UNIVERSIDAD DE JAÉN

FACULTAD DE CIENCIAS EXPERIMENTALES
DEPARTAMENTO DE CIENCIAS DE LA SALUD

TESIS DOCTORAL

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POTENCIAL BIOACTIVO DEL ACEITE DE OLIVA VIRGEN EN FUNCIÓN DE LA VARIEDAD

PRESENTADA POR:
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Fdo: ANGÉLICA QUINTERO FLÓREZ

Trabajo para optar al Grado de Doctor

Director:

Fdo: Gabriel Beltrán Maza

JAÉN, 2017

ESTE TRABAJO DE INVESTIGACIÓN HA SIDO FINANCIADO POR EL PROYECTO DE EXCELENCIA DE LAS UNIVERSIDADES Y ORGANISMOS DE INVESTIGACIÓN DE ANDALUCÍA (LÍNEA ESPECÍFICA DE INVESTIGACIÓN SOBRE DIVERSOS ASPECTOS DEL OLIVAR Y DEL ACEITE DE OLIVA) AGR 6099 CONVOCATORIA 2010.

A mi ángel, mi fuerza y mi alegría:
mi hija Alejandra;
A mi esposo Alberto
por su apoyo y paciencia;
A mis padres, hermanos, sobrinos y amigos
que en la distancia siempre me acompañan
y me animan;

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Contenido

I. RESUMEN/ SUMMARY

I. RESUMEN

Este trabajo tiene como objetivo evaluar el potencial bioactivo del aceite de oliva virgen (AOV) en función de su variedad, factor determinante en su composición, entre otros aspectos agronómicos y tecnológicos. Se estudiaron las posibles diferencias en el potencial bioactivo respecto al contenido de ácidos grasos, compuestos fenólicos y volátiles de aceites de oliva vírgenes obtenidos a partir de diferentes variedades.

Partiendo de diferentes AOV monovarietales, se ha analizado la composición de ácidos grasos en la formación de células espumosas, se ha estudiado la bioaccesibilidad de los compuestos fenólicos y su actividad antioxidante; así como un estudio preliminar sobre la bioaccesibilidad de los compuestos volátiles del aceite.

Los resultados mostraron que la composición de los AOV monovarietales son determinantes en su capacidad bioactiva. Las diferencias en la composición acídica de los AOV tuvieron efecto en la formación de células espumosas; así los aceites ricos en ácido linoleico indujeron una alta acumulación de triglicéridos dentro de los macrófagos en comparación con los aceites ricos en ácido oleico. La bioaccesibilidad de los compuestos fenólicos dependió de la variedad aunque no se relacionó con su contenido total. Así mismo, se observaron diferencias en la capacidad antioxidante de la fracción digerida de los AOV. Por último, se evidenció que algunos de los compuestos volátiles del AOV permanecieron durante el proceso de digestión.

De los resultados se desprende que la composición de ácidos grasos y fenoles de AOV, dependiendo de su variedad, podrían afectar a sus propiedades biológicas, y abren una puerta a la posibilidad de seleccionar variedades de AOV para usos nutricionales específicos según su composición es estos compuestos. Así mismo se ha dado un primer paso en la investigación de las propiedades biológicas de los compuestos volátiles del AOV que podrían llegar a ser bioaccesibles en el organismo.

SUMMARY

The aim of this work is to evaluate the bioactive potential of virgin olive oil (VOO) in terms of their variety, a determining factor in their composition, among other agronomic and technological aspects. Possible differences in the bioactive potential with respect to the content of fatty acids, phenolic and volatile compounds of olive oils obtained from different varieties were studied.

Starting from different monovarietal VOOs, the composition of fatty acids in the formation of foam cells has been analyzed, also the bioaccessibility of phenolic compounds and their antioxidant activity have been studied; As well as a preliminary study on the bioaccessibility of volatile oil compounds.

The results showed that the monovarietal VOOs composition is determinants in their bioactive capacity. Differences in the acidic composition of VOO had an effect on the formation of foam cells; thus oils rich in linoleic acid induced a high accumulation of triglycerides within the macrophages compared to the oleic acid rich oils. The bioaccessibility of the phenolic compounds depended on the variety although it was not related to its total content. Also, differences were observed in the antioxidant capacity of the digested fraction of AOV. Finally, it was evidenced that some VOO volatile compounds remained during the digestion process.

From the results it can be seen that VOO fatty acids and phenols composition, depending on their variety, could affect their biological properties, and open a door to the possibility of selecting varieties of VOO for specific nutritional uses according to their composition on these compounds. Also a first step has been taken in the investigation of the biological properties of VOO volatile compounds which could be bioaccessible in the organism.

II. ANTECEDENTES BIBLIOGRÁFICOS

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1. Aceite de Oliva Virgen

1.1 Definición y Calidad

El aceite de oliva virgen (AOV) es considerado la principal fuente de grasa de la dieta mediterránea y posee propiedades sensoriales y nutricionales únicas. A diferencia de otros aceites vegetales, el AOV se extrae sin el uso de disolventes y se consume sin refinar. Por lo tanto, se trata de un zumo obtenido del fruto del olivo (O*lea europaea, L.*), extraído exclusivamente mediante técnicas mecánicas que incluyen molienda del fruto, batido de la pasta resultante y separación de la fase oleosa [1]. Este tratamiento de extracción permite conservar una importante cantidad de componentes minoritarios presentes en el fruto que son de gran interés nutricional y sensorial.

La calidad del aceite de oliva está regulada para la Unión Europea (UE), por el reglamento CEE n° 2568/91 junto con sus últimas modificaciones, reglamento CEE n° 656/95, reglamento CEE n° 796/2002, reglamento CEE n° 1989/2003, reglamento CEE n° 640/2008, reglamento CEE n° 61/2011, reglamento CEE n° 299/2013, reglamento n°1833/2015 y reglamento n°1227/2016

El reglamento CEE n° 1512/2001 establece las denominaciones y definiciones del aceite de oliva, y el reglamento n°1227/2016 establece las características de los aceites de oliva, así:

a **Aceite de oliva virgen:** Aceites obtenidos a partir del fruto del olivo únicamente por procedimientos mecánicos u otros procedimientos físicos, en condiciones que no ocasionen la alteración del aceite, y que no hayan sufrido tratamiento alguno distinto del lavado, la decantación, el centrifugado y la filtración, con exclusión de los aceites obtenidos mediante disolvente, mediante coadyuvante de acción química o bioquímica,

o por procedimiento de re esterificación y de cualquier mezcla con aceites de otra naturaleza.

- **Aceite de oliva virgen extra**: aceite de oliva virgen con una acidez libre, expresada en ácido oleico, como máximo de 0.8 g por 100 g. No presenta defectos sensoriales y el atributo frutado es superior a cero.
- **Aceite de oliva virgen**: aceite de oliva virgen con una acidez libre, expresada en ácido oleico, como máximo de 2 g por 100 g. Puede presentar algún defecto sensorial, pero la mediana del defecto debe ser ≤ 3.5 y el atributo frutado mayor a cero.
- **El aceite de oliva lampante:** aceite de oliva virgen cuya acidez libre expresada en ácido oleico es superior a 2 gramos por 100 gramos. Presenta algún defecto sensorial y la mediana del defecto es ≥ 3.5, o cuando la mediana del defecto es ≤ 3.5 pero la mediana del atributo frutado es igual a cero.

b **Aceite de oliva refinado**: es el aceite de oliva obtenido de aceites de oliva vírgenes mediante técnicas de refinado que no provoquen ninguna modificación de la estructura glicerídica inicial. Su acidez libre expresada en ácido oleico es como máximo de 0.3 gramos por 100 gramos.

c **Aceite de oliva**: es el aceite constituido por la mezcla exclusivamente de aceite de oliva refinado y de aceites de oliva vírgenes distintos al aceite lampante, cuya acidez libre, expresada en ácido oleico, no podrá ser superior a 1 gramo por 100 gramos.

d **Aceite de orujo de oliva crudo**: es el aceite obtenido a partir del orujo de oliva mediante tratamiento con disolvente o por medios físicos, o que corresponda, con excepción de algunas características determinadas, a un aceite de oliva lampante; con exclusión de los aceites obtenidos por procedimientos de re esterificación y de cualquier mezcla con aceites de otra naturaleza.

e **El aceite de orujo de oliva refinado:** Aceite obtenido mediante refino de aceite de orujo de oliva crudo, cuya acidez libre, expresada en ácido oleico, no podrá ser superior a 0.3 g por 100 g.

f **El aceite de orujo de oliva:** Aceite constituido por una mezcla de aceite de orujo de oliva refinado y de aceites de oliva vírgenes distintos del lampante, cuya acidez libre, expresada en ácido oleico, no podrá ser superior a 1 g por 100 g.

1.2 Composición Química del AOV

La composición química del aceite de oliva virgen varía en función de diversos factores como son la variedad de aceituna, grado de maduración del fruto, condiciones agronómicas del cultivo, y condiciones tecnológicas de extracción. En el aceite se diferencian dos fracciones, fracción mayoritaria y minoritaria. La fracción mayoritaria, también llamada fracción saponificable, incluye los triacilglicéridos, que representan el 98% del peso total, diglicéridos, monoglicéridos y ácidos grasos libres; mientras que en la fracción minoritaria (insaponificable) sus componentes están presentes en cantidades muy pequeñas, representan alrededor del 2% del peso total del aceite e incluye más de 300 compuestos químicos [2, 3]. Se encuentran ésteres no glicéridos, alcoholes alifáticos y triterpenos, esteroles, hidrocarburos, pigmentos, fenoles lipofílicos e hidrofílicos y compuestos volátiles. Estos compuestos minoritarios están presentes exclusivamente en el aceite de oliva virgen.

La mayor parte de los ácidos grasos del aceite forman parte de los triglicéridos. Los triglicéridos del AOV muestran una asimetría en la distribución de los ácidos grasos, se encuentra una única molécula simétrica, la trioleina formada por el glicerol esterificado con tres moléculas de ácido oleico, el cual representa entre el 40 y 59% de estos compuestos. Otros triglicéridos encontrados en proporciones significativas en el AOV son dioleopalmitina (POO – 12-20%) y dioleolinoleina (OOL – 12.5-20%), POL (5.5-7%) y SOO (3-7%), siendo, P: palmítico, E: ácido esteárico, O: ácido oleico y L: ácido linoleico [3].

La composición característica de los triglicéridos muestra claramente el predominio del ácido oleico, aunque la variabilidad puede ser muy elevada debido a factores agroclimáticos. Pese a ésta, tan solo una proporción baja de ácidos grasos saturados (<

1.3%) ocupa la posición 2 del triglicérido. Es por ello que la fracción de triglicéridos es utilizada como criterio de pureza, para diferenciar el aceite de oliva virgen de otro tipo de aceites.

Los ácidos grasos presentes en el aceite de oliva poseen entre 14 y 24 átomos de carbono y la cantidad de dobles enlaces varía de 0 a 3. Los ácidos grasos presentes en el aceite de oliva así como los límites de calidad marcados para cada uno de ellos se muestran en la Tabla 1.

Tabla 1. Composición de ácidos grasos del Aceite de Oliva Virgen y Límites de Calidad

Ácido Graso	**Nomenclatura**	**Límite** (Reglamento UE n° 1348/2013) (% m/m metil ésteres)
Mirístico	C 14:0	≤ 0.03
Palmítico	C 16:0	7.50 – 20.00
Palmitoleico	C 16:1	0.30 – 3.50
Heptadecanoico (margárico)	C 17:0	≤ 0.30
Heptadecenoico (margaroleico)	C 17:1	≤ 0.30
Estearico	C 18:0	0.50 – 5.00
Oleico	C 18:1	55.0 – 83.0
Linoleico	C 18:2	2.50 – 21.0
Linolenico	C 18:3	≤ 1.00
Araquidónico	C 20:0	≤ 0.60
Galdoleico (eicosanoico)	C 20:1	≤ 0.40
Behenico	C 22:0	≤ 0.20
Lignocérico	C 24:0	≤ 0.20

La fracción minoritaria representa entre el 1 y 2% del aceite de oliva virgen. Sin embargo, a pesar de su baja concentración, algunos de sus constituyentes son determinantes a la hora de evaluar la estabilidad oxidativa, las características organolépticas y la calidad nutricional del aceite. Además, su análisis cuantitativo puede contribuir de forma importante a la detección de adulteraciones. En la Tabla 2 se resumen los compuestos minoritarios más significativos del aceite de oliva virgen y sus concentraciones.

Tabla 2. Componentes de la Fracción Minoritaria del Aceite de Oliva Virgen

Compuesto	Cantidad
Hidrocarbonos	
- Escualeno	200 – 7500 mg/kg
Tocoferoles	
- α-tocoferol	55 – 370 mg/kg
- β-tocoferol	~ 10 mg/kg
- δ-tocoferol	~ 10 mg/kg
- γ-tocoferol	~ 20 mg/kg
Pigmentos	
- Clorofilas	1 -20 mg/kg
- Carotenoides (β-caroteno y licopeno)	1 -20 mg/kg
Alcoholes grasos	< 150 mg/kg
Alcoholes Diterpénicos	
- Fitol	25 – 595 mg/kg
- Geranilgeraniol	< 50 mg/kg
Esteroles Totales	1000 mg/kg
- β-sitosterol	75 - 90%
- Δ5-avenasterol	5 - 20%
- Campesterol	4%
- Estigmaesterol	2%
4-metilesterol	50 – 360 mg/kg
Alcoholes triterpénicos	350 – 1500 mg/kg
Dialcoholes triterpénicos	
- Eritrodiol	6 – 10 mg/kg
- Uvaol	18 mg/kg
Ácidos triterpénicos	40 – 185 mg/kg
- Ácido oleanólico	17 – 82 mg/kg
- Ácido maslínico	20 – 98 mg/kg
Fosfolípidos	20 – 156 mg/kg
Compuestos Fenólicos	50 – 1000 mg/kg
Compuestos Volátiles*	7352 – 31187 ng/g

Fuente: Datos obtenidos de Boskou et al. [3]
* Valores obtenidos de Sánchez-Ortiz et al. [4]

1.3 Factores que afectan a la composición del AOV

La composición química del AOV depende principalmente de variables agronómicas y variables tecnológicas como: la variedad, el grado de madurez del fruto, las condiciones climáticas, el manejo del suelo y del riego y el proceso de extracción del

aceite, en particular las condiciones de molienda y batido y el tipo de sistema de centrifugación utilizado [5].

Factores Agronómicos

Las características del fruto son una de las variables más importantes involucradas en la calidad del AOV. Las propiedades químicas y bioquímicas del fruto se ven afectada por las prácticas agronómicas y por tanto, afecta a la composición del AOV. Dentro de las variables que se han estudiado se encuentran:

Variedad: cualquier variedad y medio pueden proporcionar aceites clasificados en la categoría virgen extra, siempre que procedan de aceitunas sanas, recogidas en el momento oportuno, de una forma adecuada y sean elaborados correctamente [12]. La variedad de aceituna está relacionada con los niveles y actividades enzimáticas, genéticamente determinados, responsables de la formación y posterior transformación durante el procesado de los componentes tanto mayoritarios como minoritarios del fruto del olivo [13, 14]. Las distintas variedades de aceituna pueden producir AOV de diferente composición en ácidos grasos [15, 16], polifenoles [15-21], tocoferoles [22], así como de ácido oleanólico, ácido maslínico, uvaol, eritrodiol [23], fracción esterólica (β-sitoesterol, Δ^5-avenasterol, campesterol, estigmasterol) [24] y escualeno [25]. De esta manera los aceites de oliva monovarietales se podrían caracterizar de acuerdo a su composición [26].

Grado de madurez del fruto: los procesos metabólicos y transformación que ocurren dentro del fruto hacen variar su composición química a lo largo del periodo de maduración [27, 28]. La variación en la composición y calidad del fruto así como de su correspondiente AOV a lo largo del proceso de madurez, ha sido estudiada por diferentes autores [29-33]. La composición de ácidos grasos varía a los largo de la maduración [27, 31, 32, 34], así el contenido de ácido palmítico desciende, mientras que el ácido oleico muestra pequeñas variaciones en su porcentaje y el ácido linoleico aumenta[35]. La concentración de fenoles hidrofílicos y compuestos volátiles se ven afectados por el desarrollo del fruto [36-38]; El contenido de fenoles del AOV desciende durante el proceso de maduración [11, 28]. Por otro lado, se produce un incremento del contenido del alcohol triterpénico 24-metilen-cicloartanol del aceite durante el proceso de maduración del fruto, que corresponde a una

disminución del butiroespermol y cicloartenol [39], una disminución en 4-metil-esterol citrostadienol que produce un incremento de 3-metil-esterol gramisterol, y un incremento del Δ^5-avenasterol debido a la disminución en β-sitosterol [36, 39, 40]. De los tocoferoles, el contenido de α-tocoferol del aceite desciende durante la maduración del fruto [22, 41]. Las clorofilas y los pigmentos carotenoides del AOV disminuyen con la maduración [40, 42]. Por último, la calidad sensorial del aceite se ve afectada con el proceso de maduración, así se produce un descenso de la intensidad de los atributos sensoriales positivos: frutado, verde, y amargo, y el frutado cambia de verde a maduro [43].

Condiciones pedoclimáticas: se ha observado en estudios que la localización geográfica donde se cultiva el olivo juega un rol importante en la composición del AOV [21, 44]. Varios autores han descrito que AOV de frutos cultivados a baja altitud [44, 45], contienen alta cantidad de esteroles, polifenoles [44] y tocoferoles [46], a la vez que un bajo contenido de clorofilas y ácidos grasos insaturados [39] en comparación con los aceites de montaña. En relación a los ácidos grasos, algunos estudios han demostrado que el ratio MUFA/PUFA es mayor en cultivos de altitud elevada [44-47].

Por otro lado, se han observado diferencias en la proporción de constituyentes volátiles de aceites de diferentes variedades y orígenes geográficos [44, 45, 48]; AOV obtenidos de frutos monovarietales de altitud elevada son en general más dulces y tienen fragancia herbácea respecto a los correspondientes aceites de baja altitud. Las bajas temperaturas a esta altitud pueden influir en la actividad enzimática, ya que el hexanal (percepción verde-dulce) se origina a partir de mayores cantidades, y E-2-hexenal (olor verde-sabor astringente) se origina a partir de ácido linoleico y ácido linolénico, respectivamente [39], lo que sugiere que tanto los factores genéticos como las condiciones medioambientales influyen en la formación de compuestos volátiles [48].

Riego: los componentes químicos del AOV más influenciados por la irrigación son los compuestos fenólicos, que a su vez inciden tanto en la estabilidad oxidativa como en las características sensoriales (amargo y picante), mostrando ambos una relación inversa con la

cantidad de agua aplicada [49-53]. Los compuestos volátiles también se ven afectados, así se observa una menor concentración de alcoholes y ésteres en aceites de cultivos con muy baja irrigación [53].

Factores Tecnológicos

En general, las diferentes etapas del proceso desde la recepción del fruto en la almazara hasta la obtención del AOV pueden afectar en mayor o menor medida su composición. Dentro de ellas destacan por su importancia la molienda y el batido de la pasta [5]. La molienda es un paso crítico del proceso, ya que es donde el pool enzimático involucrado en la generación y transformación de compuestos fenólicos polares y volátiles es activado a la vez que se liberan diferentes componentes presentes en las células que pueden ser incorporados en mayor o menor concentración al aceite. Dependiendo de las condiciones de molienda, la concentración de esos componentes menores pueden ser modificados [6, 7, 9, 54, 55] así como sus propiedades sensoriales y bioactivas.

La fase de batido afecta también principalmente a la fracción minoritaria del AOV y la calidad final del mismo. El batido puede afectar al contenido de fenoles [7, 8, 56-58], volátiles [8, 57-59] y triterpenos [60]. El aumento de la temperatura durante el batido, generalmente incrementa el contenido fenólicos del AOV [7, 8], mientras que el contenido de volátiles generalmente disminuye bajo esas condiciones [7]. El tiempo de batido influye en la composición de los fenoles totales del AOV, entre los primeros 30 a 45 min su contenido aumenta y después de este tiempo disminuye [7]. Respecto a los compuestos volátiles los aldehídos C6 aumentan con el tiempo de batido y mientras los alcoholes C6 no se ven afectados [8, 56, 58]. Los compuestos triterpénicos se ven afectados con el proceso de obtención del AOV, un nivel de trituración más fino resulta en incremento de ácido maslínico, además un mayor tiempo de batido resulta en incremento de la concentración de ácido oleanólico, ácido maslínico y uvaol [60]

El sistema de separación sólido/líquido influye también en estos componentes; en general, los sistemas de 2 fases (mayoritarios en España) dan lugar a aceites con mayor contenido de tocoferoles y compuestos fenólicos, especialmente *o*-difenoles [61]. La fase de clarificación del aceite se lleva a cabo de forma continua mediante centrifugación

vertical. La cantidad de agua adicionada en esta etapa y su temperatura dan lugar a un descenso en el contenido de polifenoles y de las características sensoriales del aceite [62]. Finalmente, el almacenamiento en bodega es una etapa muy importante donde se deben preservar las características cualitativas del AOV; los factores que afectan la calidad del aceite durante el almacenamiento son la temperatura, la exposición a la luz y el contacto con el oxígeno [63, 64]. En general el AOV sufre una degradación durante el periodo de almacenamiento [63]. Estos mismos factores, condicionan la conservación del aceite ya envasado hasta su consumo.

2. Compuestos Bioactivos

2.1 Aspectos generales y definición

El mayor conocimiento del impacto de la dieta en la regulación del funcionamiento del organismo a nivel genético y molecular está cambiando el rol de la nutrición y resulta en nuevas estrategias dietarias. La dieta no solo provee nutrientes adecuados para mantener los requerimientos metabólicos, también puede contribuir a la mejora de la salud humana [65].

Todos los componentes de las plantas, desde las células bacterianas hasta las millones de células que las estructuran, producen compuestos químicos para su supervivencia y sustento. Estos compuestos son usualmente divididos en dos grupos: 1). Metabolitos primarios: carbohidratos, aminoácidos, proteínas y lípidos. 2). Metabolitos secundarios: sustancias con muy bajo peso molecular, como ácidos fenólicos, alcaloides o terpenos, relacionados con la capacidad de supervivencia y protección de las plantas. Diferentes estudios han demostrado que la producción de metabolitos secundarios depende del clima, suelo y condiciones de cultivo [66]. Estos metabolitos secundarios debido a la acción que pueden ejercer en el organismo humano son denominados compuestos bioactivos.

En general, los compuestos bioactivos son compuestos esenciales y no esenciales que están en la naturaleza, son parte de la cadena alimentaria y pueden tener un efecto en la salud humana [65]. Los compuestos bioactivos pueden ser clasificados en función de la extracción, en [67]:

- Compuestos hidrofílicos o polares: ácidos fenólicos, flavonoides, ácidos orgánicos, azúcares.
- Compuestos lipofílicos o apolares: carotenoides, alcaloides, terpenoides, ácidos grasos, tocoferoles, esteroles.

Las propiedades biológicas de cualquier compuesto dependen de dos requisitos: el primero, su biodisponibilidad y metabolismo en el tracto gastrointestinal, en este caso es esencial el conocimiento de las formas químicas que finalmente van a llegar a los tejidos con el fin de obtener un mejor conocimiento acerca de su mecanismo de acción en vivo; y el segundo, la persistencia en el plasma, es decir, la estabilidad durante el proceso de digestión y las vías metabólicas, en los que se pueden formar compuestos derivados que son igualmente bioactivos [68].

2.2 Bioaccesibilidad y Biodisponibilidad

Las propiedades bioactivas de cualquier compuesto y su capacidad de llevar a cabo acciones biológicas en el organismo dependen de su biodisponibilidad, que se determina mediante el estudio de los mecanismos de absorción, distribución y metabolismo de dicho compuesto.

Las condiciones de digestión gastrointestinal pueden dar lugar a drásticos cambios estructurales que pueden afectar a la estabilidad, biodisponibilidad y bioactividad de los compuestos alimentarios [69].

Existen distintas definiciones de biodisponibilidad pero, en general, este concepto se refiere a la proporción de un nutriente que el organismo absorbe de los alimentos y que utiliza para las funciones corporales normales [70]. Versantvoort et al. [71], definen la biodisponibilidad oral como la fracción de una dosis externa que resulta en una exposición

interna. La dosis externa representa la cantidad total del compuesto ingerido. El compuesto es considerado interno si es absorbido en el tracto gastrointestinal y transportado a través del hígado hacia la circulación sistémica. La biodisponibilidad oral consiste en tres etapas (Figura 1): 1). Bioaccesibilidad: liberación de los compuestos de su matriz al lumen intestinal. 2). Permeabilidad Intestinal: es la capacidad de un compuesto para pasar el epitelio intestinal y llegar a la vía portal. 3). Metabolismo: fracción de un metabolito que pasa al hígado sin ser metabolizado que podrá alcanzar la circulación sistémica [71].

Las etapas de la vía metabólica donde pueden darse cambios en la biodisponibilidad de los nutrientes son [70]:

- La liberación del nutriente de la matriz físico-química del alimento
- La acción de las enzimas digestivas en el intestino
- La unión y utilización por parte de la mucosa intestinal
- El paso por la pared intestinal (pasando a través de las células, entre ellas o de ambas formas) a la sangre o la circulación linfática
- La distribución sistémica
- La deposición sistémica
- El uso metabólico y funcional
- La excreción (por vía urinaria o fecal)

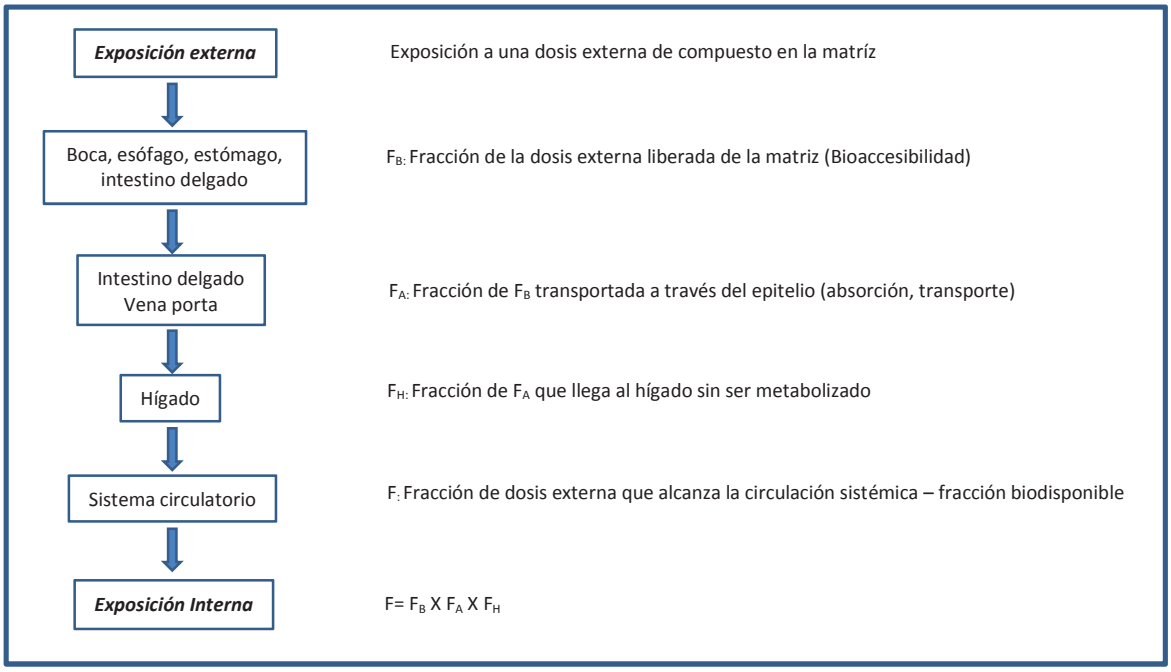

Figura 1. Representación esquemática del proceso que determina la biodisponibilidad oral

La biodisponibilidad de un nutriente se rige por factores externos e internos. Entre los factores externos se incluye la matriz alimentaria y la forma química del nutriente en cuestión, mientras que el sexo, la edad, el estado nutricional y la etapa de la vida (Ej. embarazo) son algunos de los factores internos. Dado que algunos aspectos, como el estado nutricional, también determinan la cantidad de un nutriente que el cuerpo utiliza, almacena o excreta, algunas definiciones de biodisponibilidad se limitan a la fracción del nutriente que es absorbida [72].

2.2.1 Efecto de la matriz en la biodisponibilidad y bioaccesibilidad

El primer paso para que un nutriente esté biodisponible es su liberación de la matriz alimentaria y su conversión en una forma química que pueda unirse e introducirse en las células del intestino o incluso atravesarlas lo que se denomina bioaccesibilidad [71, 73]. Los nutrientes se hacen bioaccesibles mediante los procesos de masticación y digestión enzimática del alimento en la boca, su combinación con ácidos y enzimas en los jugos gástricos en el estómago y, finalmente, su liberación en el intestino delgado, principal lugar

de absorción de los nutrientes. Una vez aquí, enzimas procedentes de los jugos pancreáticos siguen descomponiendo la matriz del alimento.

Estudios en animales y humanos sugieren que la biodisponibilidad de un compuesto puede ser significativamente diferente dependiendo de la matriz alimentaria, así como el procesado o preparación de los alimentos donde se encuentre [74]. La digestibilidad de algunas matrices alimenticias, por ejemplo de alimentos de origen vegetal, se facilita al cocinar o triturar los alimentos [75]. La biodisponibilidad del β-caroteno de los vegetales es baja (~ 14%), sin embargo, aumenta al adicionar aceite al aderezo para ensaladas [76]. La biodisponibilidad del Fe de la carne es mayor que el de la soja o el huevo [74].

Debido a que la liberación de un mismo compuesto puede ser diferente según la matriz en la que se encuentre, su biodisponibilidad oral debe tener en cuenta siempre la relación con la matriz donde se encuentre [71].

2.2.2 Efecto de la matriz en la absorción y metabolismo

Propiedades específicas de los compuestos, como peso molecular, lipofilicidad, afinidad por P450, etc., determinan su paso a través del epitelio intestinal y su susceptibilidad para ser metabolizados en el hígado [77, 78]. La matriz no afecta a las propiedades específicas del compuesto, por lo que no se espera que una vez liberado de la matriz, ésta tenga un efecto en su absorción o metabolismo. Sin embargo, en algunos casos la matriz ha mostrado afectar al transporte de los compuestos a través del epitelio intestinal [74, 79]. Así, otros constituyentes de la matriz alimentaria pueden competir con el compuesto bioactivo en el transporte a través del epitelio intestinal o por el contrario, favorecer su absorción como en el caso de minerales y algunas vitaminas. El transporte a través del epitelio intestinal y el metabolismo en el hígado depende fundamentalmente de las propiedades del compuesto específico, por lo que se puede asumir que estas etapas no se ven afectadas por la matriz ingerida [71].

2.2.3 Factor Relativo de Biodisponibilidad

Como se ha descrito anteriormente, la biodisponibilidad oral es el producto de tres procesos: bioaccesibilidad, transporte intestinal y metabolismo. La contribución de cada proceso individual es difícil de determinar en humanos [80]. El factor de biodisponibilidad relativa refleja las diferencias de la biodisponibilidad de un compuesto de dos matrices o condiciones fisiológicas diferentes debidas a diferencias en la bioaccesibilidad del compuesto de cada matriz o condición, asumiendo que el transporte y el metabolismo del compuesto no son afectados por la matriz alimentaria [71]. En los ensayos *in vivo* se toman como puntos finales para la determinación de la biodisponibilidad de un compuesto la concentración en sangre, excreción urinaria y/o rendimiento animal (ganancia de peso, mortalidad, etc.). Sin embargo, con los modelos *in vitro* es posible comparar la exposición interna de un compuesto de dos matrices diferentes (agua vs alimento sólido) o dos condiciones fisiológicas diferentes (ayuno vs con comida), que facilita la determinación del factor de biodisponibilidad relativa [71].

2.3 Métodos para determinar bioaccesibilidad y biodisponibilidad

Los métodos para determinar la biodisponibilidad o la bioaccesibilidad de compuestos implican simulación de experimentos realizados en un laboratorio (*in vitro*) o modelos en humanos o animales (*in vivo*)[81].

Los experimentos *in vivo* en humanos o en animales experimentales pueden proveer la mejor información de la biodisponibilidad (relativa) de la ingesta de compuestos [80]. Sin embargo, las técnicas *in vitro* permiten reducir la necesidad de animales experimentales y la gran cantidad de productos/matrices diferentes que demandan las técnicas in vivo, además son reproducibles y los procedimientos están estandarizados [71].

2.3.1 Métodos in vivo

Estos métodos incluyen distintos bioensayos con animales, generalmente ratas y pollos, así como, protocolos a corto plazo y a largo plazo con dosis simple, la inclusión de métodos por isótopos y estudios de perfusión intestinal todos ellos realizados en humanos y animales [82].

En los seres humanos el método de balance químico fue el primero en utilizarse antes de que el uso de radioisótopos fuera introducido. Consiste en evaluar la diferencia entre la cantidad ingerida y excretada de un determinado compuesto durante un cierto periodo de tiempo [83]. Como ventaja señalar la no exposición de los sujetos a radiaciones ionizantes y su fácil realización. Sin embargo, en la práctica, errores en la determinación de la cantidad ingerida o excretada pueden ocasionar errores significativos en la estimación de la absorción [83]. Uno de los principales inconvenientes de la técnica radica en diferenciar en la muestra de heces, el elemento no absorbido de origen dietético del procedente de la excreción endógena.

Los isótopos radioactivos han sido ampliamente utilizados, presentan como principal ventaja que, al suministrar dos isótopos radioactivos de un mismo compuesto, uno por vía oral y otro por vía parenteral, puede determinarse la excreción endógena. Sin embargo, debido a su alto costo y la dosis necesaria su uso es limitado [84]. También es utilizado otro tipo de marcaje con isótopos, denominado marcaje extrínseco en el que, el isótopo es añadido directamente a la comida completa o a uno de sus componentes mayoritarios [85].

Aunque lo ideal sería la realización de estudios *in vivo* con humanos, estos estudios son costosos, laboriosos y difíciles de llevar a cabo con precisión. Esto ha llevado al incremento del uso de animales de experimentación que presentan menos limitaciones. El principal problema de este tipo de ensayos es que los resultados obtenidos no siempre son extrapolables al ser humano debido a las diferencias existentes entre el metabolismo de animales y hombre [86].

2.3.2 Métodos in vitro

Una alternativa a los estudios *in vivo* con humanos o animales son los estudios *in vitro*. Este tipo de métodos es más rápido, sencillo y de menor coste económico. La movilización de un compuesto de la matriz alimenticia en el tracto gastrointestinal es un proceso dinámico con continuos cambios bajo las condiciones fisiológicas de éste. Con modelos de

digestión *in vitro* el proceso de digestión en el tracto gastrointestinal es simulado de una manera simplificada bajo condiciones fisiológicas, por ejemplo, composición química de fluidos digestivos, pH y tiempos de residencia típicos para cada etapa. Los modelos de digestión *in vitro* describen dos (estómago e intestino delgado) o tres etapas (boca, estómago, intestino delgado o estómago, intestino delgado e intestino grueso) del proceso. La bioaccesibilidad del compuesto puede ser determinada en cada etapa, sin embargo, la absorción de los compuestos tiene lugar en el intestino delgado, por lo que la bioaccesibilidad es determinada principalmente en el quimo o fracción bioaccesible del intestino delgado [71].

Los métodos *in vitro* pueden ser clasificados en dos grupos, según se determine la fracción soluble o la fracción de ésta que se dializa.

Métodos basados en la solubilidad: determinan la cantidad de elemento soluble presente en el sobrenadante obtenido por centrifugación [87, 88] o filtración [89] del digerido gastrointestinal del alimento.

Métodos basados en diálisis: incorporan una membrana de diálisis durante el proceso de digestión gastrointestinal para simular una difusión pasiva a través de la mucosa intestinal. De esta manera permite diferenciar entre compuestos solubles de alto y bajo peso molecular [90].

Aunque estos métodos sólo estiman la fracción soluble/dializable del compuesto disponible para la absorción y por tanto, sólo simulan el primer paso del proceso *in vivo*, son ampliamente usados por su buena correlación con estudios *in vivo* [74, 91]. Los resultados de estos modelos deben tomarse como índices relativos de la biodisponibilidad, proporcionando datos que permiten establecer tendencias, comparaciones y determinar el efecto causado por diferentes factores sobre la biodisponibilidad del nutriente [92]. En general, esta técnica es complementada con estudios de permeabilidad intestinal para alcanzar unos resultados similares a los procesos *in vivo.*

Modelos para el estudio de permeabilidad intestinal *in* vitro: la cantidad de nutriente soluble o dializable puede ser útil para la estimación de la bioaccesibilidad,

aunque no todo el nutriente soluble o dializado sea absorbido. Los métodos mencionados anteriormente carecen de un componente vivo y éste es necesario para una determinación más aproximada de la disponibilidad relativa de un compuesto.

En este sentido, se han complementado los estudios de digestión *in vitro* con estudios de permeabilidad intestinal. Varios sistemas han sido desarrollados con objeto de imitar *in* vitro las características de la mucosa intestinal. Así, se han desarrollado modelos que utilizan tejidos extirpados, células aisladas y preparaciones de membrana, y monocapas utilizando cultivos celulares, siendo este último el método más utilizado en los años recientes.

En el modelo basado en monocapas se utilizan los cultivos de células del epitelio intestinal. Las monocapas de células Caco-2 han sido utilizadas en estudios de transporte de compuestos [93]. Las células Caco-2 en cultivo, tienen gran similitud a los enterocitos intestinales, crecen adheridas a un sustrato formando una monocapa, cuando éstas alcanzan la confluencia, se diferencian espontáneamente adquiriendo las características morfofuncionales del enterocito maduro [94].

El modelo *in vitro* basado en monocapas, pretende llevar a cabo la cuantificación en un compartimento receptor, de un compuesto que ha pasado de un compartimento dador. La monocapa diferenciada forma un sistema de dos compartimentos, uno apical (Ap), que corresponde al lumen intestinal *in vivo*, y otro basolateral (BL), que corresponde al espacio intersticial en contacto con los capilares sanguíneos. Ambos se encuentran separados por el filtro que contiene la monocapa. Este paso a través de las monocapas celulares, permite calcular el coeficiente de permeabilidad aparente (Papp) [95].

No obstante, la utilización de este modelo para el estudio de la captación y transporte de nutrientes a nivel intestinal también presenta inconvenientes. Esta línea celular, al proceder de carcinoma de colon humano, puede presentar un metabolismo diferente al esperado y mostrar una mayor resistencia transepitelial [96]. Además, no presentan capacidad de

producir mucina y tiene una baja tasa de transporte de nutrientes en relación a los enterocitos normales. Probablemente, estas limitaciones con respecto a las condiciones *in vivo* se deben a una menor superficie de transporte si se compara con el intestino, la presencia en el mismo de diferentes especies celulares y la ausencia de regulación endocrina. Sin embargo, se le considera un modelo válido debido a las condiciones ya estandarizadas de los experimentos y a los resultados reproducibles, por lo que su empleo es cada vez más frecuente [97, 98].

3. Compuestos del AOV con propiedades bioactivas

La dieta mediterránea, que incluye el AOV como uno de los ingredientes más importantes, está fuertemente relacionada con la reducción de la prevalencia de enfermedad cardiovascular y Cáncer según diferentes estudios: EUROLIVE [99, 100], PREDIMED [101-103], CORDIOPREV [104].

Las propiedades nutricionales y de salud del AOV están relacionadas con la presencia de ácidos grasos monoinsaturados (MUFAs) como el ácido oleico [105] y componentes minoritarios como compuestos fenólicos (HTy, Ty, ácido caféico y oleuropeina), tocoferoles (α-tocoferol), esteroles (β-sitosterol, campesterol, Δ7-estigmasterol), hidrocarburos (escualeno, β-caroteno e hidrocarbonos aromáticos policíclicos), y pigmentos (clorofila y luteína) [106].

3.1 Ácidos grasos

Los ácidos grasos (AGs) son ácidos carboxílicos, de cadena alifática no ramificada, que se clasifican en tres grandes grupos: ácidos grasos saturados (SFA), monoinsaturados (MUFA) y poliinsaturados (PUFA). Los SFAs no contienen dobles enlaces, los principales SFAs del AOV son el ácido palmítico (16:0) y esteárico (18:0). Los MUFAs contienen un par de átomos de carbono unidos por un doble enlace *cis*. El ácido oleico (18:1n-9) es el principal MUFA y representa entre el 55 y 83% del total. La familia de los PUFAs contiene 2 o más dobles enlaces cis, con el primero localizado entre los carbonos 3 y 4 y el segundo

localizado entre los carbonos 6 y 7, son también denominados n-3 o n-6 respectivamente [107].

Como se describió en el apartado de composición química del AOV, los ácidos grasos (AG) presentes en el AOV son principalmente ácido palmítico (C16:0), palmitoléico (C16:1), esteárico (C18:0), oleico (C18:1), linoleico (C18:2), and linolénico (C18:3), ácido mirístico (C14:0), heptadecanoico (17:0) y eicosanoico (20.0), estos tres últimos se encuentran en cantidades traza [3].

La composición de ácidos grasos del AOV puede variar en función de la zona de producción, latitud, clima, condiciones de cultivo, y estado de madurez del fruto [3]. La principal fuente de variación es la variedad. Diferentes autores han descrito un importante componente varietal en la composición de los ácidos grasos, por encima del 70% de variabilidad, principalmente en ácido palmítico, esteárico, oleico y linoleico. En la Tabla 3 se muestra la composición de ácidos grasos de AOV de diferentes variedades de olivo [3].

Esas notables diferencias en la composición de ácidos grasos pueden tener efectos relevantes en las propiedades de salud que son atribuidas al AOV. Sin embargo, raramente son mencionadas en los estudios nutricionales [108, 109].

3.1.1 Aspectos nutricionales

Los ácidos grasos poliinsaturados (PUFAs) con 18 átomos de carbono como el linoleico (18:2 n-6) y α-linolénico (18:3 n-3) son conocidos como ácidos grasos esenciales (EFAs) en la nutrición humana. Esos ácidos grasos son componentes indispensables de la estructura, desarrollo y función celular, aunque no son sintetizados por el cuerpo humano [110].

Tabla 3. Composición de ácidos grasos (%) de AOV de diferentes variedades

AG	`Bianchera´[a]	`Busa´[a]	`Leccino´[d]	`Picual´[a]	`Arbequina´[b]	`Koroneiki´[b]	`Frantoio´[c]
C16:0	12.7	12.1	13.7	12.3	17.5	11.6	10.9
C16:1	0.88	0.72	1.32	1.01	2.41	1.07	0.89
C17:0	ND	ND	ND	0.03	ND	ND	0.07
C17:1	ND	ND	-	0.07	-	-	-
C18:0	3.03	1.99	1.9	2.56	1.88	2.15	1.53
C18:1	75.9	74.4	75.6	79.8	58.8	75.5	78.3
C18:2	5.35	8.56	5.65	3.06	12.9	8.56	6.79
C18:3	0.52	0.71	0.16	0.55	0.63	0.26	0.49
C20:0	0.45	0.35	0.3	0.31	0.40	0.42	0.33
C20:1	0.24	0.27	ND	0.17	ND	ND	0.27
C22:0	ND	ND	ND	0.05	ND	ND	0.18

Referencias: [a] Skevin el al. [111]; Allalout et al.[112] ; [c] Aguilera et al. [47];
ND. No detectado

Tanto el ácido linoleico (LA) como el α-linolénico (ALA) pueden ser desaturados y alargados en humanos para formar series de n-6 PUFA (γ-linolénico, ácido dihomo-γ-linolénico, ácido araquidónico, ácido docosatetraenóico y ácido docosapentaenóico) y n-3 PUFA (ácido estearidónico, ácido eicosapentaenóico, ácido docosapentaenóico y ácido docosahexaenóico) [113].

La ingesta de PUFA es necesaria a través de la dieta y deberá aportar entre el 6 y 11% de las calorías de grasa [113]. Los ácidos grasos n-6 y n-3 deben estar presentes en la dieta en un ratio correcto, ya que el exceso de ácido linoleico puede interferir la síntesis endógena de ácido eicosapentanóico y docosahexanóico con la consecuente carencia para el cuerpo [110]. Sin embargo, en el último informe de la FAO sobre grasa y ácidos grasos en la nutrición humana, no recomienda una proporción específica de ácidos grasos n-3/n-6, siempre y cuando la ingesta de éstos ácidos se sitúe en las establecidas, n-3 PUFA del 2% y para n-6 PUFA entre 2.5 y 9% de la energía total [113].

Los ácidos grasos monoinsaturados (MUFA) no son componentes esenciales de la dieta ya que el organismo los puede sintetizar. Sin embargo, están presentes en muchos alimentos, el ácido graso monoinsaturado más común es el ácido oleico y es el ácido graso mayoritario del aceite de oliva [114].

3.1.2 Metabolismo y transporte

Los ácidos grasos de la dieta son absorbidos por los enterocitos y convertidos en TG. Los TG son incorporados a los quilomicrones (QM), junto con el colesterol y las apo lipoproteínas. Los QM son secretados dentro de la linfa y pasan a la sangre vía ducto torácico. En la sangre, las partículas de QM sufren una rápida lipólisis por acción de la lipoproteína lipasa (LPL). Este proceso moviliza algunos de los TG y forma pequeños quilomicrones remanentes (QMr) que transportan los TG remanentes, colesterol y otros lípidos hasta el hígado. Los QMr tienen ApoB48 como proteína estructural, secretada como parte integral de los QM iniciales y apoE que es adquirida en la circulación y tiene un papel clave en su captación hepática. Los QMr junto con las LDL pueden atravesar la barrera endotelial y entrar a la vía arterial. Los QMr en el espacio sub-endotelial son captados por los macrófagos sin necesidad de procesos oxidativos (a diferencia de las LDL), conduciendo a la formación de células espumosas, iniciando o agravando el proceso de aterogénesis (Figura 2). Por tanto, además de los efectos en las concentraciones plasmáticas de LDL, los lípidos de la dieta estarían disponibles para influir en procesos aterogénicos en la pared arterial durante su transporte del intestino al hígado [115, 116].

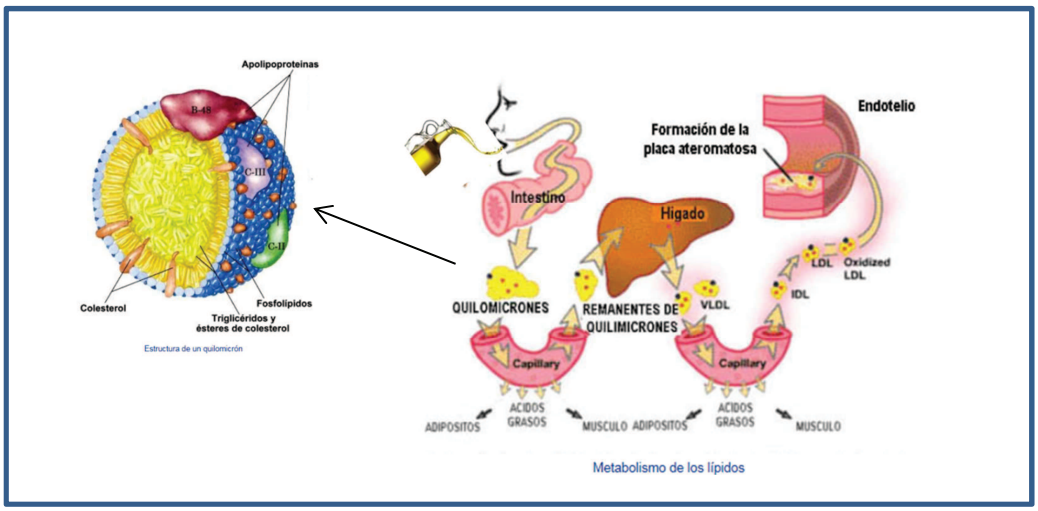

Figura 2. Metabolismo de lípidos dietarios y formación de placa ateromatosa. Modificado de ©2001 Medmovie.comTM

3.1.3 Actividad Biológica de los ácidos grasos del AOV

Existen evidencias clínicas de los efectos beneficiosos para la salud de la dieta mediterránea. El efecto protector se ha asociado a la elevada ingesta de ácido oleico y α-linoleico y la baja ingesta de ácidos grasos saturados (SFA) y ácido linolénico [117].

Varios estudios muestran que los MUFAs tienen acción protectora frente a enfermedades coronarias, diabetes y cáncer [113, 114]. Estos ácidos grasos se han relacionado con un efecto positivo frente a factores de riesgo de esas enfermedades como la hipertensión [118-123], la sensibilidad a la insulina [121] y la concentración de lipoproteínas plasmáticas[116, 122, 124-127]. Además el ácido oleico tendría efectos benéficos en el estado postprandial y en los primeros eventos de progresión de la aterogénesis, cuando forma parte de los quilomicrones (QM) [127].

Los PUFAs también se han relacionado con disminución del riesgo de enfermedades cardiovasculares cuando se sustituyen en la dieta ácidos grasos saturados por PUFAs [113]. Dietas enriquecidas con n-3 PUFAs reducen la presión arterial en humanos y de forma espontánea la hipertensión en ratas, debido a un incremento en la síntesis de eicosanoides de la serie n-3 con mayor efecto vasodilatador que su serie homóloga n-6 [119].

3.2 Compuestos Fenólicos

Químicamente los compuestos fenólicos contienen al menos un anillo aromático (benceno) al que están unidos uno o más grupos hidroxilo, incluyendo derivados funcionales [128]. Existe una gran variedad de compuestos fenólicos, que se pueden agrupar en las siguientes categorías:

- Fenoles, ácidos fenólicos, ácidos fenilacéticos
- Ácidos cinámicos, cumáricos, isocumáricos y cromanos
- Lignanos
- Flavonoides
- Ligninas

- Taninos
- Benzofenonas, santonas y estilbenos
- Quinonas
- Betacianinas

Otros compuestos fenólicos se encuentran en la naturaleza en una forma conjugada, comúnmente con molécula de azúcar [128]. Los polifenoles se suelen encontrar en los vegetales en forma de glucósidos, pero la acción de enzimas o de algunos procesos, puede liberar las correspondientes agliconas [129]. La biosíntesis de los compuestos fenólicos se lleva a cabo a partir del ciclo de Calvin, a través del ácido shikímico [130], y estos se acumulan en las vacuolas y en la pared celular.

La mayor concentración de polifenoles se encuentra en las frutas, hortalizas y productos derivados de ellas, como el aceite de oliva virgen, vino tinto, té, cerveza, cereales y las leguminosas. Los compuestos fenólicos contribuyen a la estructura de la pared celular de las plantas, a su pigmentación, protegen de los rayos solares y, en especial, participan en la defensa contra patógenos y depredadores. Dadas estas funciones, los fenoles juegan un papel en el sabor, color y textura de los alimentos [131].

3.2.1 Compuestos Fenólicos en el aceite de oliva virgen.

Hay alrededor de 36 estructuras distintas de fenoles identificados en el aceite de oliva virgen. Los polifenoles del AOV son en su mayoría productos de la hidrólisis de oleuropeína y ligustrósido, agliconas y compuestos relacionados [132], además de lignanos y flavonoides. Los compuestos fenólicos del AOV pueden ser agrupados de acuerdo a su estructura química en los siguientes grupos [133]:

Alcoholes Fenólicos: estos compuestos poseen un grupo hidroxilo unido a un grupo hidrocarbono aromático. El hidroxitirosol y tirosol son los principales alcoholes fenólicos encontrados en el aceite de oliva virgen (Figura 3). Aunque también es posible encontrar acetato de hidroxitirosol, acetato de tirosol y una forma glucosídica del hidroxitirosol [134].

Ácidos fenólicos: estos compuestos pueden dividirse en tres subgrupos; derivados del ácido benzoico, derivados del ácido cinámico y otros ácidos fenólicos y derivados [133]. En la Figura 3 se muestra la estructura química de los ácidos fenólicos encontrados en el aceite de oliva virgen, los ácidos fenólicos con la estructura C6-C1 se denominan ácidos benzoicos, mientras que los ácidos cinámicos presentan la estructura C6-C3, como el caféico, vainílico, siríngico, *p*-cumárico, *o*-cumárico, protocatéquico, sinápico y *p*-hidroxibenzoico [132].

Secoiridoides y derivados: Este grupo se caracteriza por la presencia ya sea de ácido elenólico o derivados del ácido elenólico en su estructura molecular (Figura 4) [132] . Son isómeros de las agliconas de la oleuropeína y ligustrósido presentes en la aceituna que se generan durante la extracción del aceite de oliva por acción de enzimas endógenas del fruto, dando lugar a las formas dialdehídicas y aldehídicas de la aglicona de la oleuropeína y ligustrósido [135].

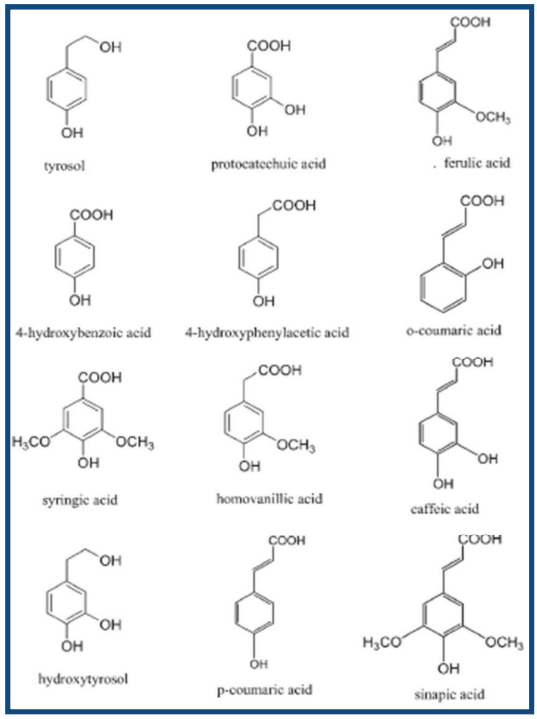

Figura 3. Estructura química de los fenoles libres y ácidos fenólicos [3]

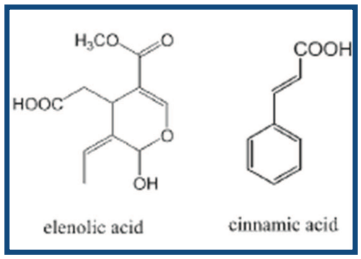

Figura 4. Estructura química del ácido elenólico y ácido cinámico [3]

Los secoiridoides constituyen la fracción fenólica mayoritaria del aceite de oliva virgen [135]. En general, el derivado secoiridoide mayoritario en el AOV es la forma dialdehídica del ácido elenólico unida al hidroxitirosol (3,4-DHPEA-EDA). También está presente la forma dialdehídica derivada del ligustrósido (*p*-HPEA-EDA), comúnmente denominada *oleocantal* [136-138]. Además de estos compuestos fenólicos mayoritarios, se han identificado otros compuestos secoiridoides como la forma aldehídica del ácido elenólico unida al hidroxitirosol (3,4-DHPEA-EA), isómero de la aglicona de la oleuropeína, y la forma aldehídica del ácido elenólico unida al tirosol (*p*-HPEA-EA), isómero de la aglicona del ligustrósido [139]. Las estructuras químicas se muestran en la Figura 5.

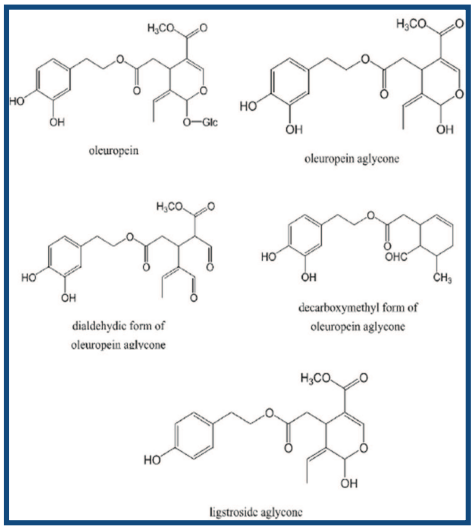

Figura 5. Estructura Química de Oleuropeína y Ligustrósido [3]

Hidroxi-Isocromanos: en el AOV se han identificado, 1-fenil-6,7dihidroxi-isocromano y 1-(3'-metoxi-4'-hidroxi)fenil-6,7dihidroxi-isocromano [140]. La estructura química de estos compuestos se puede observar en la Figura 6.

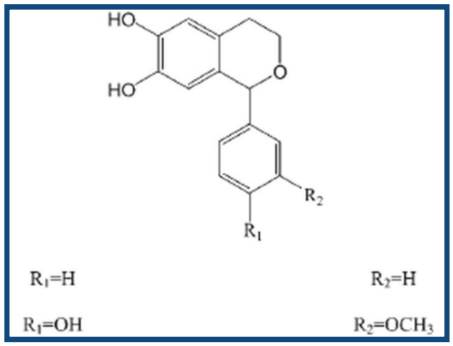

Figura 6. Estructura química del hidroxi-isocromano [3]

Flavonoides: estos compuestos contienen dos anillos benzoicos unidos por una cadena lineal de tres carbonos. Se pueden dividir en dos subgrupos: flavonas y flavonoles [133]. En el aceite de oliva virgen se han descrito flavonoides como la luteolina y apigenina (Figura 7), y (+)-Taxifolin [141].

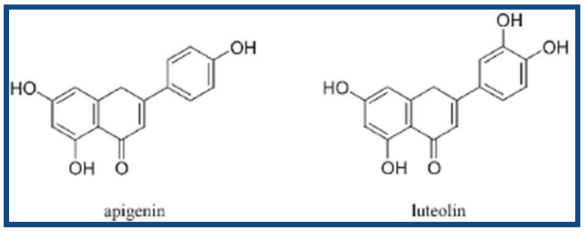

Figura 7. Estructura química de los flavonoides [3]

Lignanos: su estructura está basada en la condensación de aldehídos aromáticos. Los lignanos son encontrados como compuestos fenólicos prevalentes en el aceite de oliva virgen [132]. Se han aislado y caracterizado el (+)-1-acetoxipinoresinol, (+)-pinoresinol y (+)-1-hidroxipinoresinol [142, 143]. En la Figura 8 se presenta la estructura química del pinoresinol y del 1-acetoxipinoresinol.

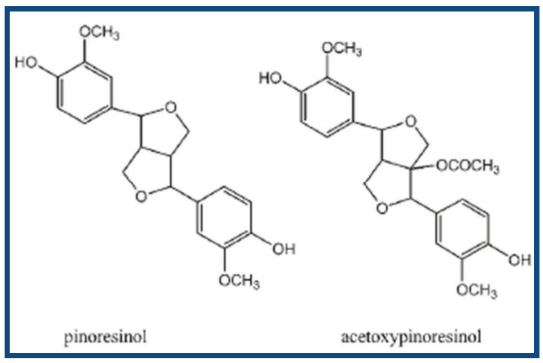

Figura 8. Estructura química de lignanos [3]

La composición de compuestos fenólicos del AOV puede variar en función de la zona de producción, latitud, clima, condiciones de cultivo, y estado de madurez del fruto [133, 144]. Las diferencias entre los aceites monovarietales se observan principalmente en su contenido de compuestos secoiridoides y derivados. En la Tabla 4 se muestra la composición de compuestos fenólicos de AOV de diferentes variedades de olivo [145, 146]. Asimismo, el contenido y composición de la fracción fenólica del AOV también puede verse afectada por las condiciones de elaboración del aceite [6, 8, 9, 58]. Esas diferencias en la composición de compuestos fenólicos pueden tener efectos relevantes en las propiedades de salud que son atribuidas al AOV.

3.2.2 Compuestos fenólicos y calidad del Aceite de Oliva Virgen.

Aunque no están incluidos en los parámetros de calidad reglamentada, la calidad del aceite de oliva virgen depende en gran medida de la cantidad y composición la fracción fenólica. Los aceites ricos en polifenoles presentan un grado mayor de estabilidad, y unas propiedades organolépticas características, como el amargo y las percepciones bucotáctiles de astringencia y picante [11, 147].

Tabla 4. Concentración de compuestos fenólicos AOV monovarietales (µg / mL)

Compuesto fenólico	**`Arbequina´**	**`Arbosana´**	**`Cornicabra´**	**`Hojiblanca´**	**`Picual´**	**`Sikitita´**
Hidroxitirosol	1.76 ± 0.01	2.07 ± 0.02	1.43 ± 0.02	1.98 ± 0.03	1.42 ± 0.08	1.82 ± 0.3
Tirosol	2.28 ± 0.12	18.7 ± 6.42	4.28 ± 0.09	4.39 ± 0.36	4.42 ± 0.13	5.49 ± 0.55
Ácido Caféico	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00
Ácido *p*-Cumárico	1.15 ± 0.01	0.24 ± 0.02	0.22 ± 0.01	0.55 ± 0.00	0.22 ± 0.01	1.06 ± 0.06
Ácido Ferúlico	0.12 ± 0.00	0.15 ± 0.00	0.10 ± 0.00	0.13 ± 0.00	0.10 ± 0.00	0.33 ± 0.02
Oleuropeina	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00
Luteolina	3.74 ± 2.24	4.14 ± 1.06	0.84 ± 0.02	3.94 ± 2.53	0.93 ± 0.02	4.93 ± 3.07
Apigenina	1.17 ± 0.01	2.91 ± 0.12	0.77 ± 0.00	1.31 ± 0.04	0.79 ± 0.00	1.04 ± 0.00
Ácido Vainílico	0.62 ± 0.02	0.80 ± 0.02	0.16 ± 0.00	0.85 ± 0.09	0.14 ± 0.01	1.06 ± 0.03
Vainillina	0.19 ± 0.01	0.10 ± 0.00	0.42 ± 0.01	0.21 ± 0.01	0.43 ± 0.00	0.17 ± 0.00
Diosmetin	3.06 ± 1.61	1.05 ± 0.01	0.48 ± 0.01	0.52 ± 0.03	0.53 ± 0.01	4.89 ± 2.83
Quercetina	0.06 ± 0.00	0.05 ± 0.01	nd	0.04 ± 0.00	0.04 ± 0.00	0.09 ± 0.01
Apigenina-7-glucosido	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00
Luteolina-7-glucosido	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Rutina	0.04 ± 0.00	0.04 ± 0.003	0.04 ± nd	0.04 ± nd	0.038 ± nd	0.04 ± 0.00
Verbascosido	0.03 ± 0.00	nd	nd	nd	nd	0.03 ± 0.00
3,4-DHPEA-AC	56.5 ± 12.8	1.09 ± 0.01	7.66 ± 1.23	17.2 ± 8.27	8.16 ± 1.84	9.14 ± 2.66
3,4-DHPEA-EDA	439 ± 159	6.96 ± 0.62	10.0 ± 2.36	82.2 ± 29.2	11.2 ± 3.68	9.03 ± 2.78
p-HPEA-EDA	56.7 ± 11.6	10.4 ± 1.72	15.7 ± 4.82	18.47 ± 9.30	17.18 ± 6.94	7.72 ± 1.84
3,4-DHPEA-EA	9.65 ± 2.95	8.55 ± 1.08	51.1 ± 25.1	66.7 ± 21.4	51.7 ± 18.5	9.32 ± 3.08
p-HPEA-EA	1.58 ± 0.01	2.23 ± 0.07	39.3 ± 12.5	17.0 ± 9.33	42.9 ± 12.7	1.59 ± 0.00

Fuente: Sánchez de Medina et al. [145]

Se ha descrito una correlación entre la respuesta positiva de los atributos astringente y amargo del aceite de oliva virgen y la concentración de compuestos secoiroideos. Algunos autores han sugerido que derivados secoiroideos de la oleuropeína como el 3,4-DHPEA-EDA y 3,4-DHPEA-EA son los que más contribuyen al amargor de aceite de oliva virgen [1, 149]. También se ha mostrado una fuerte relación entre la intensidad del amargo y del picante y el contenido de *p*-HPEA-EDA [52]. Andrewes et al. [150], describieron que *p*-HPEA-EDA produce una fuerte sensación picante en la parte posterior de la garganta. En cambio, la fracción que contiene 3,4-DHPEA-EDA produce una ligera sensación de picante percibida en la lengua. Mateos et al. [151], describieron la forma aldehídica de la oleuropeína aglicona es responsable del atributo amargo, correlacionando los niveles de

derivados secoiroideos y el amargor sensorial de aceites de oliva vírgenes de diferentes variedades.

3.2.3 Digestión, metabolismo y transporte de compuestos fenólicos.

La estructura de los compuestos fenólicos está estrechamente ligada a su acción biológica ya que influye en la bioaccesibilidad y biodisponibilidad y por tanto, en sus efectos saludables. Considerando la ingesta media de AOV en los países mediterráneos de 50 g/día y un contenido de fenoles de 180 mg/kg, Vissers et al. [152] estimó que la ingesta diaria de fenoles es de 9 mg/kg. Por tanto, conocer los cambios que sufren los compuestos fenólicos durante su digestión y la cantidad que puede estar disponible es de gran importancia.

Digestión: a nivel bucal, una vez los compuestos fenólicos del AOV y las proteínas salivares se ponen en contacto, se forman agregados complejos que pueden precipitar. Las proteínas ricas en prolina (PRP) y las histatinas son las más efectivas en precipitar fenoles. También se ha demostrado que las glicoproteínas salivares con fuerte actividad lubricante como la mucina, PRP glicosiladas y la amilasa, tienen una elevada capacidad de unirse a fenoles [153, 154]. Esta interacción de fenoles-proteínas se ha relacionado con la percepción sensorial de la astringencia.

El pH ácido (aproximadamente 2) del estómago puede afectar a la estabilidad de los compuestos fenólicos y por tanto, modificar su acción biológica. Dependiendo del tipo de compuesto, el ambiente ácido del estómago puede afectar más o menos a la estabilidad de su molécula. En el caso del tirosol e hidroxitirosol la modificación a nivel gástrico es mínima, sin embargo el hidroxitirosol-acetato es hidrolizado ligeramente dando lugar a hidroxitirosol libre [155, 156]. Las condiciones ácidas del estómago podrían afectar la integridad de la aglicona de la oleuropeína [157]. Para unos autores la aglicona de la oleuropeína no sufre modificaciones en el ambiente gástrico, mientras que otros describen una degradación en función del tiempo de permanencia, contribuyendo a un aumento del hidroxitirosol libre [157, 158].

El grado de hidrólisis que sufren las formas conjugadas de la oleuropeina, ligustrósido y sus agliconas a nivel gástrico puede condicionar la presencia de compuestos simples en el intestino delgado que es el lugar donde se produce, en mayor medida, el proceso de absorción de la gran mayoría de los nutrientes [159].

Absorción y transporte: el conocimiento sobre el mecanismo de absorción de los fenoles del aceite de oliva virgen no se ha desarrollado en su totalidad. Se ha propuesto que las diferentes polaridades de la aglicona de la oleuropeína y la aglicona del ligustrósido, tirosol e hidroxitirosol, podrían resultar en diferentes mecanismos de absorción [160]. Así el tirosol e hidroxitirosol, son compuestos polares y su transporte podría ocurrir vía difusión pasiva [161].

En cuanto a compuestos menos polares como la aglicona oleuropeína y el aglicona ligustrósido, no se ha descrito de forma definitiva su mecanismo de absorción. Vissers et al. [160] en un estudio en humanos describieron como la absorción mayoritaria de los fenoles del aceite de oliva virgen se produce en el intestino delgado. Se produciría una hidrolisis de la aglicona de la oleuropeína y del ligustrósido a hidroxitirosol y tirosol, respectivamente. Estos fenoles se metabolizarían, probablemente después de la absorción en el intestino delgado. La absorción de ligustrósido aglicona, hidroxitirosol, tirosol y oleuropeína aglicona después de la administración en humanos fue de 55-66% [160].

Pinto et al. [158] estudiaron la absorción de los secoiridoides usando una preparación intestinal *in vitro*. Los compuestos 3,4-DHPEA-EDA y 3,4-DHPEA-EA (o sus glucorónidos) no son absorbidos en la forma parental, por tanto, no tienen formas biodisponibles *in vivo*. Los principales metabolitos encontrados en el intestino delgado procedentes de estos secoiridoides fueron glucorónidos conjugados de 3,4-DHPEA-EDAH_2 y 3,4-DHPEA-EAH_2 que han experimentado reducción (probablemente enzimática) durante su transferencia en el enterocito.

Aunque se considera al intestino delgado (yeyuno e íleo) como la principal vía de entrada de la mayoría de los compuestos fenólicos, no habría que descartar la presencia de parte de ellos en el colon donde pueden desarrollarse procesos de fermentación, por acción

de la microbiota local, dando lugar a metabolitos de bajo peso molecular, sobre todo ácidos fenólicos [162].

Se ha descrito que la absorción de hidroxitirosol y tirosol en humanos es dosis dependiente [163]. La bioaccesibilidad *in vitro* de los fenoles del aceite de oliva virgen varía entre un 90% y 37%, dependiendo de la cantidad de aceite de oliva virgen ingerido [68].

Metabolismo, excreción y biodisponibilidad: Tras la absorción, estos compuestos sufren un metabolismo de fase II en el interior de los enterocitos y en el hígado, donde se llevan a cabo reacciones enzimáticas de metilación, glucuronización y/o sulfatación [162]. Un importante paso en el metabolismo de los fenoles del aceite de oliva virgen es la transformación de la agliconas de la oleuropeína y del ligustrósido a hidroxitirosol o tirosol y ácido elenólico [160]. Vissers et al. [160] encontraron que el 15% de suplemento de oleuropeína aglicona administrado a sujetos saludables fue excretado en la orina como hidroxitirosol y tirosol. Estos compuestos pueden transformarse ya sea en el tracto gastrointestinal antes de que sean absorbidos o en las células intestinales, sangre o hígado después de ser absorbidos.

Otra fase importante en el metabolismo es la conjugación con el ácido glucurónico ya que se ha encontrado que el tirosol e hidroxitirosol son principalmente excretados como glucurónidos [160, 163, 164]. La posible glucuronización tiene lugar en los enterocitos y en las células hepáticas [160, 165]. Asimismo, en estudios sobre el metabolismo del tirosol, ácido *p*-cumárico, pinoresinol, luteolina [166] e hidroxitirosol acetato [135] del AOV en monocapas de células Caco-2, se describe que los conjugados metilados fueron los metabolitos mayoritarios. La acetilación del hidroxitirosol incrementó significativamente su transporte a través de las células de barrera del intestino delgado, incrementado el transporte de hidroxitirosol a los enterocitos. Otra posible vía metabólica para los dihidroxifenoles es la *o*-metilación, que tiene lugar principalmente en el hígado. De hecho

el hidroxitirosol *o*-metilado es un importante metabolito en la orina después de la ingesta de aceite de oliva virgen [161, 167].

La parte restante de fenoles que no es detectado en orina es probablemente metabolizado en el cuerpo. Así, los fenoles del aceite de oliva virgen son modificados antes de ser excretados por los riñones [160]. Mateos et al. [168] estudiaron el metabolismo hepático *in vitro* de hidroxitirosol, tirosol e hidroxitirosol acetato, utilizando células humanas del hepatoma HepG2. Los autores describieron como el hidroxitirosol acetato fue convertido en gran parte a hidroxitirosol libre y posteriormente metabolizado ya que pequeñas cantidades de hidroxitirosol acetato glucuronizado fueron detectadas. El tirosol fue pobremente metabolizado, ya que menos del 10 % del fenol fue glucuronizado después de 18 h.

En definitiva, los polifenoles son excretados a través de la orina. Vissers et al. [160], describieron la excreción de polifenoles (principalmente como tirosol e hidroxitirosol) es de 5-16% del total ingerido. Miró-Casas et al. [169] encontraron una recuperación media en orina en torno al 25% del tirosol administrado, mientras que Visioli et al. [167] reportaron la excreción de 30-60% para hidroxitirosol y del 20-22% para tirosol. Pocos autores han identificado en orina metabolitos derivados del metabolismo hepático e intestinal de los fenoles simples debido a falta de patrones puros para su identificación. Khymenets et al. [170] encontraron que después de la ingesta de 50 mL de AOV, menos del 5% de los fenoles consumidos fueron excretados en su forma libre. Aproximadamente el 4.5% de la cantidad ingerida de hidroxitirosol se excreto como alcohol homovanílico-*O*-metilado y el 70% aproximadamente del total excretado fue en forma de 4'-*O*-glucuronido. La recuperación de fenoles como glucurónidos representó aproximadamente el 10% de la dosis consumida (10.5% para los glucurónidos del hidroxitirosol y alcohol homovanílico y 9.6% para el glucurónido del tirosol) [170].

La biodisponibilidad de los compuestos fenólicos además puede disminuir cuando son parte de una comida completa. Se ha encontrado tanto *in vitro* como *in vivo* que los compuestos fenólicos interaccionan con proteínas y otras macromoléculas encontradas en alimentos y en el tracto digestivo [171-173]. De hecho se ha demostrado la afinidad de los

fenoles del AOV con las proteínas en función de su estructura química, siendo mayor la capacidad de ligar proteínas de los compuestos secoiridoides que del hidroxitirosol y tirosol [172].

3.2.4 Actividad Biológica de los Compuestos Fenólicos del Aceite de Oliva.

La principal actividad biológica de los compuestos fenólicos del AOV está relacionada con su acción antioxidante debido a su habilidad para actuar como donadores de hidrógeno. La inhibición de la oxidación de los fenoles es mayor con el incremento del número de grupos hidroxilo. En particular, los compuestos *o*-dihidroxil han mostrado alta actividad antioxidante por la formación de enlaces hidrógeno intramoleculares durante la reacción con radicales libres; también, donando electrones sustituyentes en posición *orto*, tendiendo a debilitar el enlace O-H del fenol y proporcionar más estabilidad al radical fenoxil [132, 174]. El hidroxitirosol (3,4-DHPEA), 3,4-DHPEA-EDA y 3,4-DHPEA-EA, son los antioxidantes más importantes confirmando que la presencia de un solo grupo hidroxil confiere una cantidad limitada de actividad antioxidante; también la presencia de $–COOCH_3$, como en la aglicona de la oleuropeína puede causar una disminución en su poder antioxidante ya que no es un grupo donador de electrones [132].

Los compuestos fenólicos actúan también como antioxidantes mediante la transición de metales como el Cu y el Fe, quelando los iones metálicos, previniendo así su participación en reacciones Fenton que pueden generar altas concentraciones de radicales hidroxilo [174-176].

La actividad biológica de los compuestos fenólicos del aceite de oliva virgen no se limita a su capacidad antioxidante, ésta puede extenderse a su interacción con importantes sistemas enzimáticos. La actividad biológica de los compuestos fenólicos del aceite de oliva se puede esquematizar como se muestra en la Figura 9.

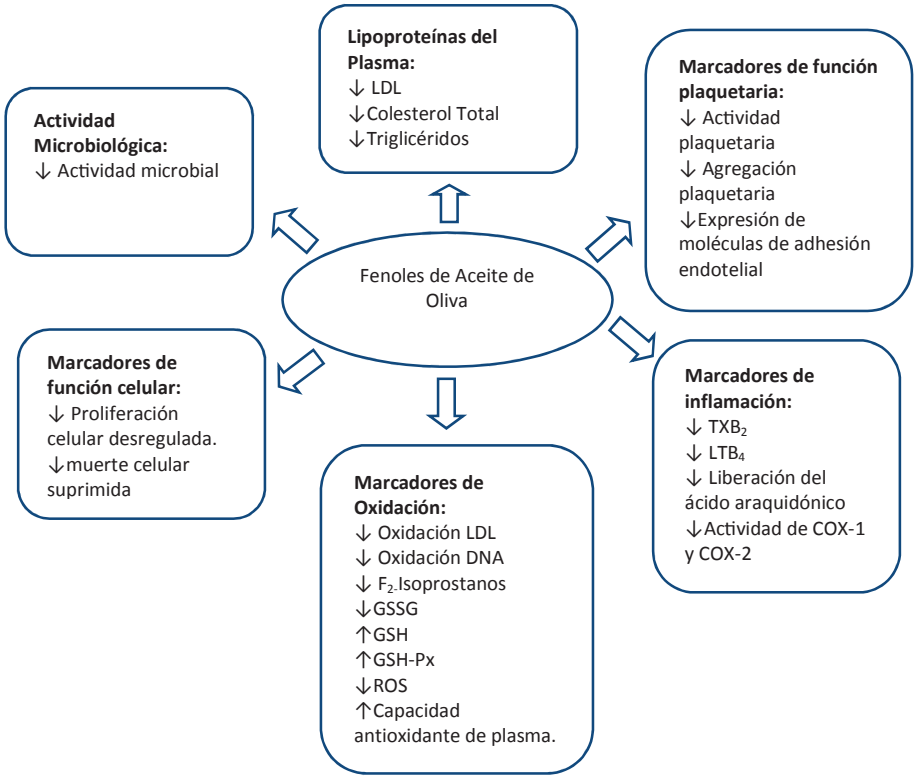

Figura 9. Actividad Biológica de los compuestos fenólicos del aceite de oliva. Adaptado de Cicerale et al. [133]

En particular se ha descrito que los fenoles del aceite de oliva virgen presentan las siguientes actividades:

- Inhiben la agregación plaquetaria [177].
- Tienen actividad antiinflamatoria [178], reducen la formación de moléculas pro-inflamatorias como tromboxano B2 y Leucotrieno B4 [174].
- Inhiben el uso de oxígeno en neutrófilos humanos.
- Incrementan la producción de óxido nítrico por los macrófagos en ratas expuestas a endotoxinas, regulando el sistema inmune [174].
- Protegen frente al estrés oxidativo por su capacidad de captación de radicales libres [179, 180].

- El tirosol e hidroxitirosol afectan a la velocidad de proliferación, el perfil del ciclo celular o apoptosis celular en células epiteliales mamarias humanas o células mamarias cancerosas. El hidroxitirosol disminuye especies intracelulares reactivas de oxigeno (ROS) en células epiteliales mamarias humanas; por otro lado, previene el daño oxidativo del ADN en tres líneas celulares mamarias [181].
- Estimulan la inducción de enzimas antioxidantes [182].
- Inhiben la oxidación de lipoproteínas de baja densidad (LDL) ricas en colesterol [183, 184].
- Previenen de la disfunción endotelial [185].
- Tienen actividad antimicrobiana y antiviral [174].
- Modulan el metabolismo [186].

Esta actividad biológica de los compuestos fenólicos se traduce en efectos beneficiosos sobre la salud humana, que se resumen en la Tabla 5.

Tabla 5. Compuestos fenólicos del aceite de oliva y sus efectos en la salud.

Efecto sobre la salud humana	Mecanismo de acción	Compuesto Fenólico
Prevención de enfermedades cardiovasculares	Inhibición de la oxidación de colesterol LDL. Inhibición de HMG-CoA reductasa. Inhibición de tromboxano B2 y consecuentemente agregación plaquetaria.	Oleuropeína, Hidroxitirosol Ácido caféico, Ácido protocatéquico 3,4-dihidroxi-feniletanil-acidoelenolico
Prevención de enfermedad tumoral	Acción inhibitoria en la actividad de la xantina oxidasa y reducción en la formación de superóxido. Lignanos actúan como anti-estrógenos e incrementan la hormona sexual ligada a globulina.	Secoiridoides (Hidroxitirosol y tirosol) Lignanos

Efecto sobre la salud humana	Mecanismo de acción	Compuesto Fenólico
Actividad anti-inflamatoria	Acción inhibitoria en ciclo-oxigenasa y lipoxigenasa. Reducción de la formación tromboxano B2 y leucotrieno B4	Hidroxitirosol y otros polifenoles
Actividad antimicrobial y antiviral	Inhibición del crecimiento y actividad viral y bacteriana	Oleuropeína, verbascosido, hidroxitirosol y tirosol.
Modulador del metabolismo	Inhibición la liberación de ácidos grasos de los triglicéridos e inhibe las enzimas involucradas en la transformación del glicerol. Estimulación la actividad enzimática de la pepsina, resultando en alta degradación de proteína.	Oleuropeína

Adaptado de Trípoli et al. [174]

3.2.5 Interacción de compuestos fenólicos con proteínas.

Los compuestos fenólicos tienen además la capacidad de interaccionar con proteínas, este ha sido tema de interés en investigación por la importancia que tiene en los alimentos. La mayoría de los estudios sobre las interacciones proteínas-fenoles, se han realizado específicamente sobre la interacción de los taninos con proteínas y, sobre los factores que afectan esta interacción como son: el pH, temperatura, composición de aminoácidos y tamaño de la proteína, estructura del compuesto fenólico y concentración de etanol en el medio [187, 188]. La interacción polifenoles-proteínas puede resultar en:

- Sensación de astringencia: debido a la interacción de los polifenoles con las proteínas salivares ricas en prolina [189].
- Turbidez y formación de haz coloidal: debido a la interacción de los polifenoles con las proteínas en la cerveza, el vino y jugos de fruta, en los que resulta una característica indeseable [190].
- Reducción del valor nutricional de las proteínas: a los compuestos fenólicos se les ha atribuido la capacidad que tienen para ligar y precipitar proteínas. El complejo formado entre los fenoles, así como de sus productos de oxidación enzimática y no

enzimática, con las proteínas de alimentos como carnes, harinas, etc., reduce el valor nutricional de la proteína de estos alimentos [189].

- Reducción de la biodisponibilidad de los fenoles y de su capacidad antioxidante: la interacción fenol-proteína forma complejo insoluble de gran tamaño que puede precipitar y afectar su biodisponibilidad [189, 191]. Se ha descrito que ésta interacción afecta la capacidad antioxidante de los polifenoles de bajo peso molecular como el ácido ferúlico, ácido caféico, catequina [192].

Formación de complejo proteína-polifenol: las interacciones proteína-fenol en principio, pueden tener lugar mediante puentes de hidrógeno, interacciones hidrofóbicas, o enlaces covalentes [193]; y se puede categorizar como interacciones reversibles o irreversibles [189]. Las interacciones irreversibles usualmente se producen autocatalíticamente en presencia de oxígeno, o por acción enzimática (presencia de polifenoloxidasas) [194]. Las interacciones reversibles conducen a la formación de complejos insolubles en la solución, que en ausencia de agentes externos (oxigeno, iones metálicos, ácido o base) y bajo condiciones adecuadas el complejo precipitado puede redisolverse [195].

Se ha propuesto que la interacción entre polifenoles-proteínas tiene tres fases: inicialmente, el polifenol se une al péptido, de forma que varias moléculas de polifenol pueden unirse al mismo péptido. Tras esta etapa, si se adiciona más cantidad de polifenol, se llega a la segunda etapa, en la que el polifenol actúa como un enlazador entre dos moléculas de péptidos. El péptido forma un dímero de polifenol recubierto que comienza a precipitar; y, en la tercera etapa, el complejo se agrega en grandes o pequeñas partículas [196-198]. Cada una de las etapas se puede observar en la Figura 10.

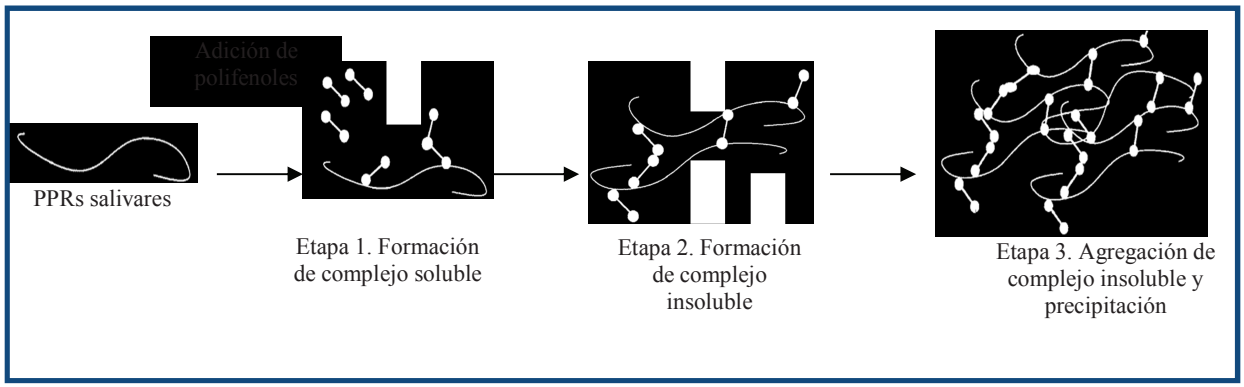

Figura 10. Representación esquemática de las etapas de unión y precipitación de polifenoles con proteínas. Adaptado de Charlton et al. [196]

Estudios realizados sobre la interacción entre fenoles simples y polifenoles con albúmina de suero bovino BSA, sugieren que los polifenoles son ligandos multivalentes con capacidad para reaccionar con dos o más moléculas de proteína. La estequiometria y tamaño del complejo proteína-fenol formado depende de las concentraciones y ratios de fenol/proteína [199].

A nivel molecular se han evidenciado interacciones hidrofóbicas al hacer reaccionar péptidos de derivados de proteínas ricas en prolina (PRPs) de ratas con pentagaloil glucosa (PGG), debido a la interacción de los grupos fenoles con el anillo pirrolico de la prolina presente en la proteína [196, 200].

Efecto de las características de las proteínas y fenoles: La mayor afinidad de los fenoles por las proteínas se encuentra en proteínas y polipéptidos de alto contenido de prolina, tales como las proteínas de la saliva [201]. Las proteínas glicosiladas también tienen afinidad y selectividad para la unión con los fenoles, esto puede ser debido a la habilidad de la porción de oligosacárido de la proteína para mantener la estructura de la proteína en una relativa conformación abierta [189]. En general, las proteínas precipitadas por fenoles suelen ser de masa molecular elevada y presentan un contenido alto de prolina [201].

Se ha sugerido que los polifenoles también se pueden ligar fuertemente a las histatinas, que son proteínas de la saliva y contienen una elevada proporción de residuos de histidina [202]. Charlton et al. [196] concluyeron que la prolina es un importante sitio de unión con los polifenoles pero que la interacción también puede ocurrir con cadenas laterales de arginina y fenilalanina. En general, la capacidad de los compuestos fenólicos de precipitar proteínas depende de su peso molecular, número y disposición del núcleo fenólico, flexibilidad conformacional, y solubilidad en agua [189].

Los polifenoles de bajo peso molecular son poco efectivos para ligar proteínas globulares. Mientras que el incremento en el número de anillos aromáticos y pirrólicos con el peso molecular de prociniadinas provee múltiples sitios de naturaleza hidrofóbica para participar en esas interacciones, presumiblemente estabilizas por puentes de hidrógeno del grupo *o*-dihidroxifenol [203].

Varios estudios han demostrado interacción de polifenoles con proteínas salivares, principalmente las mucinas, proteínas ricas en prolina (PRPs) [204-206] , histatinas (HPRs) [207] y amilasas [153].

Métodos de determinación de la interacción proteína-polifenol: La formación de complejos de los polifenoles con proteínas ha sido ampliamente estudiada y existe un gran número de metodologías para su estudio. En solución se ha estudiado su interacción por espectroscopia NMR [196, 200, 208], microcalorimetría [209], inhibición enzimática [210, 211] y electroforesis [212]; también se han estudiado la precipitación de proteínas [187, 213-215] y medida la afinidad relativa de diferentes polifenoles para formar complejos con proteínas por medio de turibidimetría o nefelometría [153, 199, 216], que es un método analítico basado en la medida de la intensidad de la luz dispersa por soluciones turbias [217].

3.3 Compuestos Volátiles

Las plantas sintetizan una gran cantidad de compuestos volátiles que facilitan las interacciones con su entorno, desde atraer a los polinizadores y dispersadores de semillas hasta protegerse a sí mismos de patógenos, parásitos y herbívoros. Basado en su origen biosintético, todos los compuestos volátiles orgánicos pueden ser divididos dentro de varias clases, incluyendo terpenoides, fenilpropanoides/benzonoides, derivados de ácidos grasos y derivados de aminoácidos, sólo pocas especies o compuestos específicos no están representados dentro de esas clases [218].

En la actualidad se ha incrementado el interés en el uso de los compuestos volátiles para extender la vida útil de los alimentos debido a sus propiedades como conservantes naturales. Los compuestos volátiles han sido ampliamente utilizados como agentes aromatizantes y la mayoría son reconocidos como seguros[219, 220].

3.3.1 Compuestos volátiles y calidad del AOV

A diferencia de otros aceites vegetales, el aceite de oliva virgen al no sufrir refinación, conserva su aroma natural, uno se los parámetros más apreciados por sus consumidores. Aproximadamente 280 compuestos han sido identificados en la fracción volátil del AOV [3]. Dicho aroma está formado por una compleja mezcla de compuestos volátiles, entre los que se encuentran aldehídos, ésteres, alcoholes, hidrocarburos y cetonas. De todos estos compuestos, los más importantes cuantitativamente son los aldehídos, alcoholes y ésteres de alcoholes de seis átomos de carbono, que representan entre el 80 y 90% del total de la fracción de volátil del AOV [221-223]. En la Tabla 6 se observan los principales compuestos volátiles y su relación con los atributos sensoriales del AOV.

Tabla 6. Compuestos volátiles del AOV y sus características sensoriales

Atributo Sensorial/Aroma	Compuestos Relacionados
Verde	Metil acetato, 1,3-hexadien-5-ine, 4-metil pentan 2-one, 2 metil-1-propanol, (Z)-3-hexenal, acetato de hexilo, 3-hexenil-acetato, (Z)-2-penten-1-ol, (E)-2-hexen-1-ol, (Z)-3-hexen-1-ol
Dulce	Etil furano, etil propanoato, 1-penten-3-ona, butil acetato, hexanal, etil butanoato
Amargo y Picante	Etil benceno, (E)-2-hexenal, (Z)-2-hexenal, 6-metil-5_hepten-2-one, quinina, cafeína, alcaloides, trideceno, 1-penten-3-ona, 1-penten-3-ona
Indeseable	1-penten-3-ol, 3-metil butanol, 2-octanona, 1-hexanol, ácido acético
Frutado	2-butanona, 3-metil butanal, 2-metil butil propanoato, etil benceno, 2-nonanona
Rancio – humedad	2-heptanona, (E)-2-nonanone
Metálico	1-penten-3-ona
Rancio	Aldehídos insaturados

Fuente: Adaptado de Morales et al. [224] y Kalua et al [225]

Diferentes factores como la volatilidad, carácter hidrofóbico, tipo y posición de los grupos funcionales, se han relacionado más con la intensidad del olor de un compuesto volátil que con su concentración [225, 226].

Está bien establecido que esta serie de compuestos volátiles se producen a partir de la oxidación de los ácidos grasos polinsaturados a través de la ruta de la lipoxigenasa (LOX) [227]. Esta ruta se induce con la rotura de los tejidos, implicando la acción de una serie de enzimas que degradan los ácidos grasos poliinsaturados y otras que modifican los productos de dicha degradación [228].

En el caso del aceite de oliva virgen la ruta LOX se activa principalmente durante la molienda de los frutos [4] y el batido de la pasta. Los volátiles así producidos se incorporan posteriormente al aceite confiriendo su aroma característico. Estos compuestos son sintetizados a partir de ácidos grasos poliinsaturados de estructura (Z,Z)-1,4-pentadiento como el ácido linoleico (LA) y linolénico (LnA) a través de las sucesivas reacciones enzimáticas de la ruta descritas en la Figura 11 [229, 230].

De esta forma, el aroma de un determinado aceite depende en gran medida de los niveles de actividad de las enzimas que componen la ruta mencionada, los cuales cambian en función de la variedad, estado de maduración del fruto [222, 229] y las condiciones utilizadas durante la extracción del aceite [56].

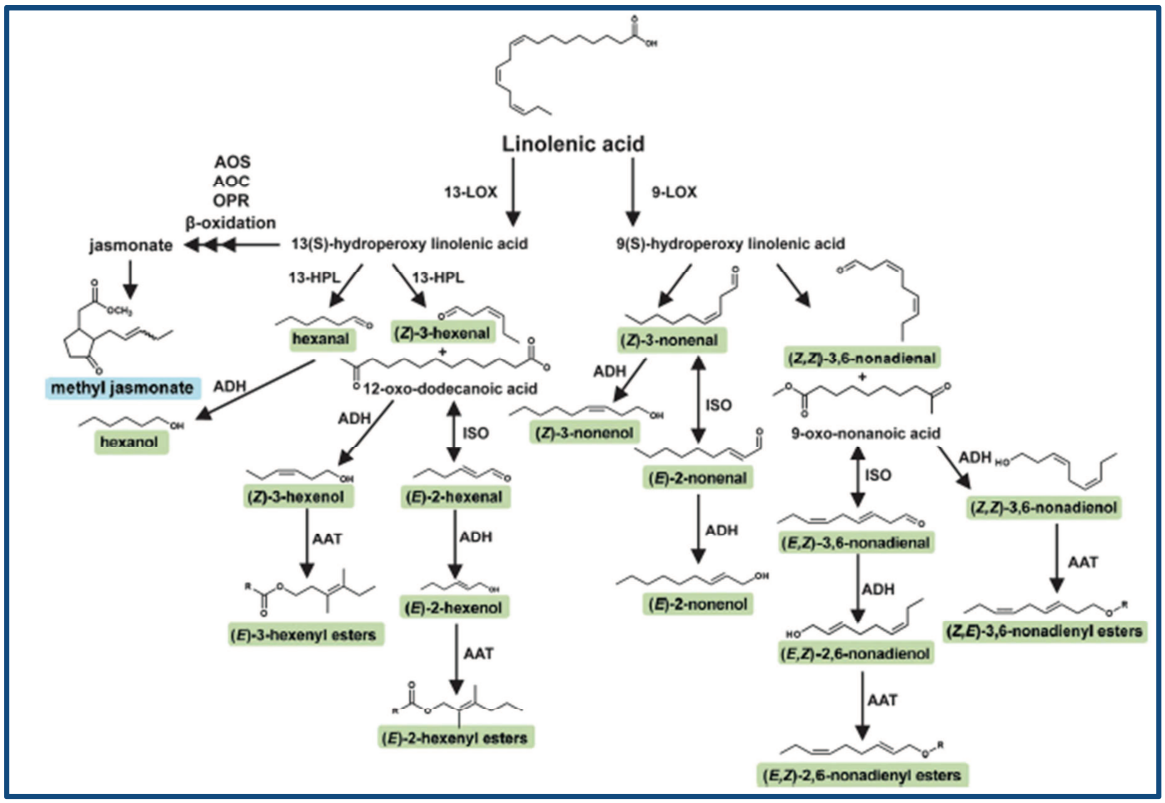

Figura 11. Síntesis de compuestos orgánicos volátiles derivados de ácidos grasos – ruta de la lipoxigenasa (LOX). AAT, alcohol aciltransferasa; ADH, alcohol desihidrogenasa; AOC, aleno oxido ciclasa; AOS, aleno oxido sintetasa; 9-HPL, 9-hydroperoxide liasa; 13-HPL, 13-hydroperoxide liasa; ISO, isomerasa; 9-LOX, 9-lipoxigenasa; 13-LOX, 13-lipoxigenasa; OPR, 12-oxofitodienoato reductasa [218].

3.3.2 Actividad Biológica de los Compuestos Volátiles

Además de sus propiedades aromatizantes, los compuestos volátiles pueden actuar como un ingrediente funcional y antimicrobiano aunque este es un aspecto que se encuentra aún en estudio [231].

Se han encontrado que los compuestos relacionados con el atributo sensorial "verde" como el hexanal y el (E)-2-hexenal tienen propiedades antimicrobianas [232-234]. (E)-2-Hexenal ha sido reconocido como el principal agente antimicrobiano frente a *Salmonella* spp., *E. coli* and *Pseudeomonas aeruginosa* [234, 235]. Otros estudios han mostrado un papel clínicamente útil de estos compuestos, cuando son usados en una concentración no tóxica, en el tratamiento de tumores debido a la inhibición de la glutatión S-transferasa [236]. Por otra parte, se ha sugerido que el uso de (E)-2-hexenal, en combinación con otros agentes antimicrobianos, contribuye a la erradicación de *helicobacter pilori* en pacientes afectados por gastritis aguda [237].

Basados en la evidencia encontrada, los compuestos volátiles del AOV podrían ejercer también un efecto beneficioso para la salud aunque falta por explorar si estos metabolitos son liberados de la matriz después de la ingesta de AOV para posteriormente ejercer su acción.

3.4 Esteroles

Los esteroles de las plantas, también llamados fitoesteroles, constituyen la mayor proporción de la fracción insaponificable de los aceites vegetales. Estos compuestos son derivados biosintéticamente del escualeno y forma un grupo de triterpenos [238]. El contenido total de esteroles del AOV varía entre 1000 y 2300 ppm [239, 240]. Están influenciados por la variedad [24] y muestran un descenso durante la maduración del fruto [36].

Los esteroles se pueden clasificar en dos grupos, los esteroles comunes (4 α-desmetilesteroles) y los 4 α-metilesteroles. Los esteroles comunes del AOV son β-sitoesterol, Δ-5-avenasterol y campesterol. En cantidades menores aparece el estigmasterol, colesterol, 24-metilencolesterol, Δ-7-campesterol, Δ-5-23-estigmastadienol, clerosterol, sitostanol, Δ-7-estigmaesterol y Δ-7-avenasterol [241, 242].

Los fitoesteroles tienen una estructura similar al colesterol pero con algunas modificaciones, como el lado de la cadena, incluir un doble enlace y un grupo etilo o metilo [243]. En el AOV el más común de los fitoesteroles es el β-sitoesterol que se corresponde entre el 75-90% de la fracción esterólica, de la que el Δ-5-avenasterol representa el 5-20%, el campesterol el 1-4% y el estigmasterol entre el 0,5 y 2% [244]. La composición y contenido de esteroles puede verse afectada de acuerdo a la variedad de cultivo, año de cosecha, grado de madurez del fruto, tiempo de almacenamiento del fruto antes de la extracción del aceite y el método de extracción del aceite [3].

Una de las principales actividades biológicas de los fitoesteroles es la reducción del colesterol LDL y colesterol total en sangre, debido a que interfieren en la solubilización del colesterol en las micelas a nivel intestinal disminuyendo así su absorción. La dosis efectiva de fitoesteroles para la disminución del colesterol LDL sanguíneo es del 8 a 15% lo que significa 1.5 a 3 g por día [245]. También se ha descrito sus propiedades antitumorales en colon, mama y próstata [246], sus propiedades antiinflamatorias (160), y su capacidad para modular el sistema inmune [247]. No existe evidencia de ninguna actividad mutagénica en estudios *in vitro* [248] o de toxicidad en estudios en animales [249]. La única limitación es su posible interferencia con la absorción de los carotenoides, aunque este efecto negativo puede ser compensado con una dieta que contenga cantidades apropiadas de estos nutrientes [245].

3.5 Tocoferoles

Los tocoferoles son considerados los antioxidantes lipídicos naturales más importantes, previenen la peroxidación lipídica por la captación de radicales en membranas y partículas lipoprotéicas [250]. En el AOV se han descrito cuatro formas del tocoferol: α-, β-, γ-, y δ- tocoferol. El compuesto mayoritario en el AOV es el α-tocoferol y su cantidad varía de pocos mg/kg hasta 300 mg/kg [3]. Se ha descrito un ratio α-TOC/ PUFAs como miligramos de vitamina E por gramo de ácidos grasos poliinsaturados, que no debería ser menor a 0.5. Este ratio es raramente encontrado en aceites vegetales, sin embargo en el AOV se encuentra en un rango de 1.5 a 2.0 [110]. El contenido de tocoferoles del AOV

puede verse afectado por la variedad y muestra una reducción durante la maduración del fruto, refinado y procesos de hidrogenación [3, 22].

Estos compuestos son conocidos por contribuir a la capacidad antioxidante del AOV [250, 251], así como incrementar la estabilidad de este durante el almacenamiento protegiéndolo de degradaciones termo-oxidativas [252]. Los tocoferoles del AOV además de captar radicales libres, también previenen la foto-oxidación [253].

En relación a sus propiedades bioactivas en la salud humana, el α-tocoferol defiende al cuerpo frente al ataque de radicales libres, previene desordenes de la piel, la aterosclerosis y el cáncer [251]. Sin embargo, hay controversia acerca de los efectos *in vivo* y a largo plazo de la vitamina E, ya que no está claro que cantidades o combinaciones pueden ser beneficiosas para prevenir enfermedades crónicas [251]. Algunas investigaciones han demostrado que estas acciones son debidas a una relación sinérgica entre la acción antioxidante de algunos compuestos fenólicos y los tocoferoles [254].

3.6 Pigmentos

El AOV, frente a otros aceites vegetales, contiene cantidades considerable de pigmentos como clorofilas y carotenoides. Las clorofilas se encuentran principalmente como feofitinas *a* y *b* [255]. La mayoría de los carotenoides presentes en el AOV son β-caroteno y luteína [256]. La clorofila, es un fotosensibilizador, y puede iniciar procesos de oxidación cuando el AOV es expuesto a la luz [257]. Por otro, lado el β-caroteno [258] mejora la estabilidad frente a la fotoxidación. El efecto del β-caroteno durante la oxidación en la oscuridad, donde las reacciones no son iniciadas por sensibilización de los pigmentos, depende de las condiciones en las que las reacciones ocurran. Por tanto, el β-caroteno puede actuar como antioxidante o pro-oxidante dependiendo del sustrato, concentración y presencia de tocoferoles [259]. La luteína tiene un efecto antioxidante y trabaja en combinación con el licopeno, como un agente altamente activo frente al envejecimiento de la piel y el riesgo de

cáncer. Una adecuada ingesta de carotenoides derivados de fuentes vegetales como del AOV pueden actuar como un factor protector de la piel [110].

3.7 Escualeno

El escualeno es un triterpeno poliinsaturado compuesto de seis unidades de isopreno. Actúa como un precursor bioquímico del colesterol y otros esteroides. Es un compuesto sintetizado tanto por las plantas como animales, y se encuentra muy extendido en la naturaleza. Se encuentran en altas cantidades en la aceituna de mesa, aceite de hígado de tiburón, germen de trigo y salvado de arroz [260]. El escualeno también es un componente mayoritario de la fracción insaponificable del AOV (alrededor del 40%) [3], su contenido está influenciado de forma muy importante por la variedad [25]. En los humanos, el escualeno es sintetizado en el hígado y en la piel, transportado en la sangre por las VLDL y LDL, y secretado en grandes cantidades por las glándulas sebáceas [261].

El escualeno ha sido considerado un importante componente de la dieta mediterránea debido a su potencial quimiopreventivo frente al cáncer. Se han alcanzado niveles de escualeno en el organismo tras la inclusión en la dieta de AOV (40 g por día, cantidad común de las personas de los países Mediterráneos) que puede tener un considerable efecto inhibitorio en el desarrollo de cáncer [262, 263]. Se ha encontrado que el escualeno del AOV tiene una composición similar a la encontrada en el sebo. El escualeno en el sebo se encuentra en altas cantidades y actúa como un potente captador del oxígeno singlete, inhibiendo la lipoperoxidación inducida por la radiación ultravioleta, y por tanto ejerciendo una influencia anti-neoplásica en colon, mama y próstata e inhibiendo el desarrollo de tumores [264]. También tiene un efecto protector frente al cáncer de piel probablemente por la captación del oxígeno singlete generado por la luz ultravioleta [265]. La ingesta oral como también el uso externo del AOV ha mostrado ejercer fotoprotección en la piel [266]. La presencia de escualeno así como de α-tocoferol y carotenoides en el AOV es un soporte del uso tópico de este aceite como ingrediente en cosmética y en cremas dermoprotectoras [110].

3.8 Dialcoholes y ácidos Triterpénicos

Los principales dialcoholes triterpénicos del AOV son el uvaol y el eritrodiol. Poseen un grupo hidroxilo funcional en el C3 en su estructura química, así como un grupo metanólico en la posición C17. De los ácidos triterpénicos presentes en el AOV, han sido identificados el ácido oleanólico, maslínico, ursólico, betulínico, 2α-hidroxiursólico y el deoxiursólico [3], siendo los ácidos oleanólico y maslínico los más abundantes. Ambos compuestos poseen un grupo hidroxilo funcional en el C3 de su estructura química, así como un grupo carboxílico en el C17. El ácido maslínico se diferencia del oleanólico por la presencia de un grupo hidroxilo adicional en el C2. La concentración de triterpenos en el AOV depende de la variedad, época de recolección del fruto y condiciones de elaboración del aceite [23, 60, 267].

En los últimos años ha crecido el interés por la actividad biológica de los compuestos triterpénicos entre las que destacan la antineoplásica, anticancerígena, anti-inflamatoria, antioxidante, hepatoprotectora, cardioprotectora, antimicrobiana, antidiabética, antiviral, anti-HIV, etc.

Se ha descrito que el ácido oleanólico y uvaol tienen efecto cardioprotector ya que son capaces de proteger las partículas de LDL frente a la oxidación inducida por el cobre (CuSO4) *in vitro* [268, 269] y que reducen la producción de dienos conjugados [269, 270]. Además son capaces de provocar relajación tras su adición acumulativa en anillos de aorta [271] y tener un efecto vasodilatador [272]. Por otro lado, se ha observado que el ácido oleanólico favorece que las células arteriales incrementen la síntesis de prostaglandina I2 (prostaciclina), conocida por su propiedad vasodilatadora y antitrombótica [272]. Se ha encontrado efecto inhibitorio de los ácidos ursólico y oleanólico sobre la proliferación celular en líneas celulares de carcinoma de colon [273-276]. El ácido oleanólico y maslínico han mostrado efecto citotóxico frente a líneas tumorales humanas de pulmón, ovario, melanoma, sistema nervioso central y colon [277]. El eritrodiol y uvaol también han presentado efecto antiproliferativo y apoptótico frente al cáncer de mama [278, 279].

4. Alegaciones Nutricionales del AOV

La Agencia Europea de Seguridad Alimentaria (EFSA *European Safety Authority*) elaboró el Reglamento CE n° 1924/2006 (modificado por 107/2008 y 109/2008), relativo a las declaraciones nutricionales y propiedades saludables de los alimentos, en vigor desde el 1 de Julio de 2007. Con este reglamento se pretende armonizar a escala comunitaria lo que se consideran declaraciones nutricionales y de salud, las cuales deben estar basadas en pruebas científicas generalmente aceptadas y ser bien comprendidas por el consumidor medio.

Se entiende por declaración cualquier mensaje o representación que no sea obligatorio, con arreglo a la legislación comunitaria o nacional, incluida cualquier forma de representación pictórica, gráfica o simbólica, que afirme, sugiera o dé a entender que un alimento posee unas características específicas. En el artículo 2 se definen tres tipos de declaraciones: nutricionales, de propiedades saludables y de reducción de riesgo de enfermedad.

- Declaración nutricional: es cualquier declaración que afirme, sugiera o dé a entender que un alimento posee propiedades nutricionales benéficas específicas con motivo del aporte energético proporcionado, proporcionado en un grado reducido o incrementando o no proporcionado y/o de los nutrientes u otras sustancias que contiene, contiene en proporciones reducidas o incrementadas o no contiene (Art.2.4).

- Propiedades saludables: son cualquier declaración que afirme, sugiera o dé a entender que existe una relación entre una categoría de alimentos, un alimento o uno de sus constituyentes, y la salud (Art.2.5).

- Reducción del riesgo de enfermedad: son cualquier declaración de propiedades saludables que afirme, sugiera o dé a entender que el consumo de una categoría de alimentos, un alimento o uno de sus constituyentes reduce significativamente un factor de riesgo de aparición de una enfermedad humana (Art. 2.6).

En los tres casos, el nutriente o sustancia acerca de la cual se hace la declaración debe estar contenido en el producto final en una cantidad significativa para generar el efecto declarado de forma representativa en la población y en una forma asimilable por el organismo. Este efecto además, siempre debe quedar establecido a través de pruebas científicas aceptadas de forma consensuada, las cuales pueden ir acompañadas de una solicitud de protección de datos sujetos a derechos de propiedad industrial (Art.18). Por otro lado, la cantidad de producto que cabe razonablemente esperar que se consuma debe proporcionar la cantidad significativa de nutriente o sustancia sobre la cual se declara el efecto nutricional o fisiológico. Por supuesto, el efecto benéfico debe ser relevante para la salud humana.

Por el contrario, estas no podrán ser falsas, ambiguas o engañosas. No podrán dar lugar a dudas sobre la seguridad y/o la adecuación nutricional de otros alimentos, así como alentar o aprobar el consumo excesivo de un alimento. No podrán hacer referencia a recomendaciones de médicos individuales u otros profesionales de la salud. Tampoco podrán sugerir que una dieta equilibrada y variada no puede proporcionar todos los nutrientes necesarios, ni afirmar, sugerir o implicar que el alimento tiene propiedades de prevención, tratamiento o curación de una enfermedad humana. Por último, ninguno de los tres tipos de alegaciones podrá referirse a cambios en las funciones corporales con términos o representaciones alarmistas.

Sin embargo, no todos los alimentos podrán utilizar una declaración nutricional. El artículo 4 del reglamento señala que los alimentos promocionados con declaraciones van a ser percibidos por los consumidores como alimentos con ventajas nutricionales, fisiológicas o de otro tipo para la salud respecto a otros productos similares o distintos a los que no se atribuyan declaraciones. Por tanto, para que un alimento pueda presentar alegaciones de salud primero debe ajustarse a unos perfiles nutricionales, teniendo en cuenta las cantidades de determinados nutrientes y otras sustancias que contenga, como por ejemplo grasas, ácidos grasos saturados, ácidos grasos *trans*, azúcares y sal o sodio.

El grupo de expertos en Productos Dietéticos, Nutrición y Alergias de la EFSA, estableció perfiles nutricionales (FSA-Q_2007-058), para los cuales se tuvo en cuenta el rol de la dieta, los diferentes grupos de alimentos existentes y la contribución de los nutrientes a la dieta total de la población, dándole gran importancia a aquellos grupos que contribuyen significativamente a la dieta de las personas, entre los que se encuentran, los aceites, los productos lácteos, los cereales y las frutas.

Dentro del este apartado se consideró la posibilidad de admitir una serie de excepciones, permitiéndose la solicitud de alegaciones de salud a determinados alimentos que no cumplen los requisitos exigidos de perfil nutricional, pero sobre los que existen numerosas pruebas científicas que avalan sus propiedades saludables. Entre las excepciones se destaca el aceite de oliva. Se considera que las grasas monoinsaturadas son beneficiosas, pero solo si se encuentran en más de un 45% de la cantidad total de ácidos grasos y además, la cantidad de grasa saturada no proporciona más de un 10% de la energía total. En este sentido, el aceite de oliva no podría obtener la alegación de "Alto contenido en ácidos grasos monoinsaturados". La Agencia Española de Seguridad Alimentaria y Nutrición propuso que la limitación no sea el porcentaje de ácidos grasos saturados si no una relación monoinsaturados/saturados superior a 4.5 (AESAN-2006-04).

Mediante el Reglamento (UE) número 432/212 de la Comisión de 16 de mayo de 2012, se estableció una lista de declaraciones autorizadas de propiedades saludables de los alimentos distintas de las relativas a la reducción del riesgo de enfermedad y al desarrollo y la salud de los niños, contiene 222 declaraciones de propiedades saludables autorizadas bajo el ámbito del artículo 13.1 del Reglamento (CE) número 1924/2006 relativo a las declaraciones nutricionales y de propiedades saludables en los alimentos. El Reglamento contiene el listado de las declaraciones permitidas, así como sus condiciones de utilización. De las declaraciones establecidas, algunas de estas declaraciones hacen referencia de forma específica al aceite de oliva o bien se podrían aplicar a él. Entre las declaraciones aprobadas figuran las siguientes:

1.- Referida al nutriente, sustancia, alimento o categoría de alimentos: ÁCIDO OLEICO.

a) Declaración: La sustitución de grasas saturadas por grasas insaturadas en la dieta contribuye a mantener niveles normales de colesterol sanguíneo. El ácido oleico es una grasa insaturada.

b) Condiciones de utilización de la declaración: Esta declaración solo puede utilizarse respecto a alimentos con alto contenido de ácidos grasos insaturados, de acuerdo con la declaración ALTO CONTENIDO DE GRASAS INSATURADAS que figura en el anexo del Reglamento (CE) n 1924/2006.

2.- Referida al nutriente, sustancia, alimento o categoría de alimentos: POLIFENOLES DEL ACEITE DE OLIVA.

a) Declaración: Los polifenoles del aceite de oliva contribuyen a la protección de los lípidos de la sangre frente al daño oxidativo.

b) Condiciones de utilización de la declaración: Esta declaración solo puede utilizarse respecto a aceite de oliva que contenga un mínimo de 5 mg de hidroxitirosol y sus derivados (por ejemplo, un complejo de oleuropeína o tirosol) por 20 g de aceite de oliva. Para que un producto pueda llevar esta declaración, se informará al consumidor de que el efecto beneficioso se obtiene con una ingesta diaria de 20 g de aceite de oliva.

3.- Referida al nutriente, sustancia, alimento o categoría de alimentos: VITAMINA E

a) Declaración: la vitamina E contribuye a la protección de las células frente al daño oxidativo.

b) Condiciones de utilización de la declaración: Esta declaración solo puede utilizarse respecto a alimentos que son como mínimo, fuente de vitaminas o minerales de acuerdo a la declaración FUENTE DE […] que figura en el anexo del Reglamento (CE) No 1924/2006.

Solamente podrá declararse que un alimento es una fuente de vitaminas o minerales, así como efectuarse cualquier otra declaración que pueda tener el

mismo significado para el consumidor, si el producto contiene como mínimo una cantidad significativa de vitaminas o minerales tal como se define en el anexo de la Directiva 90/496/CEE. En la cual por regla general, para decidir lo que constituye una cantidad significativa se considera un 15% de la cantidad recomendada específica en el anexo y suministrada por 100g o 100 mL o por envase, si éste contiene una única porción.

Para el caso de vitamina E, la cantidad diaria recomendada es de 12 mg, y el contenido total de tocoferoles del AOV varía entre 9.5 – 82 mg/100 g de AOV, lo que corresponde una cantidad diaria recomendada entre 79 - 683%. Por lo anterior se puede hacer la declaración que el AOV es fuente de vitamina E.

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III. HIPÓTESIS Y OBJETIVOS

III. HIPÓTESIS Y OBJETIVOS

Considerando la literatura disponible se plantean las siguientes hipótesis:

1. ¿Las diferencias en composición de ácidos grasos de aceites de oliva virgen monovarietales tienen efecto sobre la formación de células espumosas por parte de los macrófagos?
2. ¿El contenido de compuestos fenólicos del AOV según la variedad de aceituna de la que procede, será determinante en la bioaccesibilidad de éstos compuestos?
3. ¿Existirán diferencias en la capacidad antioxidante de los aceites de oliva virgen monovarietales después del proceso digestivo?
4. ¿Los compuestos volátiles del AOV son bioaccesibles?

Objetivo Principal

Evaluación del potencial bioactivo del aceite de oliva virgen en función de la variedad.

Objetivos específicos

1. Evaluación del efecto de la composición de ácidos grasos del aceite de oliva virgen en la formación de células espumosas.
2. Estudio de la interacción de compuestos fenólicos presentes en el aceite de oliva virgen con una proteína digestiva.
3. Determinación de la recuperación de los compuestos fenólicos durante el proceso de digestión *in vitro* de aceites de oliva virgen de diferentes variedades y su bioaccesibilidad.
4. Evaluación de la capacidad antioxidante de los aceites de oliva virgen tras el proceso digestivo *in vitro*.
5. Describir el cambio que sufren los compuestos volátiles durante la digestión *in vitro* del aceite de oliva virgen, su recuperación y bioaccesibilidad.

IV. RESULTADOS

Tabla de Contenido Resultados

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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The Fatty Acid Composition of Virgin Olive Oil from Different Cultivars Is Determinant for Foam Cell Formation by Macrophages

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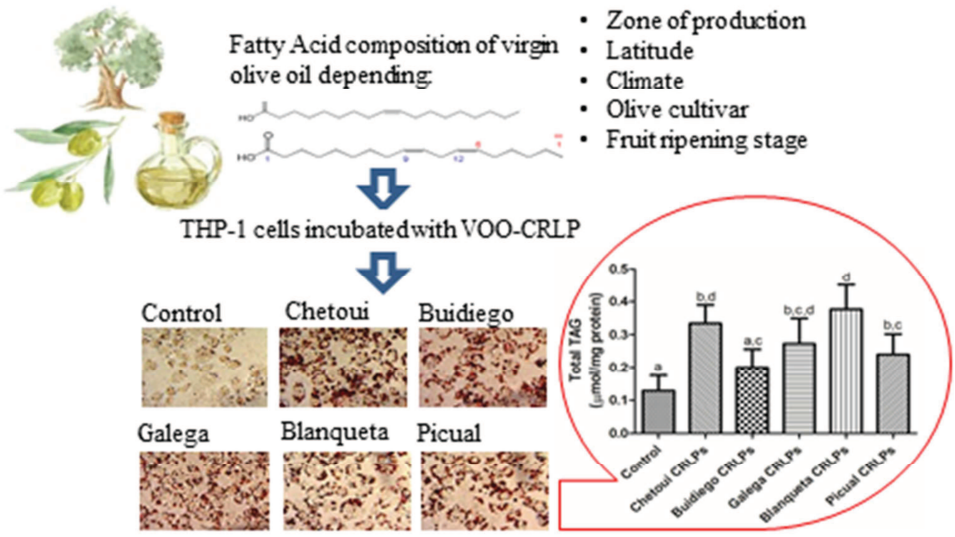

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The fatty acid composition of virgin olive oil from different cultivars is determinant for foam cell formation by macrophages

Abstract.

Although the beneficial role of VOO in the Mediterranean Diet is well-known, its effects on health cannot be attributed solely to oleic acid. In addition to the effect of minor components, the presence of other fatty acids, which depend largely on the cultivar among other factors, need to be considered. In the present study, we examined the effect of chylomicron remnant-like particles (CRLP) enriched in fatty acids of virgin olive oil (VOO) from `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ cultivars on the foam cell formation by THP-1 macrophages. THP-1 cells were incubated with VOO-CRLP for 24h. Lipid accumulation in cells was measured by measuting total triacylglycerol concentration and fatty acid composition. Intracellular TAG concentrations were higher in cells incubated with `Chetoui´ and `Blanqueta´ CRLP than with `Buidiego´ and `Picual´ CRLP. CRLP prepared from cultivars with a greater PUFA content caused more lipid accumulation in cells than those prepared from MUFA-rich cultivars. In conclusion, linoleic acid-rich VOO induced higher TAG incorporation into THP-1 macrophages compared to oleic acid-rich VOO, being 18:1/18:2 ratio consistently correlated with intracellular TAG accumulation. The results of this study demonstrate that the differences in the FA composition of VOO have effect on foam cell formation. In addition, they point out to the possibility of selecting VOO cultivars for specific nutritional uses according to their FA composition.

Keywords. Extra Virgin Olive Oil; fatty acids; lipid ; macrophages; foam cells

Abbreviations Used

CRLP: chylomicron remnant-like particles

VOO: virgin olive oil

TAG: triacylglycerols

CM: chylomicrons

TRL: TAG-rich lipoproteins

SFA: saturated fatty acids

MUFA: monounsaturated fatty acids

PUFA: polyunsaturated fatty acids

CE: cholesteryl esters

CMR: *chylomicrons* remnants

FA: fatty acid

INTRODUCTION

Virgin olive oil (VOO) is the primary source of fat in the Mediterranean diet, but it is appreciated worldwide because of its nutritional benefits. VOO is defined as: "oil-obtained from the fruit of the olive tree solely by mechanical or other physical means under conditions, particularly thermal conditions, that do not lead to alterations in the oil, and which have not undergone any treatment other than washing, decantation, centrifugation and filtration"[1]. The composition of VOO is primarily triacylglycerols (TAG) (~99%) and secondarily free fatty acids, mono- and diacylglycerols, and an array other lipophilic compounds, such as hydrocarbons, sterols, aliphatic alcohols, tocopherols, and pigments. Fatty acids (FA) present in olive oil are principally palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids. Myristic (C14:0), heptadecanoic (17:0) and eicosanoic acids (20.0) are found at trace amounts. But

FA composition may differ from sample to sample, depending on the zone of production, the latitude, the climate, the olive cultivar, and fruit ripening stage[2].

One of the most important factors affecting the FA composition of VOO is the cultivar, although it is rarely mentioned in nutritional studies[3,4]. Different authors described that FA composition of VOO has a strong varietal component, over 70% of the variability found, especially palmitic, stearic, oleic and linoleic acids. Therefore, the fatty acid composition may differ substantially, ranging from 75% of oleic and < 5% linoleic acid in the `Picual´ cultivar to 55% of oleic acid and 20% of linoleic acid in the `Blanqueta´ cultivar[5]. These notable differences in fatty acid composition might have relevant effects in the healthy properties that have been attributed to VOO.

There is ample agreement that at least part of the beneficial effects of VOO are associated with the oleic acid content[6], including the protection against oxidative stress, blood pressure lowering, reduction of total and LDL-cholesterol levels and even the reduction of the risk of breast, prostate and colorectal cancer. In addition, oleic acid has been regarded as responsible for beneficial effects also in the postprandial state[7,8] and the first events occurring during the progression of atherogenesis, when forming part of chylomicrons (CM)[9].

Dietary lipid components are absorbed by enterocytes and carried in CM, which are large TAG-rich lipoproteins (TRL) that are secreted into lymph and pass into the blood via thoracic duct. CM undergo rapid lipolysis by lipoprotein lipase (LPL), a process that removes some of their TAG and forms the smaller CM remnants (CMR) which deliver the remaining TAG, cholesterol and other lipids to the liver [10,11]. Studies have demonstrated that CMR can cross the endothelial barrier and enter into the arterial wall[12,13]. CMR have been shown to induce the formation of foam cells in a variety of macrophage populations,

including those derived from the human monocyte cell line THP-1[14-16], and furthermore, without need of further oxidation[17].

The (FA) composition of CMR influences their uptake and lipid accumulation in macrophages[9,18]. De Pascale et al.[9] studied the effects of CMR-like particles (CRLP) enriched in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), n-6 polyunsaturated fatty acids (PUFA) and n-3 PUFA, obtained by incorporating TAG from palm, olive, corn and fish oil, respectively, on THP-1 macrophage foam cell formation. They reported that CRLP enriched in SFA or MUFAs were taken up more rapidly than those enriched in n-6 or n-3 PUFA and that SFA-rich particles showed the greatest lipid accumulation.

Accordingly, the FA composition of CMR may be determinant for the accumulation of cholesterol and TAG in the macrophages, affecting their metabolism and modulating their atherogenicity[16,19]. However, the effect of different FA profiles in monovarietal oils on foam cell formation from macrophages has not been addressed so far. Therefore, in the present study we investigated the effect of fatty acid composition of VOO from five olive cultivars on foam cell formation by THP-1 macrophages.

MATERIALS AND METHODS

Plant Material and virgin olive oil extraction

Samples of of `Picual´, `Buidiego´, `Blanqueta´, `Galega´ and `Chetoui´ Olive (O. europaea, L.) cultivars were selected by their differences in oil fatty acid composition. The trees were spaced 7 x 7 m and grown in the experimental orchard of Centro IFAPA 'Venta Del Llano'- Mengibar, Jaen (Spain) using standard growing techniques. The study was carried out during the 2013/14 crop year.

VOO extraction was performed using an Abencor laboratory oil mill (Comercial Abengoa, S.A., Seville, Spain) equipped with a hammer mill, a thermobeater and a paste centrifuge; that simulates at laboratory scale the industrial process of VOO production. The extraction was repeated twice for each cultivar. The milling of the olive fruits was performed using a stainless steel hammer mill operating at 3000 rpm provided with a 5 mm sieve. The olive paste malaxation was carried out at 28 °C with kneading at 50 rpm for 45 min. Centrifugation of the kneaded olive paste was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oil obtained was decanted, filtered and stored in glass bottles at -20 °C in the dark without headspace and under N_2 until analysis.

Fatty Acid Composition of virgin olive oil

Fatty acid methyl esters (FAME) were prepared as described by the EU regulation 2568/91[20]. The chromatographic separation was carried out by a Perkin–Elmer Autosystem gas chromatograph (Perkin–Elmer, Spain) equipped with an autosampler, split/splitless injector, flame ionisation detector (FID) and a fused silica capillary BPX70 column of 50 m length x 0.25 mm i.d. and 0.25 µm of film thickness (SGE Scientific PTY Ltd., Australia). Helium was used as carrier gas and the oven temperature was maintained at 198°C throughout the analysis. The injector and detector temperatures were 235 and 245°C, respectively. The results were expressed as relative area percent of the total.

Preparation of chylomicron remnant-like particles (CRLP)

All lipid standards for the preparation of CRLP were supplied by Larodan Inc. (Malmö, Sweden), except TAG, which were isolated from `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ VOO by solid-phase extraction (SPE) using diol cartridges (Supercleane LC-Diol; Supelco, Bellefonte, PA) and hexane:methylene choride (9:1, v/v) as eluent. The eluate containing TAG was evaporated to dryness under a stream of nitrogen

and redissolved in hexane. CRLP were prepared using a lipid mixture containing 70% TAG from VOO, 2% cholesterol, 3% cholesteryl esters (CE) and 25% phospholipids (PL) (Sigma-Aldrich, St Louis, MO) as described previously[21]. The composition of the initial PL moieties was: phosphatidylcholine 70.50%, lysophosphatidylcholine 6.88%, phosphatidylethanolamine 11.00%, phosphatidylinositol 2.58%, phosphatidylserine 2.58% and sphingomyelin 6.54% (Sigma-Aldrich).

Lipids were sonicated by immersing a clean ultrasonic probe (Bandelin Electronics, Berlin, Germany) directly into the lipid mixture in 0.9% NaCl in tricine buffer (20 mM, pH 7.4), and supplying the probe with 50W for 20 min (5-min bursts with 5-min rest periods to allow for cooling) at 56°C. The resulting emulsion was brought to a density of 1.21 g/mL with KBr, layered under a stepwise density gradient (2.5 mL, d=1.065 g/mL; 2.5 mL, d=1.020 g/mL; 3 mL, d=1.006 g/mL) as described previously[21], and centrifuged at 17 000g for 20 min at 20 °C (Beckman Optima L-90K centrifuge; Beckman Coulter, Palo Alto, CA) in a SW41Ti swing-out rotor. The upper layer, which was an uneven emulsion of lipids, was discarded. The removed layer was replaced with an equal volume of NaCl solution (d=1.006 g/mL), and tubes were centrifuged at 70 000g for 1 h (20 °C) in a SW41Ti swing-out rotor. The upper layer was then collected and stored at 4 °C until use (max 2 days).

Apolipoprotein E transfer to CRLP

The apolipoprotein transfer was achieved by incubation of the lipid emulsion with a fraction of human serum (VWR International Eurolab, S.L, Spain) (1:2, vol/vol), prepared by ultracentrifugation and dialyzed before use[21] (5 h, 4°C). After incubation, the particles were ultracentrifuged (154 000 g, 16 h, 12°C) under a NaCl solution (d=1.006 g/mL). The upper layer was collected and recentrifuged (254 000g, 5 h, 12°C) under a NaCl solution (d=1.006 g/mL) to isolate the CRLP fraction. CRLP were dialyzed before use (2h, 4°C) and stored a 4°C. All preparations were used within one week.

Culture of THP-1 macrophages

THP-1 monocytes were maintained in suspension at a density of (3-8)x10^5 cell/mL in RPMI 1640 culture medium containing 10% fetal bovine serum (previously inactivated at 56°C, 30 min), 2 mM L-alanil-glutamine, 100 U/mL-1 penicillin and 100 ug/mL streptomycin at 37°C in 95% air⁄5% CO_2.

The cells were induced to differentiate into macrophages by incubation with phorbol myristate acetate (PMA) (200 ng/mL) for 72 h. After this time, the cells adhering to the culture dishes were washed with RPMI 1640 culture medium (3 x 2 mL) to remove any undifferentiated cells and traces of PMA. The viability of the THP-1 macrophages, as assessed by Trypan blue exclusion, was >90% in all experiments.

Lipid accumulation studies

Cells at a 1 x 10^6/mL concentration were incubated with CRLP (0.15 µmol TAG/mL of medium) for 24 h. For Oil Red O staining, cells were washed with PBS 1X (3 x 2 mL) and 60% propan-2-ol, then 1 mL of Oil red O (0.15% w/v) in 40% propan-2-ol/H_2O v/v was added. After 10 min, the stain was removed and cells were washed with 3 mL of PBS as many times as necessary to remove traces of the solution. Then, 2 mL of glycerol (30%) were added and images were capted using a microscope-mounted Canon digital camera.

For intracellular lipid analysis, cells were washed with PBS 1X (3 x 3 mL), harvested in 0.7 µL PBS, and kept on a bed of ice. Cells were then disrupted by sonication for 5 s (x 2, 50 W), and a sample was taken for protein determination (preserved at -70°C) and the rest were kept at –20°C for subsequent total TAG determination and FA composition.

Triacylglycerol and fatty acid composition

Total TAG in CRLP and cells were determined by enzymatic analysis using a commercial enzymatic reagent colorimetric/fluorimetric kit (BioVision Incorporated, USA). For the analysis of FA composition in CRLP, TAG were isolated by SPE diol columns as described above and were transmethylated using sodium metoxide in methanol (0.5%). For the analysis of FA composition in the cells, these were directly transmethylated using a metilation mixture (MeOH-tolueno-DMP-H_2SO_4) and heptane. The resulting FAME of CRLP and cells were analyzed by GC, using a model 5890 series II GC (Hewlett-Packard Co, Avondale, AZ) equipped with a flame ionization detector an a capillary silica column Supelcowax 10 (Supelco, Co, Bellefonte, PA) of 60 m length and 025 mm internal diameter. FAME were identified by the comparison of their retention times against those of standards and quantified by external standard using peak area integration; results were expressed as relative area percent. CRLP and cell protein content was measured by the method of Bradford with BSA as standard.

Statistical analysis

Results are expressed as means $\pm$ SD, unless otherwise stated. Statistical analyses were conducted using the GraphPad Prism v.5 statistical package (GraphPad Software, Inc.). Statistical significance was assessed by one-way or two-way ANOVA followed by Bonferroni's multiple comparison test. Correlations between variables were assessed using Pearson's correlation coefficients. Differences were considered statistically significant at $p<0.05$.

RESULTS

Chylomicron remnant-like particle TAG content and fatty acid composition

The TAG content was similar in all five types of CRLP (µmol / mL, n=3): `Chetoui´ CRLP, 2.38 ± 0.4; `Buidiego´ CRLP, 1.99 ± 0.62; `Galega´ CRLP, 2.76 ± 0.17; `Blanqueta´ CRLP, 1.93 ± 0.10; `Picual´ CRLP, 1.83 ± 0.53.

Overall, the FA profile of the TAG in the CRLP and VOO from all cultivars studies was similar (Table 1). However, significant differences were observed in the FA composition of the TAG in CRLP among cultivars for palmitic, palmitoleic, stearic, oleic and linoleic acids. Oleic acid content was the highest in all CRLP, accounting for 60-76% of all FA, with significant differences between cultivars. Likewise, linoleic acid ranged from 4 to 18%. `Picual´ CRLP showed the highest oleic acid content (76.6%) and the lowest linoleic acid content (4.1%). In contrast, `Chetoui´ CRPL had the lowest oleic acid content (60.9%) and the greatest linoleic acid content (18.9%). As a consequence, the MUFA concentration was higher in `Picual´ CRLP and PUFA concentration in `Chetoui´ CRLP. SFA content was higher in `Blanqueta´ CRLP.

Intracellular lipid accumulation in THP-1 macrophages

THP-1 macrophages were treated with `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ CRLP (0.15 µmol TAG/mL) for 24 h. Incubation of macrophages with all CRLP resulted in increased intracellular accumulation of lipids as assessed by Oil Red O staining (Figure 1). Images revealed a light staining in control cells and a strong red staining in cells incubated with CRLP, confirming the intracellular incorporation of lipids.

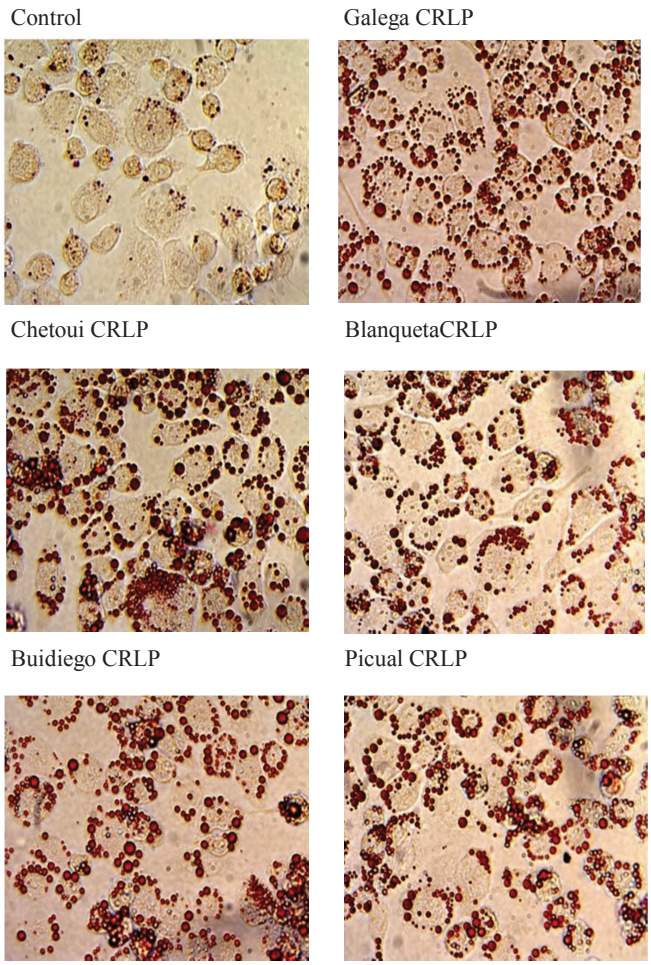

Figure 1. Lipid accumulation in Cells THP-1 macrophagues incubated with CRLPs containing TAG from `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ VOO cultivars. THP-1 macrophages were incubated with CRLPs (final concentration 0.15 µmol TAG/mL) for 24 h and stained with Oil Red O.

Table 1. Fatty Acid composition of VOO from `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ cultivars, and of TAG in CRLP which were prepared using the TAG from the same oils.

	`CHETOUI´		`BUIDIEGO´		`GALEGA´		`BLANQUETA´		`PICUAL´	
	VOO	**CRLP**	**VOO**	**CRLP**	**VOO**	**CRLP**	**VOO**	**CRLP**	**VOO**	**CRLP**
16:0	13.54 ± 0.05	13.67 ± 0.22 a	13.41 ± 0.10	13.79 ± 0.3 a	15.80 ± 0.21	15.34 ± 0.30 b	16.43 ± 0.21	16.56 ± 1.20 c	12.34 ± 0.04	12.50 ± 0.46 d
16:1	0.37 ± 0.01	0.76 ± 0.05 a	0.71 ± 0.02	1.36 ± 0.23 a,b	2.13 ± 0.03	2.62 ± 0.05 b	1.39 ± 0.05	1.40 ± 0.78 a,b	1.01 ± 0.02	1.37 ± 0.10 a,b
17:0	0.05 ± 0.01	0.07 ± 0.02	0.06 ± 0.01	0.11 ± 0.04	0.11 ± 0.01	0.15 ± 0.04	0.12 ± 0.01	0.14 ± 0.10	0.03 ± 0.01	0.07 ± 0.03
17:1	0.08 ± 0.01	0.10 ± 0.02	0.07 ± 0.01	0.25 ± 0.24	0.26 ± 0.01	0.28 ± 0.02	0.25 ± 0.02	0.14 ± 0.16	0.07 ± 0.02	0.14 ± 0.04
18:0	2.55 ± 0.02	3.52 ± 0.15 a,b	2.87 ± 0.02	4.12 ± 0.83 a	1.83 ± 0.01	2.46 ± 0.24 b	2.11 ± 0.03	2.80 ± 0.26 b	2.56 ± 0.02	3.44 ± 0.22 a,b
18:1	62.48 ± 0.08	60.90 ± 0.17 a	75.42 ± 0.19	70.58 ± 1.24 b	74.58 ± 0.23	71.71 ± 0.60 c	64.10 ± 0.23	62.86 ± 0.63 d	79.84 ± 0.06	76.64 ± 1.28 e
18:2	19.69 ± 0.01	18.91 ± 0.19 a	6.22 ± 0.04	7.33 ± 1.25 b	4.29 ± 0.02	5.69 ± 0.45 c	14.31 ± 0.01	14.28 ± 0.29 d	3.06 ± 0.03	4.11 ± 0.56 e
18:3	0.55 ± 0.04	1.06 ± 0.03	0.57 ± 0.05	1.0 4 ± 0.18	0.51 ± 0.03	0.88 ± 0.05	0.59 ± 0.02	1.05 ± 0.05	0.55 ± 0.01	0.93 ± 0.03
20:0	0.34 ± 0.03	0.47± 0.03	0.34 ± 0.01	0.49 ± 0.24	0.24 ± 0.02	0.35 ± 0.02	0.38 ± 0.03	0.35 ± 0.02	0.31 ± 0.02	0.31 ± 0.01
20:1	0.29 ± 0.01	0.13 ± 0.03	0.22 ± 0.02	0.24 ± 0.16	0.17 ± 0.01	0.11 ± 0.04	0.19 ± 0.00	0.11 ± 0.04	0.17 ± 0.01	0.13 ± 0.07
22:0	0.09 ± 0.01	0.22 ± 0.03	0.08 ± 0.00	0.31 ± 0.07	0.053 ± 0.01	0.27 ± 0.08	0.10 ± 0.01	0.25 ± 0.03	0.05 ± 0.01	0.25 ± 0.08
Others	-	0.19 ± 0.12	-	0.39 ± 0.35	-	0.13 ± 0.03	-	0.09 ± 0.02	-	0.14 ± 0.05
Total SFA	16.57 ± 0.02	17.95 ± 0.09 a	16.76 ± 0.03	18.82 ± 0.30 a	18.03 ± 0.05	18.57 ± 0.14 a	19.14 ± 0.06	20.10 ± 0.32 b	15.29 ± 0.02	16.57 ± 0.16 c
Total MUFA	63.22 ± 0.10	61.89 ± 0.25 a	76.42 ± 0.23	72.43 ± 1.75 b	77.14 ± 0.27	74.72 ± 0.68 c	65.93 ± 0.30	64.51 ± 1.58 d	81.09 ± 0.10	78.28 ± 1.44 e
Total PUFA	20.24 ± 0.03	19.97 ± 0.21 a	6.79 ± 0.07	8.37 ± 1.34 b	4.80 ± 0.04	6.57 ± 0.48 c	14.90 ± 0.02	15.33 ± 0.32 d	3.61 ± 0.04	5.04 ± 0.58 e
Unsaturated/SFA	5.04 ± 0.05	4.56 ± 0.18	4.96 ± 0.11	4.29 ± 1.13	4.54 ± 0.12	4.38 ± 0.43	4.22 ± 0.13	3.97 ± 0.74	5.54 ± 0.05	5.03 ± 0.72
18:1/18:2	3.17 ± 0.09	3.22 ± 0.27 a	12.13 ± 0.21	9.63 ± 1.87 b	17.38 ± 0.24	12.60 ± 0.83 c	4.48 ± 0.24	4.40 ± 0.78 d	26.09 ± 0.08	18.65 ± 1.56 e

*Data are expressed as area percent and are the mean ± SD from three separate preparations (n=3). Different letters in value of CRLP within a row are different ($p < 0.05$) by two-way ANOVA analysis.

Intracellular TAG concentrations differed according to the type of CRLP used. There was a significantly higher accumulation of TAG in cells incubated with `Chetoui´ and `Blanqueta´ CRLP than with `Buidiego´ and `Picual´ CRLP (Figure 2).

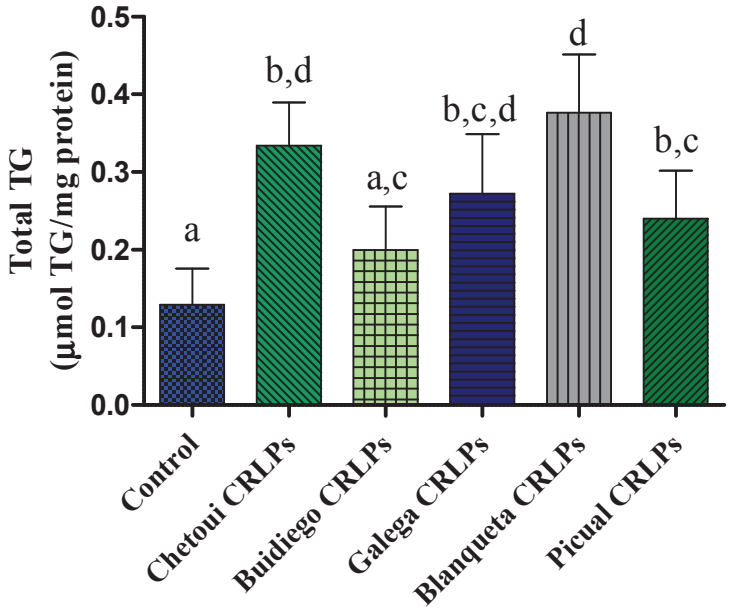

Figure 2. Total TAG accumulated after 24 h in THP-1 macrophagus incubated with `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ CRLPs. Data are the mean from three separate experiments, and error bars show the SD. Different letters indicate significant difference ($p < 0.05$) by one-way ANOVA analysis and Bonferroni's post-hoc test.

The TAG fraction of THP-1 macrophages showed significant differences for the main FA, namely palmitic, palmitoleic, stearic, oleic, linoleic and arachidonic (20:4 n-6) acids and, therefore, for the SFA, MUFA and PUFA contents (Table 2).

Table 2. Fatty acid composition of THP-1 macrophages incubated with `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ CRLP for 24h.

	Control	`Chetoui´ CRLP	`Buidiego´ CRLP	`Galega´ CRLP	`Blanqueta´ CRLP	`Picual´ CRLP
14:0	0.74 ± 0.38	0.28 ± 0.06	0.10 ± 0.03	0.22 ± 0.22	0.32 ± 0.03	0.17 ± 0.07
14:1 n-5	0.13 ± 0.07	0.31 ± 0.03	1.14 ± 0.31	0.95 ± 1.33	0.06 ± 0.02	0.06 ± 0.03
16:0	25.39 ± 1.89 a	17.34 ± 1.51 b	17.18 ± 0.50 b	19.90 ± 0.96 c	18.62 ± 0.65 b	16.55 ± 0.76 b
16:1 n-9	6.14 ± 0.30 a,d	2.04 ± 0.35 b	7.20 ± 2.49 a,c	4.48 ± 0.17 d	2.59 ± 0.10 b	2.59 ± 0.27 b
16:1 n-7	0.33 ± 0.06	0.35 ± 0.04	0.11 ± 0.02	0.19 ± 0.05	0.28 ± 0.02	0.12 ± 0.01
16:1 n-5	0.51 ± 0.14	0.66 ± 0.00	1.80 ± 0.10	0.34 ± 0.04	0.34 ± 0.07	0.21 ± 0.05
18:0	14.66 ± 0.50 a	7.94 ± 0.37 b,d	7.27 ± 0.18 b	8.58 ± 0.64 b,d	6.69 ± 0.98 b,c	9.55 ± 0.88 d
18:1 n-9	32.04 ± 0.33 a	48.27 ± 1.79 b	51.41 ± 0.86 c	49.92 ± 6.21 b,c	51.50 ± 1.38 c,d	59.33 ± 4.07 d,e
18:2 n-6	3.38 ± 0.38 a	13.33 ± 0.91 b	5.92 ± 1.28 c	6.07 ± 0.89 c	11.60 ± 0.28 b	4.43 ± 0.15 a,c
18:3 n-6	0.44 ± 0.10	0.90 ± 0.15	0.70 ± 0.09	0.52 ± 0.02	0.90 ± 0.01	0.73 ± 0.08
18:3 n-3	1.21 ± 0.11	1.27 ± 0.12	1.20 ± 0.01	0.38 ± 0.13	0.97 ± 0.13	1.24 ± 0.07
20:0	1.58 ± 0.01	0.41 ± 0.06	0.20 ± 0.02	0.45 ± 0.04	0.38 ± 0.00	0.24 ± 0.07
20:1 n-9	0.56 ± 0.07	0.22 ± 0.05	0.09 ± 0.05	0.12 ± 0.03	0.18 ± 0.03	0.11 ± 0.02
20:2 n-6	1.22 ± 0.10	0.53 ± 0.02	1.03 ± 0.13	0.67 ± 0.07	0.50 ± 0.04	0.39 ± 0.05
20:4 n-6	4.63 ± 0.15 a	1.79 ± 0.06 b	1.89 ± 0.06 b	2.52 ± 0.64 b	1.80 ± 0.14 b	1.42 ± 0.23 b
20:5 n-3	1.37 ± 0.19	0.72 ± 0.09	0.40 ± 0.08	0.74 ± 0.05	0.68 ± 0.05	0.46 ± 0.28
22:4 n-6	0.39 ± 0.00	0.47 ± 0.15	0.63 ± 0.06	0.94 ± 0.04	0.61 ± 0.10	0.50 ± 0.08
22:5 n-6	1.21 ± 0.22	0.53 ± 0.18	0.23 ± 0.03	0.85 ± 0.06	0.75 ± 0.04	0.76 ± 0.01
22:5 n-3	1.64 ± 0.31	0.49 ± 0.10	0.36 ± 0.01	0.87 ± 0.10	0.42 ± 0.03	0.40 ± 0.07
22:6 n-3	2.45 ± 0.29	2.16 ± 0.41	1.15 ± 0.58	1.30 ± 0.56	0.81 ± 0.11	0.75 ± 0.09
Total SFA	42.37 + 0.70 a	25.97 + 0.50 b	24.74 + 0.18 b	29.15 + 0.46 c	26.01 + 0.42 b	26.51 + 0.45 b
Total MUFA	39.69 + 0.16 a	51.84 + 0.38 b	61.75 + 0.64 c	56.00 + 1.31 d	54.95 + 0.27 d	62.42 + 0.74 c
Total PUFA	17.94 + 0.19 a,d	22.19 + 0.22 b	13.51 + 0.23 c	14.85 + 0.26c	19.04 + 0.09 d	11.07 + 0.11 e
Unsaturated/SFA	1.36 + 0.35	2.85 + 0.36	3.04 + 0.35	2.43 + 0.68	2.84 + 0.26	2.77 + 0.43
18:1/18:2	9.48 + 0.35 a	3.62 + 1.35 b	8.69 + 1.07 a	8.22 + 3.55 a	4.44 + 0.83 b	13.40 + 2.11 c

Data are expressed as area percent and are the mean ± SD from three separate preparations (n=3). Different letters in values within a row are different ($p < 0.05$) by two-way ANOVA analysis.

Control cells presented a higher SFA proportion compared with cells incubated with CRLP. Among cells incubated with CRLP, SFA accumulation was enhanced in cells incubated with `Galega´ CRLP (29%), while MUFA concentration was higher in those incubated with `Picual´ (62%) and `Buidiego´ (61%) CRLP and lower with `Chetoui´

CRLP (51%). Finally, PUFA content was increased in cells treated with `Chetoui´ CRLP (22%) and decreased with `Picual´ CRLP (11%). As well as in CRLP, palmitic, stearic, oleic and linoleic acids were found with the highest proportions in the intracellular TAG fraction. It was observed that oils with the greatest oleic and linoleic acid contents, led to cells engorged with TAG rich in those FA. Oleic acid was present in higher concentration in cells incubated with `Picual´ CRLP (59%) and lower in those treated with `Chetoui´ (48%) and `Galega´ CRLP (50%). Conversely, linoleic acid was found at increased amounts in cells incubated with `Chetoui´ CRLP (13%) and at lower concentration in cells incubated with `Picual´ CRLP (4%).

The oleic acid to linoleic acid ratio (18:1/18:2 ratio) of VOO was plotted against TAG accumulation in cells in order to determine the relationship between oleic and linoleic acid content in the parent oils and lipid intracellular accumulation (Figure 3A) and the FA composition in intracellular TAG (Figure 3B). A higher 18:1/18:2 ratio was associated with lower TG accumulation in the cells, and very strongly with the intracellular 18:1/18:2 ratio.

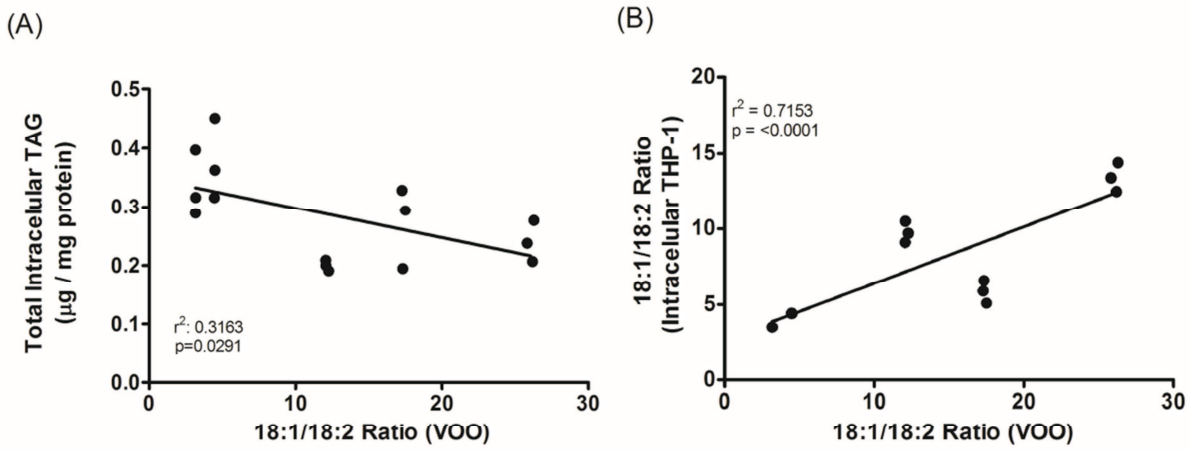

Figure 3. Correlation of oleic acid to linoleic acid ratio (18:1/18:2) of VOO with (A) TAG accumulated in THP-1 macrophages and (B) oleic acid to linoleic acid ratio (18:1/18:2) in intracellular THP-1 macrophages after incubation with `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ CRLPs.

DISCUSSION

Dietary fats enter the blood in the form of CM that undergo lipolysis in extra-hepatic capillary beds. The resulting CMR particles contain about 30% of TAG and most cholesterol mass of the parent lipoproteins and are considered atherogenic lipoproteins. CMR are taken up aorta[13], by several types of macrophagues[10] and give rise to foam cells by inducing accumulation of cholesterol, TAG or both[9,10,14,22-24].

A number of epidemiologic studies developed in different countries constitute a firm and reliable experimental base supporting the beneficial effects of the Mediterranean diet, rich in olive oil, with regard to the reduction of cardiovascular disease[25,26]. Although the benefits of VOO against cardiovascular disease cannot be attributed solely to oleic acid[27], the protective effects have been ascribed to higher intakes of oleic and α-linolenic acid (18:3, n-3) acids and lower intakes of SFA and linoleic acid (18:2, n-6)[28-31.]

The FA composition of VOO depends on the zone of production, the latitude, the climate, the variety, and the stage of maturity of the fruit. For instance, Greek, Italian, and Spanish VOO are, in general terms, low in linoleic and palmitic acids and they have a high percentage of oleic acid. In contrast, Tunisian VOO are high in linoleic and palmitic acids and lower in oleic acid[2]. Given these differences, we evaluated the uptake of CRLP prepared with TAG obtained from five varieties of VOO and the induction of lipid accumulation in macrophages derived from the human monocyte cell line THP-1. Since CMR uncontaminated with lipoproteins of a similar density, such as CM and very-low-density lipoproteins, cannot be easily obtained from human blood, we used CRLP as experimental modelP[21], considering that CRLP have been used by a number authors before to study lipid metabolism[9,23,32-35]. The composition of CRLP can be easily manipulated and they provide a suitable and convenient model for present work.

We obtained VOO from `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ cultivars, which presented differences in their FA composition and we used their TAG to prepare CRLP (Table 1). `Chetoui´, `Buidiego´, `Galega´, `Blanqueta´ and `Picual´ CRLP did not differ in their TAG content and their FA composition was similar to the parent oils. These findings are consistent with previous works using palm, olive, corn or fish oils[34,35].

The exposure of human monocytes to VOO-CRLP caused lipid to accumulate intracellularly, and thus provide evidence of CRLP uptake by monocytes and foam cell formation. It has been reported that not all CMR are equally taken up by macrophages and the particle size and lipid composition may have a significant influence on the process of uptake and lipid accumulation[36]. Batt et al[14] studied the effect of LDL and CRLP on lipid accumulation in human monocyte-derived macrophages (HMDMs) and in macrophages derived from human monocyte cell line THP-1. They found that CRLP without prior oxidation, caused lipid accumulation in human macrophages to an extent comparable to that caused by oxLDL. They also reported a greater proportion lipid accumulation in response to CRLP in the form of TAG and in the form of cholesteryl esters (CE) in response to oxLDL.

Our experiments showed as `Blanqueta´ and `Chetoui´ CRLP caused the greatest TAG accumulation in THP-1 cells. `Blanqueta´ CRLP were characterized by higher SFA content and `Chetoui´ CRLP by higher linoleic acid and PUFA content. This result can be comparable with previous works by De Pascale et al.,[9] and Song et al.[24], who added SFA, MUFA and n-6 or n-3 PUFA to THP-1 macrophages in the form of CRLP or non-esterified FA, respectively. Both confirmed that SFA are taken up more rapidly and result in greater lipid accumulation in cells, although they observed that PUFA-rich particles caused the lowest lipid accumulation. In contrast, our results showed as the CRLP with greater PUFA content caused more lipid accumulation in cells than MUFA rich-CRLP. However, in agreement with our work, Song et al.[24] described differences between incubation with oleic

or linoleic acids only for cholesterol and not for TAG. Likewise, De Pascale et al.[9], employed CRLP, reporting significant differences in intracellular TAG accumulation only between palm-CRLP and corn-CRLP although differences between corn-CRLP and olive-CRLP were not observed. Interestingly, in that study, 18:1/18:2 ratio (7.65) in CRLP was similar (9.63) to that obtained when we prepared CRLP from the ``Buidiego´´ cultivar, which caused the lowest TAG accumulation in the cells.

When FA composition of TAG accumulated in THP-1 macrophages was compared with that of CRLP we observed thatit was highly preserved, i.e. cells treated with CRLP with the highest concentrations of 18:1, 18:2 or 16:0 also exhibited the highest levels of these FA intracellularly. In fact, the 18:1/18:2 ratio in the oils correlated positively and very strongly with the same ratio in cell TAG. Also, we found that 18:1/18:2 ratio also correlated with TAG concentration in the cells, although a negative relationship was obtained. Napolitano et al.[37] studied the influence of the TAG source (trilinolein or triolein) in CRLP in human macrophages. Their results were in close agreement with ours, finding that particles enriched with both trilinolein or triolein, induced a pattern of increase in intracellular TAG synthesis, but that was higher when after incubation with trilinolein. However, an important point to consider is that despite all the CRLP of study were rich in oleic acid, they differed in every other minor FA. Slight differences in the FA can lead to a highly different TAG molecular species composition, which may have important effects in the metabolic fate of lipoproteins, as observed in elderly individuals[38].

Although the mechanisms by which TRL are taken up by macrophages have not been studied extensively, CMR are believed to be incorporated into macrophages by two main pathways: uptake of whole particles via membrane receptors and hydrolysis by macrophage-secreted LPL)[37]. Extracellular lipolysis seems particularly important for internalization of dietary fatty acids[37]. It has been suggested that Vmax of adipose LPL decreases with increasing chain length and unsaturation of monoacid CM-TAG due to

decreased fluidity of the particle[39]. However, differences between oleic and linoleic-acid rich seem to be significant only for large CM and not for VLDL[40]. On the other hand, studies carried out in THP-1 cells have proposed that oleic acid-rich dietary fats may prevent excessive lipid accumulation in monocyte/macrophage cells by down-regulating the gene expression of apolipoprotein B48 receptor[41] and that linoleic acid stimulates CD36 receptor, promoting intracellular TAG accumulation[42].

In conclusion, the results of this study demonstrate that the effect of differences in the FA composition of VOO is similar to the effect of caused by the same FA when forming part of other dietary oils. In fact, linoleic acid-rich VOO induced higher TAG incorporation into THP-1 macrophages compared to oleic acid-rich VOO, being 18:1/18:2 ratio consistently correlated with intracellular TAG accumulation. Since the oleic and linoleic acid composition of VOO is dependent on the cultivar, among other factors, this feature of the oil should be considered in nutritional studies. Moreover, the results presented here also point out to the possibility of selecting VOO cultivars for specific nutritional uses according to their FA composition.

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Research Article

Interaction between extra virgin olive oil phenolic compounds and mucin

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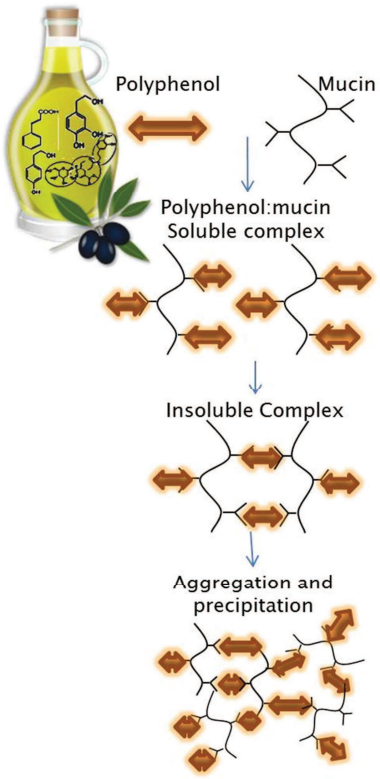

Interaction Between Extra Virgin Olive Oil Phenols Compounds and Mucin

Abstract.

EVOO phenolic compounds provide nutritional benefits because of their antioxidant capacity. The interaction between phenolic compounds and proteins may reduce their bioavailability and antioxidant capacity. The objective of this project was to evaluate the interaction between phenolic compounds from EVOO and mucin. EVOO phenolic extract and some commercially available pure phenolic present in EVOO were also used in this study: cinnamic, p-coumaric, caffeic, vanillic and protocatechuic acids, Tyrosol, Hydroxytyrosol and oleuropein.

The interaction between polyphenols and proteins was measured by the increasing of turbidity of the reaction mixture and expressed NTU. Polyphenols solutions at different concentrations were mixed with mucin solutions. Reaction mixtures were measured after 1, 30, 60, 90 and 180 minutes at 37ºC.

The results suggest that EVOO polyphenols interact with the mucin solution forming an insoluble complex after 1 minute of reaction. The highest interaction between polyphenols and proteins was observed for the EVOO phenolic extract and cinnamic, *p*-coumaric and caffeic acids. The interaction between polyphenols and mucin varied depending on the phenols concentration and chemical structure. The importance of the results is to evaluate the possible consequences of EVOO phenolic-protein interactions in the bioavailability and antioxidant capacity of the polyphenols present in the EVOO.

Keywords. Virgin olive oil; polyphenols; polyphenol-protein interactions; mucin; bioavailability.

ABBREVIATION

EVOO: Extra Virgin Olive Oil

VOO: Virgin Olive Oil

NTU: Nephelometric Turbidity Units

OA: oleuropein aglycon

Lig Agly: ligustroside aglycone

1. INTRODUCTION

Virgin olive oil (VOO) is one of the most well-known elements of the Mediterranean diet. It is appreciated because of its nutritional benefits. Since contains few, but significant phenolic components that other vegetable oils lack. The phenolic fraction of VOO is formed by a wide range of groups, including phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoid derivatives (dialdehydic and aldehydic form of elenolic acid linked to hydroxytyrosol or 3,4-DHPEA-EDA, and the dialdehydic and aldehydic form of elenolic acid linked to tyrosol or p-HPEA-EDA), phenolic acids (vanillic and p-coumaric acids), lignans (pinoresinol and acetoxypinoresinol) and flavonoids (luteolin and apigenin) [1-7].

EVOO sensory properties are largely affected by its phenolic composition. Since they are responsible for the bitter and pungent sensory [8-11].They also provides protection against oxidative process [12, 13].

VOO phenolic compounds show biological activities as inhibition of platelet aggregation [14], inhibition of low density lipoprotein (LDL) oxidation [15, 16], anti-inflammatory activities [17], antimicrobial and antiviral activities, immune system

regulation [18], protection against oxidative stress [19-21], stimulation of induction of antioxidant enzymes and modulation of the metabolism [22].

In general, polyphenols are known to interact with protein molecules creating soluble or insoluble complexes; this interaction could be a reversible process. However, it has been shown that interactions salivary proteins with tannins form complexes insoluble non-reversible [23]. These interactions are made through hydrogen binding, hydrophobic or hydrophilic interactions, or less frequently covalent bonds [24-28]. The interactions between polyphenols and proteins can be divided into three steps. First, proteins and polyphenols are combined to form soluble complexes. Then, these complexes can grow to a colloidal size, at which point they scatter light. Finally, they become larger until they form insoluble aggregates [23, 29, 30]. Polyphenol-protein interactions depend on several factors such as: chemical structure of polyphenols, structure of proteins, the protein/polyphenol ratio and some solution parameters (pH, ionic strength, temperature) [27, 31-35].

Most of the studies on interaction between phenolic compounds and proteins have been focused on tannins with protein precipitating properties. Most of research on tannin-salivary protein interaction has been linked to its role in astringency perception. Tannins interact with proline-rich proteins of saliva producing their precipitation, giving a dry mouth sensation that is referred to as astringency [36-39]. Another food technological effect of phenolic-protein interactions is haze formation in beer, fruit juice and wine [25].

Furthermore, the formation of polyphenol-protein aggregates causes an unfolding of the protein structure and also affects the bioavailability of both components proteins and polyphenols [40, 41]. This reduction in polyphenol bioavailability is also expected to affect their beneficiary properties. The interaction of salivary proteins with polyphenols affects the antioxidant capacity of the low molecular weight polyphenols such as ferulic acid,

caffeic acid and catechin [42]. In addition, polyphenols are known to mix with digestive enzymes leading to enzymatic activity modulation [43].

Few studies have been reported with low molecular weight polyphenols and phenolic compounds present in virgin olive oil. Therefore, the aim of this work was to evaluate the interaction between phenolic of EVOO and mucin (protein rich in threonine, serine and hydroxyproline). EVOO phenolic extract and some commercially available pure phenolic present in VOO were also used in this study: cinnamic, p-coumaric, caffeic, vanillic and protocatechuic acids, tyrosol (p-hydroxyphenylethanol), hydroxytyrosol (3, 4-dihydroxyphenylethanol) and oleuropein. The importance of the results is to evaluate the possible consequences of EVOO phenolic-mucin interactions in the bioavailability and antioxidant capacity of the polyphenols present in the EVOO.

MATERIALS AND METHODS

2.1 Samples

2.1.1 EVOO phenolic extract and pure phenolics compounds

The phenolic extract of VOO was obtained from `Picual´ olive cultivar in the experimental oil mill located in the Centre IFAPA "Venta del Llano". The polyphenol extract was obtained by liquid/liquid extraction with methanol-water (60:40 v/v). A sample of 100 g of EVOO was dissolved in n-hexane (50 mL). The phenolic compounds were extracted with 50 mL of methanol/water four times, mixing at 14.000 rpm during 1.30 min and then, it was left to decant. All the hydroalcoholic fractions were filtered (0.45 µm syringe filter) and then concentrated by lyophilization and reconstituted in methanol (400 µL) for phenolic compounds analysis and in ethanol (50% v/v) to evaluate the interaction with mucin.

Cinnamic, *p*-coumaric, caffeic, vanillic and protocatechuic acids (Sigma-Aldrich), tyrosol (Lancaster Chemicals), hydroxytyrosol, oleuropein (Extrasynthese) were selected as pure phenolics compounds.

Phenolic solutions were prepared by dissolving phenolic extract of EVOO or each pure phenolic compound in 1% ethanol. All samples were prepared immediately prior to testing. Solutions were prepared at the following concentrations: extract of EVOO - 80, 150 and 300 mg/L; pure phenolic compounds – 0, 80, 100, 200, 400, 500, 600, 800 and 1000 mg/L;

2.1.2 Characterization of EVOO phenolic extract

Total phenol content was determined in EVOO extract following the method described by Vázquez-Roncero, (1973) [44]: using Folin-Ciocalteau reagent and absorbance measurement at 725 nm in a Uv-Vis spectrophometer Cary 50 Bio (Varian, Spain). The results were obtained as mean values of at least three determinations and expressed as mg/kg of caffeic acid.

HPLC Analysis of Phenolic compounds were performed using an HP 1100 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump delivery system, a degasser, an autosampler, a diode array UV-Visible detector and a mass spectrometer detector. A reversed-phase C18 Zorbax Eclipse Plus (150 x 4.6 mm, 1.8 µm particle sizes) with a C18 precolumn filter was used with an injection volume of 20 µL and a flow rate of 0.8 mL/min. The mobile phase was a mixture of water/acetic acid (0.55% v/v) (solvent A) and acetonitrile (solvent B). Solvent gradient changed according to the following conditions: from 0 to 10 min, 95%A: 5%B, from 10 to 12 min, 70%A: 30%B, from 12 to 17 min 67%A: 33%B, from 17 to 20 min 62%A: 38%B, from 20 to 23 min 50%A: 50%B, from 23 to 23.5 min 5%A: 95%B, and from 23.5 to 25 min 0%A: 100%B. The wavelengths were set at 240, 280, and 330 nm. The identification was made using *MS* (Bruker Daltonic, Bremen, Germany) as well. The analyses were carried out employing an electrospray

interface (ESI) (G1607A, Agilent Technologies, Palo Alto, CA), operating in positive mode following these conditions: drying gas flow, 9 L/min; nebulizer pressure, 30 psi; drying gas temperature, 300ºC; capillary voltage, 3200 V; and fragmentor voltage, 70 V. Phenolic compounds were quantified at 280 nm, on the basis of the corresponding standard, with the exception of elenolic acid, which were quantified as hydroxytyrosol, and secoiridoid derivitatives, which were quantified as oleuropein. The results were obtained as mean values of three determinations and expressed as mg/kg of VOO.

2.2 Polyphenol-mucin interaction assay

Polyphenol-mucin interaction assay was carried out according to the method described by Monteleone et al. (2004) [45]. Polyphenols solutions (8 mL) were mixed with 2 mL of mucin from porcine stomach type III (Sigma Aldrich, M1778) solution at 0.2% using a citrate phosphate buffer pH 3.5, 0.1M. Two reference samples were used, a polyphenol solution (8 mL of polyphenol solution mixed with 2 mL of citrate phosphate buffer) and a mucin reference sample (8 mL of 1% ethanol mixed with 2 mL of mucin solution). All the reagents were thermo-stated at 37ºC.

The interaction between the phenolic compounds and the mucin was measured applying the nephelometry method [45, 46]. The turbidity values of the mixes were measured using a portable turbidimeter HACH 2100P ISO (Hach Co.) in Nephelometric Turbidity Units (NTU), from 0.01 to 1000 NTU in automatic range mode and automatic decimal point placement. The ratio optical system includes an LED lamp, a 90°detector to monitor the scattered light and a transmitted light detector. The microprocessor calculates the ratio of the signals from the 90° detector and the transmitted light detector. The instrument was calibrated before the experiment with Hach StablCal Stabilized Formazin. The measurements were made with the signal average mode on, which takes ten measurements and calculates their average.

Each phenol solution mixed with mucin was measured after 1, 30, 60 and 180 min. The polyphenol-mucin interaction was expressed as NTU, obtaining the NTU values measured for the mucin reference (NTUm) plus the polyphenol reference sample (NTUp) from the NTU value of the polyphenol-mucin sample (NTUs), so: NTU = NTUs – (NTUm + NTUp).

2.3 Data Analysis

Analysis of variance (ANOVA) was carried out in order to evaluate the effects of reaction time and concentration of phenolic compounds on polyphenol-protein interaction. The comparison of means was done by Fisher test (LSD) at a level of significance of 0.05.

RESULTS AND DISCUSSION

3.1 Characterization of EVOO phenolic extract

The lyophilized phenolic extract of virgin olive oil looked like syrup and showed a dark brown color. The total phenol content of the extract was 558 $\pm$ 0.01 mg/kg. The composition of the EVOO phenolic extract is shown in Fig. 1. The extract contained the most representative phenolic compounds of virgin olive oil. The main phenolics quantified were isomers of oleuropein aglycone (OA) and isomers of ligustroside aglycone (Lig Agly), secoiridoids derivatives from oleuropein and ligustroside, respectively (Table 1).

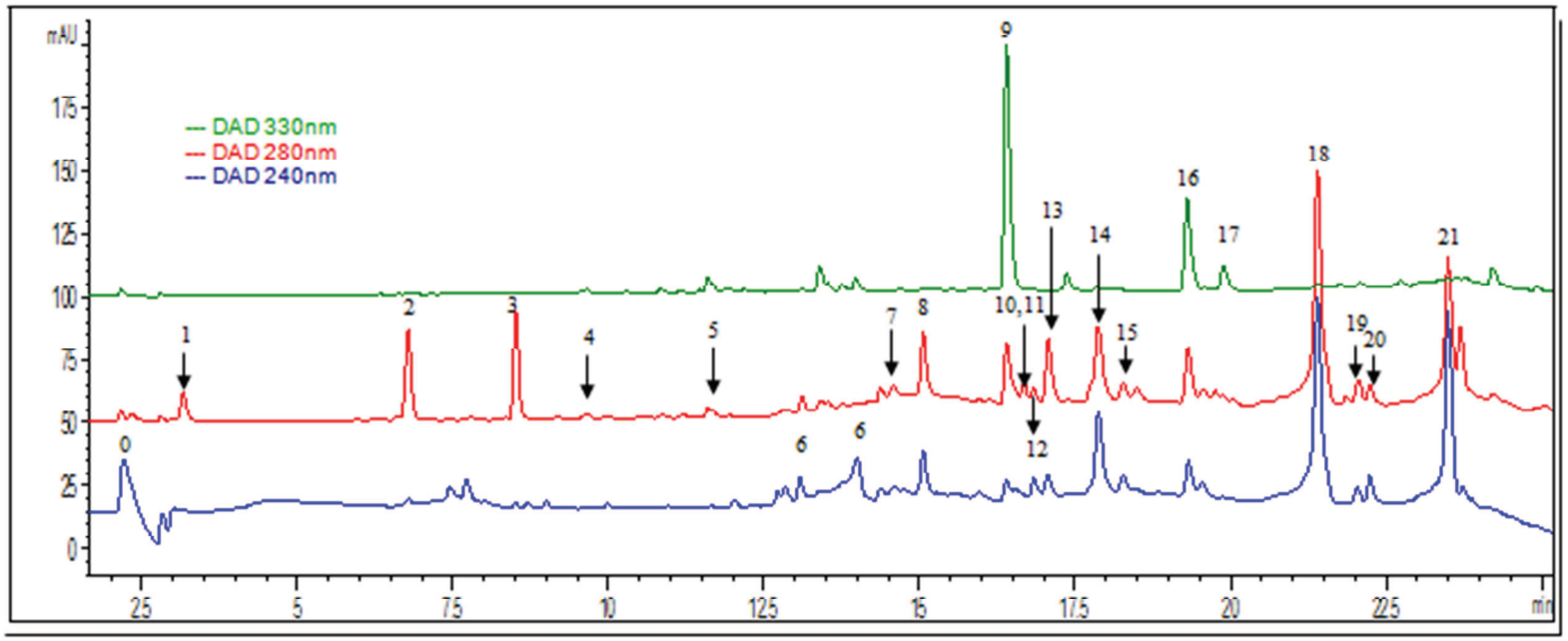

Figure 1. HPLC chromatogram VOO phenolic extract (280, 240 and 330 nm).Peak indentification numbers: 1. Gallic Acid ; 2. Tyrosol; 3. Hydroxytyrosol; 4. Caffeic Acid; 5. p-cumaric acid; 6. Elenoic Acid; 7, 8, 13, 15, 17, 18 y 19. Isomers of oleuropein aglycone ; 9. Luteolin; 10, 12, 14, 20 y 21. Isomers of ligustroside aglycone; 11. Pinoresinol; 16. Apigenine.

Table 1. Phenolic Composition (mg/kg) of lyophilized VOO phenolic extract measure by HPLC at 280 nm (mean ± SD, n = 3)

Name	Peak No.	mg/kg of VOO
Phenyl alcohols		
Hydroxytyrosol	3	10.98 ± 0.09
Tyrosol	2	12.22 ± 0.22
Phenolic Acids		
Gallic Acid	1	1.26 ± 0.09
Elenoic Acid[a]	6	65.98 ± 1.76
Caffeic Acid	4	0.27 ± 0.00
p-cumaric acid	5	0.31 ± 0.00
Secoiridoid derivatives[b]		
Isomers of Oleuropein Aglycone	7, 8, 13, 15, 17, 18, 19	296.41 ± 2.5
Isomers of ligustroside aglycone	10, 12, 14, 20, 21	194.05 ± 2.8
Lignans		
Pinoresinol	11	3.9 ± 0.09
Flavonoids		
Luteolin	9	4.62 ± 0.01
Apigenine	16	2.98 ± 0.12

[a] Quantified as hydroxytyrosol.
[b] Quantified as oleuropein.

3.2 Polyphenol-mucin interaction assay

The relative affinity of EVOO polyphenols to bind with mucin was measured according to their ability to precipitate them in a solution in which the composition of the resulting polyphenol-mucin aggregate was determined directly by nephelometry. This analytical method allows measuring the scattered light in the solution resulting from the gradual formation of a turbidity precipitate corresponding to the polyphenol-mucin aggregate [47].

3.2.1 Effect of reaction time on polyphenol-mucin interaction

The assayed phenolic compounds interacted with mucin resulting in a cloudy solution and then, solution turbidity was higher. An analysis of two-way ANOVA (reaction time and concentration of phenolic compounds) was performed in order to compare the interaction of each phenolic compound (at each concentration) with mucin during a reaction time from 1 to 180 min. Results showed a significant and positive effect of the reaction time on the interaction of VOO phenolic extract, caffeic acid, cinnamic acid, vanilic acid, hydroxytyrosol and oleuropein with mucin ($p \leq 0.05$) (Table 2). Significant differences along the reaction time were obtained for the comparison of mean turbidity values of each phenolic compound applying the LSD test, n=5 (Fig. 2).

Table 2. Two way ANOVA analysis of turbidity produced of each phenolic compound (at each concentration) with the mucin during reaction time of 1 at 180 min (n=5)

Origin of variations	F- value	Probability	Critical value for F	Significant difference *P<0.005*
VOO Phenolic Extract	3.795	0.032	3.259	Yes
Caffeic acid	5.026	0.003	2.668	Yes
p-coumaric acid	2.298	0.080	2.668	NO
Cinnamic acid	2.877	0.038	2.668	Yes
Protocatecuic acid	2.520	0.060	2.668	NO
Vanillic acid	3.795	0.012	2.668	Yes
Hydroxityrosol	13.611	0.000	2.668	Yes
Tyrosol	1.713	0.171	2.668	NO
Oleuropein	2.967	0.034	2.668	Yes

In general, the mixes of phenolic compounds and mucin showed turbidity immediately after 1 minute. An increase up to the maximum was appreciated during the incubation time reaching equilibrium later. Then, a slight decrease for pure compounds was observed.

EVOO phenolic extract showed a linear increase of turbidity during the incubation time studied (180 min). Previous works have reported that the turbidity of protein-polyphenol mixes was developed almost instantaneously and then, it increased slightly during the incubation time, reaching the equilibrium for 60 min [31, 45]. The slight decrease of turbidity of some of the pure phenolic after reaching the maximum value may be explained because polyphenol-mucin complexes started to agglomerate, becoming unstable and producing their precipitation and consequently differences in haze value. Therefore, according to Monteleone et al. (2004) a short reaction time was adopted (1 min) in which only aggregates could have been formed might just have formed [45].

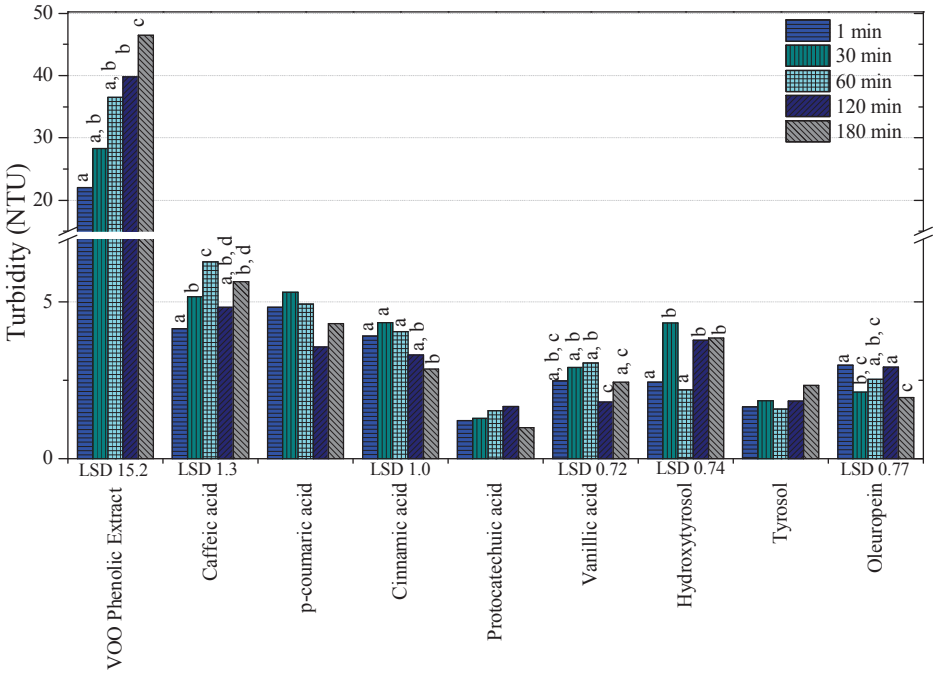

Figure 2. Turbidity (NTU) of polyphenol - mucin mixes as function of reaction time.Values are the mean of concentration. Fisher test (LSD) has been used to assess significance. Different letters in the same group indicate significant difference ($p < 0.05$) in the reaction time, n=5.

3.2.2 Effect of phenolic concentration on polyphenol-mucin interaction

In agree to literature, polyphenol-protein aggregate formation depends of several factors among them the protein and polyphenol concentration on the solution [23, 27, 39, 45, 48]. It can be explained following the model described by Siebert et al. (1996): considering that each polyphenol molecule has a fixed number of binding ends and each protein has a fixed number of polyphenol binding sites. Then, at low polyphenol concentrations respect to protein, each molecule of phenol could form a bridge between two protein molecules, forming dimers of protein, small aggregates and a slight turbidity. When the number of phenol ends equals the number of protein binding sites, each molecule of phenol bind other dimers of protein resulting on largest network of large aggregates complex and increased turbidity. An excess of polyphenols relative to protein would provoke that all of the protein binding sites were occupied; forming small aggregates due to that each free polyphenol end would have small chances of finding a free binding site on a protein molecule and not would form bridge between protein molecules. This would result in less turbidity. Therefore, turbidity is a function of polyphenol- mucin aggregate that depends on the concentration of polyphenols respect to the protein [27].

In this work, the mucin concentration was constant and chosen taking into consideration the amount used in the artificial formulations [49] versus an increasing polyphenol concentration, for to establish the minimum polyphenol concentration that forming aggregates with mucin. In order to be able to discuss the final results, the phenolic compounds were grouped according to their chemical structure as follows: cinnamic acid derivatives, benzoic acid derivatives, phenolic alcohols, secoiridoids and EVOO phenolic extract. The results of two- way ANOVA analysis showed a significant effect of increasing phenolic concentration on mixes turbidity ($p \leq 0.05$, $n=5$) (Table 3).

Table 3. Two way ANOVA analysis of turbidity of phenolic compounds (at each concentration) at 1 min of reaction whit mucin (n=5)

Origen of variations	**Sum of Squares**	**Degrees of Freedom**	**Average of Squares**	**F- value**	**Probability**	**Critical value for F**	**Significant difference *P<0.005***
Concentration	359.3265424	8	44.9158178	18.82702991	2.4116E-13	2.108688484	Yes
Phenolic Compounds	99.97715556	7	14.28245079	5.986668873	*3.14392E-05*	2.178155555	Yes
Error	133.5997132	56	2.385709164				
Total	592.9034111	71					

For the first minute (1 min) of reaction, the turbidity appeared at different concentrations depending on the phenolic molecular structure. Aggregates with mucin were formed from the lowest concentration (80 mg/kg) for *p*-coumaric acid, cinnamic acid and EVOO phenolic extract. Caffeic and vanillic acids produced aggregates from 100 mg/kg whereas for tyrosol, the lowest concentration for turbidity was 200 mg/kg. For other phenols such as protocatechuic acid, hydroxytyrosol and oleuropein the turbidity appeared from 400 mg/kg. After the initial increase, many of them achieved equilibrium, although the oleuropein showed a slight final decrease (Fig. 3). These differences could be explained by molecular structure of pure phenolic compounds and the components of EVOO phenolic extract, which determine the formation of large aggregates with different sizes and thus, with different turbidity [45].

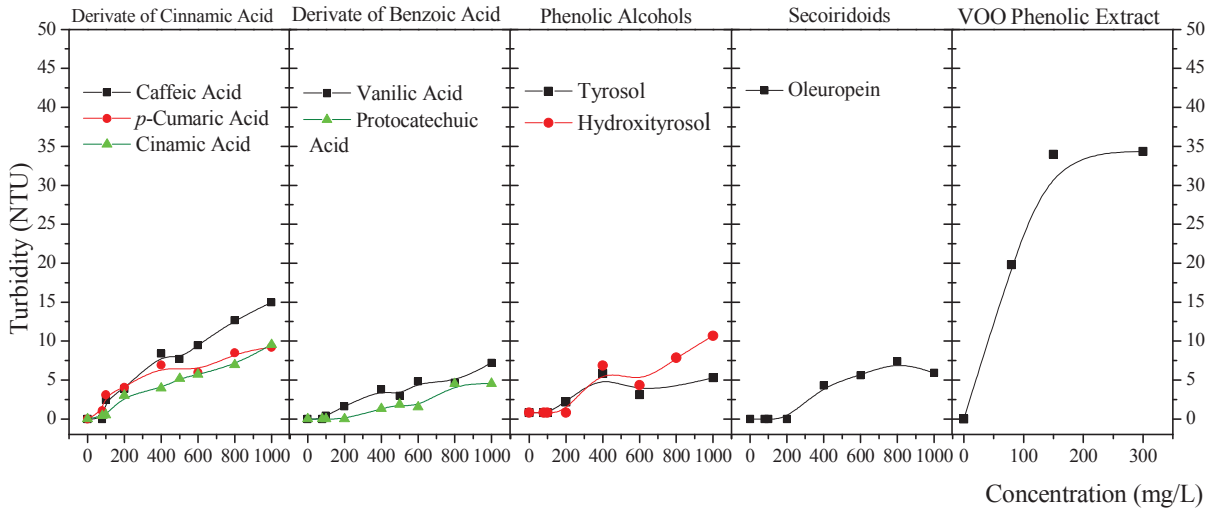

Figure 3. Turbidity (NTU) of polyphenol-mucin complex as a function of the concentration of polyphenol compound in 1 minute of reaction

3.2.3 Influence of phenolic compound

When the effect of the concentration of phenolic compounds was analyzed in the analysis of variance (ANOVA), significant differences ($p \leq 0.05$, $n=5$) were found among the phenolic compounds (Table 3). Comparing the mean turbidity generated by each phenolic compound (LSD test) significant differences was found among them (Fig. 4).

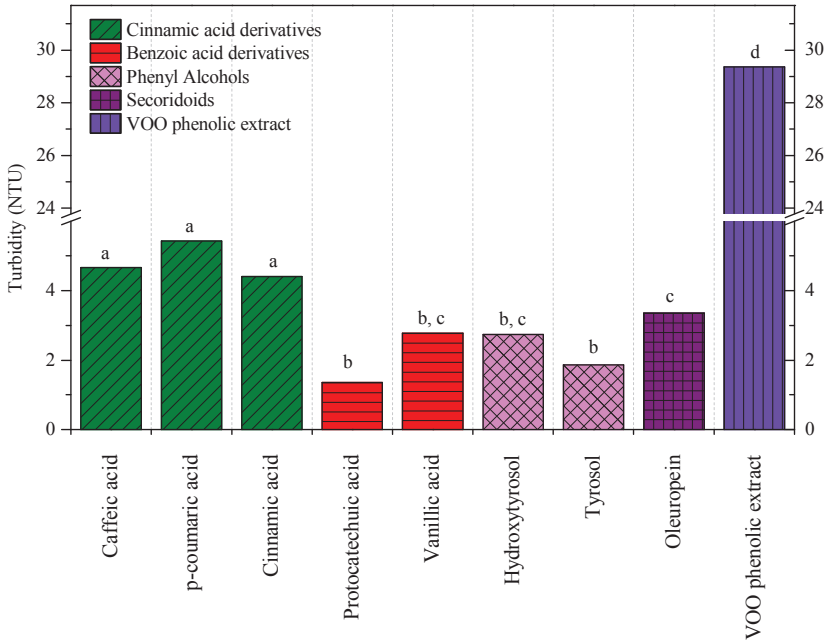

Figure 4. Turbidity (NTU) of polyphenol-mucin complex for 1 minute of reaction. Values are the mean of the concentration, n=5. Fisher test (LSD) has been used to assess significance. Different letters indicate significant difference ($p \leq 0.05$ - LSD value 1.45).

The VOO phenolic extract exhibited the highest value of turbidity, followed by cinnamic acid derivatives and oleuropein, whereas benzoic acid derivatives and phenyl alcohols showed the lowest. The highest interaction of VOO phenolic extract with mucin may be explained taking into account that their main phenols were oleuropein aglycone and lygustroside aglycone. These compounds have a higher molecular weight, a greater number of aromatic rings and more –OH groups than the individual phenolic compounds used in this study. These are some features of polyphenol structure and properties reported that influence to their capacity of protein binding [28, 30, 31, 50-53]. Pripp et al. (2005) described that hydroxytyrosol and tyrosol of the EVOO extract showed a weakly binding to

the proteins, whereas other phenolic compounds of the extract had a stronger binding to BSA and sodium caseinate [54].

Within the individual phenolic compounds, phenolic-mucin interactions were higher with caffeic, *p*-coumaric and cinnamic acid. This may be due the number of hydroxyl groups and functional groups. Interactions between low molecular weight phenolics and proteins, using G-50 Sephadex Chromatography, are higher in phenolic compounds that contain *o*-dihydroxyl groups such as protocatechuic and caffeic acids [55]. Furthermore, the compounds that contain *o*-dihydroxyl-phenolic groups can be mixed with the protein via a bi-dentate hydrogen bond with an isolated group of hydroxyl-phenolic [35].

Although oleuropein has a higher molecular weight, two -OH groups and a higher number of aromatic rings, its interaction with mucin may be affected by the glycosidic group of the molecule which may compete with phenol to bind the protein and thus, affect to particle size evolution [56].

CONCLUSIONS

The nephelometry method was used to quickly and easily determine the interaction between same phenolic compounds present in VOO and mucin. All the phenolic assayed interacted with mucin from the beginning of the reaction forming insoluble complexes. The interaction increased during the incubation time to reach equilibrium. Polyphenol-mucin aggregate formation depended on the phenolic concentration and phenol molecular structure. Among the pure phenolic compounds studied, cinnamic acid derivatives showed the highest interaction with mucin. *P*-coumaric and cinnamic acids bound to mucin at low concentrations. VOO phenolic extract showed a higher interaction with mucin than the individual compounds, even at very low concentration. This difference was related to its phenolic composition (secoiridoid derivates and elenolic acid mainly), its molecular weight,

functional groups, the number of hydroxyl groups, the position of the phenols in the molecule and interaction between them.

The results about formation of polyphenol-mucin complex are important from the nutritional aspect since it could affect the bioavailability and antioxidant capacity of the different phenolic compounds during the digestion of VOO. Furthermore, the binding of polyphenols with digestive enzymes could make the EVOO intake had a modulating effect of digestion. Secondly, the results have shown that the method used in this study to evaluate the polyphenol-protein interaction is fast and easy for its possible application as a complementary method to sensorial analysis to determine the astringency intensity of the EVOO.

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Effect of olive cultivar on bioaccessibility and antioxidant activity of phenolic fraction of virgin olive oil

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Abstract.

Aim: This study aims to characterize the phenolic profile and antioxidant capacity of seven monovarietal virgin olive oils (VOOs) and evaluate their *in vitro* gastrointestinal stability. **Methods:** `Picual´, `Blanqueta´, `Sevillana´, `Habichuelero´ and `Chetoui´ olive cultivars were selected for VOO extraction. The oils were subjected to *in vitro* digestion. The recovery index (RI) of phenolic compounds after each digestion step and the bioaccessibility index (BI) were evaluated. Also, the antioxidant activity of the bioaccessible fraction (BF) of VOOs was determined by DPPH, ABTS and ORAC assays, as well as by studying the intracellular reactive oxygen species in Caco-2 cells. **Results:** Differences were found in the composition of phenolic compounds in VOOs depending on cultivars. During the digestive process, important losses of phenolic compounds were observed between the buccal and duodenal steps, unlike HTy and Ty, which presented increased recovery due to the hydrolysis of secoiridoid derivatives. Differences in the bioaccessibility of phenolic compounds were found between varieties of VOOs. `Sevillana´ VOO had the highest total bioaccessibility (36%), followed by the `Picual´ (19%), `Chetoui´ (17%), `Habichuelero´ (10%) and `Blanqueta´ (8%) varieties. The BF of all the varieties of VOO showed similar radical ABTS scavenging capacity, `Chetoui´ and `Blanqueta´-BF having the highest radical DPPH scavenging capacity, and `Habichuelero´ and `Picual´-BF showing protective effects against the peroxyl radical measured by $ORAC_{FL}$ assay. All VOO-BFs presented decreases in ROS levels in Caco-2 cells. **Conclusions:** Our results suggest differences in the bioaccessibility of phenolics from diverse VOO varieties, which could lead to different biological properties. Therefore, this

study represents a first step toward the development of novel dietary strategies focusing on the phenolic supplementation of different VOOs in order to preserve human health.

Keywords. Extra Virgin Olive Oil; phenolic compounds; bioaccesibility; antioxidant capacity.

INTRODUCTION

Virgin olive oil (VOO) is one of the best-known elements of the Mediterranean diet. It is appreciated because of its sensory and nutritional benefits [1]. The consumption of olive oil has been associated with many desirable nutritional properties including a lower incidence of coronary heart disease and cancer [2].

The health benefits of virgin olive oil consumption are partly attributed to phenolic compounds. The VOO phenolic fraction is formed of different phenolic groups, including phenolic alcohols (hydroxytyrosol and tyrosol), secoiridoid derivatives (dialdehydic and aldehydic form of elenolic acid linked to hydroxytyrosol, and the corresponding derivatives linked to tyrosol), phenolic acids (vanillic and *p*-coumaric acids), lignans (pinoresinol and 1-acetoxypinoresinol) and flavonoids (luteolin and apigenin)[3].

The concentration of phenolics in VOO depends on several agronomic and technological factors [4, 5]. Among them, a genetic component has been described as a significant source of variability. Monovarietal VOOs show differences in their phenolic profile mainly thanks to secoiridoids and their derivatives [6, 7]. These differences between cultivars might have a significant effect on the health-promoting properties of monovarietal VOOs.

The bioactive properties of phenolic compounds have been linked to their capacity to inhibit oxidation processes [8]. Several studies have shown that VOO phenolics inhibit platelet aggregation [9] and low density lipoprotein (LDL) oxidation [10], and show anti-

inflammatory [11], antimicrobial and antiviral activities. They are involved in immune system regulation [12], protection against oxidative stress[13, 14] and modulation of the metabolism [15].

The biological effects *in vivo* of phenolic compounds depend on their bioavailability since they are quantitatively transported into the small intestine by passive diffusion [16]. Experiments on animals and humans have confirmed that olive oil phenolics are well absorbed at intestinal level [17] and that during gastric and intestinal digestion they experience hydrolysis and modifications [18, 19], being available for intestinal absorption in the forms of hydroxytyrosol (HTy) and tyrosol (Ty), which enter the circulatory system as glucuronide, sulphate and methylate phase II metabolites [20-24].

Although several studies have been published dealing with the absorption, disposition, metabolism and excretion of virgin olive oil phenolics in the human body [18, 22, 25, 26], information about the behaviour of VOOs from different olive varieties is limited [27].

The aim of this study was to characterize the phenolic composition of seven monovarietal VOOs and to evaluate their digestive stability using an *in vitro* gastrointestinal digestion method. Furthermore, the antioxidant capacity of the bioaccessible fraction of the phenolic compounds was measured by ABTS, DPPH and ORAC assays and the study of intracellular reactive oxygen species (ROS) in Caco-2 cells.

MATERIALS AND METHODS

Reagents and Standards

All the chemicals and reagents used were either analytical-reagent or HPLC grade. The water was treated in a Milli-Q water purification system (Milipore, Bedford, MA) before use. The chemicals and reagents used in the *in vitro* digestion model were: potassium

chloride (KCl), potassium thiocynate (KSCN), monosodium phosphate (NaH_2PO_4), sodium phosphate (Na_2PO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), magnesium chloride ($MgCl_2$), calcium chloride dihydrate ($CaCl_2*2H_2O$), ammonium chloride (NH_4Cl), D-(+)glucosamine hydrochloride, D–glucuronic acid, D–(+) glucose, uric acid, urea, mucin from stomach type III (M1778), α-amylase from porcine pancreas, hydrochloric acid 37% (HCl), pepsin from porcine gastric mucosa , pancreatine from porcine pancreas, lipase from porcine pancreas Type II, bile salts, and albumin from bovine serum (BSA). All the enzymes, organic and inorganic compounds used were purchased from Sigma-Aldrich Co. (St. Louis, USA). The standards used for the identification and quantification of phenolic compounds were: cinnamic, *p*-coumaric, caffeic, vanillic, homovanillic and protocatechuic acids, pinoresinol and luteolin were obtained from Sigma-Aldrich Co. (St. Louis, USA); tyrosol from Lancaster Chemicals; and hydroxytyrosol, hydroxytyrosol acetate and oleuropein from Extrasynthese. The methanol was of HPLC grade.

The following chemicals and reagents used to determine antioxidant assay were purchased from Sigma–Aldrich Co. (St Louis, MO): Hepes Buffer; Sodium Pyruvate; non-essential amino acids mixture 100x (NEAA); 20,70- dichlorofluorescein diacetate (DCFH-DA); dimethyl sulfoxide (DMSO); 5-methylphenazinium methyl sulfate; DL-all-rac-a-tocopherol (Vitamin E CAS 10191-41-0 (TOC)) purity 96%; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox™ CAS 53188-07-1 (TR)) purity 97%; 2,20-azobis (2-methylpropionamidine) dihydrochloride (AAPH) purity 97%; 2,2-diphenyl-1-picrylhydrazyl (DPPH) purity 90%; and 2,20-azino-bis(3-ethylbenzthiazoline-6-sulphonicacid) diammonium salt tablets (CAS 30931-67-0 (ABTS)); phosphate buffered saline (1x, Dulbecco's, PBS) was purchased from Applichem GmbH (Gatersleben, GERMANY); Minimum essential medium with Eagle's salts (MEM) and foetal bovine serum (FBS) were obtained from PAA Laboratories GmbH (Pasching, Austria). TrypLE Express, HuMEC ready medium kit and fluorescein (FL) were obtained from Gibco® Life

Technologies Ltd (Paisley, UK). $K_2S_2O_8$ (CAS 7727-21-1) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). CellTiter-Blue® Cell Viability Assay was obtained from Promega Corporation (Madison, WI, USA). The culture plates were obtained from NUNCtm (Roskilde, Denmark).

Plant Material and Virgin Olive Oil Extraction

The `Picual´, `Blanqueta´, `Sevillana´, `Habichuelero´ and `Chetoui´ (Olea europaea, L.) olive cultivars were selected based on their different profiles of VOO phenolic compounds. The olive trees were spaced 7 x 7 m and grown in the experimental orchard of Centro IFAPA 'Venta Del Llano'- Mengibar, Jaen (Spain) using standard growing techniques. The study was carried out during the 2014/15 crop year.

VOO extraction was performed using an Abencor laboratory oil mill (Abengoa, Seville, Spain) equipped with a hammer mill, a thermobeater and a paste centrifuge that simulates the industrial process of VOO production at laboratory scale. The oil extraction was repeated twice for each cultivar. The olive milling was performed at 3000 rpm with a 5 mm sieve. The olive paste malaxation was carried out at 28 °C for 45 min. Oil separation was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oil was decanted, filtered and stored in glass bottles in the dark at -20 °C without headspace and under N_2 atmosphere until analysis.

In Vitro Digestion Model

The study of the bioaccessibility of VOO phenolics was carried out using the method reported by Versantvoort et al. [28] with some modifications. Briefly, the model includes a three-step procedure simulating digestive processes in the mouth, stomach and small intestine.

The saliva and digestive juices were prepared artificially as described in **Table 1** and heated at 37°C ± 2°C. The digestion started by adding 6 mL artificial saliva to 5 g of VOO, incubated at 37°C ± 2°C and rotated head over heels (55 rpm) for 5 min [29]. Then, 12 mL of gastric juice was added, and the mixture was rotated head over heels for 2 h as described above. The gastric pH was between 2 and 3. Finally, 12 mL of duodenal juice, 6 mL bile, and 2 mL bicarbonate solution (1M) were added simultaneously, rotating the mixture for another 2h. The pH of the chyme (digestion mixture) ranged between pH 6.5 to 7. Each VOO sample was digested in triplicate. All the *in vitro* digestion steps were carried out in amber bottles in the dark.

At the end of each digestion step (buccal, gastric and duodenal), the digestion mixtures were centrifuged for 5 min at 2750 g [28, 29] before separating the solutions into three layers: the pellet (digested matrix), which was discarded; an aqueous phase (Wp); and an oily phase (Op) at the top of the centrifuge tube. Each sample was kept at -80°C until its analysis. To evaluate the digestion of VOO phenolic compounds, two different indexes were studied: the percentage of recovery or recovery index (RI) used to determine the amount of phenolics in the complete (Op + Wp) digest after each digestion step from 1 g of VOO; and the bioaccessibility index (BI), defined as the percentage of phenolic compound solubilized in Wp after the duodenal digestion of 1 g of VOO, which defines the proportion of the phenolic compounds available for absorption into the systemic circulation.

Table 1. Constituents and concentrations of the various synthetic juices of the in vitro digestion model representing fed conditions

	Artificial Saliva	Gastric Juice	Intestinal Juice	Bile
Inorganic Compounds				
KCL 89.6 g/L	10 mL	9.2 mL	6.3 mL	4.2 mL
KSCN 20 g/L	10 mL			
NaH_2PO_4 88.8 g/L	10 mL	3 mL		
Na_2SO_4 57 g/L	10 mL			
NaCl 175.3 g/L	1.7 mL	15.7 mL	40 mL	30 mL
$NaHCO_3$ 84.7 g/L	20 mL		40 mL	68.3 mL
HCl 37% 370 g/L		6.5 mL	0.18 mL	0.15 mL
KH_2PO_4 8 g/L			10 mL	
$MgCl_2$ 5 g/L			10 mL	
Organic Compounds				
Urea 25 g/L	8 mL	3.4 mL	4 mL	10 mL
Glucose 65 g/L		10 mL		
Glucuronic Acid 2 g/L		10 mL		
Glucosamine hydrochloride 33 g/L		10 mL		
Compound added to mixture of organic and inorganic solution				
$CaCl_2*2H_2O$ 22.2 g/L		18 mL	9 mL	10 mL
NH_4CL 30.6 g/L		10 mL		
α-amilasa	290 mg			
Uric Acid	15 mg			
Mucin	0.025 g	3 g		
BSA		1g	1 g	1.8 g
Pepsin		2.5 g		
Pancreatin			9 g	
Lipase			1.5 g	
Bilis				30 g
	pH 6.5 ± 0.1	pH 1.0 ± 0.1	pH 7.8 ± 0.2	pH 8.0 ± 0.2

The inorganic and organic solutions are augmented to 500ml with distilled water. After mixing of the inorganic and organic solutions, some further constituents are added and dissolved. If necessary, the pH of the juices is adjusted to the appropriate interval with NaOH 1M or HCl 37%.

Extraction of Phenolic Compounds

Phenolic compounds were extracted from VOO, Wp and Op samples. The VOO and Op samples were dissolved in hexane, and extracted with methanol/water (60:40), agitated for 90 secs, and then, centrifuged at 2423g for 6 min at 4°C. The supernatant was recovered and then, a second extraction was performed.

The Wp samples were passed through OASIS HLB 200 mg SPE cartridges (Waters Corp., Milford, MA) according to the method described by Suarez et al.[30] with some modifications. The retained phenolic compounds were eluted using 5 mL of methanol. To concentrate the analytes, the elution solvent was evaporated to dryness under a nitrogen stream in an evaporating unit at 30 ºC (PIERCE Model 18780, IL, USA) and reconstituted with 1 mL of methanol. All the extracts were filtered through a 0.22 µm PES syringe filter (Millex, Merk Millipore Ltd).

UHPLC-HR-MS Analysis of Phenolic Compounds

The phenolic compounds were analysed using an UHPLC with a PDA detector scanning from 200–600 nm, equipped with an autosampler cooled at 4° C (Dionex Ultimate 3000 RS, Thermo Corporation) and an ExactiveTM Orbitrap mass spectrometer fitted with a heated electrospray ionization probe (HESI) (Thermo Fisher Scientific, San José, USA). Chromatographic separation was performed at 40°C on a Kinetex 5 u 100A 100 x 2.1 mm C18 column (Phenomenex, Macclesfield, UK). Ten microliters of each extract were analysed using a 5–50% gradient of acidic methanol in 0.1% aqueous formic acid at a constant flow rate of 0.6 mL/min for 55 min. After passing through the PDA flow cell, the eluate was directed to the mass spectrometer with the HESI operating in negative ionization mode. The analysis was carried out in full-scan (100–800 m/z) and full-scan with in-source collision-induced dissociation (CID) (100–800 m/z; CID 25.0 eV).

The capillary temperature was 300 °C; the sheath gas and auxiliary gas were 60 and 20 units/min, respectively; the source voltage was 3.0 kV. Identification was achieved by comparing the exact mass and retention time with pure reference standards. In the absence of standards, compounds were tentatively identified by comparing the theoretical exact mass of the molecular ion with the experimentally measured accurate mass of the molecular ion. In addition, identification was confirmed by the appearance of typical fragments produced from the molecular ion. Quantification was performed as follows: by PDA at 280 nm for HTy, Ty, vanillic, homovanillic, cinnamic and *p*-cumaric acid, vainillin, hydroxytyrosol acetate (HTy-Ac), tirosol acetate (Ty-Ac), dialdehydic form of elenoic acid linked to HTy (3,4-DHPEA-EDA), dialdehydic form of elenoic acid linked to Ty (*p*-HPEA-EDA), pinoresinol, 1-acetoxipinoresinol; and at 360 nm for caffeic and ferulic acid and flavons; and by MS for the other secoiridoid derivatives that co-eluted with other phenolic compounds with an equal response factor. All the compounds were quantified on the basis of the corresponding standard, with the exception of elenolic acid and secoiridoid derivatives, which were quantified as oleuropein equivalents. 1- acetoxipinoresinol was quantified as a pinoresinol equivalent and the flavones were quantified as apigenin equivalents. The results were obtained as the mean values of three measurements and expressed as nmol/g of VOO.

Determination of Antioxidant Activity

The antioxidant activity of the bioaccessible fraction (BF) or duodenal Wp was measured by DPPH, ABTS and ORAC assays.

DPPH assay. The antioxidant activity of the BF against the stable radical 2,2 – diphenyl-1-picrylhydrazyl (DPPH) was measured as previously reported [14, 31] with some modifications. Briefly, 100 μM of methanolic solution of DPPH was mixed with undiluted BF or BF diluted to 1:100 v/v in methanol in 96-well plates. α-Tocopherol (TOC) was used as a standard antioxidant control at 0.06, 0.13, 0.25, 0.5 and 1 mol of TOC/mol of DPPH

and a sample of digestive juice was also measured as a blank control. The decrease in absorbance at 520 nm was determined immediately and every 5 min for 2 h in a microplate reader (TECAN, GENios Plus). The measurements were performed in triplicate. The inhibition of the DPPH radical was calculated as a percentage of the radical scavenging activity (% RSA) at 60 min according to Warleta et al. (2011)[14].

ABTS assay. ABTS cation radical scavenging activity was determined using a previously reported procedure [14, 32]. The ABTS radicals ($ABTS^{+}$) were obtained by ABTS/H_2O (0.5 mM) reaction with K_2S2O_8 for 16 h in the dark at room temperature. The $ABTS^{+}$ was diluted in ultrapure water until absorbance at 734 nm was 0.7 (±0.1). Trolox™ (as antioxidant reference) was dissolved in methanol to yield a 10 mM stock solution and diluted with ultrapure water to the assayed concentrations (50 – 800 µM). Undiluted BF,BF diluted to 1:100 v/v in ultrapure water, standard (Trolox™), blank (ultrapure water) and ethanol control (8%) were added to a 96-well plate. A sample of digestive juice was also measured as a blank control. The reaction was initiated by the addition of 50 µL of $ABTS^{+}$. Absorbance readings were taken every 5 min for 2 h at 30 °C in a microplate reader (TECAN, GENios Plus). All measurements were carried out in triplicate. The inhibition of $ABTS^{+}$ was calculated according to the percentage of radical scavenging activity (% RSA) at t = 60 min [14].

ORAC assay. Peroxyl radical scavenging activity was measured by the $ORAC_{FL}$ assay as previously described [33]. For analysis, undiluted BF and BF diluted in PBS (1:100 v/v) were used. A stock solution of Trolox™, as reference, was reconstituted in DMSO and diluted in PBS. Finally, fluorescein (FL) (48 nM) was mixed with samples of BF, standard (Trolox), blank (PBS) and a sample of digestive juice as a blank control. The plates were incubated for 15 min at 37 °C. The assay was initiated by the addition of 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (100 mM), and fluorescence readings (Ex. λ_{485}/Em. λ_{520} nm) were taken every 5 min for 160 min at 37 °C in a

microplate reader (TECAN GENios Plus). The final results were calculated based on the difference in the area under the fluorescence decay curve (AUC) between the blank and each sample. The results were expressed as micromolar Trolox™ equivalents (TE) calculated using the line equation from the standard curve[14]. All the samples were run in triplicate.

Detection of Intracellular Reactive Oxygen Species in Caco-2 cells

Cell culture and treatments. Caco-2 cells were purchased from the American Type Culture Collection (ATCC). They were routinely grown as monolayer cultures in MEM supplemented with 10% FBS, 1% Hepes Buffer, 1% Sodium Pyruvate and 1% NEAA. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2. They were routinely subcultured using TrypLE Express solution, and those in the exponential growth phase were used for all the experiments.

Cell viability assay. The cell survival assay, which measures the cellular growth of cells treated with BF versus untreated cells, was carried out using a CellTiter-Blue cell viability assay according to Sánchez-Quesada et al. [34], with some modifications. Briefly, the cells were seeded into 96-well culture plates in a total volume of 100 µL per well (8×10^3 cells per well). After overnight incubation to allow for cell attachment, the medium was removed and replaced by fresh medium containing increasing dilutions of 1:100, 1:1 and 1:0 (v/v) of BF in MEM for 24 h. Next, the cells were incubated with CellTiter-Blue (10%) for 3 h at 37 °C with 5% CO_2, and the relative fluorescence units were measured in a plate reader (TECAN GENios Plus) (Ex. λ_{485}/Em. λ_{595}, Gain 60). Moreover, viability was calculated according to Sánchez-Quesada et al.[34]. All the measurements were performed in triplicate, and each experiment was repeated at least three times. As a vehicle control, cells were treated with digestive juice.

Intracellular reactive oxygen species (ROS) levels were measured after 4 h of treatment with the BF of each variety, using the cell-permeable fluorescent probe, 2,7-dichlorofluorescin diacetate (DCFH-DA), as previously described by Sánchez-Quesada et al. [34]. Briefly, cells were seeded on a 96-well plate (8×10^3 cells per well of Caco-2) and after incubation with treatments, DCFH-DA (100µM) was added for 30 min at 37 °C with 5% CO_2. The cells were then read in a plate reader for 30 min (Ex. λ_{485}/Em. λ_{535}, Gain 40), and the intracellular ROS level percentage was calculated at 30 min. The addition of H_2O_2 has been reported to increase oxidative stress in cultured cells and to directly damage DNA. To evaluate the protective capacity of the BF against induced oxidative stress, H_2O_2 at 500 µM was added 30 min before the fluorescence quantification. All the tests were run in triplicate for each experimental condition, and each experiment was repeated at least three times. All the experiments were conducted using iron-free mediums (MEM).

Data Analysis

The results are expressed as the means (± SD) of three separate experiments, unless otherwise stated. The results of the cell assays are expressed as a percentage relative to the untreated control, which was set as 100%. Statistical significance was assessed by a one or two-way ANOVA followed by Bonferroni's multiple comparison test. Differences were considered statistically significant at $p<0.05$. Correlations between variables were assessed using Pearson's correlation coefficients at a 95% confidence interval. The statistical analyses were conducted using the GraphPad Prism v.5 statistical package (GraphPad Software, Inc.).

RESULTS

VOO Phenolic Compounds

The phenolic composition of VOOs from the five olive cultivars is shown in **Table 2.** A total of twenty six phenolic compounds were detected and quantified in the VOO samples. The typical UHPLC-HR-MS chromatogram is shown in **Figure 1**. Secoiridoids and their derivatives were critical to establish differences between VOOs from different olive varieties. In general, the `Blanqueta´ and `Chetoui´ oils showed high levels of secoiridoids; `Blanqueta´ was characterized by a high content of HTy, 3,4-DHPEA-EDA and *p*-HPEA-EDA, whereas `Chetoui´ showed high quantities of Ty, aldehydic form of elenoic acid linked to HTy (3,4-DHPEA-EA), aldehydic form of elenoic acid linked to Ty *(p*-HPEA-EA) and hydroxymethyl- decarboxymethyl ligustrosido aglycone (LA). Phenolic acids were not detected in some oils, and their content varied between olive cultivars. The main phenolic acids were cinnamic and elenolic acids, which reached the highest values in the `Blanqueta´ and `Chetoui´ oils, respectively. The `Sevillana´ and `Picual´ oils presented the highest content of flavonoids, mainly luteolin and apigenin, although small quantities of 6-methoxyapigenin and pinocembrin were observed, except in the `Chetoui´ and `Blanqueta´ VOOs. The `Sevillana´ oils presented the highest concentration of lignans.

The relationship between Hty/Ty and oleuropein/ligustroside derivatives was determined for all the oils. The `Blanqueta´ and `Habichuelero´ VOOs were the varieties with the highest Hty/Ty ratio (1.6 and 1.0 respectively), followed by `Chetoui´ with 0.4 and `Sevillana´ and `Picual´ with 0.3. Regarding the oleuropein/ligustroside derivatives ratio, `Sevillana´ and `Chetoui´ had the highest values (3.0), whereas `Habichuelero´ oil showed the lowest (2.0).

Table 2. Quantities of phenolic compounds in monovarietal VOOs.

Phenolic Compounds	`Habichuelero´	`Sevillana´	`Chetoui´	`Picual´	`Blanqueta´
Phenolic Alcohols					
Hydroxytyrosol (HTy)	24.1 ± 0.4 a	6.11 ± 0.0 b	25.3 ± 0.1 a	4.83 ± 0.1 b	43.5 ± 0.2 c
Tyrosol (Ty)	23.1 ± 0.4 a	17.7 ± 0.1 a	53.5 ± 0.2 b	16.1 ± 0.5 a	26.4 ± 0.1 a
Total Phenolic Alcohols	47.2 ± 0.7 a	23.8 ± 0.1 b	78.7 ± 0.3 c	20.9 ± 0.6 b	59.9 ± 0.4 c
Phenolic Acids					
Vanillic Acid	n.d.	3.89 ± 0.0 a	4.73 ± 0.0 b	3.52 ± 0.1 c	n.d.
Caffeic Acid	n.d.	0.52 ± 0.0 a	0.20 ± 0.0 b	n.d.	n.d.
Vainillin	1.34 ± 0.0 a	1.62 ± 0.0 b	1.53 ± 0.0 c	1.63 ± 0.0 b	n.d.
p-Cumaric Acid	0.85 ± 0.0 a	1.91 ± 0.0 b	2.99 ± 0.0 c	1.84 ± 0.1 d	0.84 ± 0.0 a
Ferulic Acid	n.d.	0.67 ± 0.0 a	0.71 ± 0. a	0.24 ± 0.0 b	n.d.
Cinnamic Acid	36.6 ± 0.6 a	13.6 ± 0.0 b	38.2 ± 0.2 a	22.4 ± 0.7 c	135 ± 0.8 d
Elenoic Acid	27.3 ± 0.5a	33.4 ± 0.1 b	225 ± 0.9 c	137 ± 4.0 d	19.8 ± 0.1 e
Total Phenolic Acids	66.1 ± 1.1 a	55.6 ± 0.2 b	273 ± 1.1 c	167 ± 5.0 d	156 ± 0.9 e
Secoiridoid Derivatives					
Oleuropein derivatives					
HTy-Ac	2.32 ± 0.0 a	2.53 ± 0.0 a	10.9 ± 0.0 b	23.4 ± 0.7 c	19.5 ± 0.1 d
Hidroxy Decarboxy methyl-OA	0.36 ± 0.0 a	0.37 ± 0.0 a	1.09 ± 0.0 b	0.30 ± 0.0 a	4.43 ± 0.0 c
3,4-DHPEA-EDA	774 ± 13 a	276 ± 0.8 b	786 ± 3.1 a	460 ± 14 c	2784 ± 16 d
Hydroxy OA	1.45 ± 0.0 a	0.31 ± 0.0 b	0.80 ± 0.0 c	0.16 ± 0.0 b	0.18 ± 0.0 b
3,4-DHPEA-EA	223 ± 3.7 a	29.4 ± 0.1 b	858 ± 3.4 c	261 ± 7.7 d	158 ± 0.9 e
Total oleuropein derivatives	1001 ± 16 a	309 ± 0.9 b	1657 ± 6.5 c	745 ± 21 d	2966 ± 17 e
Ligustroside derivatives					
Ty-AC	31.1 ± 0.5 a	10.5 ± 0.0 b	17.3 ± 0.1 c	19.9 ± 0.6 d	12.3 ± 0.1 e
p-HPEA-EDA	344 ± 5.8 a	82.4 ± 0.2 b	142 ± 0.6 c	155 ± 4.6 c	1029 ± 5.8 d
Hydroxymethyl-decarboxymethyl LA	8.75 ± 0.1 a	1.66 ± 0.0 b	26.0 ± 0.1 c	8.30 ± 0.2 a	2.77 ± 0.0 d
p-HPEA-EA	107 ± 1.8 a	9.40 ± 0.0 b	370 ± 1.5 c	104 ± 3.1 a	37.2 ± 0.2 d
Total Ligustroside derivatives	490 ± 8.2 a	104 ± 0.3 b	555 ± 2.2 c	287 ± 8.4 d	1081 ± 6.1 e
Total Secoiridoid Derivatives	1492 ± 25 a	413 ± 1.2 b	2212 ± 8.7 c	1031 ± 30 d	4047 ± 23 e
Lignans					
Pinoresinol	1.65 ± 0.0 a	22.2 ± 0.1 b	19.0 ± 0.1 c	9.08 ± 0.3 d	5.47 ± 0.0 e
1-acetoxipinoresinol	1.63 ± 0.0 a	38.6 ± 0.1 b	10.0 ± 0.0 c	3.51 ± 0.1 d	2.36 ± 0.0 e
Total Lignans	3.28 ± 0.1 a	60.8 ± 0.2 b	29.1 ± 0.1 c	12.6 ± 0.4 d	7.83 ± 0.0 e

Phenolic Compounds	`Habichuelero´	`Sevillana´	`Chetoui´	`Picual´	`Blanqueta´
Flavonoids					
Luteolin	0.71 ± 0.0 a	10.1 ± 0.0 b	6.91 ± 0.0 c	16.4 ± 0.5 d	1.21 ± 0.0 e
Apigenin	0.41 ± 0.0 a	5.93 ± 0.0 b	3.57 ± 0.0 c	4.80 ± 0.1 d	0.46 ± 0.0 a
6-methoxyapigenin	0.25 ± 0.0 a	0.71 ± 0.0 b	0.24 ± 0.0 a	1.03 ± 0.0 b	3.05 ± 0.0 c
Pinocembrin; 5,7-Dihydroxyflavon	3.47 ± 0.1 a	0.57 ± 0.0 b	n.d.	0.76 ± 0.0 b	n.d.
Total Flavonoids	4.84 ± 0.1 a	17.3 ± 0.0 b	10.7 ± 0.0 c	23.0 ± 0.7 d	4.72 ± 0.0 a
Total Phenolic Compounds	1613 ± 27 a	571 ± 1.6 b	2604 ± 10 c	1256 ± 37 d	4286 ± 24 e

*Data are expressed as nmol/g of VOO and are the mean + SD from three separate experiments (n=3). Different letters in values within a row are different ($p < 0.05$) by two-way ANOVA analysis.

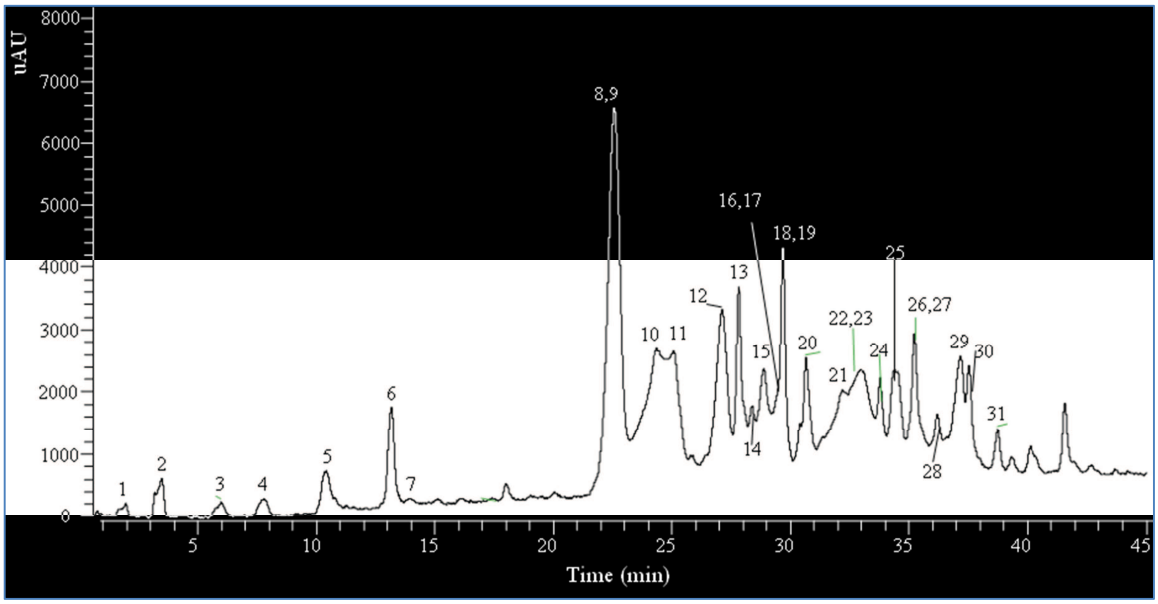

Fig. 1 UHPLC-HR-MS profile of phenolic compounds identified in virgin olive oil (`Picual´ variety). (PDA at 280). Peak identification numbers: 1, HTy; 2, Ty; 3, Vanillic Acid; 4, vainillin; 5, *p*-cumaric acid; 6 HTy-Ac; 7, ferulic acid ; 8, cinnamic acid; 9, 3,4-DHPEA-EDA; 10, hidroxy decarboxy metyl-OA; 11, 19, 20,21, 26, 3,4-DHPEA-EA and isomers; 12, *p*-HPEA-EDA; 13, pinoresinol; 14, 16, hydroxymethyl-decarboxymethyl LA; 15, 1-acetopinoresinol; 17, 29, 31, *p*-HPEA-EA and isomers; 18, elenoic acid; 22, luteolin; 23, 24, hydroxy OA; 25, Ty-Ac; 27, apigenin; 28, pinocembrin (5,7- dihydroxyflavon); 30, 6-methoxyapigenin

Stability of phenolic compounds during in vitro digestion

After the buccal, gastric and duodenal steps, the stability of each individual phenolic compound was checked by measuring the remaining amount of compounds in the Wp and Op fractions. The concentration of phenolic compounds for each digestion step and fraction was normalized with that observed in the corresponding VOO. The relative amounts of phenolic compounds (**Figure 2**) and their RI (**Table 3**) were calculated by phenolic groups. VOO phenolics did not show good stability during the *in vitro* digestion, only phenolic alcohols showing an increase in the RI at each digestive step (**Table 3**).

In general, the relative distribution of the phenolic compounds between the phases (Wp and Op) was different for each compound. After buccal digestion, an increase in phenolic alcohols was found with regard to the VOO, showing a higher solubility in the Wp during all the steps of digestion (**Figure 2A**). Their lowest recovery was observed after gastric digestion. The HTy showed a higher RI than Ty. HTy achieved the highest recovery index at the end of the *in vitro* digestion. Differences were observed between cultivars, `Sevillana´ oil showing the highest RI (693 %), followed by `Picual´ (516 %), whereas `Blanqueta´ achieved only 203% from to the initial content in the VOO (**Table 3**).

The partition coefficients of the phenolic acids between the Op and Wp showed a higher concentration in the Wp (**Figure 2B**). Important losses in phenolic acids were observed, mainly in the gastric and duodenal steps; their recovery index in the duodenal step oscillated between 6 and 22 %. Attention should be paid to the high stability of acids during the digestion in the mouth of the `Blanqueta´ VOO, although at the end of duodenal digestion, their recovery was lower (6 and 7 % respectively). The `Picual´ oil showed the highest recovery of phenolic acids. Ferulic acid was more stable during digestion for the `Sevillana´, `Chetoui´ and `Picual´ VOOs (**Table 3**).

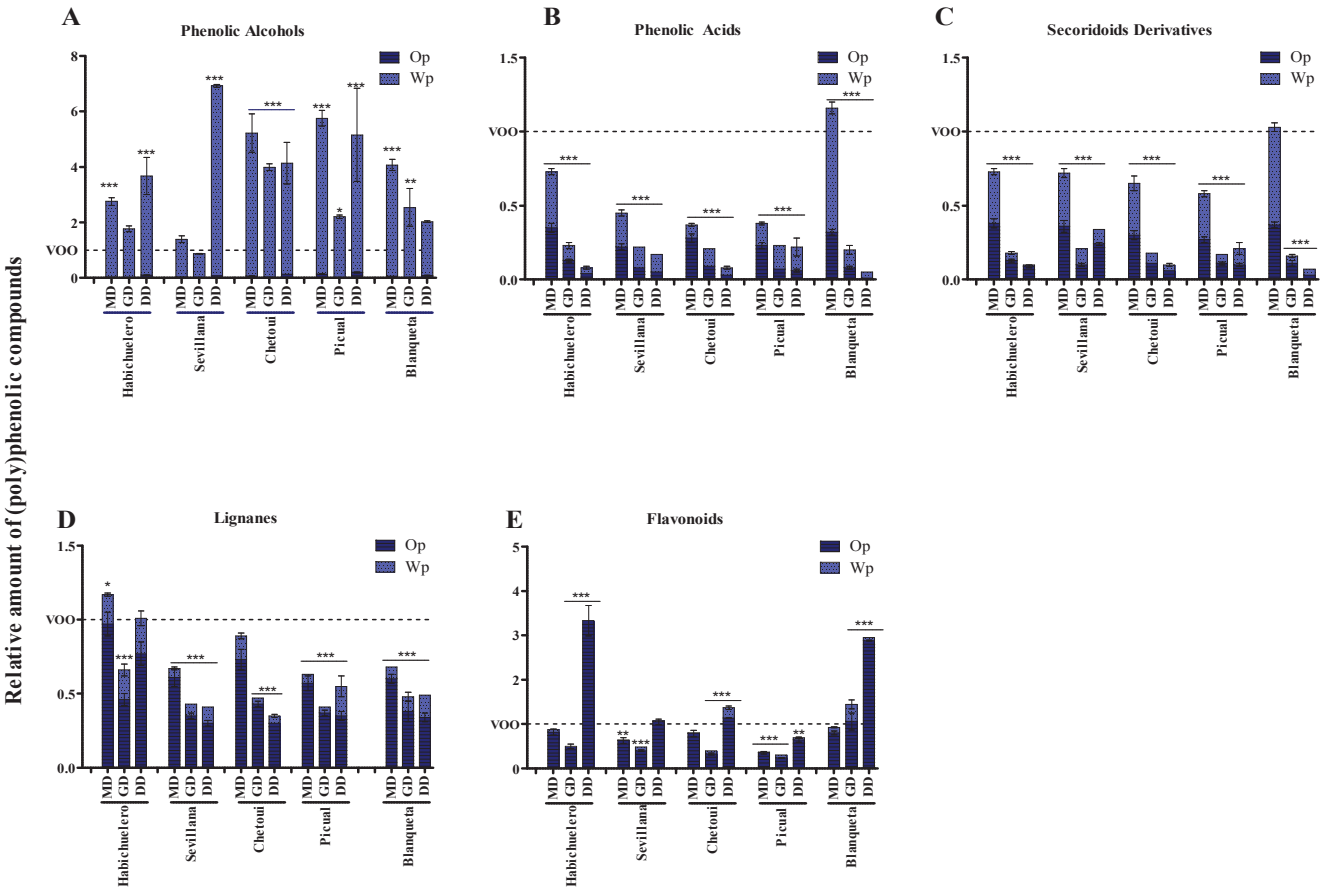

Figure 2. Relative amount of phenolic compounds refered to the initial quantities of phenolic compounds of each variety of VOO after of in vitro digestion from `Habichuelero´, `Sevillana´, `Chetoui´, `Picual´ and `Blanqueta´ VOO. MD: mouth digestion; GD: gastric digestion; DD: duodenal digestion. Data are the mean from three separate experiments, and error bars show the SD. Significant differences were determined relative to each variety of VOO, which was considered to be 1. Statistical levels of significance: * $p < 0.01$; ** $p < 0.05$; *** $p < 0.001$ by two-way ANOVA analysis and Bonferroni's post-hoc test. Op: oily phase; Wp: aqueous phase.

There was a higher concentration of secoiridoids in the Wp (**Figure 2C**). Secoiridoids had high stability during digestion in the mouth whereas in the gastric and duodenal steps these compounds showed important losses; their recovery index in the duodenal step oscillated between 7 and 34 %. Among the secoiridoids, derivatives of oleuropein (including HTy) were less stable during the digestive process than those of ligustroside (Ty). Oleuropein derivatives (3,4-DHPEA-EDA and 3,4-DHPEA-EA) achieved a RI at the end of digestion between 1 and 6 %. Ligustroside derivatives (*p*-HPEA-EDA and *p*-HPEA-EA) showed differences between monovarietal oils. *p*-HPEA-EA recovery for the `Sevillana´ VOO was 115% and 10% for `Blanqueta´. In general, a high recovery of HT and Ac was observed after duodenal digestion, values up to 440 % being obtained for the `Blanqueta´ VOO. Ty-Ac showed recoveries over 60 % in all oils. The content of tyrosol hexoside, a compound that was identified in the Wp after the mouth step, increased during the digestion process. Its recovery values in the duodenal step varied between 105 % (`Blanqueta´) and 1872 % (`Picual´) (**Table 3**).

The lignans were mainly recovered in the Op. Their content decreased after digestion in the mouth and the highest reduction was observed during the gastric step (**Figure 2D**). Their RI varied between 35 and 48 %, except for `Habichuelero´, which remained stable (102 %). In general, the recovery of 1-acetoxyprinoresinol was higher than that of pinoresinol at the end of digestion (Table 3).

During digestion, the flavonoids were mainly recovered in the Op. Flavonoids from the `Habichuelero´, `Chetoui´ and `Blanqueta´ VOOs were stable after digestion in the mouth. In general, important losses in flavonoids were observed in the gastric step. In contrast, after duodenal digestion an important increase in flavonoid compounds was observed because a new compound, 7,4'-Dihydroxyflavone, was identified (**Figure 2D; Table 3**). However, a low recovery was observed for luteolin and apigenin, between 0 – 21 % and 30 – 48 % respectively.

Table 3. Quantities of phenolic compounds in the two fractions (Op and Wp) after of *in vitro* digestion (mouth, gastric and duodenal) of monovarietal VOOs (nmol/g of VOO) and Recovery Index (RI%)

A.

Phenolic Compounds	`HABICHUELERO´										`SEVILLANA´									
	Mouth Step		RI (%)	Gastric Step		RI (%)	Duodenal Step		RI (%)	BI (%)	Mouth Step		RI%	Gastric Step		RI%	Duodenal Step		IR (%)	BI (%)
	Op	Wp		Op	Wp		Op	Wp			Op	Wp		Op	Wp		Op	Wp		
Phenolic Alcohols																				
Hydroxytyrosol (HTy)	2.04	94.6	418	0.12	57.8	251	2.85	147	649	670	0.91	22.3	380	0.41	13.0	220	0.52	150	2461	2452
Tyrosol (Ty)	n.d.	33.7	140	n.d.	25.6	106	1.73	21.9	98	104	n.d.	9.89	56	n.d.	7.20	41	1.34	13.5	84	76
Total Phenolic Alcohols	***2.04***	***128***	***276***	***0.12***	***83.4***	***177***	***4.58***	***169***	***367***	393	***0.91***	***32.2***	139	***0.41***	***20.2***	87	***1.87***	***163***	693	685
Phenolic Acids																				
Vanillic Acid	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-	n.d.	1.29	33	0.35	1.01	35	n.d.	2.09	54	54
Caffeic Acid	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-	n.d.	0.27	52	n.d.	n.d	0	n.d.	n.d.	0	0
Vainillin	0.72	n.d.	54	0.48	n.d.	36	n.d.	n.d.	0	0	0.77	n.d.	47	0.66	n.d.	41	0.36	n.d.	22	0
p-Cumaric Acid	n.d.	0.39	46	n.d.	0.33	39	n.d.	0.49	49	49	n.d.	1.80	94	0.38	0.25	33	0.20	0.70	47	37
Ferulic Acid	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-	n.d.	0.44	66	0.16	n.d.	23	0.11	0.44	81	65
Cinnamic Acid	7.98	16.5	67	1.46	3.01	12	n.d.	n.d.	0	0	3.42	0.92	32	0.51	0.66	9	n.d.	0.92	7	7
Elenoic Acid	14.7	8.04	83	6.79	2.99	36	2.89	2.20	19	9	7.94	8.06	48	2.64	6.08	26	2.08	2.35	13	7
Total Phenolic Acids	***23.4***	***25.0***	***73***	***8.73***	***6.33***	***23***	***2.89***	***2.70***	***8***	4	***12.1***	***12.8***	45	***4.70***	***8.00***	23	***2.75***	***6.49***	17	12
Secoiridoid Derivatives																				
Oleuropeinderivatives																				
HTy-Ac	n.d.	0.84	36	n.d.	2.44	105	4.95	5.12	221	243	2.28	1.06	133	n.d.	0.64	25	0.80	2.51	131	99
Hidroxy Decarboxy methyl-OA*	n.d.	0.55	155	n.d.	0.37	103	n.d.	0.18	51	51	0.02	0.36	102	0.00	0.11	30	n.d.	n.d.	0	0
3,4-DHPEA-EDA	166	337	65	32.7	22.7	7	n.d.	9.76	1	1	70.3	116	67	7.21	17.8	9	1.93	9.79	4	4
Hydroxy- OA*	0.36	0.99	93	n.d.	1.14	30	n.d.	1.30	90	90	n.d.	-	n.d.	n.d.	n.d.	-	n.d.	n.d.	-	-
3,4-DHPEA-EA	100	65.1	74	39.6	37.3	35	9.46	0.65	5	0	14.0	10.4	83	5.84	4.75	36	0.50	0.42	3	1
Total oleuropeina derivatives	*266*	*405*	*67*	*72.3*	*64.0*	*14*	*14.4*	*17.0*	*3*	2	*86.6*	*128*	69	*13.1*	*23.3*	12	*3.23*	*12.7*	5	4

A.

Phenolic Compounds	`HABICHUELERO´										`SEVILLANA´									
	Mouth Step		RI (%)	Gastric Step		RI (%)	Duodenal Step		RI (%)	BI (%)	Mouth Step		RI%	Gastric Step		RI%	Duodenal Step		IR (%)	BI (%)
	Op	Wp		Op	Wp		Op	Wp			Op	Wp		Op	Wp		Op	Wp		
Ligustroside derivatives																				
Ty-AC	26.8	1.89	92	20.8	2.45	75	15.5	3.32	60	11	8.21	0.39	82	6.98	4.06	105	6.84	9.57	156	91
p-HPEA-EDA	186	83.3	78	66.8	3.06	20	95.4	3.05	29	1	45.3	15.2	73	17.2	9.66	33	89.7	4.73	115	6
Hydroxymethyl-decarboxymethyl-LA**	5.29	2.29	87	1.45	0.29	20	n.d.	n.d.	0	1	0.17	0.78	58	0.12	0.43	34	0.03	0.31	20	19
p-HPEA-EA	76.57	28.5	99	38.3	9.44	45	8.13	0.05	8	0	7.30	1.59	95	3.49	0.58	43	0.91	0.15	11	2
Tyrosol hexoside	n.d.	0.60	60	n.d.	1.60	160	n.d.	7.20	720	720	n.d.	0.89	89	n.d.	5.24	524	n.d.	11.8	1182	1182
Total Ligustroside derivatives	*294*	*117*	*84*	*127*	*16.9*	*29*	*119*	*13.6*	*27*	3	*61.0*	*18.9*	77	*27.8*	*20.0*	46	*97.5*	*26.6*	119	26
Total Secoiridoid Derivatives	***561***	***521***	***73***	***200***	***80.8***	***19***	***133***	***30.6***	***11***	2	***148***	***147***	71	***40.8***	***43.2***	20	***101***	***39.3***	34	10
Lignans																				
Pinoresinol	1.37	0.37	105	0.81	0.35	70	1.05	0.45	91	27	13.8	0.24	63	8.67	0.78	43	6.80	0.54	33	2
1-acetoxipinoresinol	1.80	0.30	129	0.70	0.31	62	1.49	0.35	113	21	23.5	3.28	69	12.6	3.82	42	11.3	5.93	45	15
Total Lignans	***3.18***	***0.67***	***117***	***1.51***	***0.65***	***66***	***2.54***	***0.80***	***102***	24	***37.3***	***3.52***	67	***21.2***	***4.60***	43	***18.1***	***6.47***	40	11
Flavonoids																				
Luteolin	0.54	n.d.	75	0.23	n.d.	32	n.d.	n.d.	0	0	5.59	0.12	56	2.43	n.d.	24	1.46	0.33	18	3
Apigenin	0.22	n.d.	54	0.27	n.d.	66	0.14	n.d.	35	0	4.43	0.10	76	3.62	0.34	67	1.77	n.d.	30	0
6-methoxyapigenin	0.22	n.d.	87	n.d.	n.d.	0	n.d.	n.d.	0	0	0.53	n.d.	75	0.48	1.30	250	0.36	0.19	77	27
Pinocembrin (5,7-Dihydroxyflavon)	3.02	0.27	95	1.92	n.d.	55	1.19	n.d.	34	0	0.44	n.d.	78	0.36	n.d.	64	0.22	n.d.	38	0
7,4'-Dihydroxyflavone	n.d.	n.d.	-	n.d.	n.d.	-	14.8	n.d.	1480	-	n.d.	n.d.	-	n.d.	n.d.	-	14.4	n.d.	1442	0
Total Flavonoids	***3.99***	***0.27***	***88***	***2.42***	***n.d.***	***50***	***16.1***	***n.d.***	***333***	0	***11.0***	***0.22***	65	***6.90***	***1.64***	49	***18.2***	***0.53***	108	3
Total Phenolic Compounds	***593***	***675***	***79***	***212***	***171***	***24***	***160***	***203***	***22***	10	***209***	***195***	71	***74.1***	***77.7***	27	***142***	***216***	63	36

B.

Phenolic Compounds	`CHETOUI´										`PICUAL´									
	Mouth Step		RI (%)	Gastric Step		RI (%)	Duodenal Step		RI (%)	BI (%)	Mouth Step		RI (%)	Gastric Step		RI (%)	Duodenal Step		RI (%)	BI (%)
	Op	Wp		Op	Wp		Op	Wp			Op	Wp		Op	Wp		Op	Wp		
Phenolic Alcohols																				
Hydroxytyrosol (HTy)	2.83	321	1277	1.48	230	915	5.16	254	1023	1108	1.84	98.6	2079	0.31	34.3	716	1.99	78.2	1661	1332
Tyrosol (Ty)	2.54	85.3	164	1.82	81.0	155	4.85	62.0	125	129	0.99	19.0	124	n.d.	11.8	74	2.20	25.4	172	130
Total Phenolic Alcohols	***5.37***	***406***	522	***3.30***	***311***	399	***10.0***	***316***	414	443	***2.83***	***118***	576	***0.31***	***46.1***	222	***4.19***	***104***	516	408
Phenolic Acids																				
Vanillic Acid	n.d.	1.55	33	0.13	0.65	17	n.d.	1.76	37	37	n.d.	1.34	38	n.d.	0.47	13	n.d.	2.19	62	62
Caffeic Acid	n.d.	n.d.	0	n.d.	n.d.	0	n.d.	n.d.	0	0	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-
Vainillin	0.67	0.22	58	0.44	0.23	44	n.d.	n.d.	0	0	0.60	0.25	52	0.43	0.29	44	0.32	1.28	98	65
p-Cumaric Acid	0.22	1.26	50	0.37	0.22	20	n.d.	n.d.	0	0	n.d.	0.51	28	0.30	0.30	32	0.18	1.42	87	64
Ferulic Acid	0.20	0.27	66	0.19	0.03	32	n.d.	0.77	109	109	n.d.	0.13	54	n.d.	n.d.	0	n.d.	0.60	253	253
Cinnamic Acid	6.52	1.69	21	1.46	0.72	6	n.d.	n.d.	0	0	2.94	8.24	50	0.77	0.50	6	n.d.	0.35	2	2
Elenoic Acid	70.0	18.4	39	21.1	30.6	23	9.04	9.97	8	5	34.5	13.8	35	9.79	25.7	26	8.87	21.5	22	13
Total Phenolic Acids	***77.6***	***25.2***	38	***23.7***	***33.1***	21	***9.04***	***13.3***	8	5	***38.1***	***24.6***	37	***11.3***	***27.2***	23	***9.38***	***27.6***	22	13
Secoiridoid Derivatives																				
Oleuropeinderivatives																				
HTy-Ac	3.49	3.18	61	2.45	5.58	74	4.58	9.52	129	97	7.12	10.2	74	2.89	7.90	46	4.93	4.82	42	17
Hidroxy Decarboxy methyl-OA*	0.02	1.29	120	0.01	0.51	48	n.d.	0.13	12	12	0.00	0.24	84	n.d.	0.16	52	n.d.	0.13	43	43
3,4-DHPEA-EDA	134	232	47	30.00	62.01	12	3.70	33.2	5	5	60.3	169	50	15.8	16.9	7	3.62	22.5	6	4
Hydroxy- OA*	n.d.	0.47	58	n.d.	n.d.	0	n.d.	n.d.	0	0	n.d.	0.12	76	n.d.	n.d.	0	n.d.	0.15	94	94
3,4-DHPEA-EA	243	391	74	88.17	56.28	17	28.6	4.29	4	1	80.7	87.5	64	34.6	6.91	16	7.57	1.05	3	0
Total oleuropeina derivatives	*380*	*627*	61	*121*	*124*	15	*36.9*	*47.1*	5	3	*148*	*267*	56	*53.3*	*31.8*	11	*16.1*	*28.7*	6	4

B.

Phenolic Compounds	`CHETOUI´										`PICUAL´									
	Mouth Step		RI (%)	Gastric Step		RI (%)	Duodenal Step		RI (%)	BI (%)	Mouth Step		RI (%)	Gastric Step		RI (%)	Duodenal Step		RI (%)	BI (%)
	Op	Wp		Op	Wp		Op	Wp			Op	Wp		Op	Wp		Op	Wp		
Ligustroside derivatives																				
Ty-AC	10.3	4.70	87	7.06	4.00	64	6.83	4.89	68	32	11.57	1.50	66	12.1	2.04	71	8.92	15.5	122	64
p-HPEA-EDA	93.6	31.1	88	31.2	12.1	31	74.1	21.2	67	15	68.9	31.8	65	27.4	11.9	25	71.9	7.71	51	4
Hydroxymethyl-decarboxymethyl-LA**	13.5	8.13	83	4.99	1.33	24	n.d.	0.11	0	0	3.12	1.33	54	0.99	n.d.	12	n.d.	0.22	3	3
p-HPEA-EA	177	93.5	73	74.2	13.8	24	7.04	0.17	2	0	49.8	12.4	60	23.4	15.2	37	5.05	47.4	50	38
Tyrosol hexoside	n.d.	1.77	177	n.d.	8.20	820	n.d.	17.2	1720	1720	n.d.	0.81	81	n.d.	3.90	390	n.d.	18.7	1872	1872
Total Ligustroside derivatives	*295*	*139*	78	*117*	*39.4*	28	*88.0*	*43.6*	24	8	*133*	*47.9*	63	*63.8*	*33.0*	34	*85.9*	*89.5*	61	26
Total Secoiridoid Derivatives	***675***	***766***	65	***238***	***164***	18	***125***	***90.7***	10	4	***282***	***315***	58	***117***	***64.8***	18	***102***	***118***	21	10
Lignans																				
Pinoresinol	14.8	3.96	98	8.82	0.68	50	6.24	0.55	36	3	5.47	0.38	64	3.51	0.44	43	2.83	1.33	46	12
1-acetoxipinoresinol	6.55	0.61	71	3.63	0.54	42	2.50	0.78	33	8	1.74	0.33	59	1.14	0.10	35	1.58	1.21	79	28
Total Lignans	***21.3***	***4.56***	89	***12.4***	***1.22***	47	***8.73***	***1.33***	35	5	***7.21***	***0.71***	63	***4.64***	***0.54***	41	***4.41***	***2.54***	55	17
Flavonols																				
Luteolin	5.34	n.d.	77	1.60	n.d.	23	n.d.	0.71	10	10	4.60	0.11	29	2.13	0.17	14	1.12	n.d.	7	0
Apigenin	2.83	0.08	82	1.71	0.62	65	n.d.	1.30	37	37	2.38	0.14	53	2.29	0.28	54	1.23	0.32	32	6
6-methoxyapigenin	0.29	n.d.	122	0.26	0.16	176	0.33	0.43	319	182	0.56	n.d.	54	n.d.	1.08	105	0.18	0.68	83	74
Pinocembrin (5,7-Dihydroxyflavon)	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-	0.73	n.d.	97	1.17	n.d.	155	0.53	n.d.	70	0
7,4'-Dihydroxyflavone	n.d	n.d.	-	n.d.	n.d.	-	11.9	n.d.	1187	0	n.d.	n.d.	-	n.d.	n.d.	-	11.8	n.d.	1179	0
Total Flavonols	***8.46***	***0.08***	80	***3.57***	***0.78***	41	***12.2***	***2.45***	137	23	***8.27***	***0.25***	37	***5.59***	***1.54***	31	***14.8***	***1.00***	69	4
Total Phenolic Compounds	***788***	***1202***	76	***281***	***510***	30	***165***	***424***	23	17	***338***	***458***	63	***138.9***	***140***	22	***135***	***253***	31	19

C.

Phenolic Compounds	`BLANQUETA´									
	Mouth Step		RI	Gastric Step		RI	Duodenal Step		RI	BI
	Op	Wp	(%)	Op	Wp	(%)	Op	Wp	(%)	(%)
Phenolic Alcohols										
Hydroxytyrosol (HTy)	3.05	213	498	0.14	127	291	2.61	92.5	218	212
Tyrosol (Ty)	1.52	66.0	256	0.20	51.08	194	3.93	43.0	178	163
Total Phenolic Alcohols	***4.57***	***280***	407	***0.34***	***178***	254	***6.54***	***136***	203	194
Phenolic Acids										
Vanillic Acid	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-
Caffeic Acid	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-
Vainillin	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-
p-Cumaric Acid	n.d.	0.23	27	0.15	0.19	40.0	0.15	n.d.	18	0
Ferulic Acid	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-
Cinnamic Acid	41.9	101	106	6.57	0.32	5.09	n.d.	0.36	0	0
Elenoic Acid	7.26	30.1	189	6.15	18.6	125	2.06	6.37	43	32
Total Phenolic Acids	***49.2***	***133***	116	***12.9***	***19.3***	20.6	***2.20***	***6.73***	6	4
Secoiridoid Derivatives										
Oleuropeinderivatives										
HTy-Ac	4.74	19.4	124	4.52	32.6	191	16.9	68.7	440	353
Hidroxy Decarboxy methyl-OA*	0.21	10.4	240	n.d.	3.27	73.9	n.d.	1.89	43	43
3,4-DHPEA-EDA	862	2078	106	135	60.7	7.03	5.58	24.6	1	1
Hydroxy- OA*	0.15	n.d.	83	n.d.	n.d.	0	n.d.	0.12	65	65
3,4-DHPEA-EA	57.8	70.4	81	23.6	16.7	25.5	3.32	2.21	3	1
Total oleuropeina derivatives	*925*	*2178*	105	*163*	*113*	9.32	*25.8*	*97.5*	4	3

Ligustroside derivatives										
Ty-AC	9.94	6.79	136	9.59	5.17	120	7.48	10.1	143	82
p-HPEA-EDA	546	409	93	276	49.2	31.5	65.2	33.8	10	3
Hydroxymethyl-decarboxymethyl-LA**	1.05	0.95	72	0.53	0.17	25.3	n.d.	n.d.	0	2
p-HPEA-EA	22.9	55.6	211	15.2	13.6	77.5	2.80	35.5	103	96
Tyrosol hexoside	n.d.	0.72	72	n.d.	2.24	224	n.d.	1.05	105	105
Total Ligustroside derivatives	*580*	*473*	97	*301*	*70*	34	*76*	*80*	14	7
Total Secoiridoid Derivatives	***1506***	***2651***	103	***464***	***184***	16	***101***	***178***	7	4
Lignans										
Pinoresinol	3.47	0.37	70	2.03	0.33	43.2	1.09	0.75	33	14
1-acetoxipinoresinol	1.24	0.25	63	0.91	0.46	57.7	1.56	0.39	83	17
Total Lignans	***4.71***	***0.62***	68	***2.94***	***0.79***	47.6	***2.64***	***1.14***	48	15
Flavonols										
Luteolin	0.86	0.13	82	0.60	0.12	59.4	0.25	n.d.	21	0
Apigenin	0.31	0.18	105	2.74	0.14	620	0.22	n.d.	48	0
6-methoxyapigenin	2.62	0.31	96	1.68	1.56	106	0.90	0.31	40	10
Pinocembrin (5,7-Dihydroxyflavon)	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-	-
7,4'-Dihydroxyflavone	n.d.	n.d.	-	n.d.	n.d.	-	12.2	n.d.	1223	0
Total Flavonols	***3.79***	***0.63***	94	***5.01***	***1.82***	145	***13.6***	***0.31***	295	8
Total Phenolic Compounds	***1568***	***3064***	108	***485***	***383***	20	***126***	***322***	10	8

All data are expressed as the average of the three replicates of different digested VOO. To simply the results shown the SD were omitted because they were lower than 10% of the values. Op: Oily phase; Wp: aqueous phase. RI %: Recovery Index has been calculated according Eq.(1). BI %: Bioaccesibility Index has been calculated according Eq.(2). n.d.: not detected. -OA*: oleuropein aglycone. -LA**: ligustroside aglycone

Bioaccessibility of phenolic compounds after in vitro digestion

Figure 3 shows the bioaccessibility values for each phenolic group after duodenal digestion of the VOOs analysed. The bioaccessibility of total phenolic compounds varied greatly between olive cultivars. `Sevillana´ presented the highest BI (36 %), whereas `Blanqueta´ showed the lowest (8 %) (**Figure 3A**).

Phenolic alcohols showed the highest bioaccessibility values, from 194 % to 685 % for the `Blanqueta´ and `Sevillana´ olive oils respectively. HTy was the most bioaccessible (212 % to 2452 % for `Blanqueta´ and `Sevillana´ respectively), whereas Ty showed a BI > 100% for most of the olive cultivars, although the value obtained for the `Sevillana´ olive oil was below 76%. (**Figure 3B; Table 3**).

The BI of phenolic acids varied for each compound and olive variety (**Figure 3C**). In general, `Picual´ and `Sevillana´ showed a higher BI for phenolic acids. Cinnamic and elenoic were the acids with the lowest bioaccessibility for all varieties (**Table 3**).

In general, secoiridoids showed low bioaccessibility (2 – 10 %). The high level of hydrolysis of oleuropein derivatives during digestion resulted in a poor BI, lower than 5 % (**Figure 3D**). 3,4-DHPEA-EDA and 3,4-DHPEA-EA had very low bioaccessibility with values of 5 % and 1 %, respectively. The BI of HTy-Ac was over 100% in all varieties except for the `Picual´ VOO (17 %). However, ligstroside derivatives presented low bioaccessibility (7-26 %), although these values were higher than those of oleuropein. In the `Chetoui´ oils, the BI of *p*-HPEA-EDA reached 15 % while with other cultivars, values lower than 6 % were obtained; *p*-HPEA-EA had a BI of 96% for the `Blanqueta´ VOO and 38% for `Picual,´ while for the other varieties the values were lower than 2 %. Tyrosol hexoside is a compound derived from the digestion process and its content was very high in the bioaccessible fraction of all the oils, the `Picual´ and `Chetoui´ oils presenting the highest values (**Table 3**).

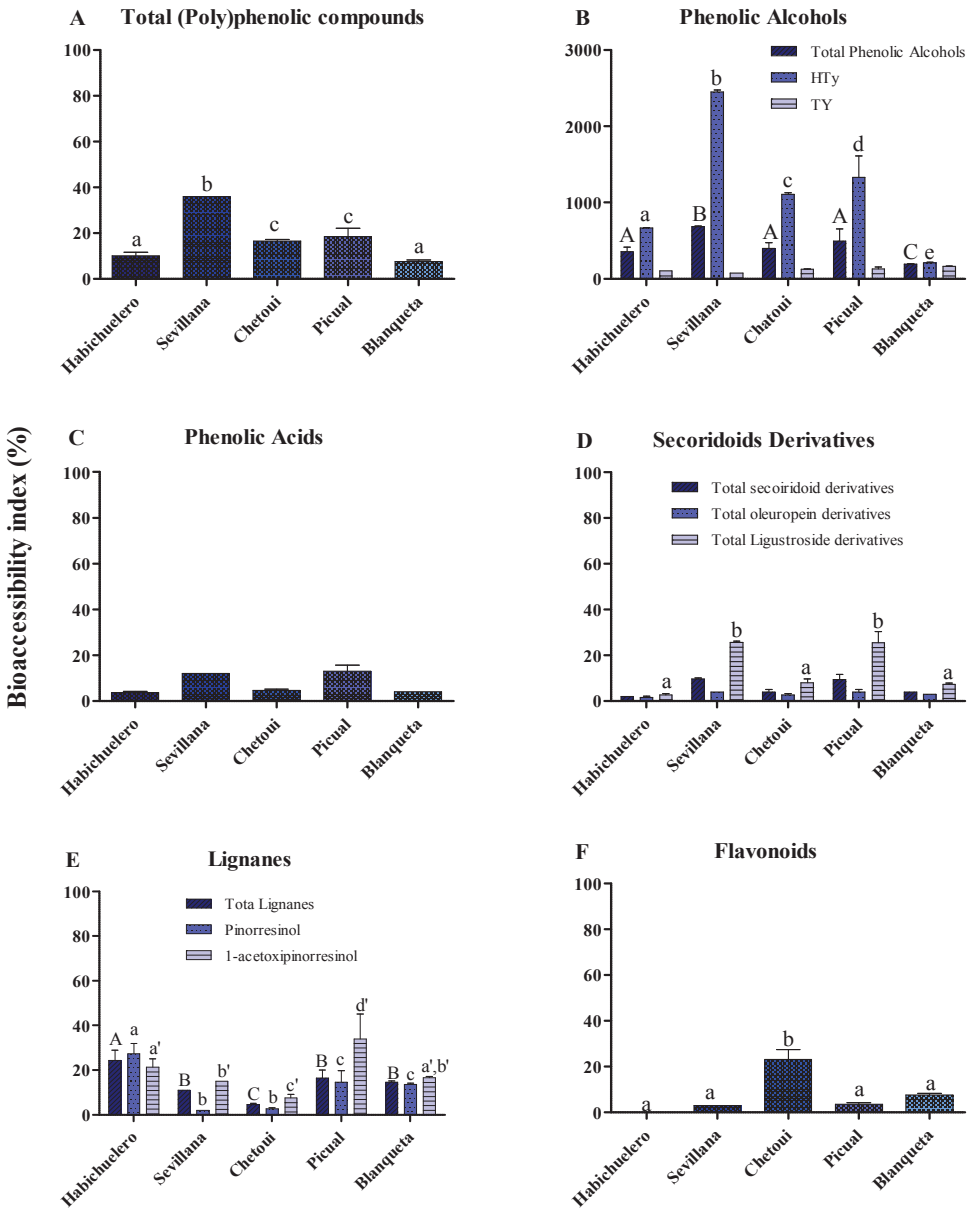

Fig. 3 Bioaccesibility Index (%) by groups of phenolic compounds of each variety of VOO after of in vitro digestion from `Habichuelero´, `Sevillana´, `Chetoui´, `Picual´ and `Blanqueta´ VOO. Bioaccesibility Index has been calculated according Eq.(2). Data are the mean from three separate experiments, and error bars show.

The bioaccessibility of lignans was low; the highest values were observed for the `Habichuelero´ VOO (24%) and the lowest for the `Chetoui´ oil (5%) (**Figure 3E**). In general, 1-acetoxipinoresinol was more bioaccessible than pinoresinol.

Finally, the flavonoids also presented low bioaccessibility, ranging from 0 to 8%, except for `Chetoui´ (23%) (**Figure 3F**). Luteolin and apigenin were not bioaccessible or had very low BI values. The flavonoid with the highest bioaccessibility value was 6-metoxyapigenin, reaching up to 182% for the `Chetoui´ and 74% for the `Picual´ VOO (**Table 3**).

Antioxidant Activity

The radical scavenging capacity of the bioaccessible fraction (BF) was measured by DPPH, ABTS and ORAC assays. The DPPH radical assay showed that the antiradical activity of the BF of all the varieties of VOOs exerted a similar antioxidant activity to TOC at the minimum ratios used (mol TOC/mol DPPH). The `Blanqueta´ and `Sevillana´ VOO-BF reached higher values, 15 and 19 % respectively. When the BF was diluted (1:100 v/v) the activity was slightly lower in all varieties (**Figure 4A**).

The ABTS assay showed that the undiluted VOO-BF reached a radical scavenger activity (RSA) $\geq$ 30%, `Sevillana´ and `Picual´ oils achieving the highest values (58 and 53%); when the BF was diluted to 1:100 v/v the RSA oscillated between 5 and 19% (**Figure 4B**).

Peroxyl radical scavenging activity measured by the $ORAC_{FL}$ assay was not detected for the undiluted BFs whereas dilution to 1:100 v/v showed a protective effect, with significant differences between samples. `Chetoui´ VOO-BF exerted higher protection against the peroxyl radical than other samples (**Figure 4C**).

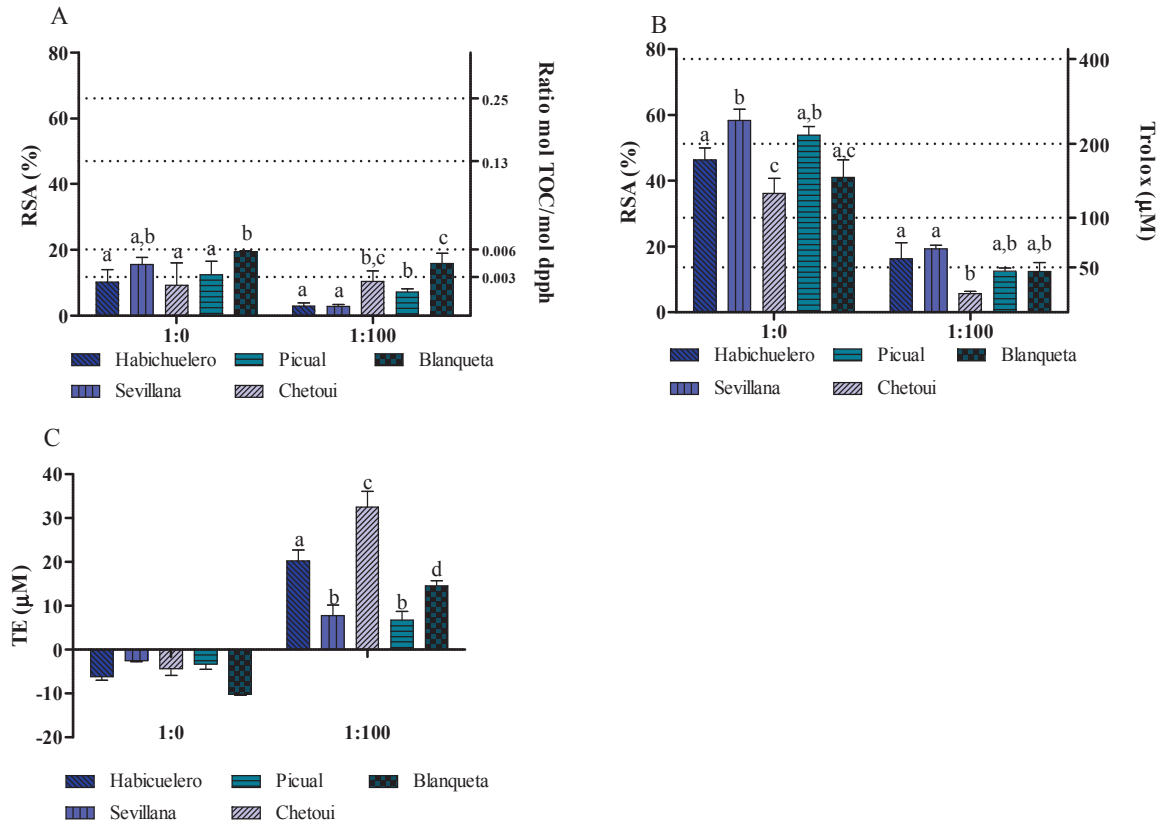

Fig. 4. Antioxidant activity from `Habichuelero´, `Sevillana´, `Picual´, `Chetoui´ and `Blanqueta´ VOO-BF quantified as Radical Scavenging Activity (RSA) by **A**. DPPH assay (%RSA at 60min) and **B**. ABTS assay (%RSA at 60 min); **C**. Antioxidant activity quantified as Trolox Equivalent (TE) by $ORAC_{FL}$ assay. Trolox and α-tocopherol (TOC) were used as antioxidant references. Wp solution or BF were used pure (1:0 v/v) and diluted (1:100 v/v). Data are the mean and error bars show the SD of three replicates. Different letters indicate significant difference ($p < 0.05$) by two-way ANOVA analysis and Bonferroni's post-hoc test.

Analysis of intracellular reactive oxygen species

In the Caco-2 cell line, cell survival decreased for both the undiluted BF (dilution 1:0) and the one diluted to 1:1 v/v in MEM. However, when the BF was diluted to 1:100

v/v in MEM, the number of cells increased (Figure 5A). Thus, the BF 1:100 dilution was selected for the ROS assays.

The BF in Caco-2 cells promoted an antioxidant effect since ROS levels decreased with regard to the untreated control. A 20% decrease in ROS was observed for the `Habichuelero´, `Sevillana´ and `Chetoui´ VOO-BFs, whereas lower values were obtained for `Picual´ and `Blanqueta´ (Figure 5B). When oxidative stress was induced by the addition of H_2O_2, the BF had no effect on the ROS level with regard to the positive control.

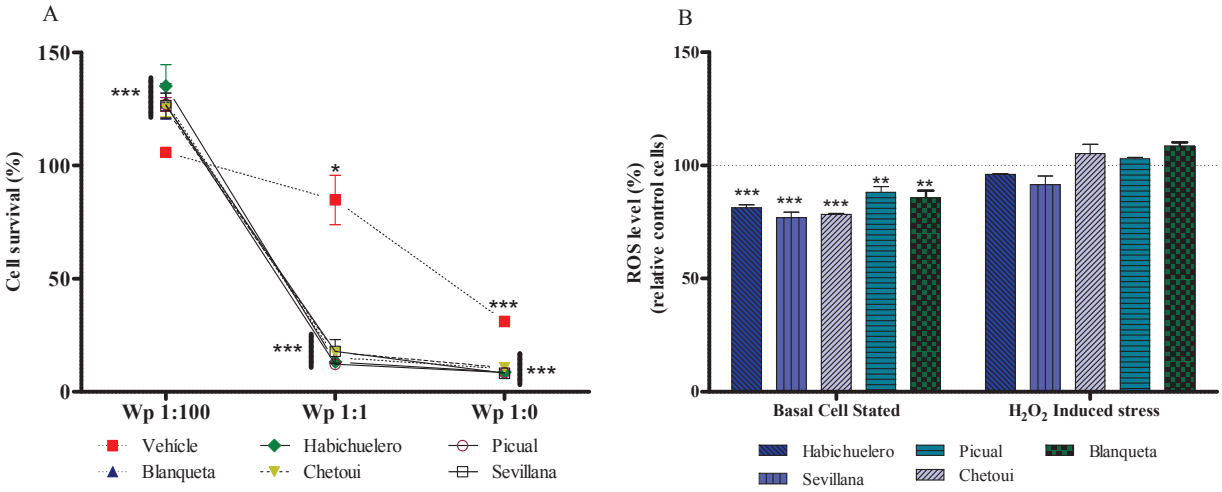

Fig. 5 A. Citotoxicity from `Habichuelero´, `Sevillana´, `Picual´, `Chetoui´, `Blanqueta´ VOO-BF and vehicle (digestive juice) at dilutions 1:100, 1:1 and 1:0 v/v in Caco-2 cells line after 24 h. **B**. ROS level of Caco-2 in basal state of the cells and after to exposure to $H_2O_2$ with treatments from `Habichuelero´, `Sevillana´, `Picual´, `Chetoui´ and `Blanqueta´ VOO-BF at dilution 1:100 v/v after 24 h. Numerical values are represented as the mean (± SEM) of three independent experiment carried out in triplicate. Significant differences were determined relative to each control, which was considered to be 100%. Statistical levels of significance: * $p < 0.01$; ** $p < 0.05$; *** $p < 0.001$ by two-way ANOVA analysis and Bonferroni's post-hoc test.

DISCUSSION

As reported, secoiridoid derivatives from oleuropein and ligustroside are the major phenolics from VOO among the varieties studied [6, 7, 35], although the relative proportion of each compound and its concentration varied between olive cultivars, and other factors such as harvesting timing and fruit ripening should be taken into account [36, 37].

The release of a compound from the ingesta to the gastrointestinal tract (bioaccessibility) is a prerequisite for its uptake and bioavailability in the human body [28, 29]. Methods for determining the bioavailability or bioaccessibility of compounds involve the simulation of experiments performed in a laboratory (*in vitro*) or human models (*in vivo*) [38]. *In vivo* experiments in humans or animals may provide the best information about the (relative) bioavailability of the intake of compounds [39]. However, *in vitro* techniques reduce the need for experimental animals and the large number of different products / matrices they demand, as well as being reproducible and the involving standardized procedures [40]. In this study, an *in vitro* digestion model involving three steps (mouth, stomach and small intestine) was used to evaluate the effect of each step of digestion on VOO phenolics and their bioaccessibility. This set of experiments made it possible to establish the extent of the degradation or transformation of phenolics from VOO along the gastrointestinal tract, revealing the amount and forms able to reach the gut epithelium for potential uptake and their possible beneficial effects, as described previously with berry phenolic compounds [41, 42].

Dinnella et al. [43] reported that VOO phenolic compounds were transferred to the aqueous fraction after digestion *in vitro* [43]. We found that the polarity of the phenolic compounds was an important factor for their distribution between the Op and Wp phases, as described by Soler et al. [27]. The more polar compounds, phenolic alcohol and acids, were detected mainly in the Wp, whereas the hydrophobic compounds (lignans and

flavonoids) were predominantly present in the Op. The distribution of scoiridoid derivatives between Op and Wp was variable during digestion (**Figure 2**).

Several authors have suggested that phenolics are transformed during digestion into other compounds with different structures, chemical properties and bioaccesibilities. These transformations are more important after the intestinal phase, since phenolic compounds are highly sensitive to alkaline conditions [44]. After mouth digestion, we observed the precipitation and hydrolysis of phenolics, with consequent reductions in their recovery, except for phenolic alcohols. We found that secoiridoids were hydrolysed (80-84%) and then the concentration of HTy, Ty and tyrosol hexoside increased during digestion. These results were similar to those reported by Corona et al [45], who found that conjugated forms of HTy and Ty underwent a rapid hydrolysis under gastric conditions, resulting in significant increases in the amount of free HTy and Ty. However, HTy-Ac and Ty-Ac showed a high stability after gastric digestion, in agreement with Pereira-Caro et al [19], who reported a higher stability of the ether functional group present in HTy-Ac.

In contrast, Soler et al [27] reported that Ty, HTy, 3.4-DHPEA-EDA, p-HPEA-EDA and elenolic acid showed good stability in the gastric phase. Pinto et al [18] studied the effect of low pH on the stability of 3,4-DHPEA-EDA and 3,4-DHPEA-EA, indicating that both may be relatively stable during their transit through the stomach and the small intestine *in vivo*.

In general, phenolic acids have low stability under gastric digestion [38], while flavonoids [27] and lignans [27] are not affected by gastric conditions; these results agree with our findings, since the recoveries varied from 41 to 66% for lignans and 31 to 117% for flavonoids depending on the VOO variety. A previous study reported high levels of interaction between phenolic acids and VOO phenolic extract and mucin for 1 min of reaction, creating complexes that could be insoluble [46].

Duodenal digestion greatly affected the recovery of the phenolic compounds. Only HTy and Ty showed higher recovery due to the hydrolysis of secoiridoids. Previous studies reported a low stability of secoiridoids in the intestinal digestion phase [18], achieving a recovery of 10% for secoiridoid derivatives, which was not compensated by free HTy and Ty [27].

The pH of the small intestine may slightly reduce the digestive stability of HTy and its alkyl derivatives depending of their lipophilic nature[19]. In agreement with our results, HTy-Ac was observed to be highly stable after the duodenal digestion of all the VOOs (except for `Picual´), as was Ty-Ac from `Sevillana´, `Picual´ and `Blanqueta´ olive oils. The higher lipophilicity of these compounds probably enhances their affinity for bile salts, micellar and vesicular structures of higher molecular weight being formed during digestion [47]. The high recovery of ferulic acid and the increase in the recovery of vanillic acid after the duodenal step in comparison with the gastric step from `Sevillana´, `Chetoui´, `Picual´ oils may be explained by the ring scission of other phenolic compounds in the food matrix (flavonoids), which leads to a higher concentration of these simple phenolic molecules and/or different rates of solubilisation [48]. The stability of lignans under duodenal conditions was higher in the `Habichuelero´ VOO, giving recoveries between 35 and 55%, as reported previously by Soler et al [27]. In this study, the main flavonoids, apigenin and luteolin, were unstable and could be hydrolysed to 7,8 dihydroxyflavone, which not was present in the VOO. Other studies have reported the instability of flavonoids [27].

The digestive stability of the VOO phenolic compounds was low. The differences observed between olive varieties may be explained by their different phenolic composition and lipid fraction (data not reported). The lipid fraction can improve the digestibility of phenolic compounds, thus giving better solubilisation from the food matrix and greater protection during digestion thanks to a greater micellarization of the solubilized phenolic [48]. The discrepancies found with other authors might be related to the different chemical

structures of the phenolic compounds studied, the food matrix characteristics or the *in vitro* digestion conditions.

The bioaccessibility index allows the evaluation of the quantity of phenolic compounds after digestion that is solubilized into the Wp and that becomes available for potential absorption in the systemic circulation. Nevertheless, most of the phenolic compounds were recovered in the Op (non-absorbable), which led to poor absorption and consequently high exposure to colonic metabolism [49, 50].

Unlike the recovery index, which was affected by the hydrophilic behaviour of phenolics, the bioaccessibility was not affected by the fat content or lipid fraction in the digestion media.[48]. However, it is necessary to know that digested Wp almost certainly contains micelles and will therefore include phenolic components associated with these hydrophobic-centred micelles. Information about whether micelle-borne phenolics are more or less bioavailable for intestinal uptake is not available [27].

High bioaccessibility and stability values have been reported for tyrosol [43] and hydroxytyrosol [51] after *in vitro* digestion of VOO, in agreement with our findings; Dinella et al [43] reported that the phenolic fraction (37-90 %) was bioaccessible after *in vitro* digestion and further dialysis processes. However, Seiquer et al [52] found that only 2.82 % of the total phenolic compounds of VOO were available to be absorbed. Most of the studies into the phenolic bioaccessibility of edible oils have been performed using single compounds [27, 43], although when they included a mixture of phenolic compounds or a food matrix their bioaccessibility showed important differences [53].

Studies *in vivo* reported that HTy and Ty are dose-dependently absorbed in humans after VOO ingestion [20]. Our results suggested that the BI% of each phenolic compound did not show a dose-dependent response when oils from different olive cultivars were compared. Few data are available about the bioaccessibility of the whole phenolic fraction

of VOO, including individual phenolic compound. To our knowledge, this study presents original data about the bioaccessibility of lignans, flavonoids, secoiridoids and phenolic acids of VOO.

As explained above, the phenolic compounds from the non-absorbable fraction (Op), mainly secoiridoid derivatives, lignans and flavonols, should not be disregarded because they reach the large intestine where they can be metabolized and/or exert biological activity. Secoiridoids that reach the large intestine are subjected to rapid degradation by the colonic microflora. Corona et al [45] reported three major degradation products produced by the microflora, one of them identified as HT. Metabolism by colonic bacterial species may have the effect of increasing overall HT bioavailability, as released HT can be absorbed into the colon. In addition, these authors concluded that secoiridoid compounds may possess potential prebiotic properties if bacterial groups such as *Bifidobacteria* and *Lactobacillus* are able to utilize it as a carbon source. The lignans are metabolized to enterodiol and enterolactone by the activity of the gut microflora in the proximal colon form, these compounds being known as “mammalian lignans” [54, 55]. Both enterodiol and enterolactone have been shown to exert estrogenic effects *in vivo* and reduce breast and prostate cancer cell growth via both estrogen-dependent [56] and independent mechanisms [57]. Flavonoids are hydrolyzed in the colon into simple phenolic acids, which are absorbed and further metabolized in the liver [58].

Few data are available about how VOO antioxidant properties are affected by *in vitro* digestion. In this study, the antioxidant capacity of the BF of VOO was evaluated. BFs are biologically active so they may exert antioxidant activity by a surface reaction phenomenon, or could even be metabolized by microflora and partially absorbed into the final segments of the intestine [59].

Phenolic compounds are considered to be the main contributors to the antioxidant activity of virgin olive oil [60, 61]. Although the antioxidant capacity observed in this study cannot be attributed solely to VOO phenolic compounds because they were not isolated from the BF, some correlations between the phenolic compounds from the oils and the antioxidant capacity of the BF were observed. The antioxidant activity of phenols is strongly influenced by their chemical structure [62]. The `Blanqueta´ VOO-BF showed the highest antioxidant capacity in the DPPH assay, a strong correlation being observed with the high levels of total phenolic compounds, secoiridoid derivatives andHTy, and with the high HTy/Ty ratio. According to the literature, compounds with an O-diphenolic structure such as HTy and oleuropein aglycon derivatives possess much higher antioxidant activity than Ty and its related compounds [63]. Moreover, synergistic effects among individual phenolic antioxidant molecules have been hypothesized [64]. The `Chetoui´ VOO-BF had the highest antioxidant capacity as determined by ORAC assay. These results would correlate with its high oil Ty content. In contrast, no correlation with VOO phenolic compounds was found with the ABTS assay. The differences found between the methods used may be due to the nature of the lipid fraction of the oils, which, due to the action of bile salts during digestion in the duodenum, form micelles and other association colloids in water that solubilize and transport hydrophobic molecules. The size and distribution of micelles and association colloids formed may vary between the different varieties of oils. Therefore, the activity of antioxidants could depend on the colloidal properties of the substrates, the conditions and stages of oxidation and the localisation of the antioxidants in different phases [27, 65].

In addition to chemical assays, the antioxidant capacity of the BF at cell level was evaluated. The intestine is the primary site where dietary antioxidants act and lipid peroxidation occurs. Thus, Caco-2 cells are a suitable model to investigate the antioxidant effect since they have previously been used to study oxidative stress prevention by olive oil [13]. We have found that the bioaccessible fraction of VOO promoted an antioxidant effect

in Caco-2 cells, reducing the ROS levels compared to control cells; differences between the VOOs from different varieties were not found. Furthermore, the BF did not protect against H_2O_2-induced oxidative stress. A relationship between ROS and the phenolic bioaccessible fraction was not observed. Very little is known about the antioxidant effect of digested foods on the oxidative markers of cells. Seiquer et al [52] studied the antioxidant effect produced by the bioaccessible fraction of extra virgin argan oil and extra virgin olive oil on Caco-2 cells, reporting that the ROS level was not affected compared with control cells; however, when the cells were treated with a pro-oxidant agent, the ROS level was significantly reduced. Isolated phenolic compounds, like HTy, reduce ROS generation after 4 h of incubation of Caco-2[13]. Lozano-Sánchez et al [35] performed a study into the influence of the phenolic composition of five monovarietal VOOs on the cytotoxic activity against human breast cancer cells, reporting that the sensitivity of breast cancer cells to phenolic extracts from VOOs was up to 12 times higher when the extracts were from VOOs with a high content of secoiridoids as compared to others with a low/nil content of these phenolic compounds.

Direct antioxidant effects of phenolic compounds in the gastrointestinal tract are more likely to be significant than those at other sites within the body post-absorption [26]. Besides protecting colonic cells against injury induced by hydrogen peroxide [52, 66], the direct antioxidant effects of the olive oil phenolic fraction in the gastrointestinal tract include: a protective effect against oxidized-LDL induced membrane damage, modifications of the cytoskeleton network, microtubular disorganization, loss of cell–cell and cell–substrate contacts, cell detachment and cell death [67]. Therefore, as well as their direct antioxidant effects, the phenolic compounds in olive oil may exert chemopreventive effects in the gastrointestinal tract via their interactions with cellular signaling pathways, which are important in controlling the growth, differentiation and metastasis of cancer cells [68]. Indeed, dietary phenolic compounds are reported to modulate a variety of intracellular signaling pathways in mammalian cells [69]. Future studies into the antioxidant capacity of

digested VOO should take into account the non-bioaccessible fraction since the gastrointestinal tract is also exposed to phenolic compounds of Op, principally lignans and flavonols.

To sum up, this study provides additional information regarding the bioaccessibility of VOO phenolic compounds. During the digestion process, VOO phenolic compounds are hydroxylated mainly to HTy and Ty. The bioaccessibility of the phenolic compounds was not dose-dependent since the VOO with the lowest phenolic content (`Sevillana´) gave the highest % BI, whereas the `Blanqueta´ VOO showed the lowest % BI. These differences may be due to differences in the hydrolysis of secoiridoid derivatives. All the cultivars showed the capacity to reduce ROS levels in Caco-2 cells. The variability found in the antioxidant capacity of the BF of the oils according to the antioxidant test used may be due to the characteristics of the micelles formed after duodenal digestion, either favouring or limiting the absorption of phenolic compounds and the antioxidant capacity. These micelles depend on the lipid matrix, which could vary between the cultivars. In conclusion, the bioaccessibility of VOO phenolic compounds is low. The differences in the phenolic composition of VOOs depend on olive cultivars and affect the bioaccessibility of the phenolic compounds. This is likely due to several factors, including the initial concentration, stability to gastrointestinal conditions and lipid matrix. It was seen that some phenolic compounds remaining in the Op (not bioaccessible) can reach the colon and be absorbed by colonocytes. Nevertheless, further research is required to determine the biotransformation of Op phenolic compounds by the colonic microbiota to metabolites with potentially protective effects *in vivo*.

Abbreviations Used

3,4-DHPEA: Hydroxytyrosol

3,4-DHPEA-EDA: dialdehydic form of elenoic acid linked to HTy

3,4-DHPEA-EA: aldehydic form of elenoic acid linked to HTy

3,4-DHPEA-AC: HTy acetate

AAPH: 2,2′-Azobis(2-methylpropionamidine) dihydrochloride

AUC: Fluorescence decay curve

BI: Bioaccessibility Index

BF: Bioaccessible fraction

DCFH-DA: 2,7 – dichlorofluroscein diacetate

DPPH: 2,2 – diphenyl-1-picrylhydrazyl

FL: fluorescein

HTy: Hydroxytyrosol

LA: Ligustroside Aglycone

LDL: Low density lipoprotein

MEM: iron-free medium

OA: Oleuropein Aglycone

Op: Oily phase of digestion

p-DHPEA: Tyrosol

p-DHPEA-EDA: dialdehydic form of elenoic acid linked to Ty

p-DHPEA-EA: aldehydic form of elenoic acid linked to Ty

p-DHPEA-AC: Ty acetate

RI: Recovery index

ROS: reactive oxygen species

RSA: radical scavenging activity

TAG: Triacylglycerols

TE: Trolox equivalents

TOC: α - tochopherol or vitamin E

Ty: Tyrosol

VOO**:** Virgin Olive Oil

Wp: aqueous phase of digestion

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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"How *in vitro* digestion affects to volatile compounds of virgin olive oil"

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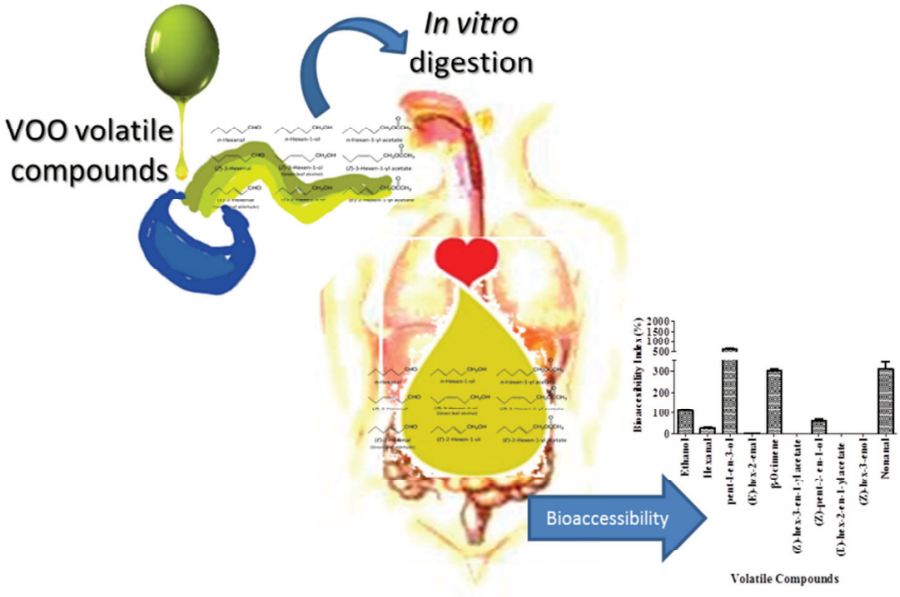

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How in vitro digestion affects to volatile compounds of virgin olive oil

Abstract.

Volatile compounds are responsible of some sensory characteristics of Virgin olive oil (VOO); however they have not been studied from the nutritional point view. In this work was studied the effect of the simulated digestion on VOO volatile compounds, analysing their changes through the three steps of an *in vitro* digestion model. Index of recovery and bioaccessibility were determined for the main volatiles of 'Picual' VOO. The results showed for the first time the recovery and bioaccessibility of several volatile compounds present in VOO and therefore their possible bioactivity in the human body.

Keywords. "*in vitro"* digestion; bioaccessibility; volatile compounds; virgin olive oil.

INTRODUCTION

Sensory characteristics let to differentiate the virgin olive oil (VOO) from other edible oils. The flavour of VOO is generated by volatile compounds; they are responsible of fruity and green odour, which are directly related to consumer acceptability[1]. The volatile compounds present in VOO are mainly aldehydes and alcohols of five and six carbons as: (E)-hex-2-enal, hexanal, (Z)-hex-3-enol, hexanol and their corresponding esters. They are the main contributors to the "unripe" component of the fruity flavour[2,3]. Among the volatiles, those related to green odour can be found at higher concentrations in VOO headspace [4].

The volatile compounds are synthesized from non-esterified polyunsaturated fatty acids containing a (*Z*,*Z*)-1,4-pentadiene structure such as linoleic (LA) and linolenic (LnA) acids by lipoxygenase (LOX) pathway. The enzyme LOX, using molecular O_2 as co-substrate, produces 13- hydroperoxide derivatives that are subsequently cleaved heterolytically by hydroperoxide lyase (HPL) to C6 aldehydes volatiles, that can be reduced by alcohol dehydrogenase (ADH) to form C6 alcohols and can finally be transformed into the corresponding esters by mean of an alcohol acyltransferase (AAT)[5-7]. Other important volatiles present in VOO are the C5 compounds[8]. The C5 compounds would be generated by LOX in a homolytic way as demonstrated in soybean preparations[9].

To date, the study of volatile fraction in VOO has been focused on the characterization of volatile profile of monovarietal oils and their relation with its sensorial notes, however for these compounds have been reported other important functions and properties[10]. In plants, volatile compounds have a key role in plant defence system against microorganisms in wounded areas[11], and show potential activity as insecticides and fungicides [12-14].In food systems, volatile compounds related to aroma can be used to improve their shelf-life[15] and health benefits[16,17]. (E)-hex-2-enal and hexanal showed strong antimicrobial properties at low concentrations against pathogen microorganisms such as *E.*

coli, *S. enteritidis* and *L. monocytogenes*[15,18]. Furthermore, hexanal have effectiveness as a metabolizable fungicide since strongly delayed the growth of mould, yeasts, mesophilic and psychotropic bacteria[19,20]. Therefore, volatile compounds may have some healthy benefits for humans although data about their bioaccessibility are scarce.

The bioaccessibility is defined as the fraction of a compound released from its matrix in the gastrointestinal tract that becomes available for intestinal absorption. During intestinal digestion of dietary oil, the intraluminal content is separated in two phases: the oily phase (Op) mainly containing undigested triacylglycerols (TAG) and released diacylglycerols (DAG), and the aqueous phase (Wp) containing bile salts and the poorly soluble end products from enzymatic hydrolysis, namely monoacylglycerol (MAG) and fatty acids structured as mixed micelles, micelles, vesicles or emulsion droplets[22,23]. The characteristics of Wp enhances the transport of lipidic compounds to enterocytes throughout the unstirred water layer close to the microvillus membrane, where they are absorbed [24].

Therefore, to know the effect of digestion on virgin olive volatiles, determining the amount available to be absorbed seems necessary. In this sense, the aim of the present study was to describe how digestion process can affect the concentration of volatile compounds of VOO. For this purpose `Picual´ VOO was supplied to *in vitro* digestion model and the by-modifications of volatile compounds are described by the first time.

MATERIALS AND METHODS

Chemicals

Chemical and reagents used to *in vitro* digestion were: potassium chloride (KCl), potassium thiocynate (KSCN), monosodium phosphate (NaH_2PO_4), sodium phosphate (Na_2PO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), magnesium chloride

($MgCl_2$), calcium chloride dihydrate ($CaCl_2*2H_2O$), ammonium chloride (NH_4Cl), D-(+)glucosamine hydrochloride, D–glucuronic acid, D–(+) glucose, uric acid, urea, mucin from stomach type III (M1778), α-amylase from porcine pancreas, hydrochloric acid 37% (HCl), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, lipase from porcine pancreas Type II, bile salts, albumin from bovine serum (BSA). All the enzymes, organic and inorganic compounds used were purchased from Sigma-Aldrich Co. (St. Louis, USA). Likewise, all standards of volatile compounds were of high-purity grade and supplied by Sigma-Aldrich: ethanol, hexanal, pent-1-en-3-ol, (E)-hex-2-enal, β-ocimene, (Z)-hex-3-en-1-yl acetate, (Z)-pent-2- en-1-ol, (E)-hex-2-en-1-yl acetate, (Z)-hex-3-enol and nonanal.

Plant Material and virgin olive oil extraction

For oil extraction, olive fruits from `Picual´ cultivar (*Olea europaea, L.)* were used. The olive trees were spaced 7 x 7 m and grown in the experimental orchard of Centro IFAPA 'Venta Del Llano' in Mengibar, Jaen (Spain) using standard growing techniques. The study was carried out during the 2014/15 crop year.

VOO extraction was performed using an Abencor laboratory oil mill (Abengoa, Seville, Spain) equipped with a hammer mill, a thermobeater and a basket centrifuge that simulates the industrial process of VOO production at laboratory scale. The oil extraction was repeated twice for each cultivar. The olive milling was performed at 3000 rpm with a 5 mm sieve. The olive paste malaxation was carried out at 28 °C for 45 min. Oil separation was performed in a basket centrifuge at 3500 rpm for 1 min. After centrifugation, the oil was decanted, filtered and stored in glass bottles at -20 °C in the dark without headspace and under N_2 atmosphere until analysis.

In vitro digestion model

The study of bioaccesibility of VOO-volatiles was carried out using the method reported by Oomen and Versantvoort et al.[25,26] with some modifications. Briefly, the model includes a three-step procedure simulating digestive processes in mouth, stomach and duodenum.

Saliva and digestive juices were prepared artificially as described in **Table 1**. Saliva and all digestive juices were heated at 37°C ± 2°C. The digestion started adding 6 mL artificial saliva to 5 g of VOO, incubated at 37°C ± 2°C and rotated head-over-heels (55 rpm) for 5 min. Then, 12 mL of gastric juice was added, and the mixture was rotated head-over-heels for 2 h as described above. The gastric pH was comprised between 2 and 3. Finally, 12 mL of duodenal juice, 6 mL of bile, and 2 mL of bicarbonate solution (1M) were added simultaneously, rotating the mixture for another 2 h. The pH of the chyme (digestion mixture) varied from pH 6.5 to 7. Each VOO sample was digested by triplicate. All the *in vitro* digestion steps were carried out in amber bottles in the dark.

At the end of each digestion step (mouth, gastric and duodenal), the digestion mixtures were centrifuged for 5 min at 2750 g, and then, separated into three layers: a). The pellet (digested matrix), it was discarded; b). Aqueous phase (Wp); c). Oily phase (Op). Both aqueous and oily phase of each sample were kept at -80°C until analysis.

To evaluate the digestion of VOO-volatiles, two different indexes were studied:

1) The recovery index (RI), defined as the amount of volatile presents in the digested (Op + Wp) after each digestion step of 1 g from VOO, so:

$$\text{RI}\ (\%) \ = \ (\text{VC}_{\text{SD}}\ /\text{VC}_{\text{VOO}}) \text{ x } 100$$

Where VC_{SD} is the volatile content of digested (mg/kg of oil) in each step of digestion (Op + Wp) and VC_{VOO} is the volatile content of VOO (mg/kg of oil).

Table 1. Constituents and concentrations of the various synthetic juices of the in vitro digestion model representing fed conditions

	Artificial Saliva	Gastric Juice	Intestinal Juice	Bile
Inorganic Compounds				
KCL 89.6 g/L	10 mL	9.2 mL	6.3 mL	4.2 mL
KSCN 20 g/L	10 mL			
NaH_2PO_4 88.8 g/L	10 mL	3 mL		
Na_2SO_4 57 g/L	10 mL			
NaCl 175.3 g/L	1.7 mL	15.7 mL	40 mL	30 mL
$NaHCO_3$ 84.7 g/L	20 mL		40 mL	68.3 mL
HCl 37% 370 g/L		6.5 mL	0.18 mL	0.15 mL
KH_2PO_4 8 g/L			10 mL	
$MgCl_2$ 5 g/L			10 mL	
Organic Compounds				
Urea 25 g/L	8 mL	3.4 mL	4 mL	10 mL
Glucose 65 g/L		10 mL		
Glucuronic Acid 2 g/L		10 mL		
Glucosamine hydrochloride 33 g/L		10 mL		
Compound added to mixture of organic and inorganic solution				
$CaCl_2*2H_2O$ 22.2 g/L		18 mL	9 mL	10 mL
NH_4CL 30.6 g/L		10 mL		
α-amilasa	290 mg			
Uric Acid	15 mg			
Mucin	0.025 g	3 g		
BSA		1g	1 g	1.8 g
Pepsin		2.5 g		
Pancreatin			9 g	
Lipase			1.5 g	
Bilis				30 g
	pH 6.5 ± 0.1	pH 1.0 ± 0.1	pH 7.8 ± 0.2	pH 8.0 ± 0.2

The inorganic and organic solutions are augmented to 500ml with distilled water. After mixing of the inorganic and organic solutions, some further constituents are added and dissolved. If necessary, the pH of the juices is adjusted to the appropriate interval with NaOH 1M or HCl 37%.

2) Bioaccessibility index (BI), defined as the percentage of volatile compound released of the matrix solubilized in Wp after of the duodenal digestion of 1 g from VOO. This index defines the proportion of the volatile compounds available for absorption into the systematic circulation, so:

$$\text{BI}\ (\%) \ = \ (\text{VC}_{\text{WP}}\ /\text{VC}_{\text{VOO}})\ \text{x}\ 100$$

Where VC_{WP} is the volatile content in the Wp (mg/kg of oil) after the duodenal step and VC_{VOO} is the volatile content of VOO (mg/kg of oil).

Analysis of Volatile Compounds

Volatile compounds in the different matrices (virgin olive oil, water and oily phases) were analysed by HS-SPME-GC/MS according to Bajoub et al.[27]. Briefly, each sample was conditioned to room temperature and then placed in a vial heater at 40°C during 10 min of equilibrium time, immediately thereafter the volatile compounds from the headspace were adsorbed onto an SPME fibre of DVB/Carboxen/PDMS 50/30 µm (Supelco Co., Bellefonte, PA). The adsorption time was 50 min at 40 °C. Volatile compounds trapped in the SPME fibre were directly desorbed in the GC system at 250 ºC. Volatile compounds were analysed in triplicate using a Bruker SCION 456 GC-TQ gas chromatograph (Bruker, Massachusetts, USA) equipped with a Supelcowax 10 capillary column (30 m x 0.25 mm, 0.25 µm, Sigma-Aldrich, St. Louis, MO); the operating conditions were as follows: helium was used as the carrier gas; the column was held for 5 min at 40 °C and then programmed to increase by 4 °C min^{-1} to 200 °C.

The identity of the volatile compounds was confirmed using the mass spectrometer Bruker SCION 456 TQ-SQ (Bruker, Massachusetts, USA) operating in EI mode (70 eV) and matched against the NIST Library by comparing the GC retention times against those of standards. The ion source and transfer line temperatures were 250°C for both. Mass spectra were obtained in full scan mode at mass-to-charge ratios ranging from 29 to 250 at

a scanning speed of 7 scan/s. Chromatograms and spectra were recorded and processed using Bruker Daltonics MS Workstation (MSWS v. 8.2). Quantification for each matrix analysed (VOO, Wp and Op) was performed using individual calibration curves for each identified compound by adding known amounts of different compounds to: refined olive oil for the identification in VOO and Op, artificial saliva, gastric and intestinal juice for the identification in Wp of each digestion step.

Data Analysis

Results were expressed as means ± SD, unless otherwise stated of three separate experiments. Statistical significance was assessed by one-way or two-way ANOVA followed by Bonferroni's multiple comparison tests. Differences were considered statistically significant at $p<0.05$. Statistical analyses were conducted using the GraphPad Prism v.5 statistical package (GraphPad Software, Inc.).

RESULTS AND DISSCUSION

HS-SPME coupled to GC-MS was utilized to characterize the volatiles of `Picual´ VOO, the results are shown in Table 2. Because of the importance of the green perception in the virgin olive oil flavour[28], the C6 compounds – also called ''green volatiles''[29] – corresponding to Hexanal, (E)-hex-2-enal, (Z)-hex-3-en-1-yl-acetate, (E)-hex-2-en-1-yl acetate and (Z)-3-hex-1-enol were quantified. Others compounds such as ethanol and β-ocimene were also quantified. Aldehydes represented more than 80% of VOO volatile fraction, being (E)-hex-2-enal the main compound. The C_5 compounds: pent-1-en-3-ol and (Z)-pent-2-en-1-ol were also quantified although they were found at lower levels.

Table 2. Volatile compound content responsible for green odour of Picual VOO (mg/kg).

Volatile compounds	mg/kg	Odour Descriptor [a]
Hexanal	2.66 ± 0.21	Green, apple, Green fruit
(E)-hex-2-enal	10.2 ± 0.13	Green, fruity, almonds
Nonanal	1.26 ± 0.29	Fatty, waxy, rancid
Aldehydes	**14.12 ± 0.63**	
Ethanol	0.39 ± 0.05	Apple, sweet
(Z)-2-penten-1-ol	0.10 ± 0.00	Banana
pent-1-en-3-ol	0.06 ± 0.00	Butter, soft green
(Z)-3-hex-1-enol	0.90 ± 0.01	Grass, banana
Alcohols	**1.45 ± 0.06**	
(Z)-3-hexen-1-yl acetate	0.02 ± 0.00	Green banana, green leaves, fruity
(E)-hex-2-en-1-yl acetate	0.65 ± 0.09	
Esters	**0.67 ± 0.09**	
β-Ocimene	0.92 ± 0.04	
Hydrocarbons	**0.92 ± 0.04**	

[a] Luna et al. 2006 and Aparicio et al. 1998 [29,49].
Data are expressed as mg/kg of VOO and are the mean ± SD from three separate experiments (n=3).

An *in vitro* digestion model, including three-step (mouth, stomach and small intestine), was used to evaluate the effect of each step of digestion on `Picual´ VOO volatiles and their bioaccessibility. After each digestion step, the digested samples were separated into three phases: a) Op corresponds to the fraction that *in vivo* would be wasted in feces or transformed by colonic flora[32]; b) Wp corresponds to the potentially bioaccessible fraction (BF); and c) The pellet.

The recovery index of VOO volatiles during *in vitro* digestion are shown in Figure 1. The stability of each compound during digestion steps were observed. Under the buccal conditions ethanol, pent-1-en-3-ol and β-ocimene showed high stability since their recoveries were higher than 100%. Nonanal achieved a recovery of 60%. The C6 compounds were less stable, their recovery varied between 34.5 % for (E)-hex-2-en-1-yl

acetate and 42.7 % for (Z)-hex-3-en-1-yl acetate. The lowest recovery was found for (Z)-pent-2-en-1-ol (14 %). The recoveries greater than 100%, obtained for ethanol and pent-1-en-3-ol may indicate oxidation of aldehydes and then, higher recoveries for both alcohols. These changes may be due to interactions or enzymatic reactions with saliva components as reported by Ployon et al.[33]

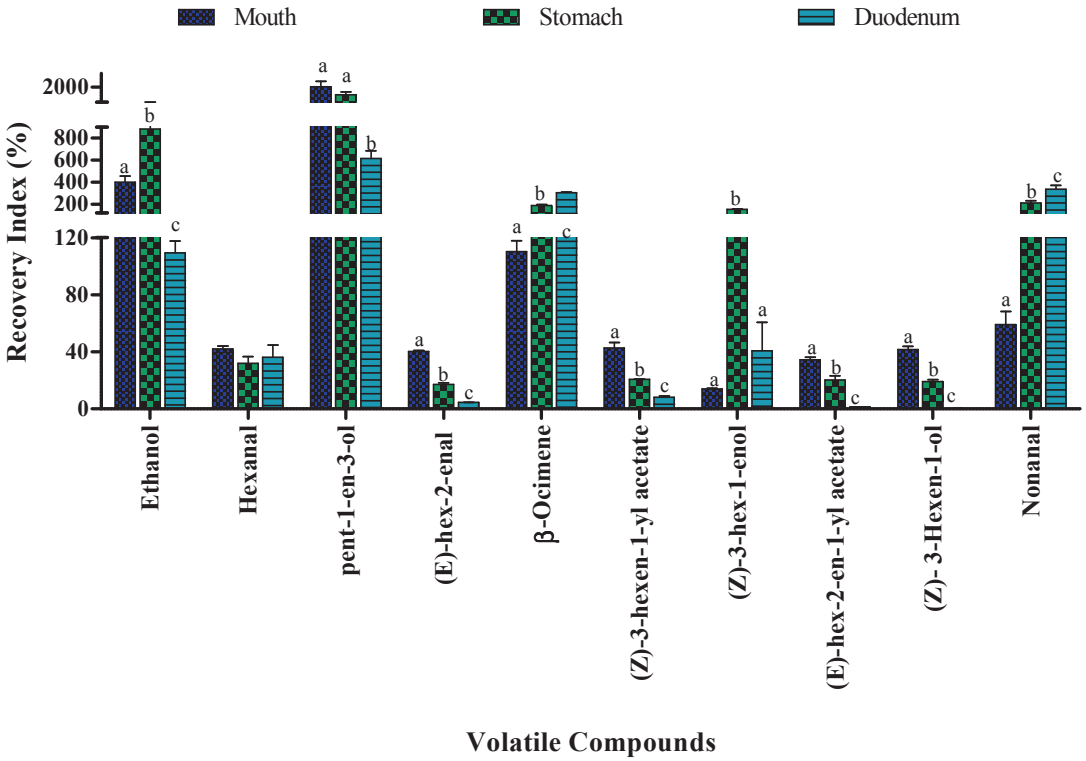

Figure 1. Recovery Index (%) of volatile compounds after *in vitro* digestion of `Picual´ VOO on each digestion step. Data are the mean from three separate experiments, and error bars show ± SEM. Different letters indicate significant difference of each compound between the digestion steps ($p< 0.05$) by two-way ANOVA analysis and Bonferroni's post-hoc test.

After gastric digestion ethanol, 1-penten-3-ol, β-ocimene, (Z)-pent-2-en-1-ol and nonanal recovery percent were higher. In the stomach, the lipids are subjected to mechanic (mixed) and enzymatic actions[34]. Because of TAG hydrolysis free fatty acids are released, they can be oxidised easily giving aldehydes that can be oxidised to alcohols. The oxidation mechanism during digestion has been reported previously by Gorelik et al. [35,36]. Degradation during digestion of other compounds, such as aldehydes, has been reported by Goicoechea et al.[37] for frying oil. In general, a loss of C6 compounds was observed regarding buccal step.

At duodenal step higher recoveries of ethanol (109%), pent-1-en-3-ol (614%), β-ocimene (303%) and nonanal (334%) were observed. Low recoveries were found for hexanal (36%) and (Z)-pent-2-en-1-ol (40,8%); very low recovery of (E)-hex-2-enal (4.7%), (Z)-hex-3-en-1-yl acetate(8,2%) were observed whereas (E)-hex-2-en-1-yl acetate and (Z)-3-hexen-1-ol were not detected.

Most of the lipid digestion occurs in the small intestine where lipid hydrolysis continues by lipase action[34], it may explain the variation of the volatiles observed at this step. Thus, the low recovery of some compounds might be due to their adsorption or breakdown by enzymes[38] and their interaction with other proteins[39-41]. The volatiles might suffer transformations by hydration of double bonds or intramolecular rearrangement of hydroxyl groups with a double bond [38]. The interactions between volatile compounds and proteins can be explained by: 1. Irreversible covalent union between aldehydes and the amino group of protein; 2. Hydrogen bonds between proteins and polar volatiles (alcohols); 3. Hydrophobic union between a-polar volatiles with proteins[42]. These interactions depend on several factors as: ionic force, pH, temperature and the structure of volatiles and proteins[43]. The pH through the *in vitro* digestion (mouth 6.7, stomach 2.2 and duodenum 6.77) can promote the binding volatile-protein according to literature[43,44] since the volatiles increase their affinity to protein as pH increased from 3 – 9, being lower for higher pH [43].

The Figure 2 shows the relative amount of digested volatiles respect to the volatiles of `Picual´ VOO and their distribution into phases, Wp and Op. Volatile distribution between aqueous (Wp) and oily phases (Op) varied because of the changes in digestion medium. In general, in the buccal step the hydrophilic compounds were distributed in the Wp whereas the hydrophobic in Op. At gastric level, the high acidity of medium plays an important physiological role, including hydrolysis of food components; in this sense fatty acids can be released from TAG increasing their concentration in both Wp and Op[37] making more lipophilic both phases. Furthermore, these fatty acids can increase the solubilization of digestion products in mixed micelles[34]. These mechanisms may explain the higher levels of hexanal, (Z)-hex-3-enol and nonanal observed at in the Wp.

The volatiles found at higher content in the duodenal Wp were ethanol, hexanal, pent-1-en-3-ol, β-ocimene, (Z)-2-penten-1-ol, and nonanal. The alcohols, because of their greater polarity, are dissolved at higher level in the Wp. The aldehydes, even though have higher lipophilicity, could be detected in the Wp, since the lipase and bile salts activities let to include them in micellar and/or vesicular structures[45]. The volatiles found in the duodenal Op (hexenal, (E)-hex-2-enal, (Z)-hex-3-en-1-yl acetate, (E)-hex-2-en-1-yl acetate and nonanal) could continue the digestive process until colon step, where they can be metabolized by intestinal microflora and then, absorbed or have beneficial effect at local level.

This set of experiments allowed for establishing degradation of VOO volatiles along the gastrointestinal tract and the amount able to reach the gut epithelium for their potential uptake and thus, produce beneficial effects. In this sense, the compounds retained into Op could have antimicrobial activity against gastrointestinal pathogens. The antimicrobial activity against microbial species in fresh food has been tested with hexanal and (E)-hex-2-enal[15,18,46]. Effect in the reduction of human and animal pathogens have been described previously with essential oils/compounds[47,48].

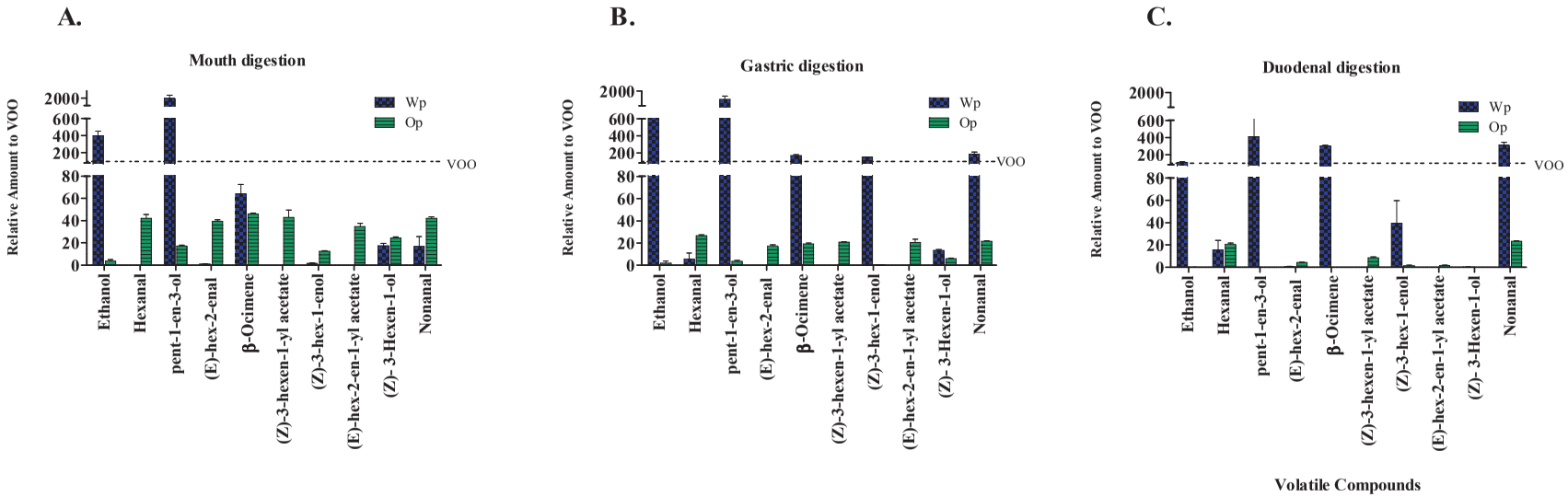

Figure 2. Relative amount of volatile compounds after *in vitro* digestion respect to volatile compounds from Picual VOO which was normalized at value 100 (horizontal line). A. Mouth digestion; B. Gastric digestion; C. Duodenal Digestion. Wp: aquose phase; Op: Oily phase. Data are the mean from three separate experiments, and error bars show the ±SEM.

The volatile compounds released from lipid matrix and solubilized in the Wp are available to be absorbed in the short intestine (Bioaccessible). Bioaccessibility index values for the volatiles selected are shown in Figure 3. From the ten volatile compounds analyzed of `Picual´ VOO, only six compounds were bioaccessible. The compounds with the highest bbioaccessibility were pent-1-en-3-ol (614%), nonanal (311%), β-ocimene (303%) and ethanol (109%), followed by (Z)-2-penten-1-ol (50%), hexanal (23%) and (E)-hex-2-enal (0.62%). As discussed above, fatty acid oxidation processes are initiated in the stomach forming hydroperoxides[36] that can be decomposed into aldehydes[49] and alcohols and then, transported to the duodenum where are available in the intestinal lumen[49]. This mechanism can explain the high bioaccessibility observed for pent-1-en-3-ol and nonanal. But the bioaccessibility of volatile compounds also would depend on the interaction with biological proteins and their capacity to be solubilized into micelles.

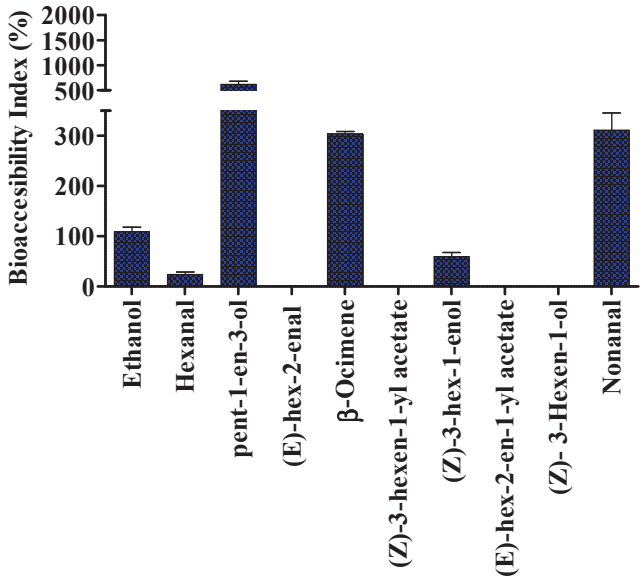

Figure 3. Bioaccesibility index (BI %) of volatile compounds of `Picual´ VOO. Data are the mean from three separate experiments and error bars show ±SEM.

The bioaccessibility observed for some of the volatiles of VOO has great importance since previous studies have noteworthy the activity of some volatile components of olive fruit and oil, against different fungal and bacterial strains. Kubo et al.[50] described the antimicrobial activity of long chain saturated and unsaturated aldehydes (hexanal, nonanal, (E)-hex-2-enal, among others) from olive fruit against a broad spectrum of food-borne micro-fungal and bacteria strains; among the microorganisms tested, fungi were the most sensitive. Besignano et al.[51] demonstrated that some α,β-unsaturated aldehydes of olive flavor may be good candidates for their application as antimicrobial agents against bacteria responsible for human gastrointestinal and respiratory tract infections. These results pointed out that unsaturated aldehydes have a broad antimicrobial spectrum and show similar activity against Gram-positive and Gram-negative bacteria. Thus the dietary intake of VOO aldehydes might help to lower the risk of bacterial infections particularly in the intestinal tract.

To the best of our knowledge, is the first time reporting the behaviour of VOO-volatile compounds along the gastrointestinal tract. Volatile compounds from `Picual´ VOO remained during digestion process, and an amount was available to be absorbed at duodenal level and reach the bloodstream. This research opens a new researcher line to study possible biological activity such as antimicrobial of volatile compounds of VOO in the human organism. Therefore, further studies are needed to elucidate the bioaccessibility of more VOO-volatile compounds and their metabolites as well as the pharmacokinetic properties of these compounds and thus the possibility that they maintain their antibacterial activity *in vivo*.

Abbreviations used.

BI: Bioaccessibility index

DAG: diacylglycerols

LA : linoleic acid

LnA : linolenic acid

LOX : lipoxygenase

MAG: monoacylglycerol

Op : oily phase

RI: recovery index

TAG: triacylglycerol

VOO: virgin olive oil

Wp: aqueous phase

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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V. CONCLUSIONES

Conclusiones

Las conclusiones de este trabajo dan respuesta a cada una de las hipótesis planteadas al inicio del trabajo de investigación, así:

1. *¿Las diferencias en contenido de ácidos grasos de aceites de oliva virgen monovarietales tendrán efecto sobre la formación de células espumosas por parte de los macrófagos?*

- Las diferencias en el contenido de ácidos grasos de los aceites de oliva vírgenes, principalmente de los ácidos linoleico y oleico, influyeron en la captación y acumulación lipídica por parte de los macrófagos. En este sentido los AOV ricos en ácido linoleico indujeron una alta incorporación de TAG dentro de los macrófagos a diferencia de los aceites con alto contenido de ácido oleico.
- Se ha encontrado que la relación entre los ácidos grasos 18:1/18:2 de los AOV puede usarse como marcador para identificar aceites con posibles efectos cardioprotectores, debido a su alta correlación con la acumulación intracelular de TAG. De los aceites estudiados, el AOV `Picual´ fue el aceite con el posible mayor efecto cardioprotector.

2. *¿El contenido de compuestos fenólicos del AOV según la variedad de aceituna de la que procede, será determinante en la bioaccesibilidad de éstos compuestos?*

- Se ha identificado que los polifenoles presentes en el aceite de oliva virgen formaron complejos insolubles con la mucina utilizando el método nefelométrico, el cual fue un método rápido y sencillo para su determinación. La formación del complejo fenol - mucina, fue mayor dependiendo de la concentración del compuesto, el número de grupos funcionales –OH y la posición de los mismos. La formación de éste y la interacción entre fenoles-mucina podrían indicar la

posibilidad que durante la digestión del AOV se pueda afectar la biodisponibilidad y la capacidad antioxidante de los compuestos fenólicos.

- En general la bioaccesibilidad de los compuestos fenólicos de los aceites de oliva vírgenes fue baja.
- Durante el proceso de digestión *in vitro* de los AOVs se observaron importantes pérdidas de compuestos fenólicos desde la etapa bucal hasta la duodenal. El HTy y el Ty fueron los únicos compuestos en los que se observó un gran incremento de recuperación, como consecuencia de la hidrólisis de los compuestos secoiridoides.
- Se encontraron ddiferencias en la bioaccesibilidad de los compuestos fenólicos dependiendo de la variedad del AOV. La bioaccesibilidad de los compuestos fenólicos no dependió de la cantidad inicial en el AOV sino del grado de hidrólisis de los derivados secoiridoides a nivel duodenal. De los aceites analizados, la bioaccesibilidad de los compuestos fenólicos fue mayor en el AOV de la variedad `Sevillana´ (36%) y menor el en AOV de la variedad Blanqueta´ (8%).
- La proporción de compuestos fenólicos que se recuperaron en la fase oleosa no se debe despreciar ya que éstos compuestos continúan el proceso digestivo hasta el colon donde pueden ser metabolizados por los colonocitos, ser reabsorbidos o ejercer alguna actividad biológica local.

3. *¿Existirán diferencias en la capacidad antioxidante de los aceites de oliva virgen monovarietales después del proceso digestivo?*

- Se encontraron diferencias en la capacidad antioxidante entre las fracciones bioaccesibles de los AOV, aunque estas no pueden ser atribuidas exclusivamente a los compuestos fenólicos debido a que en los digeridos se encuentran los demás componentes resultantes de la digestión del aceite.
- La fracción bioaccesible de `Blanqueta´ y `Chetoui´ mostraron una elevada capacidad para oxidar el radical DPPH. Estos resultados se correlacionaron con el

alto contenido en el respectivo aceite de compuestos secoiridoides, HTy y el ratio HTy/Ty. La fracción bioaccesible de `Picual´ y `Habichuelero´ mostraron alta capacidad antioxidante por el método ORAC, la cual se correlacionó con el alto contenido de Ty de los aceites.

- La variabilidad encontrada en la capacidad antioxidante de la fracción bioaccesible de los AOV se puede explicar por las características de las micelas formadas después de la digestión duodenal que dependen a su vez de la matriz lipídica inicial.
- Las fracción bioaccesible del AOV presentó capacidad de reducir los niveles de ROS en células Caco-2, sin diferencias significativas entre las variedades.

4. *¿Los compuestos volátiles del AOV serán bioaccesibles?*
    - Los compuestos volátiles del AOV `Picual´ fueron recuperados durante todo el proceso digestivo, principalmente los alcoholes y aldehídos; parte de estos compuestos se encontraron disponibles a nivel duodenal para su posible absorción y pasar al sistema circulatorio, otra porción quedó disponible para continuar su proceso digestivo en el colon, donde podrían ejercer su acción y/o ser metabolizados y reabsorbidos.

VI. Anexos

Comunicaciones Orales en Congresos

Efecto del proceso digestivo in vitro sobre los compuestos volátiles del aceite de oliva virgen de la variedad Picual

VIII Simposium Científico Técnico Expoliva, Jaén 2017

Cultivar-related differences in the fatty acid composition of virgin olive oil are determinant for foam cell formation by macrophages

XVII Congreso Latinoamericano de Nutrición (SLAN), Punta Cana, República Dominicana 2015.

Comunicaciones Póster

Efecto del proceso digestivo in vitro sobre los compuestos volátiles del aceite de oliva virgen de la variedad Picual

VIII Simposium Científico Técnico Expoliva, Jaén 2017

Obtención de aceite de oliva virgen de alta calidad nutricional y organoléptica modulando el oxígeno durante el batido de la pasta de aceituna.

IV Congreso Científico de Investigación en Formación de la Universidad de Córdoba, 2014

Efecto de la maduración y el procesamiento del fruto de olea oleuropeae sobre la biosíntesis de los compuestos volátiles y fenólicos responsables del aroma y sabor del aceite de oliva virgen

IV Jornadas Nacionales del Grupo de Olivicultura de la Sociedad Española de Ciencias Hortícolas, Baeza 2014

Effect of ripening index and cultivar on the volatile and phenolic compounds of the olive fruit pulp

12th Euro Fed Lipid Congress and 31th ISF Lecture Series: Oils, Fats and Lipids new strategies for a high quality future, Montpelier, Francia 2014

Application of High Power Ultrasounds in the Virgin Olive Oil Extraction Previous to Olive Paste Malaxation

11th Euro Fed Lipid Congress and 30th ISF Lecture Series: Oils, Fats and Lipids new strategies for a high quality future, Antalya, Turkey 2013

Interaction Between Phenolic Compounds of Virgin Olive Oil with a Salivary Protein (mucin): an approach to bioavailability

International Union of Nutritional Sciences 20th International, Granada, España 2013

Efecto de las variables tecnológicas de la molienda de fruto sobre los compuestos volátiles responsables del aroma del aceite de oliva virgen

VII Congreso Ciencia y Tecnología de los Alimentos, Córdoba 2013

Changes in the profile of phenolic compounds during virgin olive oil extraction: effect of the olive fruit maturation

7th International Society of Antioxidants in Nutrition and Health, Bonn, Alemania 2013

Cambios de los Compuestos Volátiles y Polifenólicos durante el Proceso de Obtención de Aceite de Oliva Virgen

XVI Simposium Cientifico-Técnico Expoliva, Jaén 2013

Análisis de la relación de compuestos fenólicos del aceite de oliva virgen en el desarrollo de la astringencia

Congreso Internacional de Análisis Sensorial del Aceite de Oliva Virgen, Priego de Córdoba, 2012.