % (AIE80).

2.4. Purification of raw pectin

The two samples obtained from the fractional precipitation with ethanol were purified by ethylenediaminetetraacetic acid (EDTA) complexation and dialysis through 12 kDa membrane, followed by percolation through a strong ionic exchanger resin, according to the procedure described by Cardoso, Coimbra & Lopes da Silva, (2003). The purified extracts were referred to as Alpect40 and Alpect80.

2.5. Polysaccharide analysis.

Galacturonan (anhydrogalacturonic acid) was estimated according to the m-hydroxydiphenyl method described by Blumenkrantz & Asboe-Hansen (1973) for uronic acids.

Glycosyl compositions were determined by gas chromatography (GC) after their conversion to alditol acetates, and quantified as alditol acetates. Individual neutral sugars were analyzed from duplicate samples with initial TFA hydrolysis (2 N TFA at 121 ºC for 1 h) prior to reduction, acetylation and analysis by GC (Englyst & Cummings, 1984). Inositol was used as an internal standard. Calibration was performed with a series of standard sugar solutions of L-rhamnose (Rha), L-fucose (Fuc), L-arabinose (Ara), D-galactose (Gal), D-glucose (Glc), D-mannose (Man) and D-xylose (Xyl). Chromatographic conditions utilized were described by Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent & Fernández-Bolaños (2012).

The total neutral sugars were determined colorimetrically according to the anthrone-sulfuric acid assay of Dische (1962), in triplicate and using glucose as a standard.

2.6. Determination of the total phenolic, protein and ash contents.

Total phenolic content was determined by the Folin−Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents (Singleton & Rossi, 1965). Protein was determined by the micro Kjeldahl method using the multiplication of the total nitrogen content by a conversion factor of 6.25 (Banks-Gibson, 1904). The analysis of ash was performed according to the AOAC procedure (AOAC, 1990).

2.7. High-Performance Size Exclusion Chromatography (HPSEC). Molecular Weight (MW) distribution of the purified polysaccharide-enriched extracts.

HPSEC was performed as described by Dos-Santos, Jiménez-Araujo, Rodríguez-Arcos, & Fernández-Trujillo (2011). The MW was measured using Jasco equipment (LC-Net II ADC, Kyoto, Japan) with a refractive index detector (Jasco RI-1530) and two different columns (300 X 7.8 mm i.d., Tosoh Bioscience LLC, King of Prussia, PA) in sequence TSKgel GMPWXL (dextran MW<50000KDa) and TSKgel G3000PWXL (dextran MW<60KDa). The system was calibrated with dextrans of, 252, 110, 70, 40, 6 kDa and glucose. Fractions of 250 µL were collected using a Redifrac fraction collector (Pharmacia Biotech, Uppsala, Sweden). The uronic acid and total sugar content of the fractions were determined by measuring absorbance at 520 and 620 nm respectively, in an iMark^{TM} microplate absorbance reader (Bio-Rad, Hercules, CA, USA).

2.8. Ion exchange chromatography.

The method used was described by Jiménez, Rodríguez, Fernández-Caro, Guillén, Fernández-Bolaños, & Heredia (2001) with some modifications. 5 mL Hi-Trap Columns (anionic exchanger: Q-Sepharose) from Pharmacia (Uppsala, Sweden) were used. The column was

equilibrated in a 0.01 M imidazole-HCl buffer, pH 7. The flow during injection was 1mL/min. Two steps in the fractionation were made with increasing buffer concentration of imidazole-HCl from 0.01 to 2.0 M, pH 7. In the first step, a flow of 1 mL/min was employed and neutral oligosaccharides were collected in fractions of 2 mL. In the second step, a flow of 5 mL/min was employed and acidic oligosaccharides were collected in fractions of 5 mL. All fractions were assayed for uronic acids and neutral sugars according to the colorimetric method.

2.9. Degree of methylesterification (DM) and acetylation (DAc).

The determination of the DM and DAc of the pectic polysaccharides was based on the estimated methanol and acetic acid contents released by saponification with 2M NaOH at 20 ºC for 60 min (Waldron & Seveldran, 1990). The amount of methanol released was measured enzymatically using alcohol oxidase and a spectrophotometric method, as described previously by Galanakis, Tornberg, & Gekas, (2010) and expressed as % (mol MeOH/100 mol GalA). The amount of acetic acid released after saponification was determined by HPLC (Hewlett Packard Serie 1100) on an Aminex HPX-87H (300 X 7.8 mm, Bio-Rad), according to Cardoso, Coimbra & Lopes da Silva, (2003), and expressed as % (mol AcOH/100 mol GalA). The samples were analyzed in duplicate for both methods, and compared to the values obtained for three commercial pectins with different degrees of esterification. For pectin from citrus with degrees of esterification of 89, 55-70 and 22%, the results obtained were 86, 53 and 28%, respectively.

2.10. Functional properties.

2.10.1. Water/Oil-holding capacity (WHC/OHC).

These properties were determined by centrifugation using the method described by Fuentes-Alventosa et al. (2009). 400 µL of water was mixed with 40 mg of sample in triplicate. After 5 h of stirring at room temperature, the mixture was centrifuged at 5000 g for 15 min in a Sorvall RT 6000D (DuPont, Nemours, Mechelen, Belgium). The supernatant was carefully separated and weighed. The WHC result was expressed as g water retained/g sample. The oil-holding capacity (OHC) was determined under the same conditions as WHC using soybean oil (0.92 g/mL density), and was expressed as g oil retained/g sample.

2.10.2. Emulsification activities (EA) and emulsion stability (ES).

Emulsification activities and emulsion stability were evaluated in duplicate using the method of Betancur-Ancona, Peraza-Mercado, Moguel-Ordoñez, & Fuertes-Blanco (2004). EA was expressed as a percentage of the volume of the emulsified layer with respect to the volume of the entire layer in the centrifuge tube. ES was determined using the prepared emulsions and heated at 80 ºC for 30 min. The emulsion was cooled to room temperature and centrifuged at 1200 g for 5 min in a Sorvall RT 6000D (DuPont, Nemours, Mechelen, Belgium). ES was expressed as the percentage of the remaining emulsified layer volume of the original emulsion

volume. The stability of the emulsions was also determined by letting them stand for 36 h at 4 ºC, as described by Dea & Madden (1986).

2.10.3. Binding of bile acid (BA).

The capacity of pectins to bind BA *in vitro* was evaluated following the method described by Camire & Dougherty (2003) with some modifications (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2015). The change in the BA concentration upon exposure to the pectin solution during an *in vitro* digestion process, which included an acidic digestion of the sample at pH 2 followed by pancreatine digestion, was used to estimate the binding of BA. Individual BAs such as cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) (Sigma, St. Louis, MO) were selected for analysis. The results obtained for each sample of isolated pectin were compared with the results obtained for Cholestyramine resin (Sigma C 4650), cellulose (Macherey Nagel, GmbH & Co. KG) and commercial pectin from citrus peel (Sigma P 9135). BA was analyzed by the modified Pettenkofer colorimetric method described by Boyd, Eastwood & MacLean (1966). Readings were made at the absorption maximum of 490 nm in an iMark^{TM} microplate absorbance reader (Bio-Rad, Hercules, CA, USA) and the values were determined from the obtained standard curve. The ranges for the different standards were 0-30 µg for CA, 0-750 µg for DCA and 0-750 µg for CDCA. Binding capacity was expressed as mM bile acid/100 mg of pectin and was calculated from the decrease in BA concentration (mM) in the test solution after exposure to 100 mg of dry pectin.

2.10.4. Glucose dialysis retardation index (GDRI)

GDRI was determined as described by Fuentes-Alventosa et al., (2009). The glucose concentration was determined spectrophotometrically according to the anthrone method (Dische, 1962). GDRI was calculated as follows:

$$GDRI = 100 - \left(\frac{total\ glucose\ diffused\ sample}{total\ glucose\ diffused\ control}\ x\ 100 \right)$$

2.11. Antioxidant activity.

2.11.1. Antiradical activity: 2,2-diphenyl-1-picrylhydrazyl (DPPH).

The free radical-scavenging capacity was measured using the DPPH method described in a previous study (Rodríguez et al., 2005) and expressed as EC_{50} (effective concentration, mg/mL), calculated from a calibration curve using linear regression for each antioxidant.

2.11.2. Antiradical activity: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS).

The ABTS assay was performed according to the method of Gonçalves, Falco, Moutinho-Pereira, Bacelar, Peixoto, & Correia (2009) with some modifications described in a previous work (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012). The

results were expressed in terms of the Trolox equivalent antioxidant capacity (μmol trolox/g of dry sample).

2.11.3. Reducing power

The reducing power assay was performed according to the procedure described in a previous study (Rodríguez et al., 2005). The assay was calibrated using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and the results were expressed in μmol trolox/g of dry sample.

2.12. Statistical analysis.

Results were expressed as mean values ± standard deviations. Sample comparison by Multivariate analysis of variance (ANOVA), followed by Duncan's comparison test (Statgraphics Plus program version 2.1), was used to assess the differences in the composition and functional characteristics among the different isolated pectins. The level of significance used was $p < 0.05$.

3. Results and discussion.

3.1. Purification and chemical characterization of polysaccharide-enriched extracts.

The new industrial liquid effluent obtained from the recovery of hydroxytyrosol from the liquid by-product obtained in the extraction process of alperujo gently heat treated (50-80 ºC, 1-2 h) has never been characterized. In this study, the polymers with a molecular size greater than 3 kDa were obtained by ultrafiltration through a membrane of 3 kDa, followed by precipitation with increasing concentrations of ethanol (40% and 80% ethanol (v/v), **Figure 1**). The alcohol insoluble extracts (AIE40 and AIE80) were characterized (**Table 1**) and represent 918 and 258 g of extract per 100 L of the starting material, respectively. The proportion of total compounds analyzed in both AIE was very low (58% and 35%). This may be due to polymeric polyphenols or polyphenols linked to cell wall constituent (protein and polysaccharides) that are undetectable (Obied, Allen, Bedgood, Prenzler, & Robards, 2005); however the presence of 10 and 8%, respectively, of detectable total phenolic compound, might show some interesting antioxidant activity, as will be discussed presently. The total sugar content (uronic acid and neutral sugar) was 27.7% in the first and 16.4% in the second extract, which represent 48 and 46% of the total of known compounds respectively, and were mainly composed of GalA (32 and 48%) and Ara (24 and 17%), with minor proportions of Glc (15 and 11%), Gal (9 and 10%), Rha (10 and 7%) and Man (7 and 5%). The composition of both extracts suggests the presence of pectic polysaccharides rich in arabinose, compatible with those reported for olive pomace by Cardoso, Coimbra & Lopes da Silva, (2003) and for olive mill wastewater (Galanakis, Tornberg, & Gekas, 2010), together with other polysaccharides of different nature (such as arabinoxylans,

glucomannan, and xyloglucan) described as constituents of the olive pulp (Jiménez, Guillén, Fernández-Bolaños, & Heredia, 1994).

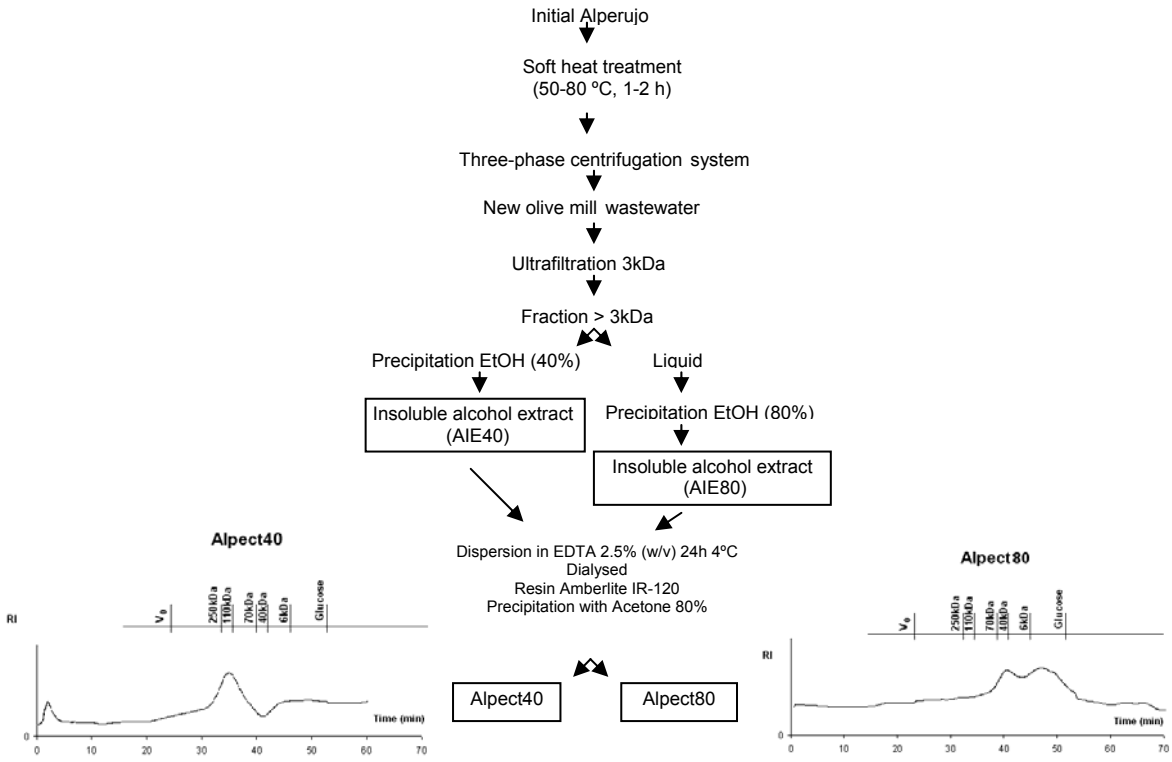


Figure 1. Scheme of recovery and purification for obtaining purified pectin from alperujo. Refractive index elution profiles obtained by HPSEC with two column TSK gel (GMPWXL and G3000PWXL) placed in a series from the purified pectin material obtained from alperujo for Alpect40 and Alpect80.

The alcohol precipitated extracts enriched for large (AIE40) and small molecular weight (AIE80) polysaccharides were partially purified according to the procedure described by Cardoso, Coimbra & Lopes da Silva, (2003), to give the purified extracts referred to as Alpect40 and Alpect80, respectively (**Figure 1**). The purification process caused an increase in the percentage of known compounds from 58% to 90% and from 35% to 73%, respectively, with an important decrease of phenols and ash (**Table 1**). With regard to protein content, Alpect40 showed a higher value (18%) compared to Alpect80 (8%). It is also worth noting the important increase of total sugar in both extracts (2 and 3 fold, respectively), with considerable quantities of total sugar (66.4% and 58.4% following purification), and with different ratios between neutral sugar and acids, similar to those obtained for pectic material isolated and purified from olive pomace by Vierhuis, Schols, Beldman, & Voragen, (2000) and Cardoso, Coimbra & Lopes da Silva, (2003). This purification process reduced the proportion of GalA in relation to the neutral sugar content in the pectic extracts, which were mostly composed of Ara (34 and 32 %), GalA (18 and 24 %), Gal (16 and 17 %), and Glc (16 and 9 %) plus Rha (11 and 7%) and Man (4 and 7 %), respectively.

Table 1: Chemical composition of alcohol insoluble extract (AIE; initial sample precipitated with EtOH 40% and EtOH 80 %) and the purified extracts of olive by-product (alperujo) (Alpect40 and Alpect80).

	AIE40	Alpect40	AIE80	Alpect80
g/100 L alperujo	918		258	
% purified extract[a]		32.9		36.2
	Composition (g/100g extract)			
Uronic acid	8.91±0.76	16.93±0.92	7.64±0.39	14.26±0.83
Neutral sugar	18.83±1.37	49.51±2.27	8.75±0.29	44.28±2.49
Phenol	10.25±0.65	5.22±0.30	7.87±1.16	5.01±0.28
Protein	11.26±0.88	17.94±0.33	5.75±0.62	8.41±0.97
Ash	8.41±0.72	0.37±0.02	5.42±0.47	0.63±0.05
Total	57.66±4.38	89.97±3.84	35.43±2.93	72.59±4.62
Total sugars [b]	27.74	66.44	16.39	58.54
	% molar			
Galacturonic acid	32.25±2.75	18.12±0.97	47.70±2.33	24.41±1.42
Rhamnose	9.56±0.61	11.06±0.74	7.04±0.40	7.33±0.93
Fucose	1.13±0.12	0.03±0.01	0.49±0.08	0.41±0.32
Arabinose	24.44±0.89	34.42±0.38	17.34±0.67	32.25±6.26
Xylose	1.42±0.01	n.d.	1.47±0.14	3.42±0.42
Mannose	6.76±1.30	4.27±0.10	4.75±0.29	6.54±0.74
Galactose	9.09±1.72	15.69±0.85	9.98±0.85	16.50±1.37
Glucose	15.32±0.31	16.41±0.34	11.24±2.01	9.14±1.11

[a] Percentage of purified pectin obtained from AIE. [b]Sum of the uronic acids and neutral sugars. n.d. Not detected.

The composition of pectin material isolated in this work is different that the composition obtained in a previous work (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2015), in which pectin materials were isolate from alperujo hydrothermally treated at high pressures and temperatures and later extraction with ethyl acetate (160 ºC, 30, 45 and 60 min). This severe condition causes numerous modifications in the raw material that generates a pectin material with different composition that the pectin material studied in this article from alperujo who has suffered a mild heat treatment.

In order to determine whether these neutral sugars were unbound fractions, present as separate polysaccharides, or were covalently linked with the galacturonan backbone, the two purified extracts, Alpect40 and Alpect80, were fractionated by anion exchange chromatography on Q-Sepharose (**Table 2**). The non-retained material (neutral polysaccharides) eluted with 0.01 M imidazole buffer solution (Fraction I), represented 10.6 and 11.7% of total sugars introduced for each sample; the retained fractions (acidic polysaccharides) eluted with 2.0 M imidazole buffer (Fraction II), represented 89.4 and 88.4 % of the total sugar introduced, respectively. The proportion of acidic sugar obtained in Fraction II was considerably higher than for Fraction I, and so it would appear that an important proportion of neutral sugar moieties were linked to the acidic pectin backbone. The major fraction, Fraction II, could be considered as soluble polysaccharides composed of pectic material, thus the two purified extracts (Alpect40 and Alpect80) consist mainly of pectin and other soluble fiber in minor proportions. Fraction I from the two purified extracts was very rich in Ara and Gal, with large proportions of Glc and Man (**Table 2**), indicating the presence of neutral polysaccharides, which could probably be

considered as separate polysaccharides or a co-extract. The analysis of Fraction II from these purified extracts indicated that the major neutral sugars were Ara, Gal and Man, followed by Glc and Rha. Therefore, it can be suggested that these polysaccharides recovered in 2.0 M of imidazole buffer eluates would appear to contain neutral sugars that are covalently linked to the acidic sugar chain, probably to give a new or atypical pectic material composed of heterogeneous polysaccharides with different structure and composition of pectin isolated from olive by-products, as described by Cardoso, Coimbra & Lopes da Silva, (2003) and Galanakis, Tornberg & Gekas, (2010). A random chemical or enzymatic hydrolysis of cell wall polymeric material from alperujo during the industrial process of pomace olive oil extraction from alperujo could explain the structural features of the pectic material solubilized.

Table 2. Distribution of the total sugars and glycosil composition of the two purified pectin extracts (Alpect40 and Alpect80) eluted by ion exchange chromatography (Neutral Fraction I, and acidic Fraction II) on Q-Sepharose.

	Alpect40		Alpect80	
	Fraction I[a]	Fraction II[b]	Fraction I	Fraction II
	% Distribution of the total sugar applied			
Total sugars	10.6±0.7	89.4±3.1	11.7±0.8	88.4±2.3
	% Distribution of composition in the elution			
Neutral sugar	90.6±0.6	51.8±2.2	93.8±4.0	63.6±1.4
Uronic acid	9.4±1.1	48.2±1.0	6.2±0.1	36.4±1.1
	% molar			
Uronic acid	9.4±1.9	48.2±5.9	6.2±2.7	36.4±1.3
Rhamnose	4.7±0.1	5.3±0.1	3.2±0.5	6.0±0.5
Fucose		0.2±0.1	0.7±0.1	0.1±0.1
Arabinose	34.4±5.2	17.9±1.9	23.9±0.6	23.8±2.1
Xylose	0.9±0.1	1.2±0.3	1.4±0.1	0.9±0.1
Mannose	11.4±0.8	13.2±3.0	9.9±1.9	11.3±1.1
Galactose	25.1±1.6	7.4±0.4	42.7±0.2	14.4±1.2
Glucose	14.1±1.3	6.7±0.7	11.9±1.0	7.2±0.6

[a]Neutral fraction eluted with 0.01M imidazole buffer and collected as Fraction I. [b]Acidic fraction eluted with 2.0M imidazole buffer and collected as Fraction II.

3.2. Determination of molecular size distribution, degrees of methylation (DM) and acetylation (DAc).

The profiles of refractive index (RI) for the two purified pectin extracts obtained for both precipitations (Alpect40 and Alpect80) following High-Performance Size exclusion Chromatography (HPSC) on two columns with TSK gel (GMPWXL and G3000PWXL) placed in a series are displayed in **Figure 1.**

The result showed that the maximum RI obtained for each extract coincides with ethyl alcohol (EtOH) precipitation and their molecular size distribution. Alpect40 revealed a broad molecular size with retention time between 30-40 min with a maximum peak at 34 min, which corresponded to an estimated molecular weight of 110 kDa. For Alpect80, the retention time was between 38-56 min with two maximums observed at 44 and 47 min, indicating that the

average size of this pectic material was around 70 kDa-180Da (Glucose), with a maximum value at 40 kDa and 6 kDa. Furthermore, these polymers appear to be composed of varying amounts of an identifiable macromolecular population, which match in amounts of uronic acid and neutral sugar, and profile of refraction index (RI). Thus, the molecular distribution observed in the pectin extracts studied seems to confirm that the majority of neutral sugars are linked to the acidic pectin backbone (data not shown).

The degree of methylation (DM) corresponds to the percentage of carboxyl groups esterified with methanol at the C-6. The degree of acetylation (DAc) is defined as the percentage of galacturonic acid units esterified with acetic acid, assuming that only the hydroxyl groups at the C-2 and/or C-3 of the carbohydrate ring are acetylated. The DM and DAc results obtained for the two samples of purified pectin material are shown in **Table 3**. The DM observed for both samples were low, Alpect40 had a DM of 24%, while Alpect80 had a DM of 2.6%, so the pectins can be classified as low methoxylated pectin (LM). These results are lower than the contents of DM in the pectin material extracted from mill wastewater, which presented a minimum DM of 59% (Galanakis, Tornberg & Gekas, 2010). On the other hand, Alpect40 had a DAc of 8.6% and this result was proximate to the value obtained for pectins isolated from olive pomace residue (DAc of 11%; Cardoso, Coimbra & Lopes da Silva, 2003),although DAc was not detected for Alpect80. The degree of esterification (DE), equating to the sum of the DM and DAc results, was 33% for Alpect40 and 2.6% for Alpect80. The DE result for Alpect40 is similar to that observed in the pectin material extracted from the residue of olive pomace, which had a DE between 31 and 43% (Cardoso, Coimbra & Lopes da Silva, 2003). Therefore, we suggest that the precipitate obtained with 40% EtOH provides a characteristic pectic material from alperujo, whereas precipitation performed with 80% EtOH yields a pectic material with very different characteristics to those recorded in the literature.

Table 3: Degree of acetylation (DAc) and methylation (DM) and functional properties of the purified pectin material (Alpect40 and Alpect80) compared with two commercial pectins (citrus and apple pectin): water holding capacity (WHC), oil holding capacity (OHC), emulsification activity (EA), stability of the emulsion (SE) and glucose degree retention index (GDRI).

	DAc[a]	DM[b]	DE[c]	WHC	OHC	EA	SE	GDRI
	%	%	%	g water holding/g sample	g oil holding/g sample	Emulsion volumen/total volumen	%	%
Alpect40	8.59±3,15	24.13±0.36	32.72±3.51	3.00±0.22	4.73±0.06	67.76±1.55	67.02±2.86	44.40±3.34
Alpect80	n.d	2.56±0.13	2.56±0.13	2.18±2.18	1.48±0.17	47.91±4.12	n.d.	29.33±3.17
Citrus Pectin	n.d	34.68±1.41	34.68±1.41	10.35±0.27	2.59±0.19	64.58±2.95	100.00±0.01	33.18±1.88
Apple Pectin	n.d	6.08±0.87	6.08±0.87	2.09±0.07	2.22±0.05	54.31±6.10	21.14±3.38	-[d]

Results expressed in [a](mol AcOH/mol Ac Gal)·100, [b](mol MeOH/mol Ac Gal)·100. [c]DAc+DM. [d]assay not realized. n.d. not detected.

In this case the soft treatment affect in small measure the pectin size and esterification degree, allowing us to recover material with high molecular size when the precipitation is carried out with EtOH 40%, and low degree of esterification in both cases studied (Alpect40 and Alpect80). These results differ with those obtained in a previous article (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fenández-Bolaños, 2015) in which the pectin material were isolated from alperujo hydrothermally treated at 160 ºC.

3.3. Functional properties.

3.3.1. Water holding capacity (WHC) and oil holding capacity (OHC). Emulsification activity (EA) and stability of the emulsion (SE).

The WHC and the OHC are the abilities of a material to retain water and oil after centrifugation, respectively (Fuentes-Alventosa et al., 2009). WHC is the ability of dietary fiber, related to soluble fiber, to absorb and retain water to form a viscous solution. Commercial citrus pectin significantly showed the highest WHC (10.4 g water/g sample; **Table 3**). The low WHC of the purified pectin material obtained, 3.0 and 2.2 g of water/g of sample, could be attributed to the relatively low contents of galacturonic acid. However, these values are comparable to the WHC obtained for commercial apple pectin (2.1 g water/g sample), which had a high content of galacturonic acid (98%). The main explanation may be a difference in the composition and the structure of these polymers.

OHC is another functional property of some ingredients used in formulated food. The purified extract Alpect40 presented a higher OHC value than the commercial pectins (4.7 compared to 2.6 and 2.2 g oil holding/g sample for citrus and apple pectin, respectively). The mechanism of oil adsorption is not well understood although high OHC has been associated with a high soluble dietary fiber content (Betancourt et al., 2004). Ingredients with a high OHC allow for the stabilization of high fat food products and emulsions, facilitating the solubilization or dispersion of two immiscible liquids (Grigelmo-Miguel, Gorinstein, & Martín-Belloso, 1999). This result shows that the new pectin materials isolated from alperujo who has suffered a mild heat treatment may have important applications in different sectors of the food industry as an emulsifier and can even act as a replacement for citrus pectin due its natural origin, an this material is easily removable from a by-product of the olive oil industry and recovery of phenol. Subsequently, we studied the emulsification activity (EA) and the stability of the emulsion (SE) in order to evaluate the functionality of the pectin samples extracted from alperujo.

The isolated polymer, Alpect40, together with the citrus pectin were found to have the highest EA of 67.8 and 64.6%, respectively (**Table 3**). The Alpect80 had a value similar to apple pectin, of 47.9 and 54.3%, respectively. These results show that polymers obtained from olive oil and hydroxytyrosol by-products can be considered as good emulsifiers, as their EA values

were proximate to or higher than 50% (Abdul-Hamid & Luan, 2000). However, only citrus pectin and Alpect40 showed good emulsification stability after heating the emulsion at 80 ºC for 30 min (100 and 67%, respectively). Nevertheless, the stability of the emulsions was also determined by standing for 24, 36 and 48 h at 4 ºC (Dea & Madden, 1986). Any separated oil layer was visible in the emulsions containing the pectin extracts for at least 48 h and therefore both Alpect40 and Alpect80 can be considered as 100% stable.

3.3.2. Glucose dialysis retardation index (GDRI).

The GDRI is a useful *in vitro* index to predict the effect that fiber has on the delay of glucose absorption in the gastrointestinal tract (Larrauri, Goñi, Martín-Carrión, Rupérez, & Saura-Calixto, 1996), this assay is realized for first time for these pectin material obtained from alperujo who has suffered a gentle warming. Alpect40 presented the highest GDRI value (44.4%), followed by citrus pectin with 33.2%, whereas the Alpect80 presented a slightly shorter delay in glucose dialysis with a value of 29.3% (**Table 3**). The citrus pectin GDRI value is in agreement with the values reported by Larrauri et al., (1996) and Adiotomre, Eastwood, Edwards, & Brydon, (1990), determined as 34.9 and 34.0% for apple pectin, respectively. High glucose retention by the pectin-enriched polysaccharide purified from alperujo may involve a favored binding or entrapping of the glucose molecules by hydrophilic groups of the polysaccharide network (Escalada Pla, Ponce, Stortz, Gerschenson, & Rojas, 2007), although other authors have pointed to a relationship between the internal structure and surface properties of fiber and glucose diffusion (López, Ros, Rincón, Periago, Martínez, & Ortuño, 1996).

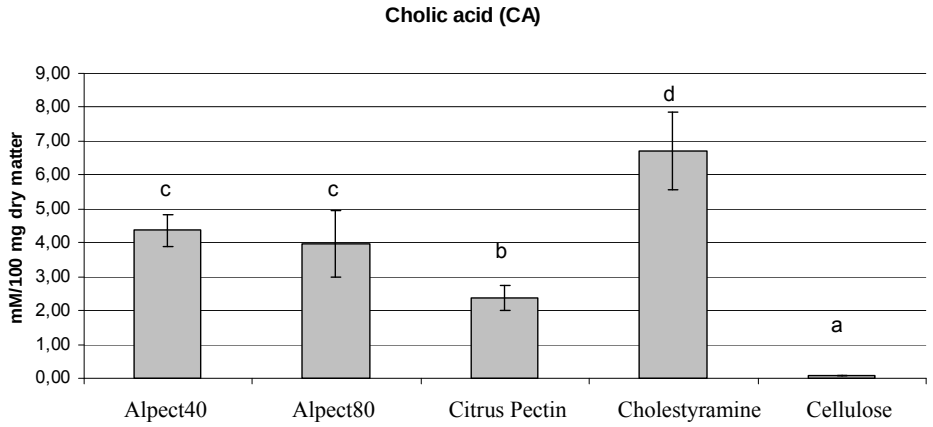
In this study the pectin studied with a higher molecular weight (Alpect40) has a higher glucose dialysis retardation index than commercial citrus pectin, and that pectin with lower molecular weight studied (Alpect80).

3.3.3. In vitro bile acid (BA) binding.

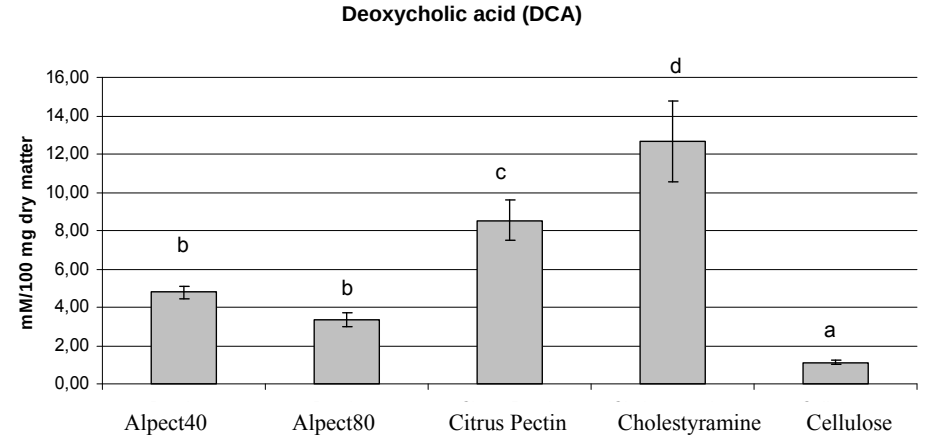
Three of the major BAs present in human bile were individually used for these binding studies. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary BAs secreted by the liver. Deoxycholic acid (DCA) is one of the secondary BAs formed through the transformation of primary acid by intestinal bacteria. The *in vitro* digestion procedure used in this study was designed to mimic aspects of the human digestive system, (Camire & Dougherty, 2003) and the BA binding of the Alpect40, Alpect80, citrus pectin, cholestyramine and cellulose were studied (**Figure 2**). Cholestyramine, a well known BA binding and cholesterol reducing agent, was used as a positive control and bound 6.7, 12.7 and 6.4 mM of CA, DCA and CDCA

in 100 mg of sample, respectively. The commercial cellulose negative control bound only 0.08, 1.12 and 0.59 mM of CA, DCA and CDCA per 100 mg of sample. These results are consistent with the published results for cholestyramine and cellulose (Kim & White, 2010). These assays are realized for first time for these pectin material obtained from alperujo who has suffered a gentle warming. The BA binding values of the Alpect40 and Alpect80 were in the range of 3.0 to 4.8 mM/100 mg of pectin depending on the type of BA. Comparing the BA binding of these pectin materials with commercial citrus pectin showed that for the two primary bile acids considered (CA and CDCA), the best results were obtained for Alpect40 and 80, presenting greater holding capacity for primary bile acids that commercial citrus pectin. However, for the secondary bile acid studied (DCA), Alpect40 and Alpect80 presented a lower BA retention capacity than citrus pectin. For the three BAs tested, no significant differences were observed between Alpect40 and Alpect80. A comparison of these results with those from previous studies on pectin and another dietary fiber (Camire & Dougherty, 2003; Kim & White, 2010) show an adequate BA binding capacity, pointing to their possible health-promoting potential.

a



b



c

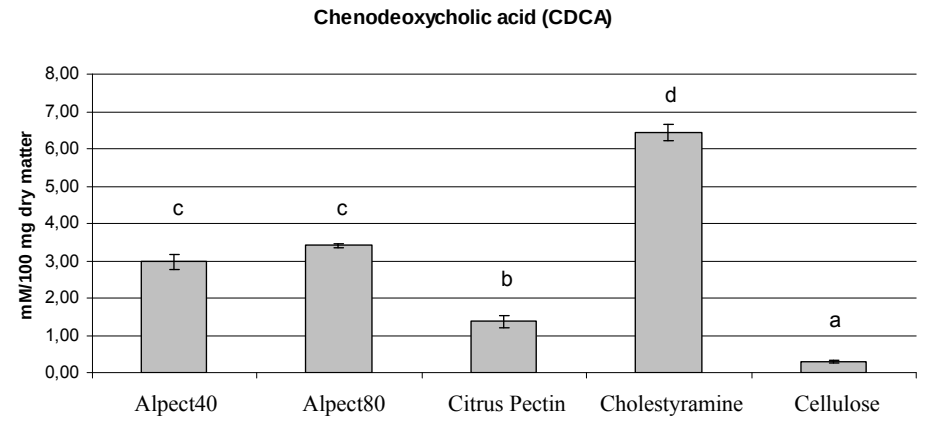


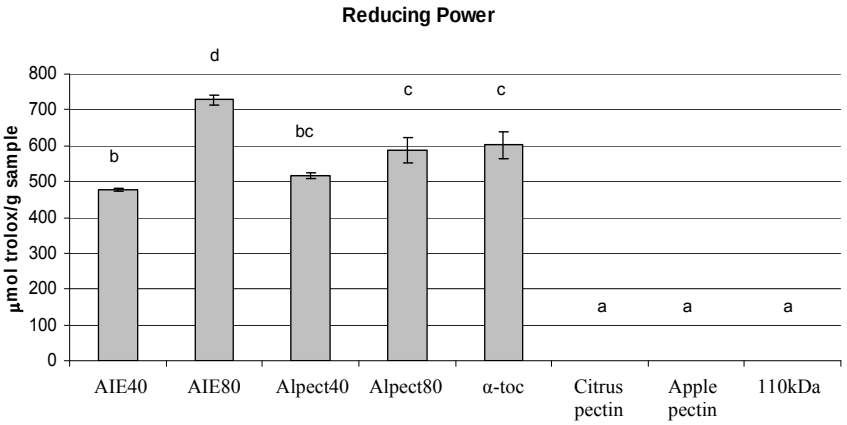
Figure 2. *In vitro* bile acid binding ability of purified pectin obtained from two different precipitations, Alpect40 and Alpect80, cellulose, cholestyramine and citrus pectin. Data are presented as the mean of three replicates ± SDs. Different letters within each bile acid indicate significant differences ($p < 0.05$).

3.3.4. Antioxidant activity.

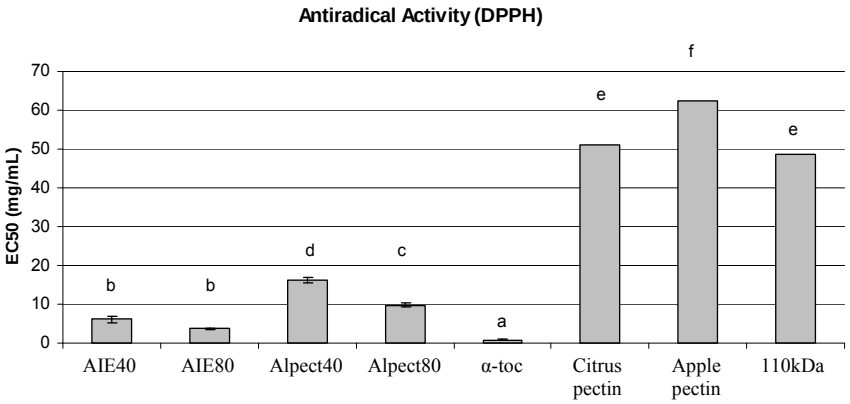
The antioxidant activity of raw polysaccharide-enriched extracts (AIE40 and AIE80) and their purified extracts (Alpect40 and Alpect80) were evaluated by DPPH, ABTS and reducing power assays (**Figure 3)**. The two initial extracts and the two purified extracts displayed higher antioxidant capacity than the commercial citrus and apple pectins and dextran (110 kDa), which exhibited very low or no activity. However, the anti-radical activities of the extracts were lower than for hydroxytyrosol, a potent natural antioxidant (data not shown), and α-tocopherol; reducing power of all the extracts was similar to α-tocopherol. The data also indicated that the raw polysaccharide extracts had higher antioxidant activity than the purified extracts. This is likely due to the higher content of polyphenols in the raw extracts, which are removed or decreased by the purification process.

Significantly, the antioxidant activity values measured as ABTS, obtained in this study for AIE40 (288.1 μmol Trolox/g sample) and AIE80 (455.3 μmol Trolox/g sample) and for the purified extracts, Alpect40 (117.1 μmol Trolox/g sample) and Alpect80 (129.6 μmol Trolox/g sample) are similar or higher than those for materials considered as important sources of antioxidant dietary fiber due to their high content of dietary fiber and associated polyphenols, such as grape pomace (284.0 μmol Trolox/g sample), focus seaweed (119.7 μmol Trolox/g sample) or the tropical fruits acai (78.2 μmol Trolox/g sample) and guava peel (226.3 μmol Trolox/g sample) (Saura-Calixto, 2010). Our data also revealed that the polysaccharide-enriched extracts from alperujo present similar o higher antioxidant activity than polysaccharides extracted from different fruits, such as cherries (159.3 μmol Trolox/g sample), cranberries (137.4 μmol Trolox/g sample), kiwi (129.0 μmol Trolox/g sample), and wolfberry (61.1 μmol Trolox/g sample). Therefore, the extracts obtained from the by-product of hydroxytyrosol recovery from alperujo can be considered as important sources of antioxidant fiber with phenolic compounds associated. This property is of nutritional significance, since such antioxidant compounds are thought to reach the colon intact, where they can produce a variety of beneficial effects (Saura-Calixto, 2011).

a



b



c

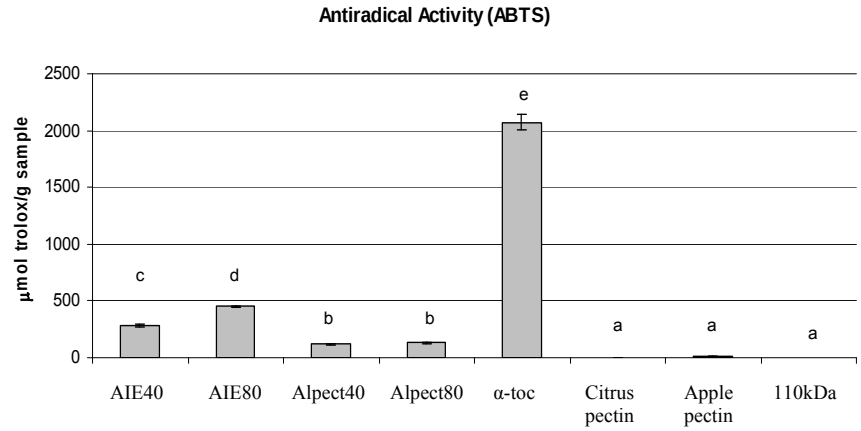


Figure 3: Reducing power (**a**), radical scavenging capacity of DPPH (**b**) and ABTS (**c**) of the pectins before (AIE40 and AIE80), and after purification (Alpect40 and Alpect80), compared to those for commercial pectins (citrus and apple pectins), dextran 110kDa, and α-tocopherol. Data are presented as the mean ± SDs. Different letters indicate significantly difference results ($p < 0.05$). The reducing power, and antiradical activity DPPH and ABTS results obtained for hydroxytyrosol were 5498 µmol trolox/g sample, 0.106 mg/mL (EC_{50}) and 6389 µmol trolox/g sample, respectively.

4. Conclusions.

Two polysaccharide-enriched extracts, obtained by ethyl alcohol precipitation with 40 and 80% (v/v) EtOH from the new liquid effluent obtained from alperujo who has suffered a mild heat treatment, and their purified extracts, present a unique composition that dictates different physical and biological properties. In comparison with commercial pectins, the extracts had low water holding capacity, high oil holding capacity and similar emulsifying activity to that of citrus pectin. These characteristics make these extracts suitable for possible use in the food industry, as emulsifiers or stabilizers.

The pectin-like polysaccharides with a high polyphenol content showed a higher capacity for binding bile acids and glucose than commercial citrus pectin. This property has been tested for the first time in this material. Pectic polysaccharides are a known component of soluble dietary fiber, with associated beneficial properties of decreasing cholesterol levels and adsorption of intestinal glucose (Rodríguez et al 2006). Therefore, the polysaccharides isolated from alperujo could have beneficial bioactive implications in cholesterol metabolism, decreasing the risk of bowel cancer, and in the control of diabetes.

The high antioxidant activities of the raw and purified polysaccharides suggests that they have potential as functional antioxidant dietary fiber, which combines in a single material the physiological effects of both dietary fiber and antioxidant, and therefore could induce beneficial effects in the colon, including the prevention of chronic diseases (Saura-Calixto, 2010).

Acknowledgements

Funding was received from the Spanish Ministry of Economy and Competitiveness and co-funded by European Social Fund (ESF) (Project AGL2013-48291-R). Dr. Rodríguez-Gutiérrez (RYC-2012-10456 contract) wish to thank to the “Ramón y Cajal” Program from, and Ms Rubio-Senent (JAE-Pre104) to the Spanish National Research Council (CSIC) program for providing financial support, both co-funded by ESF.

References.

Abdul-Hamid, A. & Luan, Y.S. (2000). Functional properties of dietary fibre prepared from defatted rice bran. *Food Chemistry*, 68, 15-19.

Adiotomre, J., Eastwood, M.A., Edwards, C.A., & Brydon, W.G. (1990). Dietary fibre: *in vitro* methods that anticipate nutrition and metabolic activity in human. *American Journal of Clinical Nutrition*, 52, 128-134.

AOAC (Official Methods of Analysis) (1990). (15th ed.). Food composition, vol. 1.(pp. 915–919)Arlington, Virginia: AOAC International.

Banks-Gibson, R. (1904). The determination of nitrogen by the Kjeldahl method. *Journal of the American Chemical Society*, 26,105 -110.

Betancur-Ancona, D., Peraza-Mercado, G., Moguel-Ordoñez, Y., & Fuertes-Blanco, S. (2004). Physicochemical characterization of lima bean (*Phaseolus lunatus*) and Jack bean (*Canavalia ensiformis*) fibrous residues. *Food Chemistry*, 84, 287-295.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484-489.

Boyd, G.S., Eastwood, M.A., & MacLean, N. (1966). Bile acids in the rat: studies in experimental occlusion of the bile duct. *Journal of Lipid Research*, 7, 83-93.

Camire, M.E., & Dougherty, M.P. (2003). Raisin dietary fiber composition and *in vitro* bile acid binding. *Journal of Agricultural and Food Chemistry*, 51, 834-837.

Cardoso, S.M., Coimbra, M.A., & Lopes da Silva, J.A. (2003). Calcium-mediated gelation of an olive pomace pectic extract. *Carbohydrate Polymers*, 52, 125-133.

Dea, I.C.M. & Madden, J.K. (1986). Acetylated pectic polysaccharides of sugar beet. *Food Hydrocolloids*, 1, 71-88.

Dische, Z. (1962). Color reactions of carbohydrates. In R. L. Whistler, & M. L.Wolfram (Eds.), Methods in carbohydrates chemistry (pp. 477-512). New York: Academic Press.

Dos-Santos, N., Jiménez-Araujo, A., Rodríguez-Arcos, R., & Fernández-Trujillo, J.P. (2011). Cell wall polysaccharides of near-isogenic lines of melon (Cucumis melo L.) and their inbred parental which show differential flesh firmness or physiological behaviour. *Journal of Agricultural and Food Chemistry*, 59, 7773-7784.

Eastwood, M. A., & Hamilton, D. (1968). Studies of the adsorption of bile salts to non-absorbed components of diet. *Biochemical et Biophysica Acta*, 152, 165-173.

Englyst, H.N., & Cummings, J.H. (1984). Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as aditol acetates, *Analyst*, 109, 937-942.

Escalada Pla, M.F., Ponce, N.M., Stortz, C.A., Gerschensona, L.N., & Rojas, A.M. (2007). Composition and functional properties of enriched fiber products obtained from pumpkin (*Cucurbita moschata* Duchesne ex Poiret). *LWT*, 40, 1176–1185.

Fuentes-Alventosa, J.M., Rodríguez-Gutiérrez, G., Jaramillo-Carmona, S., Espejo-Calvo, J.A., Rodríguez-Arcos, R., Fernández-Bolaños, J., Guillén-Bejarano, R. & Jiménez-Araujo, A. (2009). Effect of extraction method on chemical composition and functional

characteristics of high dietary fibre powders obtained from asparagus by-products. *Food Chemistry*, 119, 66-671.

Galanakis, C.A., Tornberg, E., & Gekas, V. (2010). A study of the recovery of the dietary fibres from olive mill wasterwater and the gelling ability of the soluble fibre fraction. *LWT- Food Science and Technology*, 43, 1009-1017.

Gonçalves, B., Falco, V., Moutinho-Pereira, J. M., Bacelar, E., Peixoto, F., Correia, C. (2009). Effects of elevated CO_2 on grapevine (*Vitis vinifera L*.): Volatile composition, phenolic content, and *in vitro* antioxidant activity of red wine. *Journal of Agriculture and Food Chemistry*, 57, 265−273.

Grigelmo-Miguel, N., Gorinstein, S., & Martín-Belloso, O. (1999). Characterisation of peach dietary fibre concentrate as a food ingredient. *Food Chemistry*, 65, 175-181.

Jiménez, A., Guillén, R., Fernández-Bolaños, J., & Heredia, A. (1994). Cell wall composition of olives. *Journal Food Science*, 59, 1192-1201.

Jiménez, A., Rodríguez, R., Fernández-Caro, I., Guillén, R., Fernández-Bolaños, J., Heredia, A. (2001). Olive fruit cell wall degradation of pectic polysaccharides during ripening. *Journal of Agricultural and Food Chemistry*, 49, 409-415.

Kim, H.J., & White, P.J. (2010). *In vitro* bile-acid binding and fermentation of high, medium, and low molecular weight β-glucan. *Journal of Agricultural and Food Chemistry*, *58*, 628-634.

Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Rubio-Senent, F., & Fernández-Bolaños, J. (2012). Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloids*, 28, 92-104.

Larrauri, J.A., Goñi, I., Martín-Carrón, N., Rupérez, P., & Saura-Calixto, F. (1996). Measurement of health-promoting properties in fruit dietary fibres: antioxidant capacity, fermentability and glucose retardation index. *Journal of Science and Food Agricultural*, *71*, 515-519.

López, G., Ros, G., Rincón, F., Periago, M.J., Martínez, M.C.,& Ortuño, J. (1996). Relationship between physical and hydration properties of soluble and insoluble fiber of artichoke. *Journal of Agricultural Food Chemistry*, 44, 2773-2778.

Mafra, I., Barros, A.S., &Coimbra, MA.. (2006). Effect of black oxidising table olive process on the cell wall polysaccharides of olive pulp (*Olea europaea L*. var. Negrinha do Douro). *Carbohydrate Polymers*, 65, 1–8.

Obied, H.K., Allen, M.S., Bedgood, D.R., Prenzler, P.D., & Robards, K. (2005). Investigation of Australian olive mill waste for recovery of biophenols. *Journal of Agricultural and Food Chemistry*, 53, 9911–9920.

Ou, S.Y., Kwok, K.C., Li, Y., & Fu, L. (2001). *In vitro* study of possible role of dietary fiber in lowering postprandial serum glucose. *Journal of Agricultural and Food Chemistry*, 49, 2, 1026-1029.

Pérez-Jiménez, J., Serano, J., Tabernero, M., Arranz, S., Díaz-Rubio, M. E., García-Diaz, L., et al. (2008). Effect of grape antioxidant dietary fiber on cardiovascular disease risk factor. *Nutrition*, 24, 646-653.

Ritzoulis, C., Marini, E., Aslanidou, A., Georgiadis, N., Karayannakidis, P.D., Panayotis D., Koukiotis, C., Filotheou, A., Lousinian, S., & Tzimpilis, E. (2014). Hydrocolloids from quince seed: Extraction, characterization, and study of their emulsifying/stabilizing capacity. *Food Hydrocolloids*, 42, 178-186.

Rodríguez, R., Jaramillo, S., Rodríguez, G., Espejo, J. A., Guillén, R., Fernández-Bolaños J., Heredia, A., Jiménez, A. (2005). Antioxidant activity of ethanolic extracts from several asparagus cultivars. *Journal of Agriculture and Food Chemistry*, 53, 5212−5217.

Rodríguez, R., Jiménez, A., Fernández-Bolaños, J., Guillén, R., & Heredia, A. (2006). Dietary fibre from vegetable products as source of functional ingredients. Trends in Food Science and Technology, 17, 3-15.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., & Fernández-Bolaños, J. (2012). New phenolic compounds hydrothermally extracted from the olive oil byproduct alperujo and their antioxidative activities. *Journal of Agricultural and Food Chemistry*, 60, 1175-1186.

Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J. (2015). Pectin extracted from thermally treated olive oil by-products: characterization, physico-chemical properties, *in vitro* bile acid and glucose binding. *Food Hydrocolloids*, 43, 311-321.

Saura-Calixto, F. (1998). Antioxidant dietary fiber product: A new concept and a potential food ingredient. *Journal of Agricultural and Food Chemistry*, 46, 4303-4306.

Saura-Calixto, F. (2010). Antioxidant dietary fibre: a new functional ingredient. *Science and Practice*, 34, 509-514.

Saura-Calixto, F. (2011). Dietary fiber as a carrier of dietary antioxidant: an essential physiological function. *Journal of Agricultural and Food Chemistry,* 59, 43-49.

Singleton, V.L., & Rossi, J.A., Jr. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture,* 16, 144−158.

Vierhuis, E., Schols, H.A:, Beldman, G., & Voragen, A.G.J. (2000) Isolation and characterisation of cell wall material from olive fruit (*Olea europea* cv koroneiki) at different ripening stages. *Carbohydrate Polymers*, 43, 11-21.

Waldron, K. W., & Selvendran, R. R. (1990). Composition of the cell walls of different asparagus (*Asparagus officinalis*) tissues. *Physiologia Plantarum*, 80, 568-575.

6.2.4. Capítulo 12.

Properties of Lignin, Cellulose, and Hemicelluloses Isolated from Olive Cake and Olive Stones: Binding of Water, Oil, Bile Acids, and Glucose

Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Lama-Muñoz, Aranzazu García, and Juan Fernandez-Bolaños.

Journal of Agricultural and Food Chemistry, 2014, 62, 8973-8981.

Las aceitunas de mesa y la extracción de aceite de oliva representan una importante actividad industrial social y económica que es especialmente relevante en los países mediterráneos. Los residuos sólidos que se generan a partir de la extracción de aceite de oliva desde el sistema de centrifugación de tres fases se denominan "orujo" y contiene fragmentos de huesos y pulpa del fruto de la oliva. De la industria de las aceitunas de mesa sin hueso (aceitunas rellenas), el hueso entero se recupera por separación de la pulpa. El hueso de la aceituna es un material lignocelulósico compuesto por hemicelulosa, celulosa y lignina como componentes principales, los cuales pueden ser fraccionados previo tratamiento “steam explosion”, ya que el proceso favorece la solubilización de las hemicelulosas y de la lignina en solución alcalina, dejando a la celulosa en el residuo sólido.

En trabajos anteriores de nuestro grupo de investigación se han determinado las condiciones óptimas para aplicar el tratamiento “steam explosion” sobre los diferentes residuos (orujo de tres fases, huesos de la aceituna entero y fragmentos del hueso) para obtener cada uno de los compuestos poliméricos con un alto rendimiento, obteniéndose fracciones enriquecidas en celulosa, lignina y hemicelulosa.

El objetivo de este trabajo fue estudiar estas fracciones enriquecidas en celulosa, hemicelulosas y lignina, en cuanto a sus propiedades fisicoquímicas, sus propiedades funcionales, tales como la capacidad de retención de ácidos biliares, el índice de retardo de la glucosa y su relación con la composición.

Los estudios realizados mostraron que la fracción de lignina aislada a partir de huesos de la aceituna y en menor medida a partir del orujo de tres fases, presentaba una importante capacidad de captación de ácidos biliares, entre otros importantes resultados.

Properties of lignin, cellulose and hemicelluloses isolated from olive cake and olive stones-binding of water, oil, bile acids and glucose.

Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Lama-Muñoz, Aranzazu García, Juan Fernández-Bolaños.

ABSTRACT

A process based on a steam explosion pretreatment and alkali solution post-treatment was applied to fractionate olive stones (whole and fragmented, without seeds) and olive cake into their main constitutive polymers of cellulose (C), hemicelluloses (H) and lignin (L) under the optimal conditions for each fraction according earlier works. The chemical characterization (chromatographic method and UV and IR spectroscopy) and the functional properties (water and oil-holding capacities, bile acid binding and glucose retardation index) of each fraction were analyzed. The *in vitro* studies showed a substantial bile acid binding activity in the fraction containing lignin from olive stones (L) and the alkaline extractable fraction from olive cake (Lp). Lignin bound significantly more bile acid than any other fraction and similar to cholestyramine (a cholesterol-lowering, bile acid-binding drug), especially when the cholic acid (CA) was tested. These results highlight the health promoting potential of lignin from olive stones and olive cake extracted from olive by-products.

KEYWORDS: Olive by-product; Fractionation; Steam-explosion; lignin; cellulose; hemicelluloses; Bile acid binding; Glucose retardation index.

Introduction

Table olives and olive oil extraction represent an important social and economic industrial activity that is particularly relevant in the Mediterranean countries. The solid waste generated from olive oil extraction from the three-phase centrifugation system is named “orujo” or “olive cake” and contains seed husks (fragmented olive stones), seeds, pulp and peel of the olive fruit. Olive stones without seed can be recovered by subsequent filtration of the olive cake. From the pitted table olive industry (stuffed olives), the whole stone (stone and seed) is recovered by separation of the pulp. These by-products are currently used as energy sources, and in the case of olive cake, they are also used as fertilizer and animal feed[1]. However these by-products contain valuable compounds that could be optimized to make high-valuable food products.

The olive stone is a lignocellulosic material with hemicelluloses, cellulose and lignin as main components[2]. The fractionation process allows each component to be obtained for utilization and therefore increases the value of such by-products. The steam-explosion process at high temperature (180-240 ºC) and pressure (1.47-4.12 MPa) results in the physical breakage of the cell wall by the rapid release of pressure, with the hydrolysis of glycosidic bonds in the hemicelluloses by saturated steam (autohydrolysis) or by the addition of small amounts of acid (prehydrolysis). These particulars of the steam explosion pretreatment increase the water solubility of hemicelluloses and the solubility of lignin in alkali solution, leaving the cellulose as the solid residue[3].

Previous works have determined the optimal conditions for steam explosion for the fractionation of olive by-products[2-5] to obtain each of the polymeric compounds in high yield and purity. The chemical structure and physicochemical properties of fiber, including polysaccharides and lignin, are important for functional and nutritional motives. Consequently, each fiber fraction enriched in cellulose, lignin and hemicelluloses could be useful for further application as a new source of dietary fiber due to its health benefits. The consumption of water-soluble fiber and some insoluble fibers has been shown to decrease cholesterol levels and hence reduce the risk of cardiovascular disease[6]. Dietary fiber binds bile acid and increases its fecal excretion which has been suggested as the possible mechanism of action. For this reason, dietary fiber inhibits cholesterol reabsorption and stimulates cholesterol conversion for the manufacture of additional bile acids[7]. The lignin, a component of dietary fiber, non-toxic and biocompatible, that undergoes minimal changes in the body (non-fermentable), have been proven to bind various bile acid[8] and detoxify of harmful metabolites inhibiting of colonic carcinogenesis[9].

Also, epidemiological studies suggest that dietary fiber, especially the soluble type, is associated with the regulation of serum glucose concentration, which may be useful for the control of diabetes[10]. The physicochemical properties of the fiber-rich olive by-products, related with the chemical structure of the wall polysaccharides may improve the functional and technological properties of food products. Possible uses of these components include as an enhancer of water and oil retention and to improve emulsion or oxidative stabilities[11].

Olive cake, by comparison with other residues derived from fruit, could also potentially be utilized for its phenolic content and antioxidant properties. In a previous study, low-molecular-weight phenols solubilized by the steam pretreatment were characterized[5]. The present study is focused on the residual insoluble material, polyphenolic polymerized and strongly bound to cell wall components that were extractable with an alkaline-solution post-treatment. This aromatic (phenolic) polymer was dissolved in an alkali solution, probably as occurs in alkali-extracted lignin with a stable colloid structure by the negative charge (electrostatic interactions) due to the phenolic hydroxyl and carboxyl groups in the alkaline medium which precipitated when the solution was changed to an acidic pH[12]. In addition the lignin, an amorphous polyphenol of high molecular weight, can vary in structure according to their method of extraction and their plant

source. Therefore, the properties and health benefits of the lignin will depend on the used lignocellulosic material, in this case olive stones and olive cake. The uses of fiber from new origins will probably widen the field of application for dietary fiber.

The aim of this work was the fractionation of olive stones (whole and fragmented, without seeds) and olive cake by a two-stage process based on a previous work[3], involving a steam-explosion pre-treatment and alkaline solution post-treatment. Each fraction enriched in cellulose, hemicelluloses and lignin was characterized and investigated for the potential use of these polymers. We highlight their physicochemical and functional properties, with especially focus on bile acid binding and the glucose retardation index, using *in vitro* studies.

Materials and methods

Raw material.

Whole olive stones were obtained from pitted table olives, and following incubation in an air stove at 30 ºC, were rubbed vigorously on filter paper to remove any loosely-adhering pulp tissue.

The fragments of olive stones (seed husks) with an average length of 2-6 mm were supplied by an oil-extraction plant (Oleícola el Tejar, Córdoba, Spain). The husks (9% moisture) were obtained from olive pomace after separating peel, pulp, and seeds.

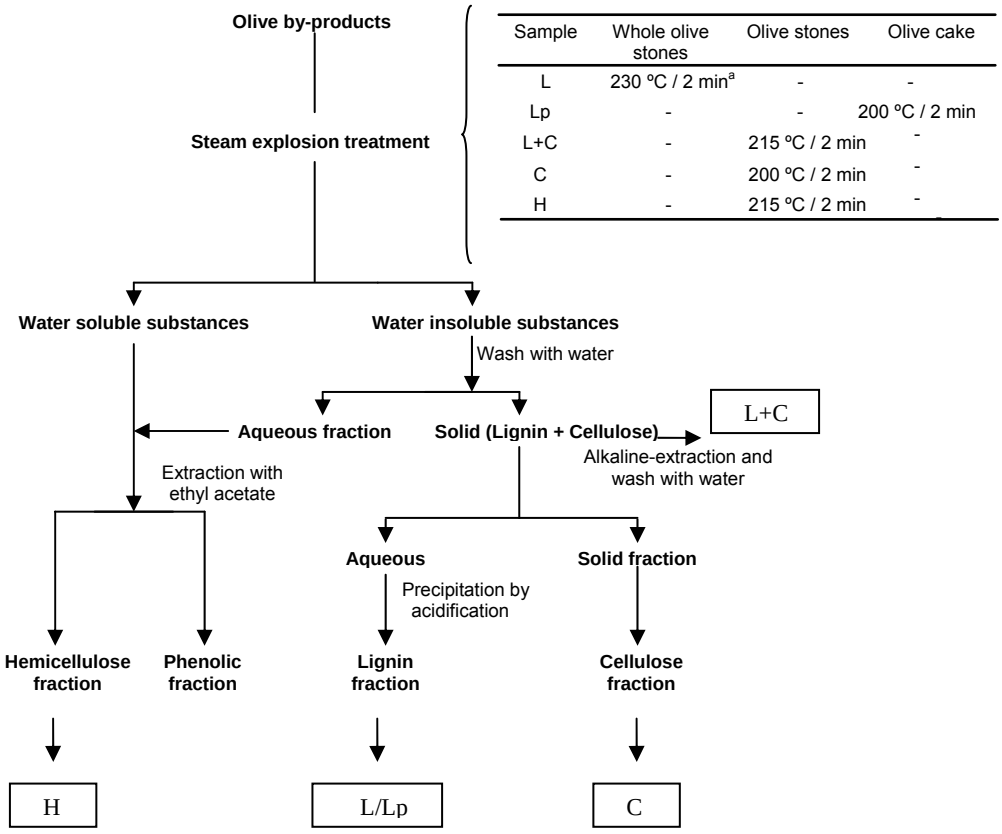
Olive cake was also supplied as pellets by “Oleícola El Tejar” (Córdoba, Spain). The olive cake, which included the residual olive stone, was subjected to vibratory ball milling before determination of its chemical composition.

Steam explosion and fractionation.

Steam explosion was carried out using a 2-L pilot-scale reactor, with a maximum operating pressure of 4.12 MPa, equipped with a ball valve opening. All experiments were carried with samples of 100 g (dry weight). The samples were steamed for varying temperatures at 2 min, prior to rapid decompression (explosion). So lignin was isolated from whole olive stone with a treatment at 230ºC/2 min (with acid), fraction of lignin from olive cake was obtained at 200 ºC/2 min, the fractions of lignin + cellulose and hemicellulose were obtained at 215 ºC/ 2 min from olive stone, and cellulose was isolated from olive stone at 200 ºC/2 min.

One treatment (whole olive stones) was carried out with previous impregnation with an acid solution (0.1% H_2SO_4 w/v) for a period of 1 h under vacuum (to remove the air from the material and facilitate the penetration of acid through the structural matrix). The material was drained in a sieve and rinsed thoroughly with distilled water to remove all traces of acid, prior to loading into the steam reactor.

After steam-explosion (**Figure 1**), the sample was vacuum filtered through Albet filter paper (weight, 73 g/cm^2; Albet, Barcelona, Spain) in a Buchner funnel. The residue was washed three times with 500 mL of distilled water for 30 min at 60 ºC, and filtered to remove the water-soluble fraction. The filtrate was concentrated to 250-300 mL by rotary evaporation at 40 ºC. The aqueous concentrate was continuously extracted for 5-6 h with ethyl acetate (refluxed at 77 ºC). The aqueous and organic phases were rotary evaporated under vacuum at 40 ºC for several hours to remove all traces of ethyl acetate. The aqueous phase was freeze-dried (fraction H). The water-insoluble material (fraction L+C) was extracted with 0.5 M NaOH (250 mL) for 15 min at room temperature to remove the lignin from the cellulose residue. The residual solid was thoroughly washed with warm water until all traces of alkali were removed or brought to a neutral pH (fraction C). The dissolved lignin was acidified with 5 M H_2SO_4 to pH 2-3. The precipitate was centrifuged, washed to neutral pH, and freeze-dried (fraction L).



Sample	Whole olive stones	Olive stones	Olive cake
L	230 ºC / 2 min[a]	-	-
Lp	-	-	200 ºC / 2 min
L+C	-	215 ºC / 2 min	-
C	-	200 ºC / 2 min	-
H	-	215 ºC / 2 min	-

Figure 1. Scheme of the fractionation treatments of whole olive stones (stone and seed), olive stones (seed husks fragments) and olive cake (residual seed husks, seed, pulp and peel) after steam explosion pretreatment. [a] Previous impregnation with dilute solution of strong mineral acid.

Ultraviolet spectroscopy.

The ultraviolet spectra of samples were recorded at a concentration of 0.0027% in methanol on a UV-VIS Hewlett-Packard (Waldbronn, Germany), model 8452 A.

For the study of difference in spectra, $\Delta\varepsilon_i$, 10 mg of lignin were dissolved in 25 mL of dioxane: water (9:1). The neutral solution was prepared by diluting 3 mL of this solution in 25 mL of dioxane. The alkaline solution was prepared by the addition of 100 mg of NaOH. The alkaline-neutral difference spectra were determined by running the alkali solution against the neutral solution.

Infrared spectroscopy.

Infrared spectra were recorded on a Bio-Rad FTS 7PC using KBr disks. Lignin (1 mg) was mixed and thoroughly ground with 300 mg of KBr to reduce particle size and to obtain a uniform dispersion of the sample in the disks. The conditions of analysis were as follows: resolution 8 cm^{-1}; co-adding 250 scans and a frequency range of 400-4000 cm^{-1}.

Analytical method.

Moisture, fat, and ash contents were determined according to the AOAC method[13] The uronic acids were estimated according to the m-hydroxydiphenyl method described by Blumenkrantz and Asboe-Hansen[14]. Total phenolic content was determined by the Folin−Ciocalteu spectrophotometric method and expressed as grams of gallic acid equivalents[15].

The content of water-soluble low-molecular weight sugars was determined by analysis with trifluoracetic acid (TFA) hydrolysis (2 N TFA at 121 ºC for 1 h) prior to reduction, acetylation and analysis by gas chromatography[16]. Inositol was used as an internal standard. The chromatographic conditions applied were described by Lama-Muñoz, et al[17].

Klason lignin was determined gravimetrically[18]. The α-cellulose determination of the insoluble fraction was carried out from bleached cellulose, which was extracted with 17.5% NaOH, and the residue was measured gravimetrically[3]. The hemicelluloses were determined as the difference between bleached cellulose and α-cellulose. Bleached cellulose was prepared by Na-chlorite delignification[19].

Functional properties.

Water/Oil-holding capacity (WHC/OHC).

These properties were determined by centrifugation using the method described by Fuentes-Alventosa, et al[20]. The oil-holding capacity (OHC) was determined under the same conditions as WHC using soybean oil (0.92 g/mL density), and was expressed as grams of oil retained/gram of sample.

Emulsification activity (EA) and emulsion stability (ES).

Emulsification activities and emulsion stability were evaluated in duplicate using the method of Betancur-Ancona et al[21]. The EA was expressed as the volume of the emulsified layer as a percentage of the volume of the entire layer in the centrifuge tube. ES was determined using the prepared emulsions and heated at 80 ºC for 30 min. The emulsion was cooled to room temperature and centrifuged at 1200 g for 5 min in a Sorvall RT 6000D (DuPont, Nemours, Mechelen, Belgium). ES was expressed as the percentage of the remaining emulsified layer volume of the original emulsion volume.

Binding of bile acid (BA).

The capacity of the fractions for binding BA *in vitro* was evaluated following the method described by Camire and Dougherty[8] with some modifications. Binding for each fraction was estimated from the change in BA concentration on exposure of the solution to the soluble fiber during an *in vitro* digestion process, which included an acidic digestion of the sample at pH 2, followed by pancreatic digestion. The individual BAs cholic acid (CA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) (Sigma, St. Louis, MO) were selected for analysis. The results obtained for each sample of isolated pectin were compared with the results obtained for Cholestyramine resin (Sigma C 4650), cellulose (Macherey Nagel, GmbH & Co. KG) and commercial pectin from citrus peel (Sigma P 9135).

(a) In vitro digestion procedure. Each BA was dissolved in 0.1 M $NaHCO_3$ to make a 31.25 mM BA solution, which, in the final mixture, will be in a range of BA concentration close to the human-body concentration in the final mixture (1.5-7mM). 100 mg of sample, pectin, cholestyramine and cellulose were added to each tube (50 mL plastic centrifuge tubes) in triplicate. The individual sample without BA was used as a blank, and a tube for reagents including BA but without sample was considered as 100% of BA concentration. Samples were digested in 1 mL of 0.01 M HCl for 1 h at 37 ºC in a shaking water bath (P-Selecta Unitronic 320 OC). After this acidic incubation, which simulated gastric digestion, the sample was neutralized with 0.1 M NaOH until pH 7.0. To each test sample, 4 mL of BA solution were added. For the sample blank, 4 mL of $NaHCO_3$ were added without bile acid. After the addition of 5 mL of porcine pancreatin (activity equivalent 8 X USP) with a concentration of 10 mg/ mL in 0.01 M buffer phosphate pH 7.0, the tubes were incubated for 1 h at 37 ºC in a shaking water bath. The mixtures were centrifuged for 10 min at 1120 g in a Sorvall RT 6000D (DuPont, Nemours, Mechelen, Belgium). The supernatants were transferred to new tubes. An additional 5 mL of phosphate buffer was used to rinse out the incubation tube, vortexed and centrifuged as before, and the supernatants were combined.

(b) Bile acid analysis. BA were analyzed by a colorimetric method (a modified Pettenkofer reaction) that was described by Boyd, et al[22]. 100 µL of supernatant (samples, blank and standards of individual BA) were collected and heated for 1 h at 137 ºC, after adding 5 mL sulfuric acid (70%). Five minutes later 1 mL of a freshly prepared solution of furfural (0.25%) was also added. The solutions were thoroughly mixed after each addition. A pink color appears which takes 60 min to reach maximum intensity. The readings were made at the absorption maximum

of 490 nm in an iMark™ microplate absorbance reader (Bio-Rad, Hercules, CA, USA) and the values were determined from the obtained standard curve. The ranges for the different standards were 0-30 µg for CA, 0-750 µg for the DCA and 0-750 µg for the CDCA. Binding capacity was expressed as mM bile acid/100 mg of fraction and was calculated from the decrease in BA concentration (mM) in the test solution after exposure to 100 mg of added fraction.

Glucose dialysis retardation index (GDRI).

GDRI was determined as described by Fuentes-Alventosa et al[20]. Samples of 400 mg were thoroughly hydrated with 15 mL of distilled water that containing 30 mg of glucose and stirring during 1 h. Then samples were transferred to dialysis bag (12000 MWCO, Sigma Chemical Co.). Each bag, blank of sample (with sample, without glucose) and a control bag (with glucose, but without sample) were put into a reservoir containing 400 mL of distilled water and held in a thermostatic water bath at 37 ºC for 1 h with constant shaking. At 10 min intervals, 0.5 mL of dialysate was collected and the glucose concentration was determined spectrophotometrically by the anthrone method[23].

$$GDRI = 100 - \left(\frac{total\ glucose\ diffused\ sample}{total\ glucose\ diffused\ control}\ x\ 100 \right)$$

Statistical analysis.

Results were expressed as mean values ± standard deviations. To assess for differences in the composition and functional characteristics among the different isolated fractions, a sample comparison was performed using the Statgraphics Plus program version 2.1. A multivariate analysis of variance (ANOVA), followed by Duncan´s comparison test, was performed. The level of significance used was $p < 0.05$.

Results and Discussion

Chemical characterization of the main fraction obtained after steam-explosion pretreatment.

Figure 1 shows the operation conditions of steam-explosion carried out for each of the samples used in the present work. These samples were chosen by the best yield and/or the more representative chemical composition of hemicelluloses, cellulose and lignin from pretreated whole olive stones (stone and seed), fragmented olive stones (seed husks) or olive cake (by-product derived from olive oil extraction by a continuous triphasic extraction system, with seed husks, seed, pulp and peel) as was described in previous works[3-5]. The lignocellulosic olive by-product materials were treated in a steam-explosion reactor, with or without a previous

impregnation with a dilute solution of strong mineral acid, followed by fractionation (**Figure 1**). The major part of hemicelluloses (**Sample H**) and a small part of lignin becomes soluble in the aqueous phase, and can be separated from the remaning lignin and cellulose insoluble material (**Sample L+C**). The lignin was recovered from the insoluble material by an alkaline-extraction followed by acid precipitation (**Sample L**), resulting in a cellulose fraction (**Sample C**) and the remaining lignin and hemicellulose.

The maximum yield of lignin from whole olive stones was achieved at 230 ºC/2 min with acid impregnation (**Sample L**). A fraction of lignin was also obtained from olive cake steam-treated at 200 ºC/2 min (**Sample Lp**). These milder conditions (lower temperature and no acid impregnation) were chosen to avoid the auto-hydrolysis of the residual fragment of olive stones present in the olive cake, obtaining the lignin from the pulp only. The lignin recovered by alkali-extraction followed by acid precipitation from steam-exploded olive stones was a de-etherified lignin with an extensive cleavage of β-aryl ether linkages, similar to exploded hardwood lignin[3]. However, the "lignin" obtained by alkaline delignification from steam-exploded olive cake did not resemble a true lignin. The parenchymal cells of olive fruit pulp are scarcely lignified[24], and the "lignin" is composed mostly of condensed and polymerized polyphenols and other polymers, with variable levels of polymerization during the processing of the olive cake. Studies on ultraviolet and infrared spectroscopy of both extracted and autohydrolyzed lignin were followed to match the difference in functional groups. The two lignin fractions (**L** and **Lp**) exhibited the basic UV spectra typical of lignin (**Figure 2a**), with an absorption maximum around 240 nm and a second maximum at 280 nm, associated to a non-conjugated phenolic group (aromatic ring), and a slight shoulder at 340-350 nm. The difference spectra, obtained by subtracting the spectra of the alkaline lignin from the neutral lignin (ionization difference, $\Delta\varepsilon_i$) are shown in **Figure 2b**. The two lignins (lignin from whole olive stones, L, and lignin from olive cake, Lp) were found to have three characteristic maximums at 254-256 nm, 298-300 nm and 360-370 nm in their ultraviolet ionization spectrum, which indicate that both contain phenolic hydroxyls conjugated to the α carbonyl group, carbon-carbon double bonds or biphenyl group[25]. A list of previously reported absorption coefficients $E(\%_{cm})$ from steam-exploded olive stones at various wavelengths in the 260 to 350 nm region[3] showed similar absorption coefficients to guaicyl-syringyl lignin of hardwoods, in contrast to the results for exploded olive cake lignin with very low absorption coefficients. The lignin isolated from steam-exploded olive stones showed a typical infrared lignin spectrum of guaicyl-syringyl lignin (**Figure 3a$_1$**), with a characteristic lignin band in the region 1327-815 cm^{-1} due to aromatic ether ring breathing and aromatic C-H deformation of the syringyl and guaicyl units[26]. In both lignin samples, a wide absorption band focused at 3396 cm^{-1}, originated from the OH- stretching vibration in the aromatic and aliphatic OH group, and a band at 2926 cm^{-1} from the C-H stretching were present. In addition, the occurrence of obvious aromatic skeleton vibration bands at 1600, 1515 and 1460 cm^{-1} confirmed the presence of "lignin bands" in both alkaline-extractable lignins, although in the case of lignin from olive cake, a lower intensity relative to other bands indicates a smaller content of aromatic ring. However, the lignin fraction isolated from olive cake showed some significant differences (**Figure 3a$_2$**), with a strong

multiple and broad absorption band between 1736-1675 cm^{-1} attributed to conjugated/unconjugated carbonyl groups, carboxylic acid and ester, indicating the presence of hemicelluloses, and a conjugated aryl carbonyl group, absent in the lignin of olive stone. These results confirm that the lignin fraction isolated from olive stones had a typical exploded hard-wood lignin structure, distinguishable from steamed olive cake lignin, although with the presence of certain similar functional groups.

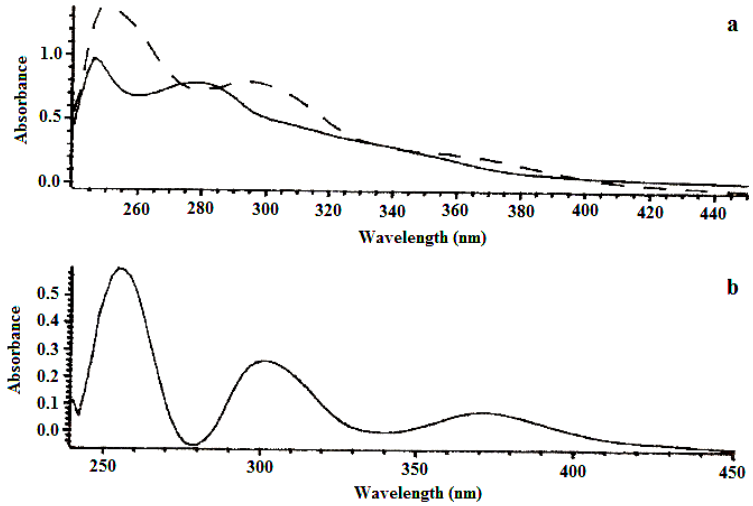


Figure 2: **a)**Ultraviolet spectrum for alkaline (dash line) and neutral solution (solid line) of alkali-extracted lignin from steam-exploded olive cake (sample Lp). **b)** The alkaline-neutral difference spectra, ultraviolet ionization, $\Delta\varepsilon_i$ (b).

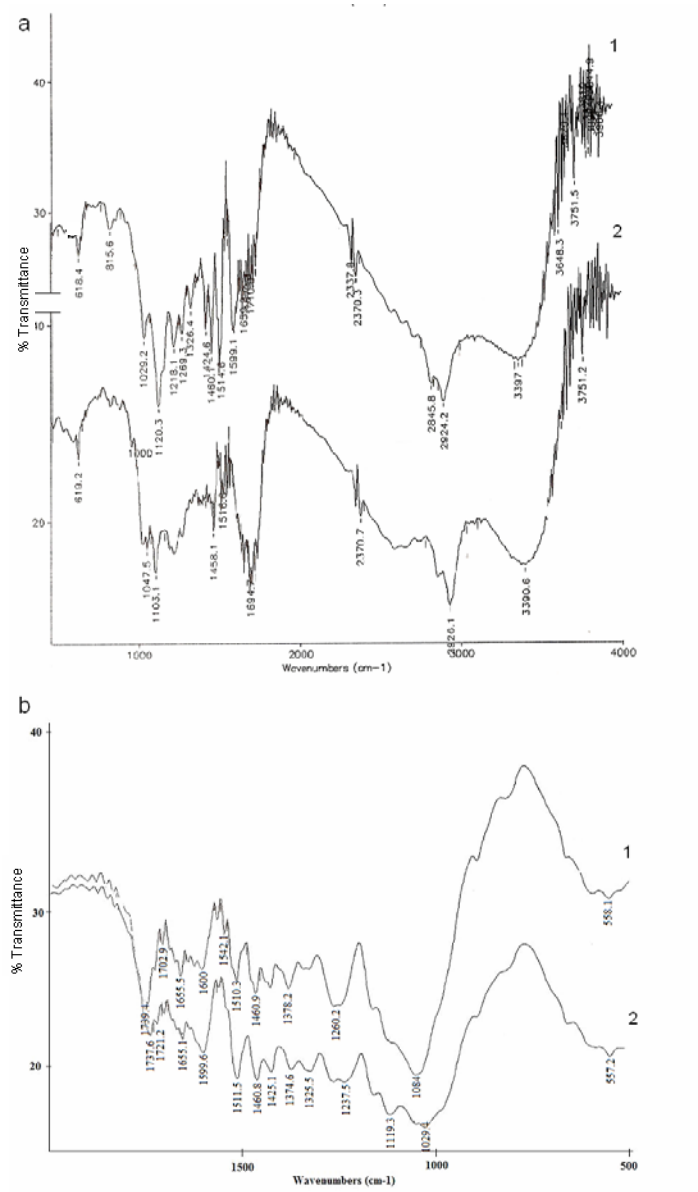


Figure 3: **a)** Infrared spectra of alkali-extracted lignin (1) from steam-exploded whole olive stones (Sample L) and (2) from steam-exploded olive cake (Sample Lp). **b)** Infrared spectra of (1) milled and steam-untreated olive stones and (2) insoluble fraction isolated from steam-exploded olive stones (Sample L+C).

The water insoluble fraction (**Sample L+C**) was recovered from olive stone steam-treated at 215 ºC/2 min (with previous acid impregnation) prior to alkali-extraction, and the overall yield represents a recovery of total solid matter, including α-cellulose and lignin (**Table** 1). However, the efficiency of the process is more associated with extent of transformation in the substrate. Since the steam-explosion results in the hydrolysis of the major part of the hemicelluloses and a small part of the lignin remains soluble in the aqueous fraction, a residue of α-cellulose and lignin remain. In these conditions the material defibrates efficiently and then favors the release of lignin in the alkaline solution to achieve a maximum yield of lignin fraction and then falls gradually with the severity of treatment[3]. The infrared spectra of the insoluble fraction isolated from steam-

exploded olive stones was compared to the initial olive stone which was milled and steam-untreated (**Figure 3b$_1$** and **3b$_2$**). The relative intensities of the bands for aromatic skeleton vibration, assigned at 1600, 1510 and 1425 cm^{-1}were rather similar in milled and steam-pretreated samples, indicating a similar core structure of the lignins[27-28]. However, it appears that olive stone exhibited considerable changes following steam-explosion, with the disappearance or decrease in characteristic absorption peaks of hemicelluloses. A wide absorption band focused on the ester group at 1740 cm^{-1} with another at 1240 cm^{-1}, and it is also possible to relate the enhancement of the cellulose peaks at 1120 and 1030 cm^{-1} to the disappearance of the characteristic vibration at 1080 and 1047 $cm^{-1}$of the C-O stretching bands of hemicelluloses.

Table 1. Composition and Recovery Yield of the Water- Insoluble and Soluble Fraction (Sample L+C; Sample H[a]) and Composition of Water-and-Alkali-Insoluble Fraction (Sample C) of Steam-Exploded Olive Stones at 215 and 200 °C during 2 min, Respectively.

	g/100 g dry weight of initial material	
Sample L+C	Water soluble substance	27.5
	Insoluble fraction Sample (L+C)	54.1
	Lignin alkali-extracted (L)	13.3
	Insoluble (C)	48.8
Sample C	Bleached cellulose	68.8±2.5
	α-Cellulose	60.7±1.5
	Hemicellulose[b]	8.1±0.9
	Klason lignin	35.7±0.8
Sample H	Total sugar[c]	61.03±5.02
	Rhamnose	0.28±0.01
	Arabinose	2.24±0.23
	Xylose	55.35±4.5
	Mannose	0.44±0.06
	Galactose	1.62±0.03
	Glucose	1.10±0.01
	Uronic acids	2.52±0.06
	Polyphenols	2.91±0.34
	Ash	4.02±0.02
	Other compounds[d]	29.6±0.25

[a]After steam explosion of olive stone at 215 °C/2 min, and after ethyl acetate extraction. [b]Hemicellulose was determined by difference between bleached cellulose and α-cellulose. [c]Sugar determined by 2 N trifluoroacetic acid (TFA) hydrolysis at 120 °C for 2 h prior to gas chromatography derivatization to alditol acetates. [d]Quantified by difference (100% − known compounds).

Cellulose was the major component in the insoluble water fraction of olive stones after the steam-explosion (200 ºC/2 min) and alkali-extraction of lignin (**Sample C**), in which the remaining lignin (36%) and hemicellulose (8%) were present (**Table 1**).

The water-soluble fraction obtained after after extraction by ethylacetate for a selected sample of olive stones steam-extracted at 215 ºC/2 min was rich in hemicelluloses, (**Sample H**). The main components were carbohydrates (61%), from polysaccharides extracted from the cell

wall with quite different structures (xylans, arabinoxylan, xyloglucan; **Table 1**), although a substantial portion of the water-soluble material produced during the pretreatment (30%) was not identified[4]. This may be explained by the high percentage of chemical transformation and by the condensation reaction between carbohydrates, their degradation products and the phenol derived from lignin degradation[29].

Functional properties: WHC, OHC, EA, and ES.

The functional properties of the fractions obtained by steam explosion and alkali delignification of olive by-products were compared to commercial citrus pectin (**Table 2**). Citrus pectin showed significantly the highest WHC (10.3 g water/g sample), which is an important property of dietary fiber (related to soluble fiber) to absorb and retain water. WHC is an important quality from both a physiological and a technological point of view, increasing the bulk volume of food, and modifying the viscosity and texture of formulated products in addition to reducing calories[30]. Sample L+C had significantly the highest WHC, compared to samples C and L, with a value similar to Sample Lp. Since WHC is related to soluble dietary fiber content, the low WHC of Sample H could be attributed to the degradation or changes in the structure of some fiber components by heating, leading to a loss of ability to retain water in the fiber.

Table 2. Functional Properties of the Main Components of the Olive Stones and Olive Cake By-Products Fractionated by Steam Explosion Pre-Treatment Combined with Alkali Delignification and Comparison with Commercial Citrus Pectin: Water Holding Capacity (WHC), Oil Holding Capacity (OHC), Emulsification Activity (EA) and Emulsion Stability (ES).

	WHC	OHC	EA	ES
	g water holding/g sample	g oil holding/g sample	Emulsion volume/Total volume	%
Citrus pectin	10.35±0.24	2.59±0.19	64.58±2.95	100±0.01
Sample L	3.18±0.25	3.37±0.06	44.96±3.76	81.36±1.21
Sample Lp	6.00±0.27	2.28±0.21	5.71±0.96	50.01±5.35
Sample L+C	6.73±0.77	2.73±0.28	13.55±0.35	75.02±0.35
Sample C	4.69±0.46	2.86±0.02	33.97±0.91	83.33±7.86
Sample H	4.63±0.76	1.97±0.69	32.18±1.63	94.44±7.86

Ingredients with a high OHC allowd for the stabilization of high fat food products and emulsions, facilitating the solubilization or dispersion of two immiscible liquids[31]. The mechanism of oil adsorption is not well characterized but a high OHC could be related to a high pectin content. The OHC of the fiber fraction studied varied between 2.0 and 3.4 g oil/g dry matter for Samples H and L, respectively. Although the values obtained for commercial citrus pectin were low, higher levels were found in the literature for dietary fiber from asparagus by-products

(around 7.5-8 g/g dry matter), with high contents in insoluble fiber, 51-67%, and 8.3-11% of soluble fiber[20].

In order to evaluate the functionality of these samples as emulsifier agents the emulsification activity (EA) and stability of the emulsion (ES) were studied. Citrus pectin had the highest EA value, 64.6%, despite the fact that the OHC of commercial pectin was lower followed by Sample L with a value around 45%. The other samples could not be considered as good emulsifiers as their EAs were lower than 50%[32]. However, almost all the samples showed good emulsification stability (75-100 %) after heating the emulsion at 80 ºC during 30 min. In spite of the low emulsifying capacity and stability indices, the use of the material obtained may stabilize foods with a high percentage of fat and emulsion as noted by Borchani, et al[33], for date fiber concentrates with an EA below 13%.

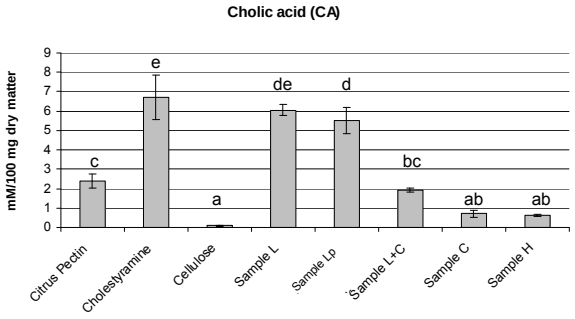
***In vitro* bile acid (BA) binding.**

The *in vitro* digestion procedure used in this study was designed to mimic aspects of the human digestion system, including acid digestion of a sample at pH 2 followed by a pancreatine digestion at pH 7[8]. Three of the major BA present in human bile were individually used for the binding assays. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two primary BA secreted by the liver. Deoxycholic acid (DCA) is one of the secondary BA formed through the transformation of primary acid by intestinal bacteria. Secondary acids are thought to be involved in the etiology and development of colorectal cancer[34].

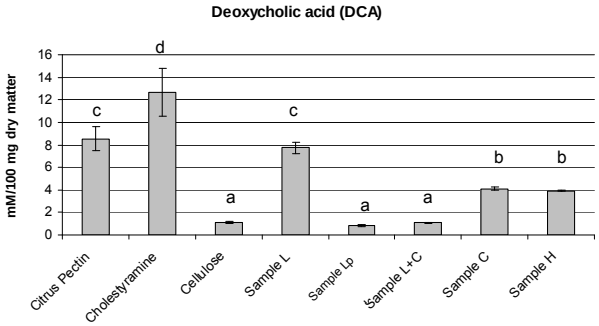
The samples prepared from olive stones and olive cake by-products on dry matter were compared to the BA binding of citrus pectin, cholestyramine, a well known BA binding anionic resin, and the negative control, commercial cellulose (**Figure 4a, b and c**). The results obtained for cholestyramine (which bound 6.7, 12.7 and 6.4 mM of CA, DCA and CDCA per 100 mg of sample, equal to 81.8, 96.6 and 91.8% of the total added BA), and cellulose (which bound only 0.08, 1.12 and 0.59 mM of CA, DCA and CDCA in 100 mg of sample, or 1.0, 9.6 and 7.6% of the total added BA), are similar to previously published results[7]. The BA binding values of the material fractionated from different steam-explosion treatments of olive by-products were in the range of 0.6 to 7.7 mM/100 mg of sample depending on the type of BA. The sample rich in lignin from olive stones, Sample L, bound similar amounts of the secondary bile acid, DCA, as citrus pectin (8.5 mM of DCA per 100 mg), a dietary fiber component that is known to interact with bile acids[35]. However, it is noteworthy that the BA binding values of Sample L, as well as Sample Lp, were significantly higher for the two primary bile acids, CA and CDCA, than citrus pectin. These results are in agreement with the fact that lignin is another of the dietary fiber components that interacts with BA[36]. The CA and CDCA binding of Sample H, C and L+C were not statistically different and had slightly lower values than those determined for citrus pectin. Although the

mechanism of interaction with BA is currently not fully understood, the structural parameter of the different polymers extracted from olive by-products may influence this interaction. It is remarkable that the fraction of the olive stone rich in lignin (Sample L) behaves similarly to Cholestyramine, commonly used in the treatment of hypercholesterolemia. However, the lignin within the matrix of cellulose (Sample L+C) showed a very small capacity for adsorption, not being statistically different from the fraction rich in cellulose (Sample C) in the case of CA and CDCA, and in the case of DCA even lower than Sample C, with values similar to the cellulose negative control. The fiber fractions studied, except Sample L+C and Sample Lp, showed higher percentages of bile acid binding with DCA (secondary BA) than CDCA and CA (primary BA). This was similar to results obtained by other authors who showed that BA adsorption decreases with the hydroxyl groups' increment in steroid nuclei[37]. In the case of the fraction of lignin from olive cake, Sample Lp, with a composition somewhat different to a true lignin, a more substantial difference in BA binding between primary and secondary BA was seen that may be related to the polymers´ structure or composition.

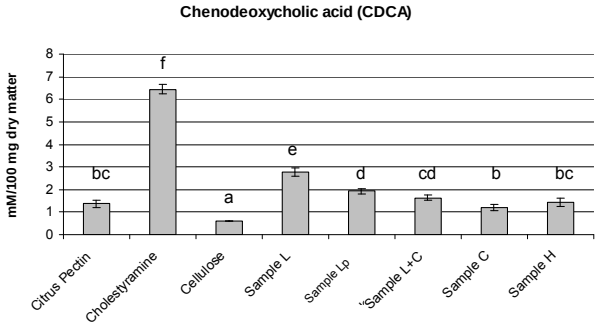
a



b



c



d

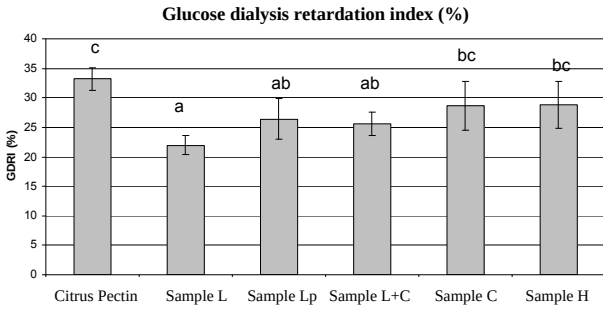


Figure 4: *In vitro* bile acid binding ability of the olive stones and olive cake by-products fractionated by steam explosion pretreatment combined with alkali delignification, compared to cholestyramine, cellulose, citrus and pectin (**a, b and c**). **d**) Glucose dialysis retardation index (%) of the samples prepared from olive stones and olive cake by-products steam-exploded on *in vitro* glucose diffusion, compared to citrus pectin. Data are presented as the means of three replicates± SDs. Different letters indicate significantly different results ($p < 0.05$).

A comparison of these results with those from previous studies on lignin[8] and other dietary fibers[7,9] shows a high bile acid binding capacity of primary and secondary acids, especially for the Sample L with CA, which point to their possible health-promoting. The captation of bile acid in the organism cause its excretion in the feces. The continued depletion of the bile acid by this way appears to be related with the levels of reduce serum cholesterol and thereby reducing the risk of cardiovascular disease[38]. This binding to bile salts (carcinogenic agents) also causes a reduction of the excessive level of bile in the colon, thus reducing the risk of developing cancer intestine[9].

Glucose dialysis retardation index (GDRI).

The GDRI is a useful *in vitro* index to predict the effect that fiber has on the delay of glucose absorption in the gastrointestinal tract[10]. The GDRI phenomenon seems to be related to the soluble dietary fiber and uronic acid contents of insoluble fiber, although other authors have pointed to the relationship between the internal structure and surface properties of fiber and glucose diffusion[39].

In **Figure 4d** the graphs for GDRI are presented. The results showed that both factors, composition and internal structure, could probably modulate this functional property, since values ranged from 22 to 29% for very different composition and nature of samples. The citrus pectin tested presented the highest value of GDRI (34%), although without significant differences between the sample of soluble fiber (Sample H) or insoluble fiber (Sample C).

The results obtained for the different components of olive stones and olive cake fractionated by steam-explosion pretreatment combined with alkali delignification were similar to those reported in the literature for mango peel (21%)[9], artichoke fiber (27%)[39] and carambola pomace (25%)[40], lower than for fiber from asparagus by-product (48%)[19] yet higher than those reported for wheat bran (5.3%)[41].

General Comments

The processing of olive by-products obtained from olive oil extraction (fragmented olive stones and olive cake) and pitted table olives (whole olive stones) by steam explosion pretreatment/alkali delignification allowed for the fractionation of the three main polymers present in the lignocellulosic matrix with a reasonable yield and purity. This study showed that the pretreatment converted the hemicelluloses into a water-soluble fraction (Sample H) rich in carbohydrates. The resulting insoluble material (Sample L+C) was characterized by the spectroscopy method and compared with the material without steam explosion (ball-milled

material). As such, we showed that the hemicelluloses were auto-hydrolyzed and the material had undergone important structural modifications during the steam explosion treatment. The cellulose was associated with a high proportion of lignin, which was partially recovered by alkaline-extraction followed by acid precipitation (Sample L), although some remains in the cellulose-rich fraction (Sample C).

The functional properties were analyzed and the fractions were found to have a water-holding capacity similar to those described for other agricultural by-products, with an average oil-holding capacity. The *in vitro* studies showed an important activity of bile acid binding for the lignin fraction from olive stones (Sample L). This lignin fraction bound significantly more bile acid than any other fraction, with values similar to cholestyramine, especially when the cholic acid (CA) was tested. For the alkaline extractable fraction from olive cake and subsequent precipitate by acid solution (Sample Lp), substantially different UV and IR spectra were obtained from those of guaicyl-syringyl lignin of olive stones; the binding values were also significantly higher than the rest of the fractions, except for the deoxycholic acid (DCA). Therefore, both lignin fractions (L and Lp) could contribute to the reduction of serum cholesterol levels and a decreased risk of bowel cancer. Furthermore, the glucose retardation index is a useful predictor of the *in vitro* health-promoting properties of the fractions, and showed that the water-soluble fraction (Sample H) and the fraction rich in cellulose (Sample C) had a high activity similar to pectin and other agricultural by-products These results would suggest the suitability of these fractions, extracted from olive by-products, for incorporation as low-calorie bulk ingredients in high-fiber foods to lower postprandial serum glucose levels.

Funding

Funding was received from the Ministerio de Economía y Competitividad of Spain and cofunded by European Social Fund (ESF) (Project AGL2013-48291-R). F.R.-S. received funding from the Spanish JAE-PRE program (CSIC-ESF) and G.R.-G. received funding from Spanish Ramón y Cajal program (RYC-2012-10456 contract) of Ministerio de Economía y Competitividad.

References

(1) Valiente, C.; Arrigoni, E.; Esteban, R.; Amadó, R. Chemical composition of olive by-product and modifications through enzymatic treatments. *J. Sci. Food Agric*, **1995,** *69*, 27-32.

(2) Heredia, A.; Guillén, R.; Fernández-Bolaños, J.; Rivas, M. Olives stone as a source of fermentable sugars. *Biomass*, **1987,** *14,* 143-148.

(3) Fernández-Bolaños, J.; Felizón, B.; Guillén, R.; Jiménez, A.; Heredia, A. Characterization of lignin steam-exploded olive stones and the cellulose residue. *Bioresour. Technol.* **1999**, *68*, 121-132.

(4) Fernández-Bolaños, J.; Felizón, B.; Heredia, A.; Rodríguez, R.; Guillén, R.; Jiménez, A. Steam-explosion of olive stones: hemicellulose solubilisation and enhancement of enzymatic hydrolysis of cellulose. *Bioresour. Technol.* **2001**, *79*, 53-61.

(5) Felizón, B.; Fernández-Bolaños, J.; Heredia, A.; Guillén, R. Steam-explosion pretreatment of olive cake. *J. Am. Oil Chem. Soc.* **2000**, *77*, 15-22.

(6) Rodríguez, R.; Jiménez, A.; Fernández-Bolaños, J.; Guillén, R.; Heredia, A. Dietary fibre from vegetable products as source of functional ingredients. *Trends Food Sci. Tech.* **2006**, *17*, 3-15.

(7) Kim, H.J.; White, P.J. *In vitro* bile-acid binding and fermentation of high, medium, and low molecular weight β-glucan. *J. Agric. Food Chem.* **2010**, *58*, 628-634.

(8) Story, J.A.; Kritchevsky, D. Comparison of the binding of various bile acids and bile salts *in vitro* by several types of fiber. *J. Nutr*. **1976**, 106, 1292-1294.

(9) Camire, M.E.; Dougherty, M.P. Raisin dietary fibre composition and *in vitro* bile acid bindig. *J. Agric. Food Chem.* **2003**, *51*, 834-837.

(10) Larrauri, J.A.; Goñi, I.; Martín-Carrón, N.; Rupérez, P.; Saura-Calixto, F. Measurement of health-promoting properties in fruit dietary fibres: antioxidant capacity, fermentability and glucose retardation index. *J. Sci. Food Agric*, **1996,** *71*, 515-519.

(11) Aguedo, M.; Kohnen, S.; Rabetafika, N.; Bossche, S.V.; Sterckx, J.; Blecker, C.; Beauve, C.; Paquot, M. Composition of by-products from cooked fruit processing and potential use in food products. *J. Food Compos. Anal.* **2012**, *27*, 61-69.

(12) Wang, G.; Chen, H. Fractionation of alkali-extracted lignin from steam-exploded stalk by gradient acid precipitation. *Sep. Purif. Technol.* **2013**, *105*, 98-105.

(13) AOAC (Official Methods of Analysis) 15th Edition. *Food Composition*, 1, **1990**; pp. 915-919.

(14) Blumenkrantz, N.; Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.* **1973**, *54*, 484-489.

(15) Singleton, V. L.; Rossi, J. A., Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Amer. J. Enol. Vitic.* **1965***, 16*, 144−158.

(16) Englyst, H.N.; Cummings, J.H. Simplified method for the measurement of total non-starch polysaccharides by gas-liquid chromatography of constituent sugars as aditol acetates, *Analyst*, **1984**, *109*, 937-942.

(17) Lama-Muñoz, A.; Rodríguez-Gutiérrez, G.; Rubio-Senent, F.; Fernández-Bolaños, J. Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. *Food Hydrocolloid,* **2012**, *28*, 92-104.

(18) Fengel, D.; Weggener, G. *Wood-Chemistry, Ultrastructure and reactions*, De Gruyter, Ed; Berlin and New York, **1984**; pp. 92-95.

(19) Browning, B.L. *Methods of wood chemistry*. *Vol II*, J. Wiley and Sons Interscience, Ed.; New York; **1967**.

(20) Fuentes-Alventosa, J.M.; Rodríguez-Gutiérrez, G.; Jaramillo-Carmona, S.; Espejo-Calvo, J.A.; Rodríguez-Arcos, R.; Fernández-Bolaños, J.; Guillén-Bejarano, R.; Jiménez-Araujo, A. Effect of extraction method on chemical composition and functional characteristics of high dietary fibre powders obtained from asparagus by-products. *Food Chem.* **2009**, *119*, 66-671.

(21) Betancur-Ancona, D.; Peraza-Mercado, G.; Moguel-Ordoñez, Y.; Fuertes-Blanco, S. Physicochemical characterization of lima bean (*Phaseolus lunatus*) and Jack bean (*Canavalia ensiformis*) fibrous residues. *Food Chem.* **2004**, *84*, 287-295.

(22) Boyd, G.S.; Eastwood, M.A.; MacLean, N. Bile acids in the rat: studies in experimental occlusion of the bile duct. *J. Lipid Res.* **1966**, *7*, 83-93.

(23) Dische, Z. Color reactions of carbohydrates. In *Methods in carbohydrates chemistry.* Whistler, R. L.; Wolfram, M. L., Eds.; Academic Press, New York; **1962**; pp. 477-512.

(24) Jiménez, A.; Guillén, R.; Fernández-Bolaños, J.; Heredia, A. Cell wall composition of olives. *J. Food Sci.* **1994**, *59*, 1192-1201.

(25) Chua, M.G.S.;Wayman, M. Characterization of autohydrolysis aspen (P. tremuloides) lignin. Part 3. Infrared and ultraviolet studies of extracted autohydrolysis lignin. Can. J. Chem. **1979,** *57,* 2603-2611.

(26) Thring, R.W.; Chornet, E.; Bouchard, J.; Vidal, P.F.; Overend, R.P. Characterization of lignin residues derived from the alkaline hydrolysis of poplar cell wall components. *Anim. Feed Sci. Tech.* **1990**, *32*, 235-242.

(27) Marchessault, R.H.; Coulombe, S.; Morikawa, H. Characterization of aspen exploded wood lignin. *Can. J. Chem.* **1982**, *60*, 2372-2382.

(28) Reeves III, J.B. Infrared spectroscopic studies on forage and by-product fibre fractions and lignin determination residues. *Vib. Spectrosc.* **1993**, *5*, 303-310.

(29) Kuznetsov, B.N.; Efrenov, A.A.; Levdanskii, V.A.; Kuznetosova, S.A.; Polezhayeva, N.I.; Shikina, T.A.; Krotova, I.V. The use of non-isobaric pre-hydrolysis for the isolation of organic compounds from wood and bark. *Bioresource Technol.* **1996**, *58*, 181-188.

(30) Rodríguez, R.; Jiménez, A.; Fernández-Bolaños, J.; Guillén, R.; Heredia, A. Dietary fibre from vegetable products as source of functional ingredients. *Trends in Food Sci. Tech.* **2006**, *17*, 3-15.

(31) Grigelmo-Miguel, N.; Gorinstein, S.; Martín-Belloso, O. Characterisation of peach dietary fibre concentrate as a food ingredient. *Food Chem.* **1999**, *65*, 175-181.

(32) Abdul-Hamid, A.; Luan, Y.S. Functional properties of dietary fibre prepared from defatted rice bran. *Food Chem.* **2000**, *68*, 15-19.

(33) Borchani, C.; Besbes, S.; Masmoudi, M.; Bouaziz, M.A.; Blecker, C.; Attia, H. Influence of oven-drying temperature on physicochemical and functional properties of date fibre concentrates. *Food Bioprocess Tech.* **2012**, *5*, 1541-1551.

(34) Baijal, P.K.; Fitzpatrick, D.W.; Bird, R.P. Comparative effects of secondary bile acids, deoxycholic and lithocholic acids, on aberrant crypt foci growth in the postinitiation phases of colon carcinogenesis. *Nutr. Cancer*, **1998**, *31*, 81-89.

(35) Sayar, S.; Jannink, J.L.; White, P.J. *In vitro* bile acid binding of flours from oat lines varying in percentage and molecular weight distribution of β-glucan. *J. Agric. Food Chem.* **2005**, *53*, 8797-8803.

(36) Kosikova, B.; Slamenova, D.; Mikulasova, M.; Horvathova, E.; Labaj, J. Reduction of carcinogenesis by bio-based lignin derivatives. *Biomass Bioenerg.* **2002**, *23*, 153-159.

(37) Drzikova, B.; Dongowski, G.; Gebhardt, E.; Habel, A. The composition of dietary fibre-rich extrudates from oat affects bile acids binding and fermentation *in vitro*. *Food Chem.* **2005**, *90*, 181-192.

(38) Eastwood, M.A.; Hamilton, D. Studies on the adsorption of bile salts to non-absorbed components of diet. *Biochim. Biophys. Acta*, **1968**, 152, 165-173.

(39) López, G.; Ros, G.; Rincón, F.; Periago, M.J.; Martínez, M.C.; Ortuño, J. Relationship between physical and hydration Properties of soluble and insoluble fibre of artichoke. *J. Agric. Food Chem.* **1996**, *44*, 2773-2778.

(40) Chau, C.-F.; Chen, C.-H.; Lin, C.-Y. Insoluble fiber-rich fraction derived from *Averrhoa caramola*: hypoglycemic effects determined by *in vitro* methods. *LWT-Food Sci. Tech*. **2004**, *37*, 331-335.

(41) Adiotomre, J.; Eastwood, M.A.; Edwards, C.A.; Brydon, W.G. Dietary fibre: *in vitro* methods that anticipate nutrition and metabolic activity in human. *Amer. J. Clin. Nutr.* **1990**, *52*, 128-134.

7. Conclusiones

7. Conclusiones

1. El método de extracción y análisis puesto a punto en el presente trabajo ha permitido la caracterización fenólica de nuevos extractos a partir de alperujo térmicamente tratado.
2. La aplicación de un tratamiento térmico al vapor (160 ºC, 15-90 minutos) sobre el alperujo y posterior extracción líquido-líquido con acetato de etilo permite obtener extractos con elevada concentración en fenoles simples. Dependiendo de las condiciones del tratamiento y del pH de la muestra se modifica la composición fenólica de los extractos.
3. El tratamiento térmico mejora significativamente las propiedades antioxidantes del nuevo extracto fenólico tanto en medio acuoso como en emulsiones lipídicas, la inhibición a la agregación plaquetaría y la inhibición de la oxidación en microsomas de ratas deficientes en vitamina E.
4. En los extractos obtenidos se ha detectado en elevadas concentraciones una nueva fracción fenólica polimérica y se han caracterizado estructuralmente dos nuevas moléculas identificadas como el ácido (E)-3-(oxobut-1-2-en-2-il)glutárico y el ácido β-D-glucopiranosil dihidroelenólico.
5. Se ha conseguido aislar, purificar e identificar dos precursores del 3,4-dihidroxifenil-glicol, los diasteroisómeros β-hidroxiacteósido y β-hidroxiisoacteósido y la 2”-hidroxioleuropeína.
6. La nueva fracción fenólica polimérica aislada presenta la capacidad de absorber y liberar hidroxitirosol, posibilitando una nueva forma para purificar este compuesto fenólico.
7. El tratamiento térmico aplicado sobre el alperujo provoca la hidrólisis de las hemicelulosas permitiendo la liberación de oligosacáridos (xilanos, xiloglucanos, glucuronoxilanos, glucuarabinoxilanos), los cuales se recuperan con un grado de polimerización entre 4 y 10 mediante un nuevo procedimiento puesto a punto.
8. El tratamiento térmico aplicado sobre el alperujo permite la recuperación de pectinas de bajo peso molecular con importantes propiedades funcionales tales como la captación de sales biliares y retardo del índice de glucosa. Las características químicas y físicas de estas pectinas posibilitan su estudio funcional como pectinas modificadas, las cuales se absorben en el intestino delgado uniéndose y bloqueando a la proteína pro-metastásica Galectina-3.
9. Se han recuperado polisacáridos pécticos de alto peso molecular a partir de alperujo sometido a un tratamiento térmico suave (50-80 ºC durante 1-2 horas), que debido a sus propiedades antioxidantes pueden ser clasificados como “fibra antioxidante”. Además presentan unas características químicas particulares que permiten una captación de sales biliares y retardo del índice de la glucosa mejor que la pectina de limón comercial.
10. La lignina recuperada a partir de un material puramente lignocelulósico presenta una capacidad de captación de ácidos biliares igual a la observada para la resina

colestiramina, la cual se emplea como fármaco en el tratamiento de hipercolesterolemia.

8. Anexos

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

New Phenolic Compounds Hydrothermally Extracted from the Olive Oil Byproduct Alperujo and Their Antioxidative Activities

Fátima Rubio-Senent, Guillermo Rodríguez-Gutíerrez, Antonio Lama-Muñoz, and Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero 4, Sevilla 41012, Spain

ABSTRACT: The application of a novel process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final liquid phase that contained a high concentration of simple phenolic compounds. This study evaluated the effects of time (15−90 min) on the composition of the phenolic compounds isolated at a fixed temperature of 160 °C. Phenolic compounds were extracted with ethyl acetate. Both qualitative and quantitative HPLC analyses of the extracts showed variation of the concentrations of phenolic compounds with time. In addition, new phenols that were not present in the untreated control have been characterized. The antioxidant activities of different phenolic extracts was measured by various assays conducted in vitro: antiradical capacity (using DPPH and ABTS radicals), ferric reducing power (P_R), inhibition of primary and secondary oxidation in lipid systems, and other tests, such as inhibition of tyrosinase activity. The results show that the phenolic extracts inhibited oxidation in aqueous and lipid systems to a significantly greater extent than the untreated control, and they performed as well as or better than vitamin E in this capacity.

KEYWORDS: *alperujo, olive oil wastes, phenols, antioxidant, hydroxytyrosol, tyrosinase, ethyl acetate extract*

INTRODUCTION

The olive oil industry is important in the Mediterranean region, and Spain produces >30% of the world's olive oil. Traditionally, the three-phase mill uses large volumes of water to aid the separation of oil and generates two byproducts. The first one is a liquid waste, which is known as olive mill wastewater, vegetation water, or alpechin. The second byproduct, a solid waste, is a combination of olive pulp and stones and is called pomace or orujo. The use of a modern two-phase processing technique, in which no water is added, generates a new byproduct called alperujo, which is a combination of liquid and solid waste. This two-phase centrifugation process is used for the separation of the oil from the vegetable material, which includes all of the mineral celluloses, hemicelluloses, pectins, gums, tannins, and polyphenols. In Spain, a massive change from the traditional three-phase process to the new two-phase process has taken place, and large volumes of waste, approximately 3.5−6 million tons/year, are generated.[1]

The Mediterranean diet has been documented by a large number of epidemiological studies.[2] It has been shown that the consumption of virgin olive oil, with high content in monounsaturated fatty acids and with an unsaponifiable fraction rich in minor components such as polyphenols, sterols, and tocopherols, leads to an increase in the total phenolic content of low-density lipoprotein (LDL) to prevent the LDL oxidation in the arterial intima.[3] Phenolic compounds from virgin olive oil may delay the progression of atherosclerosis by this mechanism. The antioxidative effects of phenolic compounds present in olive products also may contribute to the prevention of chronic diseases such as cardiovascular disease and cancer.[4] These phenolic compounds protect organisms against oxidative damage and prevent the deterioration of food by inhibiting lipid oxidation. The dialdehydic form of one of the secoiridoids, deacetoxy-ligstroside aglycone (oleocanthal), was also recently found in the olive oil. Oleocanthal has the ability to inhibit cyclooxygenase (COX-1 and COX-2), showing an anti-inflammatory effect similar to that seen with ibuprofen.[5]

After the extraction of oil, 98−99% of the phenolic compounds present in the fruit of the olive remain in the alperujo. To explore the possibility of obtaining simple phenolic compounds in high yield from two-phase olive waste, a series of hydrothermal treatments was performed. A process that allows for easy separation of the solid and liquid phases of alperujo has been developed. The process also allows for the recovery of value-added compounds in the water-soluble fraction. In this treatment, which has been patented (PCT/ES2011/070583), an autohydrolytic process occurs, resulting in the solubilization of the alperujo. Usually, whenever a lignocellulosic material is treated with water or steam at temperatures from 160 to 240 °C, an autohydrolysis process occurs.[6] Depending on the conditions used, there may be a depolymerization of polysaccharides (mainly of hemicelluloses) and a breaking of lignin structures, resulting in the solubilization of lignin fragments of low weight.

As a consequence of our hydrothermal treatment, the byproduct was partially solubilized. The simple phenols, such as hydroxytyrosol, tyrosol, and 3,4-dihydroxyphenylglycol, increased in concentration as a result of the breakdown of complex molecules, such as oleuropein, demethyloleuropein, verbascoside, and others, that contain them in their structure.[7] Interest in antioxidants for the prevention and treatment of human diseases has been sustained for at least two decades. It has been suggested that the consumption of certain foods that contain bioactive compounds, including fruits, vegetables, and

Received: October 17, 2011
Revised: December 16, 2011
Accepted: January 3, 2012
Published: January 3, 2012

dx.doi.org/10.1021/jf204223w | J. Agric.Food Chem. 2012, 60, 1175−1186

LWT - Food Science and Technology 54 (2013) 114–124

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity

Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Padre García Tejero n° 4, Sevilla 41012, Spain

ARTICLE INFO

Article history:
Received 25 October 2012
Received in revised form
25 March 2013
Accepted 11 April 2013

Keywords:
Alperujo
Phenols
3,4-Dihydroxyphenylglycol
Hydroxytyrosol
Antioxidant

ABSTRACT

Alperujo or olive oil waste was hydrothermally treated at 160 °C for 60 min to increase the phenols in the liquid phase. The extract obtained from the liquid using ethyl acetate extraction was divided into 27 phenolic fractions using adsorbent and polyamide resins. Phenolic alcohols and acids, secoiridoid molecules, elenolic acid derivatives, flavonoids, verbascoside, degradation products of sugar and a polymeric phenolic fraction (PPF) were characterized using HPLC-ESI-MS. The antiradical activity, ferric reducing power and the inhibition of primary and secondary oxidation were examined for each fraction. Hydroxytyrosol was the most abundant phenol in the ethyl acetate extract and possibly the main component responsible for the in vitro antioxidant activity of the entire phenol extract. However, other phenolic and secoiridoid molecules with interesting biological properties were also identified, and the crude extract could be considered a potential source of natural antioxidants.

1. Introduction

Many epidemiological studies show that the Mediterranean diet provides protection against a wide array of common chronic pathological conditions, including coronary heart disease, cancer and neurodegenerative disorders (Pérez-Jiménez, 2005). This effect may be attributed to many components of the diet, including the phenols that exhibit free radical scavenging activity and protect organisms against oxidative damage (Covas et al., 2006). Thus, the daily consumption of olive oil, rich in phenolic compounds improves health by reducing oxidative damage to the human body. However, after olive oil extraction, only a small percentage (1–2%) of the total phenols present in the olive fruit remain in the oil, with the majority remaining in the olive mill wastes like alperujo (two phase extraction system) that may be used as a promising source of these phenolic compounds.

To increase the concentration of these phenols and to extract them in high yield from alperujo, an environmentally friendly process based on hydrothermal treatment in which an autohydrolytic process occurs has been developed (PCT/ES2011/070583). In a previous study, phenols were selectively extracted from the autohydrolysis liquid using ethyl acetate, yielding extracts with antioxidant activity levels comparable to vitamin E (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012).

The aim of this study was to determine the phenolic composition of the ethyl acetate extract obtained after hydrothermal treatment of the alperujo at 160 °C for 60 min. Fractionation of the extract is used to facilitate the characterization and identification of the main phenolic and secoiridoid components. A second purpose of this study was to assess the antioxidant activity of each fraction to determine the relative contribution of the various compounds to the above activities for the entire phenolic extract. This study will help to evaluate the use of this extract as a potential source of natural antioxidants.

2. Materials and methods

2.1. Materials

The sample of alperujo was obtained in March 2009 from Picual olives processed at a Spanish oil mill (Instituto de la Grasa, Seville).

2.2. Standard compounds

Hydroxymethylfurfural, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, p-coumaric acid, caffeic acid and 3,4-dihydroxyphenylglycol were obtained from Sigma–Aldrich (Deisenhofer, Germany). Tyrosol and 3,4-dihydroxyphenylacetic acid

* Corresponding author. Tel.: +34 954691054; fax: +34 954691262.
E-mail address: jfbg@cica.es (J. Fernández-Bolaños).

0023-6438/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lwt.2013.04.011

Food Research International 54 (2013) 2122–2129

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste

Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños *

Departamento de Biotecnología de Alimentos, Instituto de la Grasa (Consejo Superior de Investigaciones Científicas, CSIC), Avda. Padre García Tejero, 4, 41012 Seville, Spain

ARTICLE INFO

Article history:
Received 4 December 2012
Received in revised form 4 March 2013
Accepted 6 March 2013

Keywords:
Alperujo
Antioxidant
Autohydrolysis
Ethyl acetate extracts
Hydroxytyrosol
Polymeric phenols
Sorption

ABSTRACT

Polymeric phenolic fraction (PPF) was isolated by ethyl acetate extraction from hydrolyzed liquids from steam-treated alperujo and characterized. PPF is composed mainly of phenolic compounds with small amounts of carbohydrates, protein and ash. Phenols such as hydroxytyrosol (HT) and tyrosol were bound to PPF during the ethyl acetate extraction. Acid hydrolysis (3 N HCl for 10 min at 100 °C) was used to liberate HT from the PPF. The properties of both sorption and desorption of the PPF were studied to enhance the purification of HT, up to 100%. The antioxidant activities of the PPF were evaluated in vitro using ferric reducing power and DPPH radical scavenging, showing activity but lower than HT and TROLOX. The inhibition of primary oxidation of PPF was similar to both HT and vitamin E. These antioxidant activities of the PPF were not a result of the presence of HT or similar phenols.

1. Introduction

The olive oil industry has received an increased attention in the last several decades with the growing interest in the consumption of olive oil as an inherent part of a Mediterranean diet, which has been linked with low incidence of cardiovascular disease and cancer. These effects have been ascribed to the high content of phenolic compounds and other antioxidants, such as tocopherols (Pérez-Jiménez, 2005). The phenolic compounds in olives are potentially bioactive. Olive oil contains only 1–2% of the total phenol content of the entire olive. The remaining 98–99% is present in the olive mill waste, which is available in large quantities. The residue obtained from olive oil extraction by a two-phase technique, termed "alperujo", is a valuable starting material for the production of phenols characterized by a wide array of biological activities (antioxidant, cardioprotective, antimicrobial, antihypertensive, anti-inflammatory and anticancerigenic) that could be used in the pharmaceutical, cosmetic and food industries.

These phenolic compounds present in alperujo can be recovered by a novel process based on hydrothermal treatment (Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, & Sánchez, 2011). This treatment, which is performed in a discontinuous reactor using steam at high pressures and temperatures (170 °C, 8 kg/cm^2), allows the separation of alperujo into two phases (liquid and solid). While it is practically impossible without the pretreatment process, this operation produces a high solubilization of phenolic compounds into the liquid phase.

In a previous study, phenolic compounds were extracted with ethyl acetate from the aqueous fraction of steam-treated alperujo. This process was selective for small and medium molecular weight phenols. These compounds were fractionated by an adsorption column to characterize the composition and to determine the specific antioxidant potential of the extracts in each fraction. A fraction of phenolic polymerics has been previously identified in these extracts (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012).

Many different polymeric phenolic compounds have been identified in the fruit of *Olea europaea* (Oleaceae). The antioxidative abilities of these oligomeric (oxidized phenolics) have also been characterized (Cardinali et al., 2010). The pigments from olive mill waste have been identified as catechol-melanin macromolecules derived from a polymerization of the phenolic compounds linked to sugars, proteins and fatty acids (Obied, Allen, Bedgood, Prenzler, & Robards, 2005). Phenolic antioxidants generally undergo a polymerization during the oil extractive process or even during ripening of the fruit (Bianco et al., 2006). A polymeric organic fraction formed from phenols, polysaccharides, proteins and inorganic substances, which was named "polymerin", has also been isolated from olive oil mill waste water (Capasso, De Martino, & Arienzo, 2002).

Among the phenolic compounds present in olives, olive oil and olive by-products, hydroxytyrosol (HT) is the greatest by quantity; moreover, it possesses noteworthy antioxidant activities and potentially

* Corresponding author. Tel.: +34 954 691054; fax: +34 954 691262.
E-mail address: jfbg@cica.es (J. Fernández-Bolaños).

0963-9969/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodres.2013.03.003

Eur J Nutr
DOI 10.1007/s00394-014-0807-8

ORIGINAL CONTRIBUTION

Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E-deficient rats

Fátima Rubio-Senent · Baukje de Roos · Garry Duthie · Juan Fernández-Bolaños · Guillermo Rodríguez-Gutiérrez

Received: 2 September 2014 / Accepted: 24 November 2014

Abstract
Purpose This study explored the in vitro antioxidant and anti-platelet activities of hydroxytyrosol, hydroxytyrosol acetate, 3,4-dihydroxyphenylglycol and two phenolic olive extracts. These compounds and extracts were obtained from a new industrial process to hydrothermally treat the alperujo (160 °C/60 min), a by-product of olive oil extraction.
Methods The extracts and the purified compounds were obtained chromatographically using both ionic and adsorbent resins. The antioxidant activity was determined by measuring inhibition of human platelet aggregation and inhibition of lipid peroxidation in liver microsomes of vitamin E-deficient rats.
Results The positive effect of the extracts on the inhibition of platelet aggregation is showed, being higher in the case of hydroxytyrosol acetate up to 38 %, and for the first time, its synergist effect with hydroxytyrosol has been proved, obtaining more than double of inhibition. The phenolic extracts and the isolated phenols showed good results for inhibiting the lipid oxidation, up to 62 and 25 %, respectively. A synergistic effect occurred when the hydroxytyrosol acetate and the 3,4-dihydroxyphenylglycol were supplemented by hydroxytyrosol.
Conclusion These results suggest the extract and these compounds obtained from a novel industrial process could be natural alternatives for the prevention of diseases related to cardiovascular disorder or oxidative damage.

Keywords Alperujo · Olive oil wastes · Phenols · Platelet function · Lipid peroxidation · Vitamin E

F. Rubio-Senent · J. Fernández-Bolaños · G. Rodríguez-Gutiérrez (✉)
Food Phytochemistry Department, Instituto de la Grasa (CSIC), Avda. Padre García Tejero 4, Apartado 1078, 41012 Seville, Spain
e-mail: guirogu@cica.es

B. de Roos · G. Duthie
Rowett Institute of Nutrition and Health, University of Aberdeen, Greenburn Road, Bucksburn, AB21 9SB Aberdeen, UK

Introduction

Consumption of the Mediterranean diet, characterized by high consumption of olive oil, fruits, vegetables, grains and legumes, reduces the incidence of major cardiovascular events [1] and is associated with a lower risk of peripheral artery disease [2]. The health benefits of the Mediterranean diet have been attributed to high concentration of free radical-scavenging polyphenols such as flavonoids. Virgin olive oil is rich in unsaponifiable minor components such as sterols, tocopherols and polyphenols. The polyphenols are natural antioxidants that not only contribute to the stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties [3]. Dietary polyphenols have been shown to inhibit LDL oxidation, scavenge superoxide and other ROS, and increase plasma antioxidant capacity [4]. Furthermore, some dietary phenolic compounds, mainly polyphenols, have been shown to affect human platelet function in vitro and in vivo [5, 6]. Platelets play a central role in the formation of plaques within blood vessels, contributing to early inflammatory events [5]; so, the observed cardiovascular benefits attributed to olive oil may be linked to the anti-platelet activity of olive oil polyphenols and thus to the suppression of platelet activation.

The ability of many flavonoids and phenols to inhibit peroxidation of hepatic microsomal preparations from vitamin E-deficient rats might indicate that these dietary compounds could have significant "vitamin E-like" antioxidant activity in biological systems [7]. The ability of dietary

Published online: 14 December 2014

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Isolation and Characterization of a Secoiridoid Derivative from Two-Phase Olive Waste (Alperujo)

Fátima Rubio-Senent,[†] Sergio Martos,[‡] Aránzazu García,[†] José G. Fernández-Bolaños,[‡] Guillermo Rodríguez-Gutiérrez,[†] and Juan Fernández-Bolaños*[,†]

[†]Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero 4, Seville 41012, Spain

[‡]Departamento de Química Orgánica, Facultad de Química, Universidad de Sevilla, Profesor García González 1, Seville 41012, Spain

Supporting Information

ABSTRACT: A secoiridoid derivative was isolated from the ethyl acetate extract of two-phase olive waste (alperujo). The structure of this compound was fully characterized as *s-trans*-(*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid. The spectroscopic data, including one- and two-dimensional nuclear magnetic resonance, mass spectrometry, infrared analysis, and ultraviolet spectrum, were showed. The origin of this compound has not been previously studied, although it most likely results from the breakdown of the oleuropein (or ligstroside) secoiridoid skeleton via oxidation and decarboxylation of the dialdehydic form of elenolic acid, with this transformation being enhanced by extraction of phenolics with ethyl acetate. In addition, the bactericidal activity of (*E*)-3-(1-oxobut-2-en-2-yl)glutaric acid and extracts containing it was evaluated against two phytopatoghenic microorganisms *Pseudomonas syringae* and *Agrobacterium tumefaciens*.

KEYWORDS: *alperujo, antimicrobial activity, ethyl acetate extract, elenolic acid, glutaric acid derivatives*

1. INTRODUCTION

Olive oil production is a very important and traditional industrial activity in the countries of the Mediterranean area, which account for 95% of the worldwide olive oil production.[1] The olive oil manufacturing process by a two-phase centrifugation system generates huge quantities of a semi-solid byproduct called alperujo, recognized as a valuable source of natural phenol antioxidants. After olive oil extraction, only a small percentage (1−2%) of the total phenols present in the olive fruit remain in the oil, with 98−99% of the phenol remaining in the alperujo.[2]

The phenolic compounds of olives and olive products have been extensively studied because of their biological properties.[3,4] To increase the concentration of these phenols and facilitate their extraction from alperujo, an environmentally friendly process based on hydrothermal treatment was developed.[5] This process led to an easy separation of the solid and liquid phases of alperujo and allows for the recovery of value-added compounds in the water-soluble fraction. The alperujo is partially solubilized in water by the hydrothermal treatment, with the liquid fraction being enriched in phenolic compounds that are easily extractable with ethyl acetate.[6]

The ethyl acetate extract was an interesting fraction composed of numerous phenolic compounds.[6−8] Many of the phenolic compounds present in the extract have been identified, and their structures have been elucidated; however, some compounds remain unidentified, including different derivatives of elenolic acid present in large amounts. In this work, we have isolated and identified a compound whose structure, derived from elenolic acid, has been well-established.

It has been shown that compounds with elenolic acid derivative structures, such as glutaraldehyde-like compounds, exhibit important antimicrobial activities. Numerous works have studied the antimicrobial activity of olive mill wastewaters and alperujo[9,10] because of the high concentrations of phenolic compounds present in these byproducts. For many years, this activity was attributed to oleuropein and its hydrolysis products (oleuropein aglycon, elenolic acid, and hydroxytyrosol), and the antimicrobial activity of these substances has been extensively studied.[11] Medina et al.[12] demonstrated that the main antimicrobials in olives are the dialdehydic form of decarboxymethyl elenolic acid, either free (EDA) or linked to hydroxytyrosol (HT−EDA), because of their content in glutaraldehyde-like compounds. Phytopathogenic bacteria, such as *Pseudomonas syringae* and *Agrobacterium tumefaciens*, can cause severe disease in a wide range of plants cultivated in the Mediterranean basin. *P. syringae* contributes to many diseases in tomato, peas, maple, kiwifruit, wheat, barley, and others, and *A. tumefaciens* produces tumors known as crown galls in a diverse group of dicotyledonous plants. The use antimicrobials from olive mill wastewaters against these bacteria would be of great interest.

In this work, an elenolic acid derivative was isolated and its structure was elucidated by nuclear magnetic resonance (NMR) spectroscopy. Moreover, the role of ethyl acetate in the formation of this compound was analyzed. Finally, because the structure of the compound derives from a glutaraldehyde-like compound, an antimicrobial assay was realized to evaluate its bactericidal activity.

Received: September 24, 2014
Revised: January 8, 2015
Accepted: January 9, 2015

DOI: 10.1021/jf5057716
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Food Chemistry 140 (2013) 154–160

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste

Antonio Lama-Muñoz, Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Rafael Palacios-Díaz, Juan Fernández-Bolaños *

Departamento de Biotecnología de Alimentos, Instituto de la Grasa (Consejo Superior de Investigaciones Científicas, CSIC), Avda. Padre García Tejero, 4, 41012 Seville, Spain

ARTICLE INFO

Article history:
Received 27 September 2012
Received in revised form 30 January 2013
Accepted 11 February 2013
Available online 24 February 2013

Keywords:
Alperujo
Antioxidants
3,4-Dihydroxyphenylglycol
Hydroxytyrosol
Phenols
ESI mass spectrometry
Oleuropein
Verbascoside

ABSTRACT

3,4-Dihydroxyphenylglycol (DHPG) is a potent antioxidant recently found in the free form in olive oil and table olives. DHPG can be recovered from olive oil solid waste by a hydrothermal treatment. It was observed that an increase in the concentration of DHPG occurred when alperujo aqueous extracts were subjected to mild thermal conditions (post-treatment). This fact indicates that certain solubilized compounds or precursors containing DHPG which is released with the post-treatment. In the present study, the precursors of DHPG were identified and characterized after extraction from alperujo using thermal treatment and purification by fractionation on Amberlite® XAD16 polyamide and semi-preparative reverse-phase HPLC columns. Their structures were elucidated using HPLC coupled to diode array detector (DAD) and electrospray ionization mass spectrometry (ESI-MS). The results identified three compounds as precursors, and their structures can be attributed to the diastereoisomeric forms of the two β-hydroxy derivatives of verbascoside and isoverbascoside (β-hydroxyacteoside and β-hydroxyisoacteoside), and 2″-hydroxyoleuropein, all of which contain a DHPG moiety, potentially explaining the increases in the concentration of this phenolic compound in olive oil waste.

1. Introduction

In Spain, olive oil is commonly extracted using a two-phase centrifugation system, a process that generates a semisolid waste that is called two-phase pomace or "alperujo". It has been estimated that the generation of alperujo as olive processing by-product in Spain is approximately 4–6 million tonnes every year. However, alperujo presents a serious environmental problem for Mediterranean countries due to its highly polluting organic load that includes lipids, pectins, polyalcohols, sugars, tannins and phenolic compounds, which limit its biodegradation because of their high toxicity (Roig, Cayuela, & Sánchez-Monedero, 2006). However, alperujo can provide a large, inexpensive, and unexploited source of chemicals: alperujo is rich in phenolic compounds (it contains 98–99% of the available phenols in olive fruit) with strong antioxidant properties and well-recognized health-beneficial activities (Cicerale, Lucas, & Keast, 2010).

The phenolic compounds present in alperujo can be recovered by a novel process based on hydrothermal treatment (Rubio-Senent, Rodríguez-Gutiérrez, Lama-Muñoz, & Fernández-Bolaños, 2012). This treatment is conducted in a discontinuous reactor using steam at high pressures and temperatures (170 °C, 8 kg/cm^2) and allows the separation of alperujo into two phases (liquid and solid), an operation that is practically impossible without pretreatment. The result is a significant solubilization of the phenolic compounds in the liquid phase. The simple phenol 3,4-dihydroxyphenylglycol (DHPG) is structurally similar to hydroxytyrosol (HT) (the major phenolic in olive fruit) and has an additional hydroxyl group in the β-position. DHPG is found in vegetation water of the olive fruit (Limiroli, Consonni, Ranalli, Bianchi, & Zetta, 1996), table olives (Rodríguez, Lama, Jaramillo et al., 2009) and olive oil (Medina, de Castro, Romero, & Brenes, 2006) and has been recovered from alperujo using the above treatment (Rodríguez, Lama, Trujillo et al., 2009). A patented system for purifying DHPG (>90% dry weight) from this natural source has been developed by our group and utilizes ion-exchange resins and adsorbent polymers (Fernández-Bolaños et al., 2008). Despite the many studies that show the health-beneficial biological activities of HT (Granados-Principal, Quiles, Ramirez-Tortosa, Sanchez-Rovira, & Ramirez-Tortosa, 2010; Killeen, Pontoniere, & Crea, 2011), only a few studies have been conducted on the functional properties of DHPG. DHPG has higher antioxidant and antiradical capacities and reducing power than hydroxytyrosol (HT) and prevents lipid peroxidation to a degree that is comparable with vitamin E (Rodríguez, Rodríguez, Fernández-Bolaños, Guillén, & Jiménez, 2007). Furthermore, DHPG is bioavailable and has antioxidant properties in vitamin E-deficient rats (Rodríguez-Gutiérrez et al., 2012); it may also protect against

* Corresponding author. Tel.: +34 954 691054; fax: +34 954 691262.
E-mail address: jfbg@cica.es (J. Fernández-Bolaños).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.02.063

Chapter

PRODUCTION OF OLIGOSACCHARIDES WITH LOW MOLECULAR WEIGHTS, SECOIRIDOIDS AND PHENOLIC GLYCOSIDES FROM THERMALLY TREATED OLIVE BY-PRODUCTS

Juan Fernández-Bolaños*, Fátima Rubio-Senent, Antonio Lama-Muñoz, Aránzazu García and Guillermo Rodríguez-Gutiérrez

Instituto de la Grasa. Consejo Superior de Investigaciones Científicas (CSIC). Avda. Padre García Tejero no. 4, Sevilla-41012, Spain

ABSTRACT

Alperujo was hydrothermally pre-treated to solubilize the oligosaccharides from the cell wall material of olive pulp. A protocol for the isolation of oligosaccharides of low molecular weight has been developed, which involves thermal treatment, mild acid hydrolysis, separation by ultra-filtration, adsorption chromatography and size exclusion chromatography using Superdex Peptide HR. Three groups of fractions with common characteristics were obtained and studied in two

* Corresponding author: Tel: 34-954691054; Fax 34-954691262; E-mail: jfbg@cica.es.

Food Hydrocolloids 43 (2015) 311–321

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Pectin extracted from thermally treated olive oil by-products: Characterization, physico-chemical properties, *in vitro* bile acid and glucose binding

Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Padre García Tejero n° 4, Sevilla 41012, Spain

ARTICLE INFO

Article history:
Received 11 February 2014
Accepted 2 June 2014
Available online 18 June 2014

Keywords:
Alperujo
Steam treatment
Pectin
Glucose retardation index
Water/oil holding capacity
Bile acid binding

ABSTRACT

The pectin fraction released from steam-treated alperujo at 160 °C for 30, 45, and 60 min was purified and characterized. Differences were observed in the composition, and physical and biological activities of the pectin extracts, depending on the length of the thermal treatment. The extracts presented a low molecular weight in the range of 2–40 KDa, a high content of neutral sugars, and a high percentage of acetylation. In comparison with commercial pectins, the extracts had low water and high oil holding capacities, a normal emulsifying activity and a similar emulsion stability to that of apple pectin. For the three pectin extracts studied, the *in vitro* analyses showed considerable bile-acid binding activity and a glucose retardation index similar to the values obtained for citrus pectins. Thus, the pectins isolated from olive oil waste have suitable physical and biological properties for commercial use. Pectin extraction from alperujo, a sizeable by-product of the olive oil manufacturing process, would facilitate the use and subsequent reduction of this environmentally damaging organic waste.

1. Introduction

The olive oil manufacturing process, by the continuous biphasic extraction system, generates oil and a by-product known as alperujo. Alperujo is a combination of liquid (olive vegetative water) and solid (skin, seed, pulp and pieces of stones) olive-pomace mill waste. Approximately 4–6 million tonnes of alperujo are produced by the olive oil extraction process every year in Spain. The production of such high quantities of alperujo has important environmental consequences, due to its high organic content and the presence of phytotoxic components, which makes it difficult to use in further bioprocesses.

The combustion of alperujo as a fuel is commonly used as a procedure to eliminate its harmful effect on the environment. However, several studies have investigated ways to exploit this polluting by-product in order to obtain high value products, besides a waste reduction strategy. With the aim of improving the utilization of alperujo, we have recently developed an innovative steam treatment (Patent no. ES2374675; Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, & Sánchez, 2010), which allows for an easy separation of the solid and liquid phases. In this process, an auto-hydrolysis of material occurs and facilitates the recovery of added-value compounds in the water-soluble fraction. Recoverable compounds include natural antioxidants (biophenols such as hydroxytyrosol and 3,4-dihydroxyphenylglycol), neutral and acid oligosaccharides, and phenolic glucosides (Rubio-Senent, Lama-Muñoz, Rodríguez-Gutiérrez, & Fernández-Bolaños, 2013). In addition, considerable amounts of pectic polysaccharides can be recovered by this system, because one third of the olive-pulp cell wall is made up of arabinose-rich pectic polysaccharides (Coimbra, Cardoso, & Lopes-da-Silva, 2010).

Pectins are natural hydrocolloids that are found in higher plants as principal structural elements of cell walls, and have been widely used as gelling agents, stabilizers, and emulsifiers in the food industry. Pectin is also a component of soluble dietary fibers, which is poorly digested in the small intestine but ferments in the colon, and plays a significant role in many physiological processes. It acts as a prebiotic, anti-inflammatory, anti-diarrheal, and can prevent constipation; it is also beneficial to the control of diabetes, and in the prevention of obesity and cancer (Markov, Popov, Nikitina, Ovodova, & Ovodov, 2011; Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006). In addition, interest has

* Corresponding author. Tel.: +34 954691054; fax: +34 954691262.
E-mail address: jfbg@cica.es (J. Fernández-Bolaños).

http://dx.doi.org/10.1016/j.foodhyd.2014.06.001
0268-005X/

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Properties of Lignin, Cellulose, and Hemicelluloses Isolated from Olive Cake and Olive Stones: Binding of Water, Oil, Bile Acids, and Glucose

Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Lama-Muñoz, Aránzazu García, and Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero 4, Sevilla 41012, Spain

ABSTRACT: A process based on a steam explosion pretreatment and alkali solution post-treatment was applied to fractionate olive stones (whole and fragmented, without seeds) and olive cake into their main constitutive polymers of cellulose (C), hemicelluloses (H), and lignin (L) under optimal conditions for each fraction according to earlier works. The chemical characterization (chromatographic method and UV and IR spectroscopy) and the functional properties (water- and oil-holding capacities, bile acid binding, and glucose retardation index) of each fraction were analyzed. The in vitro studies showed a substantial bile acid binding activity in the fraction containing lignin from olive stones (L) and the alkaline extractable fraction from olive cake (Lp). Lignin bound significantly more bile acid than any other fraction and an amount similar to that bound by cholestyramine (a cholesterol-lowering, bile acid-binding drug), especially when cholic acid (CA) was tested. These results highlight the health-promoting potential of lignin from olive stones and olive cake extracted from olive byproducts.

KEYWORDS: *olive byproduct, fractionation, steam explosion, lignin, cellulose, hemicelluloses, bile acid binding, glucose retardation index*

INTRODUTION

Table olives and olive oil extraction represent an important social and economic industrial activity that is particularly relevant in Mediterranean countries. The solid waste generated from olive oil extraction from the three-phase centrifugation system is named "orujo" or "olive cake" and contains seed husks (fragmented olive stones), seeds, pulp, and peel of the olive fruit. Olive stones without seed can be recovered by subsequent filtration of the olive cake. From the pitted table olive industry (stuffed olives), the whole stone (stone and seed) is recovered by separation of the pulp. These byproducts are currently used as energy sources, and olive cake is also used as fertilizer and animal feed.[1] However, these byproducts contain valuable compounds that could be optimized to make high-value food products.

The olive stone is a lignocellulosic material with hemicelluloses, cellulose, and lignin as main components.[2] The fractionation process allows each component to be obtained for utilization and therefore increases the value of such byproducts. The steam explosion process at high temperature (180−240 °C) and pressure (1.47−4.12 MPa) results in the physical breakage of the cell wall by the rapid release of pressure, with the hydrolysis of glycosidic bonds in the hemicelluloses by saturated steam (autohydrolysis) or by the addition of small amounts of acid (prehydrolysis). These particulars of the steam explosion pretreatment increase the water solubility of hemicelluloses and the solubility of lignin in alkali solution, leaving the cellulose as the solid residue.[3]

Previous works have determined the optimal conditions for steam explosion for the fractionation of olive byproducts[2−5] to obtain each of the polymeric compounds in high yield and purity. The chemical structure and physicochemical properties of fiber, including polysaccharides and lignin, are important for functional and nutritional motives. Consequently, each fiber fraction enriched in cellulose, lignin, and hemicelluloses could be useful for further application as a new source of dietary fiber due to its health benefits. The consumption of water-soluble fiber and some insoluble fibers has been shown to decrease cholesterol levels and hence reduce the risk of cardiovascular disease.[6] Dietary fiber binds bile acid and increases its fecal excretion, which has been suggested as the possible mechanism of action. For this reason, dietary fiber inhibits cholesterol reabsorption and stimulates cholesterol conversion for the manufacture of additional bile acids.[7] The lignin, a component of dietary fiber, nontoxic and biocompatible, which undergoes minimal changes in the body (nonfermentable), has been proven to bind various bile acids[8] and detoxify harmful metabolites inhibiting colonic carcinogenesis.[9]

Also, epidemiological studies suggest that dietary fiber, especially the soluble type, is associated with the regulation of serum glucose concentration, which may be useful for the control of diabetes.[10] The physicochemical properties of the fiber-rich olive byproducts, related with the chemical structure of the wall polysaccharides, may improve the functional and technological properties of food products. These components could possibly be used to enhance water and oil retention and to improve emulsion or oxidative stabilities.[11]

Olive cake, by comparison with other residues derived from fruit, could also potentially be utilized for its phenolic content

Received: May 6, 2014
Revised: August 11, 2014
Accepted: August 20, 2014
Published: August 20, 2014

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

New Hydrothermal Treatment of Alperujo Enhances the Content of Bioactive Minor Components in Crude Pomace Olive Oil

Antonio Lama-Muñoz, Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Antonio Gómez-Carretero, and Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero 4, Sevilla 41012, Spain

ABSTRACT: The application of a new process based on the hydrothermal treatment of olive oil waste (alperujo) led to a final solid rich in pomace olive oil (POO) enriched in minor components with functional activities. The effects of the time (15−90 min) and the temperature (150, 160, and 170 °C) of the thermal processing of alperujo on the yield, quality, and enrichment of minor components of crude POO were evaluated. The final treated solid had an increase in oil yield up to 97%, with a reduction in solids up to 35.6−47.6% by solubilization. Sterols increased up to 33%, aliphatic alcohols increased up to 92%, triterpenic alcohols increased up to 31%, squalene increased up to 43%, tocopherols increased up to 57%, and oleanolic acid increased up to 16% by the new treatment. The increase maintains a high concentration of functional substances probably even in the refining POO.

KEYWORDS: Alperujo, olive oil, minor components, tocopherols, sterols, squalene, triterpenic acids and alcohols, steam treatment

INTRODUCTION

Recently, interest in pomace or "orujo" olive oil (POO) has grown. Economic advantages make the price of this oil, made from the byproduct of virgin olive oil (VOO), cheaper than VOO. Despite POO presenting important disadvantages versus VOO such as organoleptic characteristics and acidity values, POO also contains all of the functional compounds in VOO, including the glyceridic fraction, except the polyphenols, in addition to other biologically active components that are present in the leaves, skin, or seeds of olives, depending on the extraction system. Some of the minor components, present in the unsaponifiable matter, with interesting health properties[1] are present at a higher concentration in POO than in VOO. For instance, elevated amounts of sterols, tocopherols, waxes, and triterpenic acids and alcohols are found in POO.[2]

Phytosterols are probably the most important of the minor components and comprise a major portion of the unsaponifiable matter of POO. They are structurally similar to cholesterol, and their role in nutrition is based on their cholesterol-lowering effects in human blood, a result of their competitive inhibition of intestinal cholesterol uptake.[3] Phytosterols are also considered to be potentially cytostatic agents in inflammatory and tumoral diseases.[4] The tocopherol group (α-, β-, and γ-tocopherols) is another class of compounds present at high levels in POO. α-Tocopherol is an essential micronutrient involved in various oxidative stress-related processes (atherosclerosis, Alzheimer's disease, accelerated aging and cancer).[5] POO contains squalene, which has been shown to have a beneficial effect on atherosclerosis lesions,[6] dermatitis, and cellular proliferation and apoptosis in skin and intestinal cancers.[7] Squalene is not easily oxidized, and it appears to function within the skin after absorption, acting as a first line of defense for the human skin surface against oxidative stress due of exposure to ultraviolet (UV) radiation from sunlight. Squalene is the first lipid targeted during free radical or singlet oxygen attacks.[8]

Long-chain fatty alcohols isolated from POO (hexacosanol, octacosanol, and triacontanol; C26, C28, and C30, respectively) have been reported to be effective in reducing the release of different inflammatory mediators.[9] Other beneficial activities have also been attributed to these compounds, such as reducing platelet aggregation and lowering cholesterol.[10] Long-chain fatty alcohols, together with pentacyclic triterpenoids, are present in the cuticular lipid layer of the olive fruit. The ingestion of POO with a high proportion of oleanolic acid attenuated the endothelial dysfunction associated with hypertension.[11] Moreover, olive triterpenic acids (oleanolic and maslinic) have been associated with a wide variety of biological activities, including anti-inflammatory,[12] antihyperglycemic,[13] and anticancer activities,[14] among others. Olive triterpenic alcohols (uvaol and erythrodiol) have beneficial effects on the inflammatory process,[12] potent antioxidant effects on the microsomal membranes of rat liver,[15] and anticarcinogenic effects that may have potential uses in the prevention and treatment of brain tumors and other cancers.[16] The four triterpenes (oleanolic acid, maslinic acid, uvaol, and erythrodiol) were shown to have vasorelaxant capacity and may have a protective role against cardiovascular risk factors such hypertension.[17]

Crude POO is obtained by extraction of dried "alperujo" with commercial hexane or by centrifugation from wet olive paste (moist pomace), and a refining process to adapt POO for consumption is necessary. The refining (physical or chemical) process eliminates undesirable compounds (peroxides, degradation products, volatile compounds responsible for off-flavors, free fatty acids, etc.) but also results in the loss of valuable bioactive compounds and natural antioxidants.[18] In this work, we improved

Received: September 14, 2010
Accepted: December 21, 2010
Revised: December 21, 2010
Published: January 25, 2011

dx.doi.org/10.1021/jf103555h | J. Agric. Food Chem. 2011, 59, 1115–1123

Food Hydrocolloids 28 (2012) 92–104

Contents lists available at SciVerse ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd

Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated

Antonio Lama-Muñoz, Guillermo Rodríguez-Gutiérrez, Fátima Rubio-Senent, Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avda. Padre García Tejero n° 4, Sevilla-41012, Spain

ARTICLE INFO

Article history:
Received 16 September 2011
Accepted 17 November 2011

Keywords:
Alperujo
Steam treatment
Pectic oligosaccharides
Enzymatic hydrolysis
Xylo-oligosaccharides
Xyloglucan

ABSTRACT

Alperujo was pre-treated in a hydrothermal reactor to solubilize the oligosaccharides. The liquid was extracted with ethyl acetate to remove the phenols, and then its sugar content was sequentially precipitated with ethanol:water solutions and ultrafiltered, obtaining oligosaccharide fractions in the range of 3000 to 1000 Da that represent 23% of the total. One of the two major fractions was rich in pectic polymers, and the composition of the other fraction indicated the possible presence of xyloglucans and xylo-oligosaccharides. Hydrolysis was used to decrease the molecular weight of oligosaccharides by means of TFA or HCl for the neutral or enzymes for the pectic oligosaccharides. They were isolated by adsorption and size exclusion chromatography and identified by HPLC, GC, ESI-MS and MS/MS. The presence of tetra-, tri- and di-galacturonic acids was confirmed, and different structures for neutral and acidic xylo-oligosaccharides and oligosaccharides of xyloglucan with low molecular weight have been proposed.

1. Introduction

Alperujo is a semisolid, residual by-product of virgin olive oil produced by a continuous, two-phase extraction system. The amount of alperujo produced in Spain alone reaches $3.5–6 \times 10^6$ tons/season. It is a moist residue with the consistency of thick sludge that contains 80% of the olive fruit, including skin, seed, pulp and pieces of stone, and it is a significant pollutant due to the presence of large amounts of organic substances (e.g., oil, polyphenols, proteins and polysaccharides). The combustion of alperujo as a fuel is a commonly used procedure to eliminate its harmful effects on the environment. However, several researchers have studied ways to exploit this olive-mill waste and have produced some products with high added-value, including some that are an economical source of natural antioxidants (Lafka, Lazou, Sinanoglou, & Lazos, 2011; Takac & Karakaya, 2009).

We continue this work on the utilization of alperujo, which will be revalued after the hydrothermal treatment because of an integrated process that upgrades all of the fractions of the material (Fernández-Bolaños et al., 2004). Recently, we have developed a simple new process, adaptable to olive pomace oil extractors in the olive oil refining industry, that allows for easy separation of the solid and liquid phases. In this new steam treatment, which has been patented (Fernández-Bolaños, Rodríguez-Gutiérrez, Lama-Muñoz, & Sánchez, 2011), an auto-hydrolytic process occurs, resulting in the solubilization of the alperujo. It also facilitates the recovery of added-value compounds in the water-soluble fraction (Rodríguez, Lama, Trujillo, Espartero, & Fernández-Bolaños, 2009), leaving a solid residue enriched in residual oil with a high concentration of bioactive compounds (Lama-Muñoz, Rodríguez-Gutiérrez, Rubio-Senent, Gómez-Carretero, & Fernández-Bolaños, 2011).

Recent studies have shown that an important part of hemicelluloses present in alperujo could be recovered as oligosaccharides by steam treatment or autohydrolysis of the water-soluble fraction (Rodríguez, Rodríguez, Guillén, Jiménez, & Fernández-Bolaños, 2007). Cell wall material for olive pulp comprises a number of molecular components, with considerable quantities of cellulose (30%), pectic polysaccharides (39%) and hemicellulosic polymers rich in xylans and glucuronoxylans (14%), xyloglucans (15%) and mannans (2%) (Jiménez, Guillén, Fernández-Bolaños, & Heredia, 1994; Jiménez et al., 2001; Vierhuis, Schols, Beldman, & Voragen, 2001), which could provide a wide range of functional components. The optimization and integration of the process for revaluing alperujo, which in this case can be also used for production of oligosaccharides for intermediate pharmaceutical or food use, could be an important and economical improvement.

* Corresponding author. Tel.: +34 954691054; fax: +34 954691262.
E-mail address: jfbg@cica.es (J. Fernández-Bolaños).

0268-005X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodhyd.2011.11.008

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Isolation and Identification of Phenolic Glucosides from Thermally Treated Olive Oil Byproducts

Fátima Rubio-Senent, Antonio Lama-Muñoz, Guillermo Rodríguez-Gutiérrez, and Juan Fernández-Bolaños*

Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), Avenida Padre García Tejero 4, Sevilla 41012, Spain

ABSTRACT: A liquid phase rich in bioactive compounds, such as phenols and sugars, is obtained from olive oil waste by novel thermal treatment. Two groups of fractions with common characteristics were obtained and studied after thermal treatment, acid hydrolysis, and separation by ultrafiltration, chromatography, and finally Superdex Peptide HR. In the first group, which eluted at the same time as oligosaccharides with a low DP (4−2), an oleosidic secoiridoid structure conjugated to a phenolic compound (hydroxytyrosol) was identified as oleuropeinic acid, and three possible structures were detected. In the second group, glucosyl structures formed by hydroxytyrosol and one, two, or three units of glucose or by tyrosol and glucose have been proposed. Verbascoside, a heterosidic ester of caffeic acid, in which hydroxytyrosol is linked to rhamnose−glucose or one of its isomers was also identified. Neutral oligosaccharides bound to a phenol-containing compound could be antioxidant-soluble fibers with bioactive properties.

KEYWORDS: *alperujo, phenol, steam treatment, oligosaccharides, secoiridoids, hydroxytyrosol*

INTRODUCTION

Olive oil production is a very important and traditional activity in the countries of the Mediterranean area, which account for 95% of worldwide olive oil production.[1] The manufacturing process of olive oil generates oil and a byproduct that is a combination of liquid and solid waste, called "alperujo" or "two-phase olive mill waste". This byproduct is a high-humidity residue with the consistency of thick sludge that contains 80% of the olive fruit, including the skin, seed, pulp, and pieces of stones, which is later separated and usually used as solid fuel.[2] In Spain, the annual production of this byproduct is approximately 2.5−6 million tons, depending on the season. Alperujo has many inconvenient and environmental problems due its high organic content and the presence of phytotoxic components, which makes it difficult to use in further bioprocesses.[3] Most of these components, mainly the phenolic compounds, confer the bioactive properties of olive oil; however, during olive oil processing, most of these compounds remain in the alperujo, making it a promising source for substances with high value.[4] Therefore, new strategies are emerging for the utilization of this byproduct, but the main challenges are separating its phases by pretreatments, eliminating the phytotoxic components for bioprocess applications and for bioactive molecule production.

One of the more attractive processes employs thermal pretreatments that allow for recovery of the bioactive compounds and valuable fractions, making it possible to utilize alperujo.[5] Recently, we have developed a simple new process, adaptable to the pomace-processing mill and refining olive oil industry, which allows for easy separation of the solid and liquid phases. In this new, patented[6] steam treatment, an autohydrolytic process occurs and solubilizes part of the alperujo. It also allows the recovery of added-value compounds in the water-soluble fraction, to create a fraction rich in interesting phenols (hydroxytyrosol, 3,4-dihydroxyphenylglycol), sugars, and oligosaccharides,[7,8] leaving behind a solid residue enriched in residual oil with an increased concentration of minor component (sterols, triterpenic acids and alcohols, squalene, and tocopherols) with functional activities.[9]

The cell wall material of olive pulp is mainly composed of cellulose, pectic polysaccharides rich in arabinose, xylans, glucuronoxylans, and xyloglucans, whereas mannans, glycoproteins, enzymes, and phenols occur as minor components.[10−14] The analysis of the phenolic compounds in the olive cell wall mainly showed the presence of *p*-coumaric acid (95%) and small amounts of vanillic acid and *p*-hydroxybenzaldehyde as well as traces of vanillin, protocatechuic acid, and protocatechuic aldehyde. Hydroxytyrosol, tyrosol, or secoiridoid derivatives are very abundant in olive pulp but were not identified in the olive cell wall.[14] However, it has been reported that interaction between polysaccharides and the hydrophilic compounds present in the olive paste may be involved in the loss of the phenolic compounds from the oil during crushing and malaxation.[15] Thermal treatment can provide a large number of bioactive compounds when applied for a short time, including oligosaccharides, that would promote the growth of beneficial bacteria in the large bowel,[16] and phenolics associated with polysaccharides, with potential activities in the food or nutraceutical industry. The production and use of this type of oligosaccharides could enhance the economical viability of the integral process to thermally treat the alperujo.

In this paper, we have characterized the oligosaccharide compounds released from alperujo steam-treated in a bath pilot reactor (100 L capacity) following mild acid hydrolysis according to a previous published procedure.[8] After the

Received: May 22, 2012
Revised: January 11, 2013
Accepted: January 16, 2013
Published: January 16, 2013

13

New Olive-Pomace Oil Improved by Hydrothermal Pre-Treatments

G. Rodríguez-Gutiérrez, A. Lama-Muñoz, M.V. Ruiz-Méndez,
F. Rubio-Senent and J. Fernández-Bolaños
Departamento de Biotecnología de los Alimentos,
Instituto de la Grasa (CSIC), Avda, Seville,
Instituto al Campus de Excelencia Internacional Agroalimentario, ceiA3
Spain

1. Introduction

The health properties of virgin olive oil (VOO) are well known in the Mediterranean Diet, in which VOO is the main source of fat (Boskou, 2000). The Mediterranean area provides 97% of the total olive production of the world and represents a major industry in the region (Aragon & Palancar, 2001). The fatty acid composition is not the only healthy component of olive oil; in addition, minor components have high biological activities (Pérez-Jiménez et al., 2007). From the olive oil by-product, the olive-pomace oil (OPO) is obtained. Recent studies have demonstrated the positive benefits of OPO on health, and these effects are due mainly to the presence of minor components (Ruiz-Gutiérrez et al., 2009). The new olive oil extraction processes in the olive mills make the extraction of OPO and the general utilisation of wastes more difficult. New thermal systems are proposed to pre-treat the olive oil wastes to facilitate their utilisation and OPO extraction.

1.1 Olive oil extraction systems

The manufacturing process of olive oil has undergone evolutionary changes. The traditional discontinuous pressing process was initially replaced by continuous centrifugation, using a three-phase system and later a two-phase system. Depending on the different olive oil production method, there are different kinds of wastes. The classic production of olive oil generates three phases and two wastes: olive oil (20 %), solid waste (30 %) and aqueous liquor (50 %). The solid waste (olive cake or "orujo") is a combination of olive pulp and stones. The aqueous liquor comes from the vegetation water and the soft tissues of the olive fruits, with water added during processing (so-called "alpechin" or "olive-mill waste water"). The presence of large amounts of organic substances (oil, polyphenols, protein, polysaccharides, etc.) and mineral salts represents a significant problem for the treatment of wastewater (Borja et al., 1997).

The use of a modern two-phase processing technique to which no water is added generates oil and a new by-product that is a combination of liquid and solid waste, called "alperujo", "alpeorujo" or "two-phase olive mill waste". This by-product is a high-humidity residue

MINISTERIO
DE INDUSTRIA, TURISMO
Y COMERCIO

Oficina Española
de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P201131041	
Fecha de recepción:	21 junio 2011, 10:42 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	0413	
Solicitante:	CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS (CSIC)	
Número de solicitantes:	1	
País:	ES	
Título:	EXTRACTO FENÓLICO DE LOS SUBPRODUCTOS DE LA ACEITUNA TRATADOS TÉRMICAMENTE.	
Documentos enviados:	Descripcion.pdf (21 p.) Reivindicaciones.pdf (3 p.) Dibujos.pdf (1 p.) Resumen.pdf (1 p.) OLF-ARCHIVE.zip	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=NOMBRE UNGRIA LOPEZ JAVIER - NIF 05211582N,OU=500050022,OU=FNMT Clase 2 CA,O=FNMT,C=ES	
Fecha y hora de recepción:	21 junio 2011, 10:42 (CEST)	
Codificación del envío:	67:BD:A8:59:51:DF:8A:DC:DD:1E:8E:56:02:5F:AE:DC:11:AA:BD:C3	

/Madrid, Oficina Receptora/

MINISTERIO
DE INDUSTRIA, TURISMO
Y COMERCIO

Oficina Española
de Patentes y Marcas

Justificante de presentación electrónica de solicitud de patente

Este documento es un justificante de que se ha recibido una solicitud española de patente por vía electrónica, utilizando la conexión segura de la O.E.P.M. Asimismo, se le ha asignado de forma automática un número de solicitud y una fecha de recepción, conforme al artículo 14.3 del Reglamento para la ejecución de la Ley 11/1986, de 20 de marzo, de Patentes. La fecha de presentación de la solicitud de acuerdo con el art. 22 de la Ley de Patentes, le será comunicada posteriormente.

Número de solicitud:	P201131161	
Fecha de recepción:	08 julio 2011, 10:27 (CEST)	
Oficina receptora:	OEPM Madrid	
Su referencia:	509	
Solicitante:	CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS (CSIC) 66%	
Número de solicitantes:	2	
País:	ES	
Título:	PROCEDIMIENTO PARA LA OBTENCION DE EXTRACTO DE HIDROXITIROSOL, EXTRACTO MEZCLA DE HIDROXITIROSOL Y 3,4-DIHIDROXIFENILGLICOL, Y EXTRACTO DE ACETATO DE HIDROXITIROSILO, A PARTIR DE SUBPRODUCTOS DEL OLIVO Y SU PURIFICACION	
Documentos enviados:	Descripcion.pdf (19 p.) Reivindicaciones.pdf (4 p.) Resumen.pdf (1 p.) OLF-ARCHIVE.zip	package-data.xml es-request.xml application-body.xml es-fee-sheet.xml feesheet.pdf request.pdf
Enviados por:	CN=NOMBRE UNGRIA LOPEZ JAVIER - NIF 05211582N,OU=500050022,OU=FNMT Clase 2 CA,O=FNMT,C=ES	
Fecha y hora de recepción:	08 julio 2011, 10:27 (CEST)	
Codificación del envío:	2F:8D:58:8A:ED:1B:D4:05:15:2A:EF:E9:3B:BC:CB:E3:E9:06:75:48	

CURRICULUM VITAE

DATOS PERSONALES:

- Apellidos y nombre: RUBIO SENENT, Fátima.
- Dirección: c/ Salvador Dalí nº13
  C.P:41909, Salteras, SEVILLA.
- Fecha de nacimiento: 12 de junio de 1985.
- Teléfonos: 955707304/625287069.
- Correo electrónico: fatimars_85@hotmail.com.

DATOS ACADÉMICOS:

FORMACIÓN ACADÉMICA.

- Licenciada en Química, especialidad "Química aplicada". Facultad de Química. Universidad de Sevilla.
  Fecha de inicio: Septiembre de 2003; Fecha de finalización: Junio 2008.

FORMACIÓN COMPLEMENTARIA:

ESPECÍFICA.

- Master "Ciencia y Tecnología Química". Impartido por la Universidad Nacional de Educación a Distancia. Proyecto realizado fin de master: "Obtención y caracterización de un extracto fenólico obtenido a partir de alperujo sometido a un tratamiento térmico. Estudio de actividad antioxidante, inhibición de la tirosinasa y actividad antiagregante plaquetaría".
  Duración: 600 horas
  Fecha de inicio: 1 de Noviembre de 2010; Fecha de finalización: Julio 2011.
- Master "Gestión de la calidad y medio ambiente". Impartido por el Colegio Oficial de Ingenieros Técnicos Forestales. Proyecto realizado fin de master: Plan de implantación de Sistema de Gestión Medioambiental en una empresa metalúrgica.
  Duración: 600 horas.
  Fecha de inicio: 30 de Agosto 2008; Fecha finalización: 1 de Septiembre de 2009.
- Curso de "Formación básica en seguridad y salud para los laboratorios de la facultad de química".
  Organizado por la Facultad de Química y patrocinado por la Fundación Cámara Urzáiz.
  Duración: 15 horas.

Fecha de inicio: 10 de Noviembre de 2003; Fecha de finalización: 17 de Noviembre de 2003.

IDIOMAS:

- Inglés:
  - Curso de Inglés II (Nivel B1) por el Instituto Oficial de Idiomas.
    Duración: 60 horas.
    Fecha de inicio: 1 de Octubre de 2009. Fecha de finalización 30 de Junio de 2010.
  - Curso de Inglés I (Nivel A2) por el Instituto Oficial de Idiomas.
    Duración: 60 horas.
    Fecha de inicio: 1 de Octubre de 2008; Fecha de finalización 30 de Junio de 2009.
  - Curso intensivo de ingles nivel upper intermediate.
    Academia OPEN (Open Schools of Languages).
    Duración: 24 horas.
    Fecha de inicio: 1 de Septiembre de 2007; Fecha de finalización: 16 de Octubre de 2007.

EXPERIENCIA PROFESIONAL.

- Consejo Superior de Investigaciones Científicas (CSIC), Instituto de la grasa. Ctra. Utrera, Km 1, Campus Universitario Pablo de Olavide, Edf. 46. CP 41013, Sevilla.
  Cargo: Personal licenciado, contrato asociado a proyecto de investigación.
  Fecha de inicio: 1 de enero de 2015.
- Tesis Doctoral en el Consejo Superior de Investigaciones Científicas (CSIC), Instituto de la grasa, con una beca JAE-predoctoral asociada al proyecto "Valorización de las fracciones líquidas, sólidas y volátiles derivadas del aprovechamiento del alperujo sometido a un nuevo tratamiento térmico a alta presión.", código de proyecto AGL2009-12352. Beca seleccionada para ser cofinanciada en el marco del Programa Operativo FSE 2007-2013 Plurirregional de adaptabilidad y Empleo dentro del Eje 3 del Fondo Social Europeo.
  Fecha de inicio: 1 de septiembre del 2010. Fecha de finalización: 31 de agosto de 2014.
- Consejo Superior de Investigaciones Científicas (CSIC), Instituto de la grasa. Avd. Padre García Tejero nº 4, Sevilla.
  Cargo: Personal licenciado, contrato asociado a proyecto de investigación de la Junta Andalucía: "Optimización del Aislamiento de Polifenoles de la Aceituna. Complejación con Ciclodextrinas, Derivatización y Evaluación Como Agentes Antioxidantes", código proyecto: AGR-3751.
  Fecha de inicio: 1 de mayo de 2009. Fecha de finalización: 31 de agosto de 2010

- Labs & Technological Services AGQ, S.L. Dirección: A-433, KM.24,3, Burguillos.
  Cargo: Técnico de Laboratorio.
  Fecha de inicio: 25 de agosto de 2008. Fecha de finalización: 30 de abril de 2009.
- Realización de prácticas de empresa en el Consejo Superior de Investigaciones Científicas (CSIC), Instituto de la grasa, bajo la supervisión de Antonio Pérez, Juan Garrido y Manuel Arenas, pertenecientes al departamento de Biotecnología de Alimentos, línea de investigación: "Modificaciones químicas y bioquímicas de pigmentos en relación con la calidad y valor nutritivo del producto".
  Fecha de inicio: 1 de Julio de 2008; Fecha de finalización: 22 de agosto de 2008.

PARTICIPACIÓN EN PROTECTOS.

- TITULO DEL PROYECTO: Caracterización química y funcional de compuestos y fracciones bioactivas especificas aisladas a partir del alperujo sometido a un nuevo tratamiento térmico a alta presión.
  ENTIDAD FINANCIADORA: Plan Nacional I+D+I
  DURACION DESDE: 2010 HASTA: 2012
  INVESTIGADOR PRINCIPAL: Juan Fernández-Bolaños Guzmán

ASISTENCIA A CONGRESOS.

- II Seminario Microencapsulación. Conceptos básicos y demostración práctica de microencapsulación monodispersa. Biomedal. Facultad de Farmacia, Universidad de Sevilla. 11 de Junio 2012.
- IX Congreso Internacional Dieta Mediterránea. Fundación Dieta Mediterránea, Barcelona. 27 y 28 de Marzo de 2012.
- Seminario Simple Preparation Tips and Tricks. Agilent Technologies. Consejo Superior de Investigaciones Científicas (CSIC), Instituto de la grasa. Avd. Padre García Tejero nº 4. Sevilla 13 de Abril de 2011.
- XXXIV Asamblea del Instituto de la grasa. Consejo Superior de Investigaciones Científicas (CSIC), Instituto de la grasa. Avd. Padre García Tejero nº 4. Sevilla 18 y 19 de Noviembre de 2010.
- 1ª Jornada de extractos vegetales: Ciencia, Regulación y Mercado. Universidad de Alcalá de Henares (Madrid). 17 y 18 de Junio de 2010.

APORTACIÓN A CONGRESOS.

- Expoliva XVI Feria Internacional del aceite de Oliva, Mayo 2013. Póster "Characterization and antioxidant capacity of a phenolic extract from steamtreated alperujo". Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños.
- Expoliva XVI Feria Internacional del aceite de Oliva, Mayo 2013. Póster "Properties and antioxidant capacity of a polymeric phenolic fraction extracted from steam-treated alperujo". Fátima Rubio-Senent, Guillermo Rodríguez-Gutiérrez, Antonio Lama-Muñoz, Juan Fernández-Bolaños.
- II Iberoamerican Congress on biorefineries, Abril 2013. Póster: "Pretreatment for bioprocess application of olive oil byproducts". Rodríguez-Gutiérrez, G., Fernández-Bolaños Guzmán, J., García-Borrego, A., Lama-Muñoz, A., Rubio-Senent, F.
- IX Congreso Internacional Dieta Mediterránea, Marzo 2012. Póster: "Effects of hidroxitirosol acetate, polimeric phenolic fraction and phenolic extract obtained from alperujo on ex vivo platelet aggregation". Rubio-Senent, F., Dauthie, G., de Rooos, B., Rodríguez-Gutiérrez, G., Lama-Muñoz, A. and Fernández-Bolaños, J.
- Expoliva XV Feria Internacional del aceite de Oliva, Mayo 2011. Póster "Nuevos antioxidantes de la aceituna ensayos in vitro de actividad biológica". Rodríguez-Gutiérrez, G., Duthie, G., de Roos, B., Lama-Muñoz, A., Gómez Carretero, A., Rubio-Senent, F. y Fernández-Bolaños, J.
- Expoliva XV Feria Internacional del aceite de Oliva, Mayo 2011. Póster "Mejora del aceite de orujo mediante un pretratamiento térmico". Lama-Muñoz, A., Rodríguez-Gutiérrez, G., Gómez-Carretero, A., Rubio-Senent, F., Fernández-Bolaños, J.

PUBLICACIONES CIENTÍFICAS.

Artículos

- Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Munoz, A., Fernández-Bolaños, J. 2015. Pectin extracted from thermally treated olive oil by-products: characterization, physico-chemical properties, in vitro bile acid and glucose binding. Food Hydrocolloids, 43, 2015, 311-321.
- Rubio-Senent, F., de Roos, B., Duthie, G., Fernández-Bolaños, J., Rodríguez-Gutiérrez, G. Inhibitory and synergistic effects of natural olive phenols on human platelet aggregation and lipid peroxidation of microsomes from vitamin E deficient rats. European Journal of Nutrition, DOI: 10.1007/s00394-014-0807-8. December, 2014
- Rodríguez-Gutiérrez, G., Rubio-Senent, F., Lama-Muñoz, A., García, A., Fernandez-Bolaños, J. 2014. Properties of lignin, cellulose, and hemicelluloses isolated from olive

cake and olive stones: binding of water, oil, bile acids, and glucose. Journal of Agricultural and Food Chemistry, 62, 8973-8981.

- Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Munoz, A., Fernández-Bolaños, J. 2013. Chemical characterization and properties of a polymeric phenolic fraction obtained from olive oil waste. Food Research International, 54, 2122-2129.
- Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Munoz, A., Fernández-Bolaños, J. 2013. Phenolic extract obtained from steam-treated olive oil waste: Characterization and antioxidant activity. LWT- Food Science and Technology, 54, 114-124.
- Rubio-Senent, F., Lama-Munoz, A., Rodríguez-Gutiérrez, G., Fernández-Bolaños, J. 2013. Isolation and identification of phenolic glucosides from thermally treated olive oil byproducts. Journal of Agriculture and Food Chemistry, 61, 1235-1248.
- Lama-Múñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Palacios-Díaz. R., Fernández-Bolaños, J. 2013. A study of the precursors of the natural antioxidant phenol 3,4-dihydroxyphenylglycol in olive oil waste. Food Chemistry, 140, 154-160.
- Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Munoz, A., Fernández-Bolaños, J. 2012. New phenolic compounds hydrothermally extracted from the olive oil by-Product alperujo and their antioxidative activities. Journal of Agrucultural and Food Chemistry, 60, 1175-1186.
- Lama-Múñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Fernández-Bolaños, J. 2012. Production, characterization and isolation of neutral and pectic oligosaccharides with low molecular weights from olive by-products thermally treated. Food Hydrocolloids, 28, 92-104.
- Lama-Múñoz, A., Rodríguez-Gutíerrez, G., Rubio-Senent, F., Fernández-Bolaños, J. 2011. New hydrothermal treatment of alperujo enhances the content of bioactive minor components in crude pomace olive oil. Journal of Agricultural and Food Chemistry, 59, 1115-1123.

Capítulos de libros

- Rodríguez-Gutíerrez, G., Lama-Múñoz, A., Rubio-Senent, F., A., Fernández-Bolaños, J., Borja, R., Fermoso, F. G. 2013. Workshop Intentacional y Taller Nacional. Valorización de residuos oportunidad para la innovación. Aprovechamiento de subproductos sólidos de la elaboración del aceite de oliva. ISBN: 978-956-236-241-2.
- Fernández-Bolaños, J., Rubio-Senent, F., Lama-Muñoz, A., García, A., Rodríguez-Gutiérrez, G. January 2014. Oligosaccharides: Food Sources, Biological Roles and Health Implications. Cap 6: Production of oligosaccharides with low molecular weights, secoiridoids and phenolic glycosides from thermally treated olive by-products. ISBN: 978-1-62948-328-3.
- Rodríguez-Gutíerrez, G., Lama-Múñoz, A., Ruiz-Muñoz, M.V., Rubio-Senent, F., A., Fernández-Bolaños, J. February 2012. Olive oil, Constituent, Quality, Health Propierties

and Bioconversions, Cap 13. New Olive-Pomace Oil Improved by Hidrotermal Pre-tratments. ISBN 978-953-307-921-9.

- Ruiz, E., Romero, I., Cara, C., Castro, E., Moya, M., Espínola, F., Fernández, D., de Torres, A., Domínguez, H., Moure, A., Conde, E., Fernández-Bolaños, J., Rodríguez, G., Rubio F. Proyectos de investigación 2008-2009. Universidad de Jaén. Actividad antioxidante de la fracción liquida obtenida del pretratamiento de la biomasa del olivar para la obtención de etanol. Propuesta de valorización, pag 7 - 26. Deposito legal GR-4184-11, ISBN 978-84-8439-607-9.

ESTANCIAS EN EL EXTRANJERO.

- Estancia en Rowett Institute of Nutrition &Health, Aberdeen, Reino Unido. Desde el 2 de Septiembre de 2011 al 3 de Diciembre de 2011
  Actividad realizada: Estudio de la actividad antiagregante plaquetaria *ex vivo* e inhibición de la oxidación de microsomas de rata con deficiencia en vitamina E.

PATENTES.

- Extracto fenólico de los subproductos de la aceituna tratados térmicamente. Número de solicitud P201131041.
- Procedimiento para la obtención de extracto de hidroxitirosol, extracto mezcla de hidroxitirosol y 3,4-dihidroxifenilglicol, extracto de acetato de hidroxitirosol y posterior purificación de todos ellos partir de subproductos del olivo. Número de solicitud P201131061.