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ESCUELA INTERNACIONAL DE DOCTORADO
Programa de Doctorado Ciencias de la Salud

Influencia de la matriz alimentaria en la biodisponibilidad de metabolitos de hidroxitirosol y su capacidad moduladora de biomarcadores de estrés oxidativo e inflamación

Autor:
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Directores:
Dra. Dª. Sonia Medina Escudero
Dr. D. Raúl Domínguez Perles
Dr. D. Ángel Gil Izquierdo

Murcia, noviembre de 2021

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AUTORIZACIÓN DE LO/S DIRECTOR/ES DE LA TESIS PARA SU PRESENTACIÓN

La Dra. Dª. Sonia Medina Escudero, Dr. D. Raúl Domínguez Perles y el Dr. D. Ángel Gil Izquierdo como Directores de la Tesis Doctoral titulada Influencia de la matriz alimentaria en la biodisponibilidad de metabolitos de hidroxitirosol y su capacidad moduladora de biomarcadores de estrés oxidativo e inflamación, realizada por Dª. Carolina Alemán Jiménez en el Departamento de Ciencias de la Salud, **autoriza su presentación a trámite** dado que reúne las condiciones necesarias para su defensa.

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SIGLAS Y ABREVIATURAS

AA, Ácido araquidónico

AESAN, Agencia Española de Seguridad Alimentaria y Nutrición

COTM, Enzima catecol-ortometil-transferasa

COX, Enzima ciclooxigenasa

CYP, Enzima del complejo citocromo P-450

DGLA, Ácido dihomogamma-linolénico

DM, Diabetes Mellitus

DOPAC, Ácido 3,4-dihidroxifenilacético

ECV, Enfermedad Cardiovascular

EFSA, Autoridad Europea de Seguridad Alimentaria

EPA, Ácido eicosapentaenoico

FAO, Organización de las Naciones Unidas para la Alimentación y la Agricultura

FRCV, Factor de Riesgo Cardiovascular

FUFOSE, Comisión Europea de Acción Concertada sobre Bromatología Funcional en Europa

GRE-AED N, Grupo de Revisión, Estudio y Posicionamiento de la Asociación Española de Dietistas-Nutricionistas

HDL-colesterol, Lipoproteína de alta densidad

HT, Hidroxitirosol

HT-LA, Hidroxitirosol esterificado con ácido linoleico

HT-OA, Hidroxitirosol esterificado con ácido oleico

HTA, Acetato de hidroxitirosol

HT-ALA, Hidroxitirosol estrificado con ácido α-linolénico

IsoPs, Isoprostanos

LDL-colesterol, Lipoproteína de baja densidad
MUFAs, Ácidos Grasos Monoinsaturados
NAOS, Estrategia para la Nutrición, Actividad Física y prevención de la Obesidad
PGs, Prostaglandinas
PREDIMED, Estudio de Prevención con Dieta Mediterránea
PUFAs, Ácidos Grasos Poliinsaturados
ROS, Especies reactivas de oxígeno
TX, Tromboxanos
Tyr, Tirosol
WBOO, Los mejores aceites de oliva del mundo
WHO, Organización Mundial de la Salud0

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Anexo I. UHPLC chromatogram of hydroxytyrosol and hydroxytyrosol esterified with fatty acids (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA) from *in vitro* culture of THP-1 cells under experimental conditions., correspondiente al *Artículo 1*.. 155

RESUMEN

El hidroxitirosol (HT) es uno de los principales compuestos fenólicos del aceite de oliva. En los últimos años, de la mano de diversos estudios clínicos, se ha demostrado la asociación del consumo dietético de fuentes naturales de HT con beneficios para la salud, relacionados con su capacidad antioxidante y protectora frente a factores de riesgo cardiovascular (FRCV). Estos efectos beneficiosos dependen de su concentración en la matriz alimentaria, así como de su bioaccesibilidad y su biotransformación durante el proceso de digestión gastrointestinal en compuestos derivados que pueden contribuir a su biodisponibilidad total y al efecto biológico de esta molécula. Por tanto, resulta importante determinar la cantidad de HT y sus derivados como resultado de los diversos procesos fisiológicos (digestión) y reacciones metabólicas que darán lugar a metabolitos biológicamente activos. En este contexto, uno de los propósitos de este estudio fue analizar el efecto de la matriz alimentaria sobre la farmacocinética y biodisponibilidad del HT, destacando el aceite de oliva virgen extra como la matriz que proporcionó los mejores valores. Este trabajo ha contribuido a destacar la importancia de la matriz en la absorción final del HT. Sin embargo, recientemente se han identificado en el aceite de oliva derivados de HT esterificado con diferentes ácidos grasos (lipofenoles) que se caracterizan por un mayor carácter lipófilo. Esta propiedad redunda en una absorción celular y estabilidad metabólica mejoradas, de modo que ejercen su actividad biológica en tejidos u órganos diana de forma más eficiente. La presente Tesis Doctoral investigó mediante un modelo *in vitro* de simulación de digestión gastrointestinal el efecto de la matriz alimentaria y las enzimas digestivas sobre la bioaccesibilidad de lipofenoles de HT, concluyendo que su estabilidad depende en gran medida tanto de la matriz alimentaria como del ácido graso formando la esterificación. Asimismo, un enfoque lipidómico en un modelo de células mieloblásticas (línea monocítica THP-1) permitió investigar la actividad antioxidante y antiinflamatoria de las moléculas esterificadas en comparación con la molécula nativa, a través del estudio de la modulación de la síntesis de isoprostanos y prostaglandinas, marcadores de estrés oxidativo e inflamación, respectivamente. Los resultados obtenidos han permitido demostrar la función biológica de los lipofenoles de HT, siendo necesario la realización de estudios adicionales *in vivo* para la confirmación del potencial biológico de estos compuestos. Asimismo, seleccionar aquellos alimentos de origen vegetal que tengan un adecuado perfil de lipofenoles de HT en función de su actividad biológica, que permitan utilizarlos como coadyuvantes en la prevención de patologías cardiovasculares asociadas al estrés oxidativo y la inflamación.

Palabras clave: Aceite de oliva, Hidroxitirosol, Lipofenol, Biodisponibilidad, Bioaccesibilidad, Estrés oxidativo e inflamación.

ABSTRACT

Hydroxytyrosol (HT) is one of the main phenolic compounds in olive oil. Recently, resorting to an array of clinical studies it has been suggested the association of dietary consumption of HT with health benefits, mostly related to its antioxidant and protective capacity against cardiovascular risk factors (CVRF). These beneficial effects depend on the concentration in the food matrix, as well as the bioaccessibility and biotransformation rates during the gastrointestinal digestion that could give rise to new compounds contributing to the final bioavailability and biological effects of HT. Therefore, it is important to determine the stability of HT and the tentative formation of derivatives as a result of the various physiological processes (digestion) and metabolic reactions. In this frame, one of the purposes of this study was to analyse the influence of the food matrix on the pharmacokinetics and bioavailability of HT, which allowed highlighting extra virgin olive oil as the matrix that provided the best values. These results have helped to stress the importance of the matrix in the final absorption of HT. However, recently derivatives of HT esterified with different fatty acids (lipophenols) have been identified in olive oil. These compounds are characterized by a greater lipophilic character. This property results in improved cellular absorption and metabolic stability, so that they exert their biological activity on target tissues or organs more efficiently. This Doctoral Thesis investigated through an *in vitro* simulation of gastrointestinal digestion the effect of the food matrix and digestive enzymes on the bioaccessibility of HT lipophenols, concluding that their stability depends largely on both the food matrix and the fatty acid forming the esterification. Likewise, a lipidomics approach in a myeloblastic cell model (monocytic THP-1 line) allowed unravelling the antioxidant and anti-inflammatory activity of the esterified molecules compared to the native molecule, through the study of the modulation of the synthesis of isoprostanes and prostaglandins, markers of oxidative stress and inflammation, respectively. The results obtained have allowed to demonstrate the biological function of HT lipophenols, being necessary to carry out additional studies *in vivo* to confirm the biological potential of these compounds and to choice rationally those foods of plant origin that have an adequate profile of HT lipophenols, which would allow designing interventions aimed at preventing of cardiovascular pathologies associated with oxidative stress and inflammation.

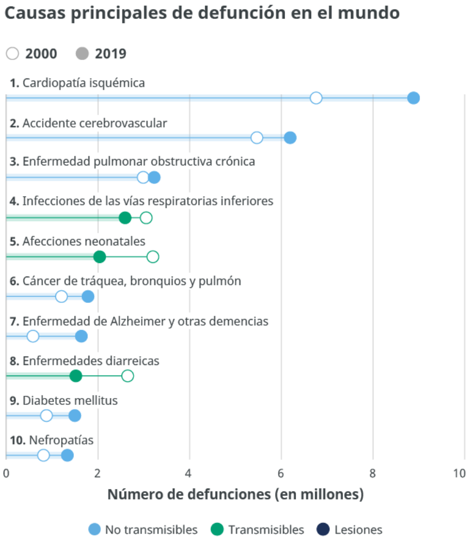
Keywords: Olive oil, Hydroxytyrosol, Lipophenol, Bioavailability, Bioaccessibility, Oxidative stress, Inflammation.

CAPÍTULO I - INTRODUCCIÓN

1. NUTRICIÓN Y SALUD CARDIOVASCULAR

1.1. FACTORES DE RIESGO DE LA PATOLOGÍA CARDIOVASCULAR

Las enfermedades cardiovasculares (ECV) están representadas por un conjunto de patologías que afectan al corazón y/o a los vasos sanguíneos. Entre aquellos procesos fisiopatológicos que producen alteraciones de la estructura tisular/celular del corazón o su normal funcionamiento cabe destacar la cardiopatía isquémica, la insuficiencia cardiaca, la cardiopatía reumática y las miocardiopatías. Por otro lado, en relación con las enfermedades vasculares, es decir, aquellas que afectan a los vasos sanguíneos, destacan la hipertensión arterial, enfermedades cerebrovasculares y la arteriopatía periférica, entre otras (1,2). Las ECV representan un problema de salud de primer orden en todo el mundo, siendo la principal causa de mortalidad en la última década (**Figura 1**). Desde una perspectiva epidemiológica, estas patologías son responsables del 28,3% de las defunciones anuales en España (3) y se caracterizan por una elevada prevalencia.



Fuente: WHO Global Health Estimates.

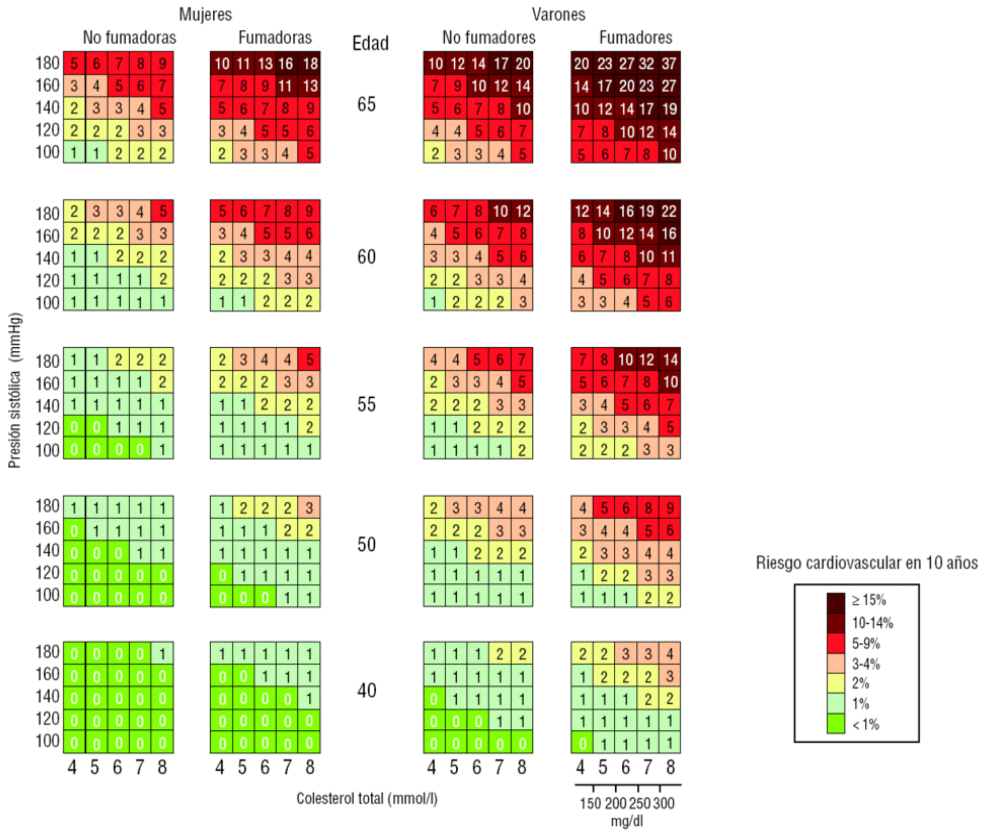
Figura 1. Principales causas de defunción en el mundo en 2019 (Organización Mundial de la Salud (WHO, del inglés *World Health Organization*): The top 10 causes of death (4)).

Estos indicadores epidemiológicos informan acerca del alto grado de discapacidad que ocasionan las ECV en los pacientes afectados, estando asimismo asociados a un aumento significativo de los costes asistenciales y, por tanto, causando un grave perjuicio, no solo a la población afectada como resultado de la evolución de la patología ECV específica y sus efectos incapacitantes, sino también a la sostenibilidad del sistema de salud y por tanto a la distribución de recursos en el tratamiento y la prevención de otros procesos fisiopatológicos (5). De acuerdo con estos indicadores epidemiológicos y la tendencia registrada en los últimos años, las perspectivas de mortalidad atribuible al conjunto de las ECV para el año 2030 son alarmantes.

Dichos referentes sugieren que cerca de 23,6 millones de personas morirán como consecuencia de este grupo de patologías, principalmente por enfermedades cardíacas y accidentes cerebrovasculares. Por tanto, se prevé que continúen siendo las principales causas de muerte (2). Por ello y para intentar paliar esta evolución epidemiológica, es imprescindible implementar estrategias de promoción de la salud cardiovascular en la población, así como identificar medidas de distinta naturaleza que permitan prevenir y reducir la incidencia y la prevalencia de este conjunto de patologías, así como la severidad de los efectos colaterales, contribuyendo a una mayor esperanza y calidad de vida de la población (6). Sin embargo, para implementar de forma eficiente tales medidas es imprescindible identificar aquellos factores de riesgo sobre los que es posible y necesario actuar en el escenario actual.

Pese a que los pacientes afectados por ECV suelen debutar en la edad adulta, los factores de riesgo están presentes desde edades tempranas. Dichas causas ejercen su efecto pernicioso sobre la salud ya en estas primeras etapas del desarrollo (infancia y adolescencia), estableciendo las bases fisiopatológicas de lo que en el futuro se convertirá en un daño vascular de entidad clínica. Así, un factor de riesgo cardiovascular (FRCV) es una característica biológica o un hábito o estilo de vida que aumenta la probabilidad de padecer o de morir a causa de una ECV en aquellos individuos que lo presentan. Sin embargo, es necesario entender que, al tratarse de una probabilidad (y de acuerdo con la idiosincrasia de este indicador estadístico), la ausencia de causas de riesgo no excluye la posibilidad de desarrollar una ECV en el futuro, de igual manera que la presencia de dichos factores de riesgo tampoco implica necesariamente su aparición (7,8).

Los FRCV se dividen en modificables y no modificables en función de la capacidad que ofrecen para interferir en ellos y, por tanto, influir en su efecto sobre los indicadores epidemiológicos de este conjunto de patologías (**Figura 2**).



Edad	Presión sistólica (mmHg)	Mujeres										Varones									
		No fumadoras					Fumadoras					No fumadores					Fumadores				
		4	5	6	7	8	4	5	6	7	8	4	5	6	7	8	4	5	6	7	8
65	180	5	6	7	8	9	10	11	13	16	18	10	12	14	17	20	20	23	27	32	37
	160	3	4	5	6	7	7	8	9	11	13	7	9	10	12	14	14	17	20	23	27
	140	2	3	3	4	5	5	6	7	8	9	5	6	7	8	10	10	12	14	17	19
	120	2	2	2	3	3	3	4	5	5	6	4	4	5	6	7	7	8	10	12	14
	100	1	1	2	2	2	2	3	3	4	5	2	3	3	4	5	5	6	7	8	10
60	180	2	3	3	4	5	5	6	7	8	9	6	7	8	10	12	12	14	16	19	22
	160	2	2	2	3	3	3	4	5	5	6	4	5	6	7	8	8	10	12	14	16
	140	1	1	2	2	2	2	3	3	4	4	3	3	4	5	6	6	7	8	10	11
	120	1	1	1	1	2	2	2	2	3	3	2	2	3	3	4	4	5	6	7	8
	100	1	1	1	1	1	1	1	2	2	2	1	2	2	2	3	3	3	4	5	6
55	180	1	1	2	2	2	2	3	4	4	5	4	4	5	6	7	7	8	10	12	14
	160	1	1	1	1	2	2	2	2	3	3	2	3	3	4	5	5	6	7	8	10
	140	1	1	1	1	1	1	1	2	2	2	2	2	2	3	3	3	4	5	6	7
	120	0	0	1	1	1	1	1	1	1	2	1	1	2	2	2	2	3	3	4	5
	100	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	2	2	3	3
50	180	1	1	1	1	1	1	2	2	2	3	2	3	3	4	4	4	5	6	8	9
	160	0	1	1	1	1	1	1	1	2	2	2	2	2	3	3	3	4	4	5	6
	140	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	3	3	4	4
	120	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2	3	3
	100	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	2	2
40	180	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	2	3	3	4
	160	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	2	2	2	3
	140	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	1	1	2	2
	120	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1

Figura 2. Tabla SCORE: estima el riesgo de muerte por enfermedad cardiovascular en sujetos de 40 a 65 años, en función del sexo, la presión arterial sistólica, el hábito tabáquico y los niveles de colesterol total. Considera un riesgo alto cuando la probabilidad de muerte a los 10 años es mayor o igual al 5% (11).

Los factores de riesgo no modificables están representados por la edad, el sexo, la predisposición genética o historia familiar, en tanto que el grupo de los factores modificables englobarían aquellos sobre los que se puede y se debe ejercer una acción preventiva, siendo por ello los de mayor interés desde la perspectiva de la profilaxis de las ECV. Estos factores modificables incluyen: tabaquismo, consumo de bebidas alcohólicas, sobrepeso/obesidad, inactividad física, hábitos alimentarios, hipertensión arterial, hipercolesterolemia y diabetes mellitus (DM). Adicionalmente a estos factores, recientemente se han identificado un conjunto de factores globales que, si bien se podrían considerar modificables, su ajuste escapa tanto de la capacidad del propio paciente como del equipo médico responsable de su cuidado. Estos están representados por la contaminación ambiental, el cambio climático y el estrés psíquico, que a la vez intervienen en la morbilidad y mortalidad asociadas a estas patologías (9,10).

De acuerdo con esta clasificación, la intervención posible y fundamental se centra en reducir el grado de exposición de los individuos y poblaciones (especialmente aquellos que cuentan en su historial clínico con uno o varios factores no modificables) a los FRCV modificables, al tiempo que se fortalece mediante formación y regulaciones legislativas la capacidad tanto individual como poblacional para tomar decisiones saludables y adoptar así conductas que favorezcan la salud cardiovascular, como se está haciendo recientemente en relación con la población infantil mediante campañas de promoción de una alimentación saludable, educación nutricional y fomento de la actividad física apoyadas por la Ley 17/2011 de Seguridad Alimentaria y Nutrición (12). En este sentido, es necesario crear entornos propicios que contribuyan a preservar la salud mediante el fomento de hábitos saludables, ayudándonos de la educación sanitaria y prestando especial atención a los hábitos de alimentación saludable (9,10,13,14).

De acuerdo con estas premisas, la Organización Mundial de la Salud y la Organización de las Naciones Unidas para la Alimentación y la Agricultura WHO and FAO (del inglés, *World Health Organization* and *Food and Agricultura Organization*), respectively) publicaron en 1990 un documento que trata sobre "La Dieta, Nutrición y prevención de Enfermedades Crónicas", que fue actualizado posteriormente en 2003. Dicho documento identifica factores como los hábitos dietéticos y de ejercicio físico que pueden reducir la probabilidad de una epidemia mundial de enfermedades crónicas, tanto en términos de incidencia como de severidad (15).

Conscientes de la importancia de la dieta en la prevención de enfermedades no transmisibles como las ECV, se han seguido celebrando desde hace años Conferencias Internacionales de Salud Cardiovascular. La primera tuvo lugar en 1992, y han permitido continuar mejorando las recomendaciones no sólo dietéticas, sino referidas a estilos de vida saludables en general, hasta establecer una aproximación más real a las necesidades y capacidades de los individuos (16).

1.2. ALIMENTACIÓN SALUDABLE

De entre todos los FRCV modificables referidos en la sección anterior, los hábitos dietéticos constituyen un factor paradigmático en relación con la diversidad de patologías cardiovasculares referidas. En este sentido, el Grupo de Revisión, Estudio y Posicionamiento de la Asociación Española de Dietistas-Nutricionistas (GRE-AED N) define alimentación saludable como "*…aquella que permite alcanzar y mantener un funcionamiento óptimo del organismo, conservar o restablecer la salud, disminuir el riesgo de padecer enfermedades, asegurar la reproducción, la gestación y la lactancia, y que promueve un crecimiento y desarrollo óptimos. Debe ser satisfactoria, suficiente, completa, equilibrada, armónica, segura, adaptada, sostenible y asequible*" (17).

A esta definición es necesario añadirle el perfil epidemiológico nutricional, que se encuentra estrechamente ligado con los hábitos dietéticos del individuo, por lo que resulta de gran interés conocer el patrón de dietético de un individuo, familia o grupos de personas. Esto permite crear guías nutricionales/dietéticas y desarrollar estrategias encaminadas a promover hábitos de alimentación saludable (18). En este sentido, desde la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) se inició en 2005 la Estrategia NAOS (Estrategia para la Nutrición, Actividad Física y prevención de la Obesidad). Dicha acción persigue invertir la tendencia de la prevalencia de la obesidad, como FRCV modificable, mediante el fomento de una alimentación saludable y de la práctica de la actividad física y, con ello, reducir significativamente las altas tasas de morbilidad y mortalidad atribuibles a las ECV (19). En el año 2011, la Estrategia NAOS fue consolidada e impulsada por la Ley 17/2011, de 5 de julio, de seguridad alimentaria y nutrición, nombrada anteriormente (12).

Así, en términos generales, la ingesta diaria debe proporcionar la cantidad suficiente de macronutrientes y micronutrientes, de acuerdo con las necesidades fisiológicas de los distintos grupos de población, en consonancia con sus hábitos de vida. Este hecho sugiere que las necesidades nutricionales dependerán de las características específicas de cada individuo, como son la edad, sexo, estado fisiológico y grado de actividad física (20).

En todas sus variantes culturales, la alimentación va a condicionar la salud de la población como parte fundamental de los factores modificables. En los países que forman parte de la cuenca mediterránea existen diferentes patrones dietéticos, reflejo de la evolución que ha tenido lugar a lo largo de los años en base, fundamentalmente, a las principales producciones agro-pecuarias locales. Aunque actualmente, dichas producciones y consecuentemente los patrones dietéticos se encuentran condicionadas por el cambio climático y la globalización, hay ingredientes cuya presencia se ha mantenido de forma constante en las distintas regiones del arco mediterráneo independientemente de estos condicionantes, como son las aceitunas de mesa y el aceite de oliva, entre otros. Estas matrices alimentarias son de especial relevancia, ya que constituyen un componente dietético con importantes propiedades biológicas, considerándose una de las fuentes de grasas dietéticas más saludables debido a su contenido en ácidos grasos mono- y poliinsaturados. En este sentido, el aceite de oliva es la principal fuente de grasa en la dieta mediterránea y diversos estudios de intervención nutricional realizados hasta la fecha actual han permitido establecer una correlación significativa e inversa entre su consumo y la tasa de mortalidad a consecuencia de ECV (21). Estos datos se han visto reforzados recientemente por el Estudio de Prevención con Dieta Mediterránea (PREDIMED), que ha proporcionado evidencias de la contribución de una dieta rica en aceite de oliva en la reducción de la incidencia de eventos cardiovasculares en personas con factores de riesgo (22).

De acuerdo con estos antecedentes, la dieta mediterránea tradicional se asocia con una optimización de los indicadores epidemiológicos en relación con la ECV, tanto en la población general como en individuos con una predisposición genética o familiar (20). Así, en la última década se ha incrementado la evidencia científica en torno al vínculo entre los patrones dietéticos y el estado de salud o el desarrollo de enfermedades crónicas como, por ejemplo, la asociación entre el consumo de granos refinados y el riesgo de mortalidad y ECV (23), la relación existente entre el consumo de frutas, verduras y legumbres y un menor riesgo de mortalidad (24,25,26), o la ingesta de carnes blancas como alternativa más saludable frente al consumo de carnes rojas y productos cárnicos procesados (22,27).

Los resultados de estos estudios evidencian los posibles efectos protectores de diversos componentes alimentarios frente a ECV, ejercidos a través de diferentes mecanismos de acción, como la mejora del perfil lipídico, la disminución de la presión arterial, una menor incidencia de procesos inflamatorios y la protección frente al estrés oxidativo. Queda claro, por tanto, el importante papel de la alimentación (*per se* o como coadyuvantes del tratamiento farmacológico), tanto en la reducción de la incidencia, como de la severidad y mortalidad de patologías instauradas.

2. COMPUESTOS BIOACTIVOS CARDIOSALUDABLES PRESENTES EN MATRICES OLEOSAS

Las propiedades biológicas atribuibles a los alimentos de origen vegetal y más específicamente a las aceitunas y al aceite de oliva, se han reforzado en las últimas décadas con el desarrollo de ambiciosos estudios clínicos que han atribuido dichas funciones biológicas a su composición en nutrientes y no nutrientes bioactivos (28).

En relación con los nutrientes presentes en el aceite de oliva con actividades biológicas promotoras de la salud, cabe destacar su elevada concentración en ácidos grasos mono- y poliinsaturados, junto con micronutrientes antioxidantes como la vitamina E (principal fuente de protección frente a los radicales libres que provocan oxidación celular), así como las vitaminas A, D y K (29).

El ácido graso monoinsaturado más abundante en la dieta mediterránea es el ácido oleico, siendo la fuente de grasa que predomina en el aceite de oliva. Tanto los ácidos grasos monoinsaturados como los poliinsaturados reducen significativamente los niveles de LDL-colesterol (del inglés *low-density lipoprotein*), principal partícula transportadora de colesterol en el plasma relacionado con la aparición de aterosclerosis y cardiopatías coronarias. Simultáneamente, estos ácidos grasos aumentan los niveles de HDL-colesterol (del inglés *high-density lipoprotein*) capaces de proteger frente al desarrollo de cardiopatías coronarias (30).

Por otro lado, los compuestos fitoquímicos bioactivos descritos en los alimentos de origen vegetal son compuestos esenciales y no esenciales, como los polifenoles, localizados en los distintos tejidos de plantas superiores (comestibles y no comestibles) y que, tras su ingesta, ejercen efectos beneficiosos sobre la salud más allá de las meramente nutricionales (31).

Los estudios disponibles actualmente en relación con los compuestos fitoquímicos han permitido sugerir su interés como compuestos responsables en mayor o menor medida del beneficio para la salud asociado al consumo de frutas y hortalizas, siendo las actividades biológicas desarrolladas dependientes de la estructura química de dichos compuestos (polifenoles, compuestos con base de azufre (glucosinolatos e isotiocianatos), terpenos, oxilipinas y hormonas vegetales, entre otros) (32).

En relación con la actividad biológica, se han atribuido numerosas propiedades saludables a dichos compuestos fitoquímicos. Entre ellos, los compuestos fenólicos han sido promovidos en relación con su capacidad de prevenir el estrés oxidativo mediante la captación de radicales libres. Más allá de los mecanismos moleculares, esta capacidad biológica se ha asociado con la reducción de la incidencia y prevalencia de ECV, así como algunos tipos de cáncer (32,33,34,35).

2.1. COMPUESTOS BIOACTIVOS DEL ACEITE DE OLIVA

De acuerdo con la distribución de los nutrientes y no nutrientes bioactivos en los alimentos de origen vegetal, éstos se encuentran presentes de manera natural en nuestra dieta en mayor o menor concentración dependiendo de los hábitos dietéticos específicos. Como mencionamos previamente, uno de los ingredientes tradicionales de la dieta mediterránea es el aceite de oliva, caracterizado por una alta concentración de ácidos grasos mono- y poliinsaturados. De los ácidos grasos monoinsaturados (MUFAs del inglés *monunsaturated fatty acids*), el ácido oleico es el mayoritario, representando entre el 55% y el 83% del total de ácidos grasos. Por otro lado, entre el 4% y el 20% de la fracción grasa del aceite de oliva corresponde a ácidos grasos poliinsaturados (PUFAs del inglés *polyunsaturated fatty acids*), entre los que destacan los ácidos linoleico y α-linolénico como los más abundantes (37). Finalmente, los ácidos grasos saturados, fundamentalmente el ácido palmítico, representan entre el 8% y el 14% (36,37) (**Figura 3**).

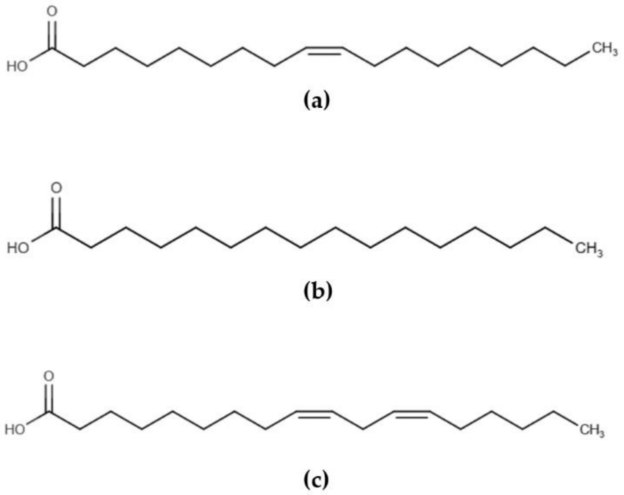


Figura 3. Ácidos grasos mayoritarios en el aceite de oliva: (a) ácido oleico, (b) ácido palmítico y (c) ácido linoleico**.**

En el aceite de oliva pueden distinguirse la fracción no saponificable y la fracción saponificable, que constituyen el 1-3% y el 97-99% del aceite de oliva, respectivamente.

La fracción no saponificable es, en parte, la responsable de la estabilidad y la calidad sensorial del aceite de oliva, afectando a factores tales como su color, su aroma y su sabor, aparte de otras propiedades beneficiosas para la salud como su capacidad de captación de radicales libres (37). El contenido de los componentes minoritarios o no saponificables del aceite de oliva depende de las condiciones edafoclimáticas, de la variedad de la aceituna, del estado de madurez de las aceitunas en la cosecha y del sistema de procesado utilizado para producir el aceite de oliva. La mayoría de estos compuestos son degradados durante el proceso de refinado, por lo que fundamentalmente se encuentran en la composición de los aceites de oliva vírgenes. Las diversas clases de componentes presentes en la fase no saponificables se pueden dividir en dos grupos; el primero formado por derivados de ácidos grasos, como los fosfolípidos, ceras y ésteres de esteroles, y el segundo grupo (que no incluye ácidos grasos en sus estructuras), se encuentra formado por hidrocarburos, alcoholes alifáticos, esteroles libres, tocoferoles, clorofilas, carotenoides y polifenoles, reconocidos por sus propiedades antioxidantes (37).

Por otro lado, la fracción saponificable está formada en su mayor parte por triglicéridos, y en menor proporción por diglicéridos, monoglicéridos y ácidos grasos libres (36,37,38).

2.2. POLIFENOLES DEL ACEITE DE OLIVA

Los polifenoles son el grupo más amplio de no nutrientes presentes en los alimentos de origen vegetal (37). Están formados por estructuras moleculares que contienen un grupo fenol, un anillo aromático unido al menos a un grupo funcional hidroxilo. Desde el punto de vista de la estructura química, son un grupo muy diverso que comprende desde moléculas sencillas hasta polímeros complejos de alto peso molecular. Son los responsables del sabor amargo, característico del aceite de oliva. Los polifenoles presentes en el aceite de oliva están representados por flavonoides, lignanos, alcoholes fenólicos, secoiridoides y ácidos fenólicos. (39,40) (**Figura 4**).

Dentro del grupo de los ácidos fenólicos y sus derivados destaca la presencia del ácido 3,4-dihidroxifenilacético (DOPAC, del inglés *3,4-dihydroxyphenyl acetic acid*), 4-hidroxi-3-ácido metoxibenzoico (ácido vanílico), ácido 3,4,5-trihidroxibenzoico (ácido gálico), el ácido 4-hidroxifenilacético y el ácido 2,5-dihidroxibenzoico (ácido gentísico), entre otros.

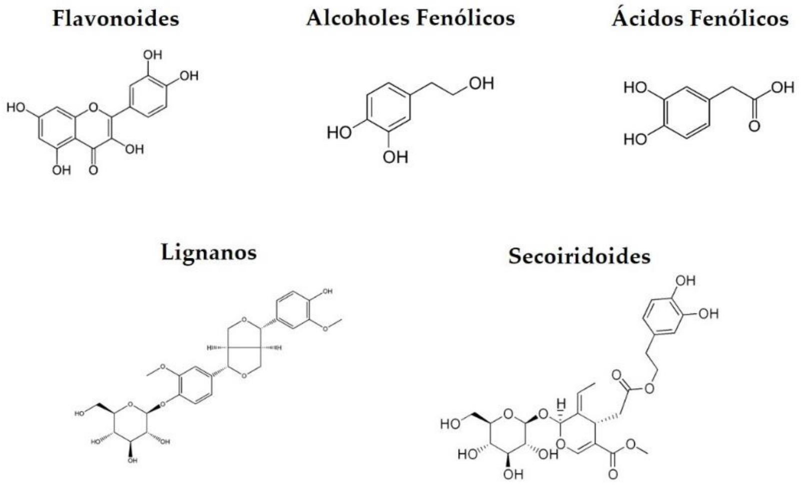


Figura 4. Estructura química de las distintas clases de polifenoles presentes en el aceite de oliva.

Por otro lado, en el grupo de alcoholes fenólicos cabe destacar el hidroxitirosol (HT, del inglés *hydroxytyrosol*), acetato de hidroxitirosol (HTA, del inglés *hydroxytyrosol acetate*) y el tirosol (Tyr, del inglés *tyrosol*). Finalmente, entre los secoiridoides y sus derivados se encuentra la oleuropeína (40,41).

3. HIDROXITIROSOL

3.1. HIDROXITIROSOL: RELACIÓN ESTRUCTURA ACTIVIDAD

El hidroxitirosol (HT), también conocido como 3,4-dihidroxifeniletanol, es el alcohol fenólico más abundante en el material vegetal (comestible y no comestible) obtenido del olivo, concentrándose principalmente en las hojas, aunque también se encuentra presente en aceitunas y, consecuentemente, en el aceite de oliva. En estas matrices vegetales, el HT se encuentra en forma libre o conjugada (40). Está considerado uno de los antioxidantes naturales más potentes, dado que su estructura química le confiere una alta capacidad de captación de radicales libres, vinculado con la cesión de hidrógeno, es decir, con su capacidad para estabilizar radicales mediante la formación de un enlace de hidrógeno intramolecular entre los hidrógenos libres de su grupo hidroxilo y sus radicales fenoxilos (38). Esta capacidad de bloquear radicales libres le confiere características muy relevantes y beneficiosas como agente regulador el daño oxidativo a nivel celular. El HT no se acumula en el organismo tras su absorción intestinal. Se excreta de forma rápida, por lo que no se ha asociado a efectos tóxicos (42,43). Basado en su capacidad de captación de radicales libres, interviene en el equilibrio corporal de producción/eliminación de especies reactivas de oxígeno (ROS, por sus siglas inglesas *Reactive Oxygen Species*) que dan lugar a fenómenos de estrés oxidativo implicados en cambios moleculares a nivel celular. Este es un factor de riesgo que condiciona el origen y la evolución de diversas enfermedades degenerativas, enfermedades crónicas y envejecimiento, así como diferentes enfermedades que cursan con inflamación (37,38,44).

La realización de diversos estudios pre-clínicos, así como de intervenciones dietéticas en humanos, ha permitido demostrar que el consumo de aceite de oliva contribuye a reducir la incidencia y severidad de ECV a través de la inducción de cambios positivos en el perfil lipídico, la disminución de la oxidación del colesterol y el aumento de los niveles plasmáticos de lipoproteínas de alto peso molecular (HDL-colesterol), actuando positivamente sobre diversos FRCV (45). En este sentido, los resultados del estudio EUROLIVE ('The effect of olive oil on oxidative damage in European population') han permitido demostrar los efectos beneficiosos de los compuestos fenólicos del aceite de oliva sobre el daño oxidativo, la inflamación y la disfunción endotelial, así como una valiosa actividad antitrombótica (46). Igualmente, el HT es capaz de proteger a las células nerviosas frente a la peroxidación lipídica, ya que es capaz de atravesar la barrera hematoencefálica y ejercer su capacidad antioxidante en las células del sistema nervioso (47). En consecuencia, el HT contribuye a reducir la incidencia de enfermedades neurodegenerativas como el Alzheimer y el Parkinson, así como el declive cognitivo asociado al envejecimiento (47,48).

También se han descrito efectos saludables del HT en la mejora de procesos inflamatorios digestivos, obesidad, diabetes y síndrome metabólico. Además, potencia el sistema inmunitario, previene la osteoporosis y se han podido demostrar efectos protectores frente a la proliferación de células cancerígenas

(48,49,50,51) (**Tabla 1**). En resumen, las propiedades beneficiosas del HT para la salud humana están fuertemente relacionadas con su capacidad para eliminar ROS, así como de activar los mecanismos moleculares antioxidantes endógenos (50).

Tabla 1. Actividad biológica y beneficios para la salud asociados al consumo aceitunas / aceites de oliva.

Actividad biológica	Marco fisiopatológico	Ref.
Actividad antioxidante	Enfermedades degenerativas y cardiovasculares	(47)
Actividad anti-inflamatoria	Inhibición de enzimas pro inflamatorias	(46)
Actividad anti-microbiana	Enfermedades infecciosas	(40)
Actividad anti-aterogénica	Enfermedades coronarias y cerebrovasculares	(49)
Actividad anti-tumoral	Cáncer	(50)
Actividad anti-agregación plaquetaria	Enfermedades coronarias y cerebrovasculares	(46)
Actividad anti-hipertensiva	Hipertensión arterial	(48)
Aumento de vitamina a y actividad de β-caroteno	Antienvejecimiento / protección de la piel	(40)
Aumento de la actividad inmune	Enfermedades infecciosas, cáncer	(50)
Reducción en los niveles de colesterol y LDL en plasma	Enfermedades coronarias	(40)

En el aceite de oliva se pueden encontrar pequeñas cantidades de derivados de HT y ésteres grasos de HT (lipofenoles de HT) (52). El HT es un compuesto muy polar, de baja solubilidad en solventes apolares y fácilmente oxidable en soluciones acuosas. Esta naturaleza hidrofílica provoca que, durante el proceso de extracción del aceite de oliva un porcentaje importante de HT se pierda, quedando disuelto en los subproductos acuosos de la elaboración del aceite (53).

Debido a la limitada solubilidad del HT en medios lipídicos debido a su naturaleza polar del HT, en los últimos años se ha investigado el aislamiento y síntesis química o enzimática de compuestos que conserven la bioactividad del compuesto fenólico, pero que posean un mayor carácter lipófilo, de modo que puedan atravesar más fácilmente membranas lipídicas (membranas celulares) y ejercer su actividad biológica en tejidos u órganos diana. Así pues, la introducción en la cadena lateral de un acilo de diferente longitud genera moléculas más lipófilas, sin que por ello se altere la bioactividad inherente a la cadena catecol. La caracterización de estas entidades químicas ha permitido definir una nueva clase de compuestos potencialmente bioactivos llamada lipofenoles o fenolípidos.

En 2016, la investigación llevada a cabo por Lee *et al*. permitió identificar por primera vez el HT en su forma conjugada a ácidos grasos mono- y poli-insaturados a través del alcohol primario del derivado fenólico en aceites de oliva. El establecimiento de la estructura química de estos compuestos llevó a plantear un eventual aumento de la biodisponibilidad del HT en esta forma química debido a la polaridad diferencial conferida por la conjugación con ácidos grasos existentes en los propios aceites. Estas moléculas se encuentran en concentraciones elevadas en aceite de oliva virgen extra (52). Consecuentemente, el referido aumento de biodisponibilidad del HT junto a la funcionalidad del ácido graso, contribuiría dar lugar a moléculas que aunarían las características químicas y biológicas propias de los ácidos grasos y las propiedades moleculares y biológicas de los polifenoles en un único compuesto (55). Además, existe evidencia de que la presencia de esteres grasos de HT contribuyen a una mayor capacidad antioxidante y un mayor efecto en la reducción del estrés oxidativo en comparación con los polifenoles no esterificados, aunque los mecanismos responsables de estos efectos beneficiosos necesitan ser estudiados en profundidad (52,55).

El carácter lipófilo de estos compuestos esterificados juega un importante papel en la absorción celular y su estabilidad metabólica, (54) existiendo una relación directa entre la longitud de la cadena alquílica y el grado de insaturación del ácido graso con la estabilidad de los ésteres de HT a nivel intestinal, de forma que las cadenas alquílicas con mayor número de carbonos y con menos insaturaciones parecen ser hidrolizadas en menor grado (53,56). Sin embargo, es necesario seguir investigando sobre en la estabilidad y biodisponibilidad de los lipofenoles tras su administración en humanos (55).

3.2. HIDROXITIROSOL Y SUS DERIVADOS ESTERIFICADOS EN LA DIETA

España produce cerca del 50% de los aceites de oliva de todo el mundo, situándose a la cabeza de este sector a nivel mundial. En la clasificación que cada año elabora la organización internacional *World's Best Olive Oils* (WBOO), los aceites de oliva de España aparecen como lo más premiados, encontrándose en primer lugar en la edición 2019/2020 (57).

Durante los últimos años, se ha demostrado la asociación del consumo dietético de fuentes naturales de HT, como es el aceite de oliva y las aceitunas de mesa con numerosos beneficios para la salud, habiéndose descrito de forma específica una acción protectora frente a los FRCV a través de estudios pre-clínicos. La Autoridad Europea de Seguridad Alimentaria (EFSA, del inglés *European Food Safety Authority*) ha avalado publicaciones científicas que sugieren la importancia del HT en la protección de las lipoproteínas de baja densidad y, por consiguiente, su participación en la reducción del riesgo cardiovascular, recomendando un consumo diario de 5 mg de HT y sus derivados para alcanzar este resultado a nivel fisiológico requerido para el desarrollo de los efectos biológicos deseados (58). Esto es de especial relevancia debido a las ventajas demostradas acerca de la actividad biológica del HT frente a otros antioxidantes, en especial, debido a su elevada biodisponibilidad y actividad biológica (59).

Dados los beneficios para la salud descritos y confirmados de la mano de diversos estudios de intervención nutricional, en la actualidad, existe un aumento en la utilización de suplementación alimenticia con antioxidantes para contrarrestar la acción de los radicales libres y el estrés oxidativo (60) y destaca la síntesis de ésteres de HT para una potenciación en su carácter antioxidante gracias a un mejor balance hidrofílico/lipofílico (53). Sin embargo, se continúa investigando la posibilidad de incrementar la afinidad por medios lipídicos del HT sin comprometer su valioso potencial biológico, dado que, al administrarlo en humanos como complemento en disoluciones acuosas, su absorción se ha revelado como muy baja. De este modo, se favorecería la absorción gastrointestinal y su capacidad de atravesar las membranas lipídicas celulares, aumentando la capacidad de acceder a sus dianas moleculares. Estas ventajas se obtendrían de la mano de la síntesis de ésteres de HT, lo que permitiría su mejor aprovechamiento tanto en alimentación como en cosmética (61).

Además, algunas investigaciones han demostrado la existencia de altos niveles de derivados esterificados de HT en el aceite de oliva virgen y virgen extra, entre los que destacan: hidroxitirosol conjugado con ácido α-linolénico (HT-ALA, del inglés *hydroxytyrosyl* α*-linolenate*), hidroxitirosol conjugado con ácido linoleico (HT-LA, del ingñes *hydroxytyrosyl linoleate*) y hidroxitirosol conjugado con ácido oleico (HT-OA, del inglés *hydroxytyrosyl oleate*) (52). (**Figura 5**), así como en varias matrices alimentarias de naturaleza oleaginosa como aceite de oliva refinado, aceite de semilla de uva, aceite de lino, o margarina (55), además de identificar oleato de hidroxitirosol en subproductos de la industria oleícola (54).

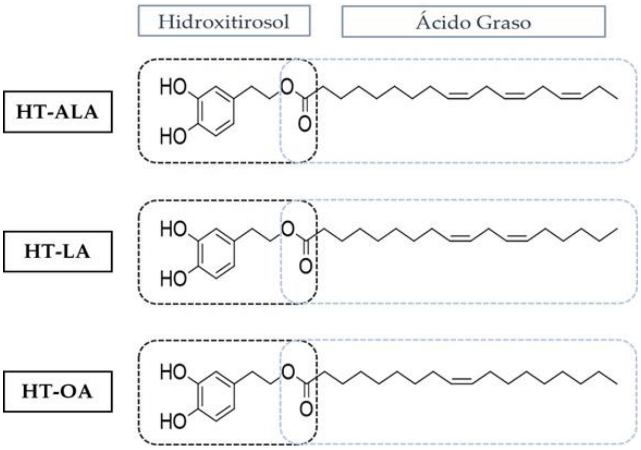


Figura 5. Lipofenoles de hidroxitirosol presentes en el aceite de oliva: HT-ALA (hidroxitirosol esterificado con ácido α-linolénico), HT-LA (hidroxitirosol esterificado con ácido linoleico) y HT-OA (hidroxitirosol esterificado con ácido oleico).

Estas caracterizaciones sugieren que la alta concentración de estos derivados de HT en los aceites podría aumentar la biodisponibilidad del HT, mejorando su capacidad de acceder a las células diana a través de membranas celulares y, por tanto, de establecer las interacciones moleculares responsables de su actividad biológica (52,55). En este sentido, Medina *et al*. han descrito recientemente que la formación de derivados esterificados de HT aumenta cuando las matrices se fortifican con el compuesto fenólico nativo, en este caso HT (55). El estudio de los mecanismos moleculares responsables de estos hallazgos permitiría la formulación de nuevos productos con importantes aplicaciones nutracéuticas y farmacéuticas (55).

4. FARMACOCINÉTICA Y BIODISPONIBILIDAD DE DERIVADOS DEL HIDROXITIROSOL

4.1. BIOACCESIBILIDAD DE LOS DERIVADOS DE HIDROXITIROSOL

Como se ha referido anteriormente, los efectos biológicos del HT dependen de su capacidad para alcanzar los órganos diana donde va a realizar su función. A su vez, esta capacidad está asociada con la capacidad del proceso de digestión gastrointestinal para extraer el HT de la matriz alimentaria, y condicionada por su estabilidad frente a las condiciones físico-químicas características del proceso de digestión gastrointestinal (bioaccesibilidad), quedando disponible para su absorción (62).

El proceso de digestión se inicia en la boca con la masticación y continua en el estómago como resultado de la acción de diferentes enzimas que se encargan de ir descomponiendo la matriz alimentaria (63). La bioaccesibilidad está influenciada por la composición de la matriz alimentaria, las sinergias y antagonismos entre los diferentes elementos, las propiedades fisicoquímicas, como el pH, la temperatura y las características de la matriz, pero también, por la idiosincrasia fisiopatológica de la persona que lo consume (64). Algunos autores consideran que el concepto de bioaccesibilidad abarca también la modificación de los compuestos bioactivos durante la digestión que da lugar a otras sustancias que puedan ser asimiladas por el organismo, así como los compuestos resultantes de su metabolismo pre-sistémico en el epitelio intestinal y en las células hepáticas (65).

Secundariamente, los compuestos extraídos y presentes en los digestatos deben atravesar el epitelio intestinal para ser llevado a través de la sangre a los distintos órganos, tejidos y tipos celulares, donde podrán ejercer sus actividades biológicas (biodisponibilidad). En este sentido, en comparación con el HT libre, los derivados lipofílicos representados por los lipofenoles de HT tienen varias ventajas, como la mejora del perfil farmacológico y la biodisponibilidad, lo que estaría asociado a una mayor bioactividad. Por otro lado, es importante tener en cuenta que la bioactividad que puede ejercer un compuesto no será dependiente exclusivamente de su concentración, sino también de sus características químicas. En este sentido, como se ha referido previamente, los lipofenoles combinarían las propiedades del compuesto fenólico (HT) y el ácido graso en una única molécula con características anfifílicas.

Actualmente, no existe información en la literatura científica sobre el mecanismo y el proceso de absorción intestinal de derivados esterificados de HT presentes en matrices alimentarias. Sin embargo, de acuerdo con estudios recientes realizados en base a modelos *in vitro* de simulación gastrointestinal sobre lipofenoles de Tyr, el grado de hidrólisis de las moléculas esterificadas por acción de las enzimas pancreáticas se correlaciona negativamente con la longitud de la cadena alquílica de la parte lipídica y positivamente con el grado de instauración del ácido graso (66). Además, estudios realizados en modelos pre-clínicos acerca de los mecanismos de transporte de lipofenoles de Tyr, corroboró estas conclusiones, describiendo asimismo un comportamiento de liberación sostenida durante el proceso de digestión (67). Esta circunstancia se encuentra asociada con una extensión del tiempo de acción del compuesto fenólico, lo que contribuiría a mejorar el interés biológico descrito para estos compuestos (67). En este contexto, tanto el HT libre como sus derivados lipofenólicos pueden sufrir diversas reacciones de hidrólisis y conjugación, aunque actualmente no se conocen con precisión los mecanismos exactos de los derivados esterificados de HT para ejercer sus actividades biológicas *in vivo*. De este modo, los efectos beneficiosos del HT y sus derivados vienen determinados no solo por su concentración en los alimentos, sino que es fundamental conocer su bioaccesibilidad y biodisponibilidad para poder entender en toda su extensión su actividad biológica.

4.1.1. Impacto del proceso digestivo

La bioaccesibilidad del HT puede verse afectada por las condiciones físico-químicas inherentes al proceso digestivo oro-gastro-intestinal. Como resultado de este proceso, una vez liberado de la matriz alimentaria, el HT y sus derivados pueden sufrir diferentes cambios estructurales por la influencia de diversos factores físico-químicos (pH y temperatura), enzimáticos (actividad de las diferentes enzimas salivares, gástricas, pancreáticas e intestinales) y mecánicos (masticación, deglución y peristalsis) (68).

Para entender el impacto que la digestión gastrointestinal ejerce sobre los componentes de la matriz alimentaria, se han desarrollado un conjunto de modelos experimentales con características y ventajas complementarios los unos respecto a los otros. Así, los efectos de la digestión gastrointestinal pueden determinarse mediante modelos *in vitro* estáticos y dinámicos, así como modelos *in vivo* (pre-clínicos y estudios de intervención dietética en humanos). Los estudios *in vivo* permiten obtener una información más consistente, robusta y específica sobre la bioaccesibilidad y biodisponibilidad de un compuesto determinado, permitiendo monitorizar fenómenos de competencia entre compuestos o de regulación nerviosa u hormonal, entre otras ventajas. Sin embargo, este modelo está también sujeto a inconvenientes que no permiten su utilización generalizada, al tratarse de modelos muy costosos y complejos, y estar sometidos a consideraciones éticas no siempre eludibles.

Para superar las limitaciones referidas en relación con los modelos *in vivo* (especialmente aquellas relacionadas con las intervenciones nutricionales en humanos), recientemente se han desarrollado nuevos modelos *in vitro* (estáticos y dinámicos), rápidos y seguros, que no están afectados por las restricciones éticas de los modelos *in vivo*. En este sentido, los modelos *in vivo* aportan información sobre factores asociados a los sujetos, en tanto que los modelos *in vitro* permiten explorar más en profundidad aspectos relacionados con el alimento y con los mecanismos moleculares responsables de los resultados obtenidos para los diferentes compuestos. Los procesos realizados en laboratorio se simulan bajo condiciones controladas de tiempo, pH, temperatura, etc., utilizando enzimas digestivas comerciales, como la pepsina y la pancreatina, para mimetizar las condiciones fisiológicas que se producen *in vivo*. Por otro lado, para evaluar la eficiencia del proceso de absorción intestinal se utilizan modelos de barrera epitelial basados en una monocapa de células epiteliales productoras y/o no productoras de mucus (cultivo individual o mixto) que permiten explorar la capacidad de los compuestos de interés de ser absorbidos (62,69,70). La absorción de los compuestos fenólicos a través del epitelio intestinal puede producirse a través de diversos mecanismos, que van a depender principalmente de la naturaleza química del compuesto para finalmente alcanzar los tejidos diana donde ejercerán su bioactividad a través de la circulación sistémica.

Aquellas moléculas que no pueden atravesar la membrana epitelial debido a sus características químicas, continúan el tránsito intestinal alcanzando el colon, donde son metabolizados por la microbiota colónica.

A través de estudios postprandiales se ha descrito la excreción de hasta un 90% de HT ingerido y sus metabolitos a través de orina y un 5% en heces (71,72), no obstante, actualmente, es necesario profundizar más acerca de las concentraciones plasmáticas de moléculas esterificadas de HT para clarificar con precisión los mecanismos de absorción de lipofenoles.

Así, la biodisponibilidad y bioactividad de estos compuestos fenólicos y sus derivados dependen de diversos factores, algunos de ellos intrínsecos al alimento, otros relacionados con los procesos tecnológicos, de elaboración/cocinado, asociados a los hábitos alimentarios de los consumidores y con la fisiología del sistema gastrointestinal (digestión, absorción y metabolismo de la microbiota) (73).

4.1.2. Influencia de la matriz alimentaria

En los últimos años se están realizando numerosos avances en relación con el uso potencial del HT como componente funcional en suplementos alimenticios y nutracéuticos, de utilidad para la reducción del riesgo de ECV (74). Sin embargo, para optimizar los resultados en relación con la concentración de HT en las células diana resulta imprescindible identificar aquellos vehículos especialmente apropiados para asegurar una óptima concentración en el producto de la digestión gastrointestinal y la mayor absorción de HT, maximizando así sus efectos beneficiosos en la salud.

En relación con estas premisas, las primeras evidencias acerca de la absorción de HT en humanos datan del año 2000 (75), y permitieron identificar la presencia de HT en orina en una cantidad proporcional a la suministrada vía oral, a través de diferentes aceites de oliva. Estos resultados sugirieron que la cantidad de HT absorbida dependía del vehículo en el que se administraba a los sujetos. Así, la excreción urinaria de HT fue mayor cuando se administró aceite de oliva virgen en comparación a los aceites de oliva refinados enriquecidos con compuestos fenólicos o cuando se añadió HT a otro alimento de naturaleza no oleaginosa (yogurt) (76,77). Este resultado fue confirmado por Tuck *et al*. (2001), quienes evidenciaron una absorción intestinal superior cuando se administraba HT a través de una matriz oleaginosa (90%) en comparación con otra de naturaleza acuosa (75%) (78).

El HT, por su carácter polar, tiene dificultades para atravesar la membrana lipídica, lo que ha despertado el interés actual por la esterificación de dicho compuesto fenólico con determinados ácidos grasos. En la industria alimentaria, el desarrollo de antioxidantes lipídicos podría realizarse para proteger la matriz alimentaria de procesos de oxidación. Mientras que, con fines nutracéuticos, aumentar la lipofilia de compuestos fenólicos polares podría realizarse para mejorar su perfil farmacológico (79). De este modo, profundizar en el conocimiento sobre la naturaleza de las matrices para saber cuáles son las más efectivas para el uso potencial del HT y sus derivados lipofenólicos como componentes funcionales, será de gran utilidad para la disminución del riesgo cardiovascular y prevención de enfermedades ocasionadas por procesos oxidativos.

4.2. METABOLISMO DE HIDROXITIROSOL Y LIPOFENOLES DE HIDROXITIROSOL

Después de ser liberados de la matriz alimentaria, la fracción bioaccesible del HT y es absorbida en el tracto gastrointestinal e inician su metabolismo en las células epiteliales intestinales y los hepatocitos fundamentalmente, con el objetivo de conseguir una detoxificación metabólica. La necesidad de metabolizar dichas moléculas está fundamentada por su condición de agentes xenobióticos, que deben ser transformados para aumentar su polaridad y así acelerar su excreción urinaria al tiempo que se reduce su bioactividad, regulando su efecto potencialmente "tóxico" sobre el organismo. Por otro lado, aquellos compuestos que retornan a la luz intestinal como resultado del reflujo epitelial o a través de la circulación enterohepática, son susceptibles de ser metabolizados por parte de la microbiota colónica, dando lugar a nuevos metabolitos potencialmente bioactivos que acaban definiendo la biodisponibilidad final de los compuestos de interés (80). No obstante, la aplicación de estos mecanismos de digestión, absorción o metabolismo a los derivados esterificados de HT requiere de caracterizaciones adicionales.

En relación con las reacciones responsables de la metabolización del HT y sus derivados, estos sufren transformaciones cuya naturaleza es dependiente de sus características químicas (polaridad y lipofilia). Dicha metabolización es dependiente de reacciones enzimáticas de fase I y de fase II. En este sentido, la detección inmediata de estos derivados metabólicos de HT sugiere que su metabolismo comienza en las propias células intestinales por la acción de la enzima catecol-ortometil-transferasa (COTM, del inglés *catechol O-methyltransferase*) (70). La actividad de dicha enzima depende tanto de la naturaleza del compuesto fenólico como de la dosis administrada, pudiendo estar además influenciada por circunstancias fisiológicas y diversidad genética que ocasionan diferencias importantes interindividuales en la capacidad de metabolizar los compuestos fenólicos y sus derivados (56). Durante el metabolismo de fase I, los compuestos fenólicos son comúnmente sometidos a reacciones de oxidación, reducción e hidrólisis, mediadas por enzimas del complejo citocromo P-450 (CYP) principalmente con actividad glucosidasa y esterasa. Las reacciones de metabolización de fase II son reacciones de conjugación catalizadas por enzimas con actividad transferasa presentes principalmente en las células intestinales y hepáticas, para transformar los compuestos en derivados más hidrofílicos y con menor actividad biológica, de forma que se favorezca su excreción renal (**Figura 6**). El balance entre sulfatación y glucuronidación varía en función de la especie y el sexo (82).

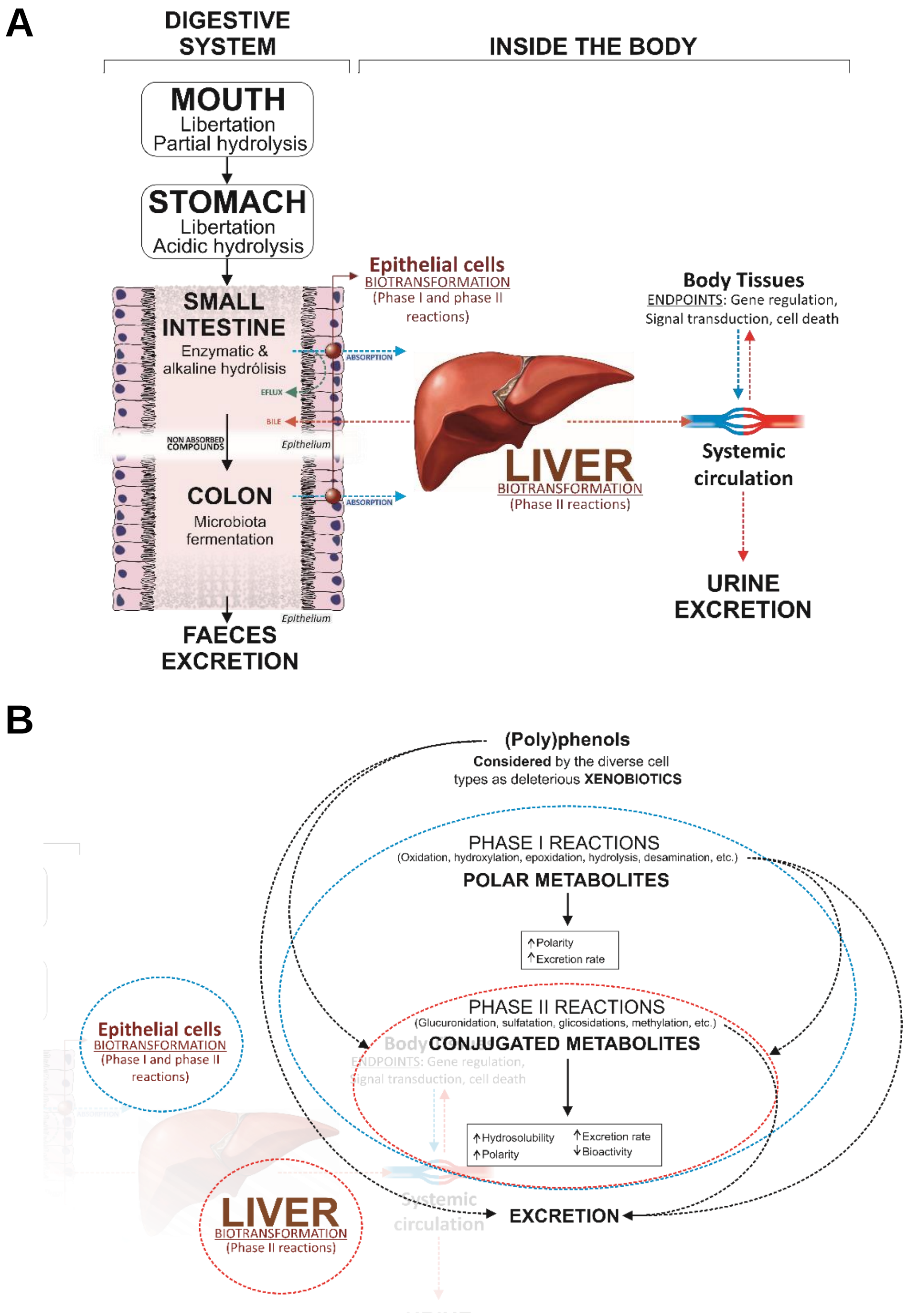

A
DIGESTIVE SYSTEM
INSIDE THE BODY
MOUTH
Libertation
Partial hydrolysis
STOMACH
Libertation
Acidic hydrolysis
SMALL INTESTINE
Enzymatic & alkaline hydrólisis
ABSORPTION
EFLUX
BILE
NON ABSORBED COMPOUNDS
Epithelium
COLON
Microbiota fermentation
ABSORPTION
Epithelium
FAECES EXCRETION
Epithelial cells
BIOTRANSFORMATION
(Phase I and phase II reactions)
LIVER
BIOTRANSFORMATION
(Phase II reactions)
Body Tissues
ENDPOINTS: Gene regulation, Signal transduction, cell death
Systemic circulation
URINE EXCRETION
B
(Poly)phenols
Considered by the diverse cell types as deleterious XENOBIOTICS
PHASE I REACTIONS
(Oxidation, hydroxylation, epoxidation, hydrolysis, desamination, etc.)
POLAR METABOLITES
↑Polarity
↑Excretion rate
PHASE II REACTIONS
(Glucuronidation, sulfatation, glicosidations, methylation, etc.)
CONJUGATED METABOLITES
↑Hydrosolubility
↑Polarity
↑Excretion rate
↓Bioactivity
Epithelial cells
BIOTRANSFORMATION
(Phase I and phase II reactions)
LIVER
BIOTRANSFORMATION
(Phase II reactions)
EXCRETION

Figura 6: Esquema de los mecanismos de absorción (**A**) y metabolización (**B**) de los compuestos fenólicos implicados en su biodisponibilidad. Agulló *et al*. (2021) (81).

Por otro lado, se ha descrito que la absorción del HT presente en aceite de oliva virgen es dependiente de la dosis. El primer estudio donde se observó el HT en plasma fue publicado en 1998 por Chen Bai *et al.*, describiendo la detección de concentraciones máximas a los 5-10 minutos tras su ingesta vía oral, disminuyendo su concentración hasta ser apenas detectable a las 3 horas post-ingesta (77). En 2001, Miro-Casas *et al.* describió con base en un estudio de intervención en humanos, las mayores concentraciones de HT en orina en las primeras 4 horas tras la ingesta de aceite de oliva virgen, así como sus derivados metabólicos (alcohol homovalínico) (85, 86). De forma complementaria, Vissers *et al.* (2002) describió cómo durante la metabolización se producía la metilación del HT en el epitelio intestinal y los hepatocitos (87). Asimismo, resultados derivados de un estudio pre-clínico, esclareció que el HT puede verse afectado por diferentes actividades enzimáticas y originar metabolitos como el ácido 3,4-dihidroxifenil acético (DOPAC), ácido homovanílico y alcohol homovanílico, pudiendo alcanzar diferentes tejidos como el cerebro al atravesar la barrera hematoencefálica, y también otros como corazón, riñón, pulmón e hígado (88).

Como resultado de los procesos de absorción intestinal y metabolización, en torno al 55-66% de los polifenoles consumidos a través de aceite de oliva se absorben satisfactoriamente, siendo excretado entre un 5 y un 16% como HT y tirosol vía urinaria (87).

4.3. FARMACOCINÉTICA Y BIODISPONIBILIDAD

La biodisponibilidad de nutrientes y compuestos bioactivos presentes en alimentos de origen vegetal es hoy en día un área importante de investigación en el campo de la alimentación y nutrición. Con estos estudios se pretende indagar más sobre el papel real de los componentes alimenticios sobre los esperados beneficios en la salud (62).

La bioaccesibilidad descrita anteriormente, junto con el proceso de absorción intestinal, son dos procesos clave que condicionan la biodisponibilidad del HT y sus derivados (79). Se puede definir la biodisponibilidad como la mayor parte de la fracción de un compuesto ingerido (nutriente o no nutriente) que posteriormente es digerido, absorbido y metabolizado, alcanzando la circulación sistémica y los tejidos concretos en los que pueda ejercer su acción biológica

(**Figura 7**). En el caso de los compuestos biológicamente activos, se pueden encontrar diferencias en la proporción en la cual se absorben.

Aunque todo nutriente es potencialmente bioaccesible, no todos son absorbibles tras la digestión gastrointestinal. Es importante conocer tanto la cantidad total de compuesto que está presente en el alimento, como la fracción biodisponible del mismo (72,89). Así, la biodisponibilidad de un nutriente se rige por factores intrínsecos como la edad, sexo, estado nutricional o las condiciones físico-químicas asociadas al proceso digestivo y factores extrínsecos representados por las características de la matriz alimentaria y su resistencia al proceso de digestión gastrointestinal, así como la forma química del nutriente en cuestión (**Figura 7**) (91,92).

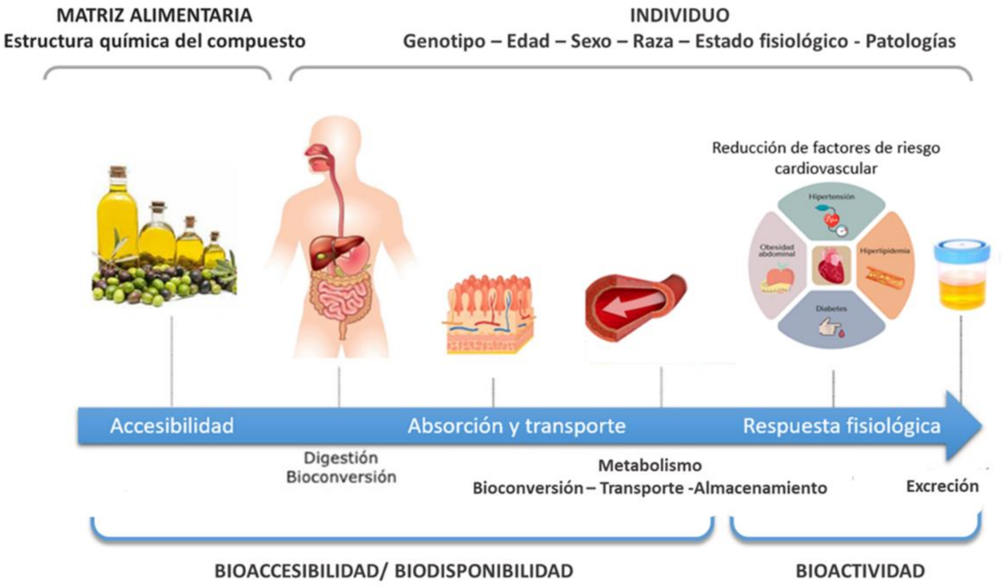

MATRIZ ALIMENTARIA
Estructura química del compuesto
INDIVIDUO
Genotipo – Edad – Sexo – Raza – Estado fisiológico - Patologías
Reducción de factores de riesgo cardiovascular
Hipertensión
Obesidad abdominal
Hiperlipidemia
Diabetes
Accesibilidad
Absorción y transporte
Respuesta fisiológica
Digestión
Bioconversión
Metabolismo
Bioconversión – Transporte -Almacenamiento
Excreción
BIOACCESIBILIDAD/ BIODISPONIBILIDAD
BIOACTIVIDAD

Figura 7. Factores que intervienen en la biodisponibilidad de los nutrientes.

El análisis de la biodisponibilidad de un compuesto determinado incluye, por tanto, el estudio del efecto de la digestión gastrointestinal sobre la estabilidad química del compuesto de interés, la ratio de absorción de la fracción bioaccesible, el metabolismo (no solo intestinal y hepático, sino también por parte de la microbiota intestinal que da lugar a nuevos compuestos bioactivos) y finalmente la distribución por los diversos tejidos y tipos celulares. De este modo, los principales objetivos de los estudios de biodisponibilidad son determinar cuáles son los compuestos presentes en los alimentos que mejor se absorben y valorar cuáles dan lugar a metabolitos más activos (84). Además, se ha demostrado que varía en gran medida dependiendo de las características físico-químicas de la matriz en la que estos compuestos son administrados, siendo para el caso del HT y sus derivados, el aceite de oliva virgen extra la matriz más efectiva descrita hasta la fecha (84).

Una elevada biodisponibilidad implica una mayor capacidad de participación en los procesos bioquímicos. Este hecho es de especial relevancia dada la bioactividad potencial del HT y sus derivados, lo que le otorgan un gran poder de modulación de diversos procesos fisiopatológicos al participar en diversas rutas metabólicas del organismo como la de las catecolaminas (**Figura 8**), además de desarrollar funciones protectoras de distintos tipos celulares y tisulares (93).

Como referimos previamente, para establecer la biodisponibilidad de un compuesto dado existen distintas alternativas y modelos experimentales, cada uno de ellos caracterizados por diferentes ventajas e inconvenientes. Los modelos *in vitro* resultan de gran interés dado que permiten establecer de manera rápida el perfil cuantitativo de los componentes alimentarios que atraviesan la barrera intestinal, así como la proporción en que lo hacen. Esta información permite seleccionar los alimentos candidatos a ser considerado en el marco de eventuales ensayos *in vivo*. Por otro lado, los ensayos *in vivo* (pre-clínicos y clínicos), a partir de constantes de velocidad de absorción determinadas, permiten recabar datos directos de biodisponibilidad y proporcionan información acerca de eventuales fenómenos de competición con otros componentes alimentarios o de saturación de los mecanismos de absorción, contribuyendo a establecer la relación dosis-efecto (62). En este sentido, el trabajo realizado por Mamajón *et al.* (2016) sobre el efecto producido por una dieta suplementada con compuestos fenólicos procedentes del aceite de oliva, corrobora la relación dosis-efecto tras la administración de HT y destaca la influencia del sexo en su biodisponibilidad (91).

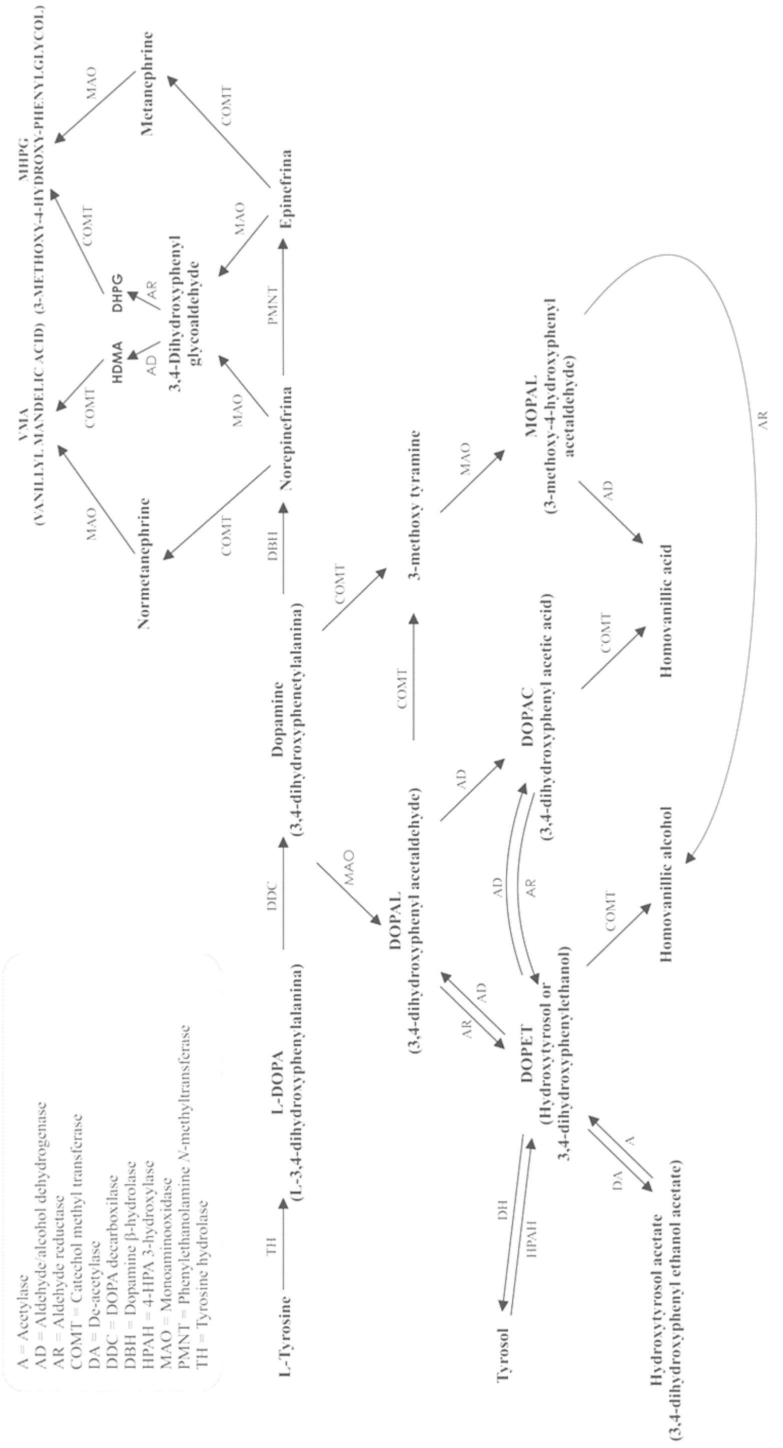

A = Acetylase
AD = Aldehyde alcohol dehydrogenase
AR = Aldehyde reductase
COMT = Catechol methyl transferase
DA = De-acetylase
DDC = DOPA decarboxilase
DBH = Dopamine β-hydrolase
HPAH = 4-HPA 3-hydroxylase
MAO = Monoaminooxidase
PMNT = Phenylethanolamine N-methyltransferase
TH = Tyrosine hydrolase
L-Tyrosine
TH
L-DOPA
(L-3,4-dihydroxyphenylalanina)
DDC
Dopamine
(3,4-dihydroxyphenetylalanina)
DBH
Norepinefrina
PMNT
Epinefrina
COMT
Normetanephrine
MAO
VMA
(VANILLYL MANDELIC ACID)
COMT
HDMA
AD
3,4-Dihydroxyphenyl
glycoaldehyde
AR
DHPG
MAO
MHPG
(3-METHOXY-4-HYDROXY-PHENYLGLYCOL)
Metanephrine
MAO
COMT
3-methoxy tyramine
MAO
MOPAL
(3-methoxy-4-hydroxyphenyl
acetaldehyde)
AD
Homovanillic acid
COMT
DOPAC
(3,4-dihydroxyphenyl acetic acid)
AD
DOPAL
(3,4-dihydroxyphenyl acetaldehyde)
AR
AD
DOPET
(Hydroxytyrosol or
3,4-dihydroxyphenylethanol)
COMT
Homovanillic alcohol
AR
DH
HPAH
Tyrosol
DA
A
Hydroxytyrosol acetate
(3,4-dihydroxyphenyl ethanol acetate)

Figura 8. Participación de los derivados del hidroxitirosol en la ruta sintética de las catecolaminas (94).

De igual manera, Domínguez-Perles *et al.* (2015) describieron diferentes concentraciones excretadas de HT en ratas machos con respecto a hembras, sugiriendo que el metabolismo diferencial de circulación entero-hepática en el patrón de excreción asociado al sexo podría constituir un factor determinante crucial para la biodisponibilidad final de HT (96). Dicho estudio, tras medir los niveles plasmáticos y urinarios de metabolitos de HT después de la ingesta de diferentes dosis del compuesto fenólico, describió que el incremento en las concentraciones del compuesto ingerido no daba como resultado un aumento de sus concentraciones plasmáticas. Los autores de este estudio concluyeron qué, aunque los compuestos se absorbieron de manera eficiente en el tracto gastrointestinal y muestran un metabolismo similar, la farmacocinética y biodisponibilidad resultan complejas debido probablemente a las interacciones de la microbiota colónica con los diferentes transportadores intestinales (96).

Actualmente, no se dispone de información acerca del mecanismo de absorción de los derivados lipofenólicos de HT, por lo que resulta imprescindible realizar nuevos estudios en este campo que permitan arrojar luz sobre los procesos de hidrólisis y absorción intestinal de dichos compuestos y que, más tarde, puedan ser corroborados en ensayos de intervenciones dietéticas en humanos. En este sentido, hasta la fecha, existen diversos estudios realizados en modelos *in vitro* de simulación gastrointestinal que han aportado información muy útil en relación con la estabilidad e hidrólisis de moléculas esterificadas de Tyr (56). Los mismos autores han descrito en modelos animales, los mecanismos de transporte de moléculas de Tyr esterificadas a través de epitelio intestinal, concluyendo que la longitud de la cadena alquílica y el grado de insaturación del ácido graso interfieren en la absorción de dichos derivados de HT, observando a su vez, que las moléculas esterificadas de Tyr muestran un comportamiento de liberación sostenida, prolongando el tiempo de acción del compuesto fenólico (66), derivándose en una biodisponibilidad mejorada de los ésteres de Tyr en comparación con la molécula libre sin esterificar a causa de los mecanismos de liberación sostenida (67).

5. CITOTOXICIDAD Y ACTIVIDAD BIOLÓGICA DE DERIVADOS DE HIDROXITIROSOL

En las últimas décadas, las nuevas necesidades detectadas en relación con la salud de la población, en una sociedad cada vez más envejecida y en la que los indicadores epidemiológicos de patologías degenerativas no dejan de aumentar, la investigación en Tecnología de los Alimentos y Nutrición se encuentran cada vez más centradas en desarrollar herramientas (ingredientes y alimentos) que contribuyan a fomentar la salud. Este interés ha dado lugar a la aparición de alimentos saludables, funcionales y nutracéuticos, los cuales aportan efectos beneficiosos para la salud o para la prevención de ciertas enfermedades (97). En este sentido, el desarrollo de nuevos productos alimenticios o suplementos a los que se les añade algún componente o se modifican para mejorar su biodisponibilidad, constituye una nueva tendencia de investigación (98).

Con respecto a las ventajas proporcionadas por los nuevos compuestos bioactivos descritos recientemente, además de los efectos beneficiosos que aportan, también es importante tener claro otros factores como la cantidad y frecuencia de consumo recomendada para obtener los efectos saludables y/o coadyuvantes deseados y evitar efectos biológicos contraproducentes, así como posibles interacciones con otros constituyentes dietéticos y su impacto en las vías metabólicas. Por estos motivos se creó la Comisión Europea de Acción Concertada sobre Bromatología Funcional en Europa (FUFOSE, del inglés *Functional Food Science in Europe*) destinada a proteger a los consumidores y potenciar el desarrollo de nuevos productos con una fuerte base experimental y científica (99).

Dentro de estas nuevas sustancias presentes de forma natural en algunos alimentos o añadidos artificialmente y siguiendo la línea de este trabajo, se encuentran las recomendaciones de uso y consumo de HT, actualmente en investigación en relación con las dosis seguras que permitan evitar efectos perjudiciales, así como posibles combinaciones con otros compuestos que potencien sus beneficios (100).

La evaluación de la seguridad de estos compuestos o el análisis de su citotoxicidad debe pasar por diferentes fases que incluyen pruebas químicas de su actividad teórica, ensayos *in vitro* sobre modelos biológicos y ensayos *in vivo* en animales y humanos tras la ingesta de los mismos (101).

Uno de los primeros estudios de toxicidad aguda con HT, realizado en animales de experimentación, fue llevado a cabo por D'Angelo *et al*. (2001), que describieron como la ingesta de una dosis diaria de 2 g/kg no producía ningún efecto tóxico o alteración macroscópica de órganos internos (88). Años más tarde, en 2004, se realizó otro ensayo sobre la toxicidad aguda y crónica a 90 días de un producto hidrolizado del extracto acuoso de la pulpa de aceituna, donde se confirmó la ausencia de efectos tóxicos a dosis de 200 mg/kg/día, así como la ausencia de efectos teratogénicos y mutagénicos (101). Estos estudios se centran en el análisis de los efectos perniciosos de HT ingerido a través de matrices alimentarias y en las concentraciones aproximadas que proporcionaría un consumo dietético adecuado; no obstante, actualmente hay pocos trabajos publicados en relación al uso de HT sintético. Uno de ellos el realizado por Auñón *et al*. (2013), en el que estudiaron el consumo de 500 mg/kg de peso corporal al día en modelos murinos, no encontrando efectos tóxicos adversos significativos (101). Por tanto, actualmente se requieren más estudios *in vivo* a largo plazo para evaluar la dosis óptima requerida para alcanzar los beneficios para la salud, evitando al mismo tiempo cualquier toxicidad o potencial efecto secundario no deseado (103).

5.1. ACTIVIDAD BIOLÓGICA DEL HIDROXITIROSOL Y SUS DERIVADOS ESTERIFICADOS: MECANISMOS DE ACCIÓN

Actualmente se dispone de evidencias científicas que atribuyen los efectos beneficiosos en la salud de determinados patrones dietéticos y/o alimentos a la presencia de compuestos bioactivos (nutrientes y no nutrientes) que ejercen una acción beneficiosa. En este sentido, el HT ha despertado un gran interés en el ámbito de investigación en Tecnología de los Alimentos y Nutrición debido a su potencia biológica y al amplio espectro de actividades biológicas asociadas, destacando su capacidad antioxidante, seguida de propiedades antiinflamatorias, anticancerígenas, neuroprotectoras, inmunomoduladoras, cardioprotectoras, citoprotectoras, antimicrobianas y antivirales, reguladoras endoteliales y vasculares y protectoras de la piel (104).

En relación con la actividad antioxidante, el HT tiene la capacidad de interferir en la secuencia de las reacciones en cadena del proceso de peroxidación, como ocurre en la lipoperoxidación, así como la captación de radicales libres y la captación de iones metálicos que catalizan la producción de ROS. Estas acciones son realizadas gracias a su grupo catecol y a su capacidad para actuar como agente quelante de hierro intracelular. Esta actividad antioxidante también se produce gracias a su capacidad para estimular la síntesis y actividad de enzimas antioxidantes, como catalasa, superóxido dismutasa y glutatión peroxidasa, todas ellas enzimas reguladoras clave de los procesos de estrés oxidativo e inflamación. Entre los metabolitos de HT, también caracterizados por una actividad antioxidante significativa, encontramos el ácido homovanílico, HTA y DOPAC (105).

En cuanto a su actividad preventiva en el desarrollo de ECV, además de su capacidad antioxidante, recientemente se ha demostrado que el HT inhibe la agregación plaquetaria, disminuye los mediadores pro-inflamatorios como la ciclooxigenasa-2 (COX-2) e interleucina-6, reduce la expresión de proteínas relacionadas con el envejecimiento celular y atenúa las alteraciones metabólicas ejerciendo un efecto favorable sobre la arteriosclerosis (105).

Recientemente, en el marco de un estudio llevado a cabo por Caroleo *et al.* (2021) se describieron las propiedades antidiabéticas del oleato de hidroxitirosilo, un lipofenol derivado de la conjugación de ácido oleico con HT, caracterizado por estimular las células β del páncreas encargadas de la producción de insulina. Asimismo, este lipofenol contribuye a conservar la función de las células β a través de la activación de vías antiinflamatorias y antioxidantes. Complementariamente a estas funciones, destaca la ventaja farmacodinámica asociada a este compuesto, la cual es debida a la cadena de ácido oleico. En conjunto, estas propiedades funcionales y químicas han llevado a proponerlo como compuesto de interés para el desarrollo de una posible futura terapia antidiabética (106). En consonancia con estas conclusiones, estudios adicionales han permitido identificar el HT o suplementos enriquecidos con él, como agentes relacionados con una mejora en la secreción de insulina y el control glucémico en pacientes diabéticos (107).

Otra molécula lipofenólica de reciente descubrimiento es el oleato de tirosilo, que se encuentra presente en el aceite de oliva y posee características similares al oleato de hidroxitirosilo entre las que destacan propiedades antiinflamatorias, antioxidantes y regeneradoras de tejidos. La descripción de estas funcionalidades refuerza la idea de que los ésteres de HT aumentan las propiedades antioxidantes, con una potencial biodisponibilidad mejorada gracias a su carácter lipofílico (100).

Funakohi-Tago *et al*. (2018) investigaron los efectos del HT y sus derivados, destacando el butirato de HT por su mayor acción de protección en células neuronales frente a la apoptosis inducida y contra al daño oxidativo, debido a su mayor solubilidad en grasas, proponiendo la posible utilidad de este compuesto para la modulación de la progresión y severidad de trastornos neurodegenerativos (109).

En relación a las ventajas funcionales asociados a la esterificación de HT con ácidos grasos, aunque actualmente no existen evidencias en relación a los lipofenoles de HT manteniéndose esta hipótesis sin contrastar, esta conjetura está apoyada por investigaciones sobre lipofenoles formados a partir de otros compuestos fenólicos. Así, Oh *et al*. (2021) investigaron como la esterificación de la quercetina mostraba una mejor actividad antioxidante que el compuesto fenólico (flavonol) nativo, respaldando lo expuesto por otros estudios acerca del efecto de la esterificación de los compuestos fenólicos ya que mostraron una mayor capacidad de inhibición de la oxidación de LDL en comparación con las moléculas sin esterificar debido al aumento de la lipofilia (110). Asimismo, Moine *et al*. (2021), en su investigación sobre moléculas esterificadas de quercetina, demostraron cómo estos compuestos son capaces de mejorar la actividad preventiva frente al estrés oxidativo al mejorar las propiedades antioxidantes y por tanto de prevenir patologías asociadas al envejecimiento (111). Por tanto, las evidencias obtenidas hasta ahora permiten sugerir que los lipofenoles mejoran las características biológicas de los compuestos fenólicos de partida (112) y, por tanto, suponen una alternativa en futuras aplicaciones como antioxidantes alimentarios, farmacéuticos o cosméticos (108).

6. EICOSANOIDES COMO MARCADORES DE LA FISIOPATOLOGÍA CARDIOVASCULAR

Los eicosanoides son metabolitos de carácter lipídico de diversas estructuras y actividades biológicas derivados de la oxidación de ácidos grasos de 20 átomos de carbono, como el ácido araquidónico (AA, del inglés *arachidonic acid*). Estas oxilipinas poseen una vida media corta, con una alta capacidad de acción a nivel local y generan una amplia variedad de efectos al unirse a receptores celulares específicos (113). Entre los precursores de los eicosanoides encontramos el AA, ácido dihomogamma-linolénico (DGLA, del inglés *dihomo-γ-linolenic acid*) y ácido eicosapentaenoico (EPA, del inglés *eicopentanoic acid*) (114).

Los eicosanoides se clasifican en diferentes clases en función de la vía de oxidación del AA, bien enzimáticamente en la ruta catalizada por la enzima COX-2, generando prostaglandinas (PGs) y tromboxanos (TX) conocidos como prostanoides, o mediante rutas no enzimáticas mediadas por ROS, responsable de la síntesis de isoprostanos (IsoPs) (113).

6.1. ISOPROSTANOS, PROSTAGLANDINAS Y TROMBOXANOS

6.1.1. Isoprostanos

Los IsoPs son oxilipinas que se producen mediante la peroxidación no enzimática del AA inducida por radicales libres. Son marcadores estables del estrés oxidativo, se producen a partir de los fosfolípidos de la membrana celular y son liberados a la circulación sistémica en su forma libre. A pesar de la diversidad de IsoPs identificados y de su reconocida función como marcadores de peroxidación lipídica debido a su gran estabilidad, entre los diferentes IsoPs identificados actualmente, el marcador por excelencia del daño oxidativo causado por ROS es el 8-iso-$PGF_{2\alpha}$ (15-F_{2t}-IsoP). Desde una perspectiva funcional, este IsoP es un potente vasoconstrictor, mediador del crecimiento celular del músculo liso, activador de las plaquetas e inductor de alteraciones en la función de la barrera celular endotelial. La elevación de sus valores en concentraciones urinarias se asocia con enfermedades como hipercolesterolemia y diabetes, entre otras (115).

Estos compuestos se localizan en una variedad de fluidos biológicos como el plasma, orina y líquido cefalorraquídeo, y en tejidos corporales como el gástrico, cerebral y vascular. Además de sus niveles fisiológicos, resultado del normal metabolismo celular, en el curso de diferentes procesos patológicos se han detectado elevados niveles de estas oxilipinas, por ejemplo, en el marco de enfermedades cardiovasculares, pulmonares, neurodegenerativas y diabetes tipo 2 (116).

Además de ser útiles como marcadores de estrés oxidativo, también se están utilizando como marcadores pronósticos en ciertas patologías como predictores de riesgo de eventos cardiovasculares, puesto que se han descrito altos niveles de IsoPs en patologías como el infarto agudo de miocardio, la angina inestable, hipercolesterolemia, eclampsia y aterosclerosis, así como en ciertas enfermedades metabólicas como la diabetes (117).

6.1.2. Prostanoides

Los prostanoides son una serie de compuestos con carácter autacoide (sustancia formada metabólicamente por un grupo de células que altera la función de otras a nivel local). Estos compuestos pertenecen a la familia de los eicosanoides y se encuentran ampliamente distribuidos por diferentes tejidos y tipos celulares.

Los prostanoides interfieren en la patogénesis de enfermedades cardiovasculares a través de una serie de procesos como la agregación plaquetaria, la vasodilatación o vasoconstricción y la respuesta inflamatoria local, llegando a desarrollar procesos como la aterosclerosis (119). Basados en estas bioactividades, los prostanoides se han monitorizado como marcadores de enfermedad temprana en personas con diversas patologías, detectando niveles elevados en enfermedades autoinmunes, cáncer, enfermedad inflamatoria intestinal, enfermedad de Alzheimer, enfermedades cardiacas, etc. (118).

Dentro de su estructura, los prostanoides están compuestos por un anillo ciclopentano que puede tener diferentes configuraciones y que a su vez condiciona su actividad biológica, pudiendo dividirse en dos grupos:

- Ciclopentano de 5 átomos de carbono: prostaglandinas (PGE, PGD, PGF) y prostaciclinas (PGI).

- Ciclopentano interrumpido con un átomo de oxígeno: tromboxanos (TXA, TXB) (116).

6.1.2.1. Prostaglandinas

Las PGs fueron las primeras oxilipinas derivadas del AA que se descubrieron, por lo que actualmente existe una gran variedad de investigaciones sobre ellas. Encontramos cuatro PGs con actividades biológicas: la D_2 (PGD_2), la E_2 (PGE_2), la I_2 (PGI_2), y la $F_{2\alpha}$ ($PGF_{2\alpha}$), pudiendo ser originadas en distintos órganos y tejidos (**Figura 9**) (120). Estos mediadores lipídicos desempeñan un importante papel en la comunicación y control de las funciones biológicas en el cuerpo, destacando su actividad como mediadores de la inflamación, especialmente la PGD_2 que actúa sobre células endoteliales de vasos sanguíneos provocando vasodilatación y aumento de la permeabilidad de la pared vascular, con el consecuente aumento del flujo sanguíneo a la zona (113). Pese a que la PGD_2 es conocida por su actividad proinflamatoria, cabe destacar también su capacidad para ocasionar resultados antiinflamatorios a nivel inmunológico (121).

Asimismo, se ha reportado el interés biológico de PGE_2, mediadora de varias funciones biológicas como la respuesta inmune, la presión arterial, la integridad gastrointestinal y la fertilidad. La PGE_2 también posee capacidad para generar inflamación y puede además igualmente evitarla, compartiendo ambas PGs la capacidad dual de actuar como pro/ antiimflamatorias dependiendo de su interacción con los distintos receptores (120).

Las PGs tienen una vida media corta antes de inactivarse y excretarse, y ejercen sólo una función paracrina (a nivel local) o autocrina (en la misma célula de la que se sintetiza) (113,117).

6.1.2.2. Tromboxanos

Los TX proceden principalmente de la acción de la COX sobre el AA. El TXA2 es un metabolito del AA generado por la acción de la COX y, en un paso posterior, por la tromboxanosintasa (**Figura 9**). Las oxilipinas pertenecientes a esta familia de eicosanoides presentan principalmente acciones cardiovasculares y hemodinámicas, estando involucrados en procesos inflamatorios endoteliales y procesos de adhesión/agregación plaquetaria involucrados en la coagulación sanguínea. La síntesis aumentada de esta molécula se vincula con enfermedades cardiovasculares como la isquemia miocárdica o el fallo cardiaco (113).

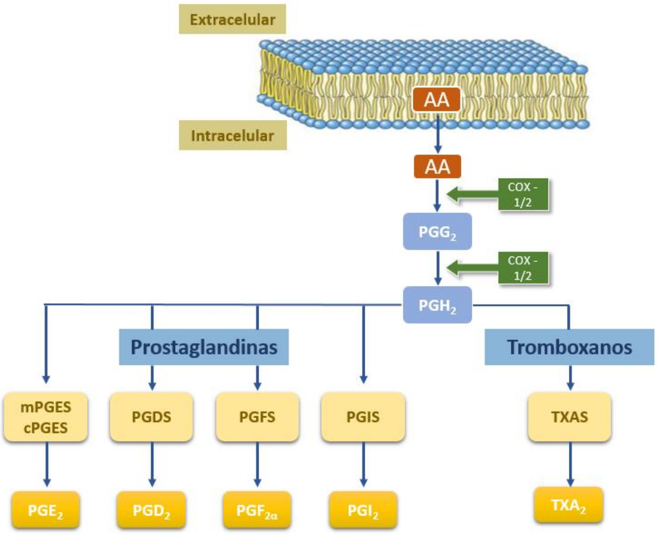

Extracelular
AA
Intracelular
AA
COX - 1/2
PGG2
COX - 1/2
PGH2
Prostaglandinas
Tromboxanos
mPGES cPGES
PGDS
PGFS
PGIS
TXAS
PGE2
PGD2
PGF2α
PGI2
TXA2

Figura 9: Biosíntesis de prostaglandinas y tromboxanos.

6.2. EFECTO MODULADOR DE LOS NIVELES DE EICOSANOIDES DE LOS COMPUESTOS BIOACTIVOS PRESENTES EN ALIMENTOS VEGETALES

Los compuestos bioactivos presentes en la dieta representan un factor que define en gran parte la composición de ácidos grasos de los fosfolípidos que constituyen las membranas celulares. El AA se encuentra normalmente esterificado en los fosfolípidos de estas membranas, siendo el sustrato principal para la síntesis de oxilipinas (118).

Los ácidos grasos Ω-3 y Ω-6 presentes en los alimentos pasan a formar parte de las membranas celulares de los tejidos, siendo responsables de diversas funciones como el mantenimiento de la estructura de las membranas biológicas, precursores de los eicosanoides con actividades antiinflamatorias y reguladores de los lípidos hemáticos (en especial del colesterol y los triglicéridos).

Dependiendo del tipo de ácido graso ingerido (mono- o poliinsaturado) se determinará la presencia de AA o ácido eicosapentaenoico (EPA) y a través de la acción de las fosfolipasas de la membrana celular se sintetizarán los diferentes eicosanoides. El ácido graso más abundante en la membrana celular es el AA, que será el que mediante la acción de la COX-1 y COX-2 darán origen a los diferentes prostanoides (122).

Diferentes estudios epidemiológicos y de intervención realizados han permitido sugerir que la ingesta de ácidos grasos omega-3 afecta favorablemente a la salud cardiovascular, aunque hoy en día todavía no se conoce en profundidad el mecanismo exacto mediante el cual los ácidos grasos ejercen su efecto protector. Entre los posibles mecanismos relacionados con el efecto cardiosaludable de los MUFAs y PUFAs se ha descrito la capacidad de estos para interferir en la coagulación sanguínea, la formación de trombos, el perfil de los lípidos plasmáticos, la presión sanguínea, y en procesos inflamatorios. Por otro lado, los efectos protectores frente a la formación de la placa de ateroma derivados del consumo de PUFAs se deben principalmente a su incorporación a los fosfolípidos de las membranas de las células, sustituyendo parcialmente el AA como sustrato inicial para la producción de eicosanoides (123,124).

También existen evidencias sobre la ingesta de HT y la modificación posterior de prostanoides, como es el caso de un estudio en el que se administró HT en modelos animales y se observó una reducción dosis-dependiente de la producción plaquetaria de TX, produciendo un efecto antitrombótico (124). Del mismo modo, la producción de prostaciclinas en el ser humano depende en gran medida de la activación de la COX-2, la cual también es inhibida por el HT, reduciendo (o incluso inhibiendo completamente) la síntesis de la prostaciclina

PGI_2, relacionada con la agregación plaquetaria y el mantenimiento de la integridad de las células endoteliales (125).

Asimismo, algunos compuestos fenólicos presentes en el aceite de oliva virgen ofrecen unos efectos inhibidores del funcionamiento plaquetario y la producción de TX, tales como el propio HT, la oleuropeína y otros compuestos fenólicos isocrománicos (125).

La producción de los diferentes tipos de oxilipinas con sus diversas acciones sobre muchos tejidos del organismo; así como los efectos de la inhibición de su liberación producida por los compuestos bioactivos presentes en los alimentos nos demuestran la importancia de estas moléculas en la regulación de procesos biológicos, especialmente los relacionados con el estrés oxidativo y la respuesta inflamatoria (113).

La dieta representa uno de los factores principales que regulan la concentración plasmática de oxilipinas y otras moléculas como las lipoproteínas. La importancia de una dieta adecuada y equilibrada en la prevención y disminución de los principales factores de riesgo asociados a la arteriosclerosis y las ECV es muy conocida y se apoya en importantes estudios llevados a cabo hasta la fecha (126). Sin embargo, la búsqueda de nuevos compuestos bioactivos que sirvan como coadyuvantes en la prevención de enfermedades no trasmisibles será un desafío crítico para la investigación alimentaria en las próximas décadas.

CAPÍTULO II - JUSTIFICACIÓN

La Dieta Mediterránea es uno de los patrones de alimentación más estudiados, destacando por sus beneficios para la salud. Está demostrado que la Dieta Mediterránea disminuye la morbimortalidad asociada a la patología cardiovascular, reduciendo la incidencia de enfermedades metabólicas como diabetes y obesidad. Estos beneficios son debidos a la alta proporción de alimentos de origen vegetal, siendo el aceite de oliva la principal fuente de grasas de este patrón dietético. El aceite de oliva, con importantes efectos beneficiosos para la salud, es rico en compuestos fenólicos, entre los que destaca el HT tanto en su forma libre como conjugada con ácidos grasos (lipofenoles).

En este contexto, el presente estudio pretende profundizar en el conocimiento acerca del efecto de la matriz alimentaria sobre la bioaccesibilidad y biodisponibilidad del HT libre y esterificado, obteniendo evidencias de su biotransformación durante la digestión gastrointestinal. En base a una eventual biodisponibilidad mejorada de los derivados esterificados de HT, conferida por la mayor capacidad de traspasar la membrana celular debido a la presencia de ácidos grasos en su estructura química, nos propusimos estudiar mediante ensayos *in vitro* la capacidad moduladora del estrés oxidativo del hidroxitirosol libre y esterificado con ácidos grasos en un modelo de células mieloblásticas (línea monocítica THP-1). Los resultados obtenidos permitirán aumentar el conocimiento actual acerca sus mecanismos de acción, en la prevención de enfermedades cardiovasculares y otras patologías asociadas al estrés oxidativo mediante la evaluación de marcadores lipídicos de inflamación y estrés oxidativo (prostaglandinas e isoprostanos).

Los resultados obtenidos del presente estudio permitirán, por tanto, el desarrollo de nuevos productos alimenticios con nuevas capacidades funcionales, así como fomentar el uso de estos compuestos como elementos centrales de nuevos suplementos de una alimentación saludable y equilibrada, contribuyendo a reducir el riesgo cardiovascular y mejorando así la salud poblacional y la sostenibilidad de los sistemas de salud pública.

CAPÍTULO III -
OBJETIVOS

3.1. OBJETIVO GENERAL

El presente estudio tiene como objetivo principal evaluar la farmacocinética de la del HT libre procedente de diferentes matrices alimentarias de naturaleza oleaginosa y determinar la bioaccesibilidad de las formas químicas de HT esterificadas con ácidos grasos. Asimismo, se investigará la bioactividad de la molécula libre *versus* esterificada, en condiciones pro-oxidativas, mediante la aplicación de modelos *in vitro* aplicando un enfoque lipidómico.

3.2. OBJETIVOS ESPECÍFICOS

Para la consecución del objetivo general de la presente Tesis Doctoral, se establecieron los siguientes objetivos secundarios:

1. Estudiar la absorción y bioconversión de la molécula de HT, en humanos, tras la ingesta de diversas matrices alimentarias de naturaleza acuosa y oleosa enriquecidas con HT.

2. Evaluar el efecto de la matriz alimentaria y de las enzimas digestivas en la bioaccesibilidad de la molécula de HT libre y esterificada con ácidos grasos, mediante la simulación *in vitro* de la digestión gastrointestinal.

3. Investigar la capacidad moduladora del estrés oxidativo del HT libre y sus derivados esterificados en un modelo de células mieloblásticas, *in vitro*, mediante la evaluación de marcadores de estrés oxidativo e inflamación.

CAPÍTULO IV -
RESULTADOS Y DISCUSIÓN

ARTÍCULO I

Pharmacokinetics and bioavailability of hydroxytyrosol are dependent on the food matrix in humans

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ORIGINAL CONTRIBUTION

Pharmacokinetics and bioavailability of hydroxytyrosol are dependent on the food matrix in humans

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Abstract

Purpose Several studies have demonstrated the properties of hydroxytyrosol, a phenolic compound present in olive oils and olives with a well-characterized impact on human health. Nevertheless, some knowledge gaps remain on its bioavailability and metabolism; overall concerning to the real rate per cent of absorption and biovailability of dietary hydroxytyrosol and the influence of the dietary food-containing hydroxytyrosol on it.

Methods A double-blind study was performed including 20 volunteers who ingested 5 mg of hydroxytyrosol through diverse food matrices, to discover the influence on pharmacokinetics and bioavailability of HT metabolites (hydroxytyrosol acetate, 3,4-dihydroxyphenylacetic acid (DOPAC), tyrosol, and homovanillic alcohol) of the distinct matrices by UHPLC–ESI–QqQ–MS/MS.

Results The HT pharmacokinetics after consumption of different food matrices was strongly dependent on the food matrix. In this aspect, the intake of extra virgin olive exhibited significantly higher plasma concentrations after 30 min of oral intake (3.79 ng/mL) relative to the control. Regarding the hydroxytyrosol bioavailability, the intake of extra virgin olive oil, as well as fortified refined olive, flax, and grapeseed oils provided significantly higher urinary contents (0.86, 0.63, 0.55, and 0.33 µg/mg creatinine, respectively) compared with basal urine, whereas hydroxytyrosol metabolites showed no significant changes. No differences were found between men and women.

Conclusions The metabolic profile of hydroxytyrosol is influenced by the food matrix in which is incorporated, with the oily nature for the final bioavailability being relevant. Extra virgin olive oil was identified as the best matrix for this compound. The results described contribute to the understanding of the relevance of the food matrices for the final absorption of hydroxytyrosol and hence, the achievement of the highest health protection potential.

Keywords Hydroxytyrosol metabolites · Human · In vivo · Food matrix · Bioavailability · Pharmacokinetic

Abbreviations

Amu	Atomic mass unit
DOPAC	3,4-Dihydroxyphenylacetic acid
HT	Hydroxytyrosol

Carolina Alemán-Jiménez and Raúl Domínguez-Perles contributed equally to the present work.

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HTA	Hydroxytyrosol acetate
HValc	Homovanillic alcohol
MRM	Multiple reaction monitoring
SPE	Solid phase extraction
Tyr	Tyrosol
UHPLC–ESI–QqQ–MS/MS	Ultra-high performance liquid chromatography coupled to electrospray ionization and triple quadrupole mass spectrometry

Introduction

Traditionally, the health benefits attributed to the Mediterranean diet have been associated with a reduction in the consumption of saturated fats and their replacement with an increased intake of olive oils, which feature high concentrations of bioactive compounds [1]. In this respect, one of the most deeply characterized phytochemicals in these plant-foods is hydroxytyrosol (HT), a phenolic compound that is naturally present in olive oils and table olives. Its importance as a healthy replacement is supported by a number of reports demonstrating the properties of HT as a powerful antioxidant, with broad positive impacts on human health [2, 3]. Based on these experimental characterizations, the interest on this compound has grown in the last few decades, as it also features outstanding anti-inflammatory, antimicrobial, and neuroprotective activities, which confer HT with a central role in the prevention of cardiovascular and neurodegenerative diseases, and metabolic syndrome as well [4–6]. These attributes have prompted the European Food Safety Authority to publish positive scientific opinions highlighting the interest of dietary HT to protect low density lipoproteins (LDL) and thus, to reduce the incidence of cardiovascular pathologies, pointing out that the dietary consumption of 5 mg is enough to achieve these benefits [7].

To understand the biological activity of HT, some light must be shed on its metabolism and distribution over diverse tissues, as well as on its capacity to cross the blood–brain barrier [8]. In this regard, the bioavailability and bioactivity of phenolic compounds depend on a myriad of factors associated to human physiology, such as their stability under gastrointestinal conditions, the efficiency of intestinal absorption, the composition of the intestinal microbiota, and the post-absorption metabolism, among other issues [9]. At present, it has been demonstrated that after dietary ingestion, phenolic compounds are metabolized in two phases: the first phase is the hydrolysis that occurs in the stomach and small intestine, and where most HT is rapidly absorbed; and the second phase, a conjugation reaction that occurs in the intestinal epithelium and hepatocytes [10, 11]. Moreover, additional factors, such as the physico-chemical characteristics of the food matrix in which bioactive compounds are present, seem to critically influence its bioavailability, although these factors have not yet been fully explored as of today [12, 13], and less so at dietary concentrations.

In addition, sex has been proposed as a critical factor which conditions the final availability of HT, which seems to be associated to a differential metabolism and enterohepatic circulation which in turn has a direct impact on the bioavailability of these compounds. These differences have been associated to higher urine concentrations in female plasma and urine, which was demonstrated upon an in vivo assay in a murine model [10]. Nonetheless, to date, no references of this are available regarding humans.

To provide a new perspective for understanding the mechanism through which bioactive compounds of olive oil act in vivo, the present article uncovers the ratio of absorption of HT and its conversion into its main metabolites, hydroxytyrosol acetate (HTA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic alcohol (HValc), and tyrosol (Tyr), in humans, throughout the 24 h following the ingestion of 5 mg of HT through diverse oily and aqueous matrices (extra virgin olive oil, refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice). Hence, metabolites were analysed in plasma and urine using the robust and sensitive method of Ultra-high performance liquid chromatography coupled to a Mass Spectrometer equipped with an electrospray ionisation (ESI) chamber and triple quadrupole mass analyser for tandem analysis (UHPLC-ESI-QqQ-MS/MS) [10].

Material and methods

Reagents

Hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), and 3,4-dihydroxyphenylacetic acid (DOPAC) were provided by SEPROX Biotech S.L. Tyrosol (Tyr) and homovanillic alcohol (HValc) were purchased from Sigma-Aldrich (St. Louis, MO, USA), as well as the hydrolytic β-glucuronidase, type H2 from *Helix pomatia* and Bis–Tris (bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane). All LC–MS grade solvents were obtained from J.T. Baker (Phillipsburg, New Jersey, USA). Formic acid and hydrochloric acid were purchased from Panreac (Castellar del Vallés, Barcelona, Spain). The solid phase extraction (SPE) cartridges used in this study (Strata X-AW, 100 mg/3 mL) were obtained from Phenomenex (Torrance, California, USA).

Study design

We hypothesized that the food matrix has a direct influence on the intestinal absorption of HT and its metabolites, and thus on the plasma and urine concentration of these compounds. To test this initial hypothesis, the design was outlined as a double-blind study in which 20 human volunteers ingested 5 mg of hydroxytyrosol through a single dose of diverse food matrices (extra virgin olive oil, and five fortified food matrices (refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice)). After each intervention, all the volunteers underwent a wash-out period of 96 h to avoid interferences among the different matrices assayed. Pharmacokinetic and bioavailability results were compared with the baseline control (BC) values, recorded from samples collected before ingesting each matrix, and an additional intervention day, when volunteers ingested non-fortified refined olive oil. Peripheral blood samples were collected at time-points (min) 0 (BC), 30, 60, 120, 180, and 240. Urine samples were collected at rest (basal urine) and under fasting conditions (24-h urine) (Fig. 1). The study was carried out on 20 Caucasian volunteers (10 men and 10 women), aged 19–23 years with normal BMI > 18.5 and < 25 kg/m^2 from the San Antonio Catholic University of Murcia (Spain), who agreed to participate in the project. All subjects fulfilled the following eligibility criteria: non-smokers, follow stable food habits, and did not receive any medication during the experimental procedure. The study was approved by the Bioethics Committee of the Catholic University "San Antonio" of Murcia in accordance with the Declaration of Helsinki., and all volunteers signed written informed consent forms. The dietary habits of the subjects were monitored during the entire assay, including wash-out and baseline periods, to avoid the ingestion of polyphenol-containing foods such as tea, chocolate, coffee, fruits or juices, vegetables, soya, berries, EVOO (extra virgin olive oil) and alcoholic drinks (including wine and beer) during the 3 days previous to each intervention, in agreement with previous reports including nutritional intervention studies with human volunteers who consumed HT-based foods that could interfere with the results obtained [14, 15].

Sample collection and preparation

A range of clinical analyses (including haematology, biochemistry, and urine chemical analysis) were performed to monitor the health status of the volunteers. Peripheral blood samples were collected at the University Hospital

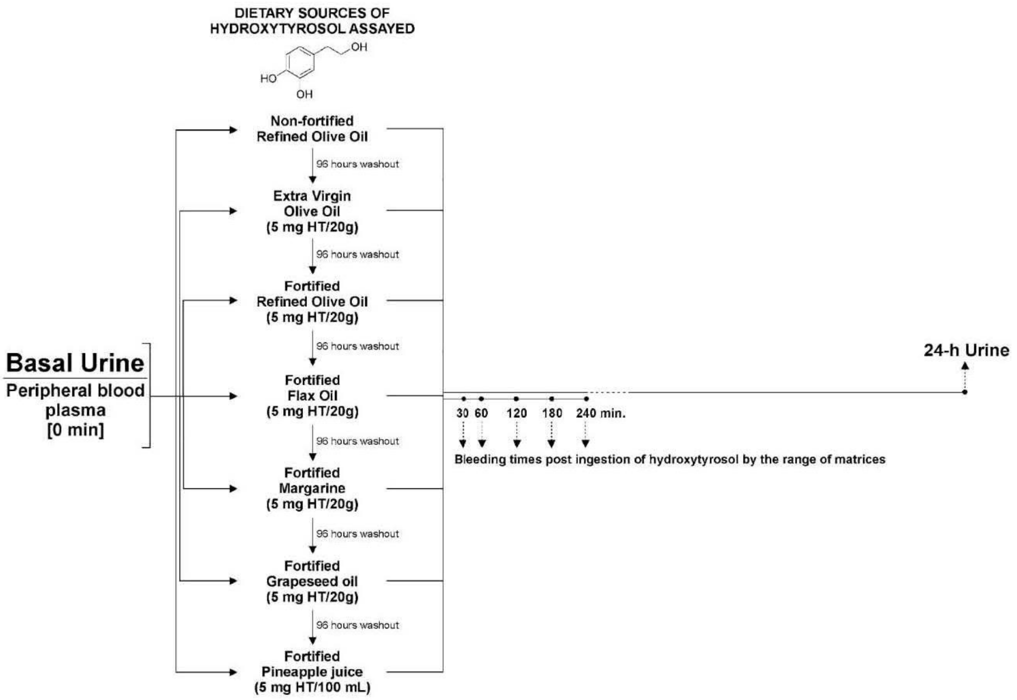
DIETARY SOURCES OF
HYDROXYTYROSOL ASSAYED
OH
HO
OH
Non-fortified
Refined Olive Oil
96 hours washout
Extra Virgin
Olive Oil
(5 mg HT/20g)
96 hours washout
Fortified
Refined Olive Oil
(5 mg HT/20g)
96 hours washout
Fortified
Flax Oil
(5 mg HT/20g)
96 hours washout
Fortified
Margarine
(5 mg HT/20g)
96 hours washout
Fortified
Grapeseed oil
(5 mg HT/20g)
96 hours washout
Fortified
Pineapple juice
(5 mg HT/100 mL)
Basal Urine
Peripheral blood
plasma
[0 min]
24-h Urine
30 60 120 180 240 min.
Bleeding times post ingestion of hydroxytyrosol by the range of matrices

Fig. 1 Study design. This was a double-blind study. Twenty volunteers (10 men and 10 women) ingested 5 mg of hydroxytyrosol added to 6 diverse food matrices (extra virgin olive oil, refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice). After each intervention, all volunteers underwent a wash-out period of 96 h to avoid interferences among the different matrices assayed. Two controls were included in the experimental design, baseline control (BC) and non-fortified refined olive oil. Peripheral blood samples were collected at time-point (min) 0 (BC), 30, 60, 120, 180, and 240. Urine samples were collected at rest (basal urine) and under fasting conditions (24-h urine)

under fasting conditions at the beginning of each day of intervention. Hence, blood samples at rest and at the different time-points described in the experimental design (Fig. 1) were obtained by venipuncture and placed in different tubes according to the analytical procedures foreseen. For the biochemical analysis, glucose (mg/dL), urea (mg/dL), total cholesterol (mg/dL) and HDL- and LDL-cholesterol (mg/dL), as well as haematological analysis were carried out using a Cobas 8000 c702 module (Roche Diagnostic, Mannheim, Germany). Immediately upon collection, for HT and HT derivatives analysis, blood from citrated blood vacutainers was centrifuged at 2000×*g* for 10 min at 4 °C to obtain plasma and then transferred to polypropylene tubes with 10% L-ascorbic acid and 0.58 M acetic acid as preservatives and stored at − 80 °C until final processing and analysis.

Twenty-four-hour urine samples were collected before and after each intervention day in sterile, dark polystyrene, tubes (2 L) with screw caps with 10% L-ascorbic acid as the chemical preservative. The urine analysis was also performed in a Cobas 8000 c702 modular analyser (Roche Diagnostic, Mannheim, Germany) to test the content of creatinine (mg/dL) and albumin (mg/L). The concentration of HT and HT derivatives analysed in the 24-h urine was normalised as micrograms per milligram of creatinine (μg/mg creatinine).

For the analysis of the concentration of HT, HTA, DOPAC, Tyr, and HValc, plasma and urine samples were thawed at room temperature and centrifuged (8538 ×*g* for 5 min). Samples (100 and 400 μL, respectively) were processed following the methodology previously described by Dominguez-Perles et al. [10]. Briefly, the supernatants were hydrolysed by incubation with 300 UI (plasma) and 1500 UI (urine) of β-glucuronidase from *Helix pomatia* for 2 h at 37 °C, clarified with 200 μL of MeOH/HCl (200 mM) and centrifuged at 8538× *g* for 5 min. The supernatants were cleaned up by SPE, using Strata X-AW cartridges, which were conditioned and equilibrated with 2 mL of MeOH/formic acid (98:2, *v/v*) and 2 mL of water/ formic acid (98:2, *v/v*), respectively. Then, the samples were loaded and the SPE cartridges were washed with water/formic acid (98:2, *v/v*). The target analytes were eluted with 1 mL of MeOH/formic acid (98:2, *v/v*) and dried with a SpeedVac concentrator (Savant SPD121P; Thermo Scientific, Waltham, MA). After that, the extracts were reconstituted with 200 μL of solvent A/B (90:10, *v/v*) used as the mobile phases for the UHPLC/MS/MS analyses. Quality control (QC) samples were prepared with HT and HT derivatives spiked in baseline control (BC) plasma and urine samples at two different concentrations (1 ng/mL (corresponds to the limit of quantification (LOQ)) and 5 ng/mL). Both precision (CV < 15%) and accuracy (80–120%) parameters in three replicates were acceptable according to ICH and FDA guidelines (data not shown).

UHPLC–ESI–QqQ–MS/MS analysis

The plasma and urinary metabolites (HT, HTA, DOPAC, Tyr, and HValc) were analysed using the previously cited methodology [10]. In brief, plasma and urinary HT and HT derivatives were analysed using an UHPLC–MS/MS with triple quadrupole technology (Agilent Technologies, Waldbronn, Germany). The chromatographic separation was carried out on the analytical column BEH C18 1.7 μm (2.1 × 50 mm) (Waters, Milford, M.A.) using water/formic acid (99.9:0.1, *v/v*) (A) and MeOH (B) as mobile phases. The flow rate was 0.4 mL/min, using a linear gradient (t; %B): (0; 20), (6; 95), (7; 100), (10; 20), and the injection volume was 10 μL. For the qualitative analyses, the target analytes were identified according to the most-abundant product ions detected by multiple reaction monitoring (MRM) in positive and negative modes depending on the analyte considered (Table 1). To avoid that the use of β-glucuronidase (with sufatase activity) from *Helix pomatia* may result in an underestimation of the bioavailability of phenolic compounds upon oral administration due to incomplete hydrolysis of conjugate compounds, previous assays with different times of hydrolysis and enzymatic units of this enzyme have been carried out and have already been used in other bioavailability studies of hydroxytyrosol performed at our laboratory [10, 16] (In this sense, the current study also monitored the potential residual conjugate compounds by theoretical MRM transitions because of the lack of commercially-available standards). Thus, $[M–H]^-$ 176 atomic mass units (amu) (glucuronide), 352 amu (diglucuronide), 80 amu (sulfate), 160 amu (disulfate), and 256 amu (sulfoglucuronide) were checked. The quantitation of HT, HTA, DOPAC, Tyr, and HValc was achieved by comparison with standard curves which were freshly prepared each day of analysis using authentic standards.

Statistics

All assays for each volunteer ($n = 20$) were performed in triplicate and concentrations were provided as means ± standard

Table 1 UHPLC-ESI-QqQ-MS/MS parameters for the quantification of hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), 3,4-dihydroxyphenylacetic acid (DOPAC), tyrosol (Tyr), and homovanillic alcohol (HValc)

Analyte	Retention time [min]	ESI mode	MRM transition
DOPAC	0.535	Positive	169 > 123
HTA	0.542	Positive	195 > 91
HT	1.214	Negative	153 > 123
HValc	1.603	Positive	169 > 151
Tyr	1.848	Positive	139 > 121

deviation (SD). Differences concerning plasma and urine concentration of HT derivatives were analysed with unpaired *t*-Student tests and analyses of variance (ANOVA). The fulfilment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested with the Kolmogorov–Smirnov (with Lilliefors correction) and Levene's tests, respectively. When statistical differences were identified, the variables were compared using Tukey's multiple range test by utilizing the separate food matrices as the sources of variation. The analyses were carried out with IBM SPSS statistics 24.0 (SPSS Inc., Chicago, IL, USA). Significant differences among means were considered at $p < 0.05$.

Results

The qualitative analysis of the biological samples (plasma and urine) collected for detecting the presence of HT and HT metabolites (HTA, DOPAC, HValc, and Tyr) was carried out by an ultra-high performance chromatography coupled to electrospray ionization and mass spectrometry with triple quadrupole technology (UHPLC-ESI-QqQ-MS/MS). Their assessment was performed after enzymatic hydrolysis of plasma and urine, which allowed monitoring free HT, HTA, DOPAC, HValc, and Tyr, after the intake of the different matrices considered. Furthermore, conjugate compounds after enzymatic hydrolysis were not present and therefore, no incomplete hydrolysis by β-glucuronidase was observed.

The retention times matched for both authentic standards and compounds present in the biological samples (0.535, 0.542, 1.214, 1.603, and 1.848 min for DOPAC, HTA, HT, HValc, and Tyr, respectively) (Table 1). For this, the parent mass and fragmentation pattern for HT metabolites were recorded by applying positive or negative ionization modes, depending on the metabolite monitored, in agreement with the method described by Dominguez-Perles et al. (2017) [10], which allowed their accurate quantification (Table 1).

Plasma kinetics of hydroxytyrosol after its oral administration through diverse food matrices

The UHPLC–ESI–QqQ–MS/MS-based assessment of peripheral blood plasma on the concentration of HT was performed at 0 (BC), 30, 60, 120, 180, and 240 min post-intake of 5 mg of HT in the array of matrices considered, corresponding to the equivalent dose recognized to promote human health according to EFSA [7]. To contrast the pharmacokinetic effect of the oily matrices, non-supplemented refined olive oil was also administered to the volunteers.

The plasma concentration of HT (Fig. 2) was achieved after enzymatic hydrolysis, which allowed registering the total amount of the target analyte. This approach provided plasma concentrations that were higher to the quantification limit of the method utilized (1 ng/mL) [10], which was the same as another methodology published for the analysis of free HT in human plasma following the administration of olive oil [17]. The highest plasma concentration of HT was achieved at 30 min after its oral intake, in agreement with previous studies found in the literature reviewed [15, 18]. Hydroxytyrosol was rapidly removed from the plasma, showing no significant differences with the plasma concentration values found in the negative control (non-supplemented refined olive oil) after 1 h.

The evaluation of the HT pharmacokinetics after its administration through the use of different food matrices evidenced that plasma concentrations were strongly dependent on the matrices used for its consumption (Fig. 2). Hence, with the intake of extra virgin olive oil, the highest average values were found after 30 min (3.79 ng/mL), being the only time-point exhibiting significant differences relative to the control, which showed trace levels below the LOQ ($p < 0.001$). However, HT plasma levels above the quantification limit of the method were found at 30, 60, 120, and 240 min post ingestion, with concentrations ranging between 1.24 and 3.79 ng/mL. A similar trend was observed when comparing the plasma concentration of HT, after the administration of fortified refined olive oil, with the control, which informed on matching times relative to extra virgin olive oil, for achieving the peak concentration of HT, but below the LOQ (traces levels). Again, significant differences with the control were found ($p < 0.01$). The remaining matrices assayed (fortified flax oil, grapeseed oil, margarine, and pineapple juice) did not provide significant increases of the HT plasma concentration HT throughout the 240 min post-intake monitored (Fig. 2).

Urinary excretion of hydroxytyrosol and its metabolites after intake of enriched matrices

As described for plasma samples, the quantification of HT metabolites (HT, HTA, DOPAC, HVAlc, and Tyr) in 24-h urine was performed on hydrolysed samples, which allowed determining the total HT absorbed.

To understand the extent to which the physico-chemical properties of the food matrix influences the bioavailability of HT, results on urine concentration of HT metabolites were statistically processed to determine: (1) the absolute concentration provided by the separate dietary interventions upon the administration of HT in the different matrices (Fig. 3), (2) the increase of HT concentration in urine relative to the basal levels in men and women (Fig. 4a), and (3) the increase of the urine concentration of HT when ingesting it through diverse food matrices (Fig. 4b).

The comparative analysis of the urinary HT after the oral intake of the diverse fortified and non-fortified

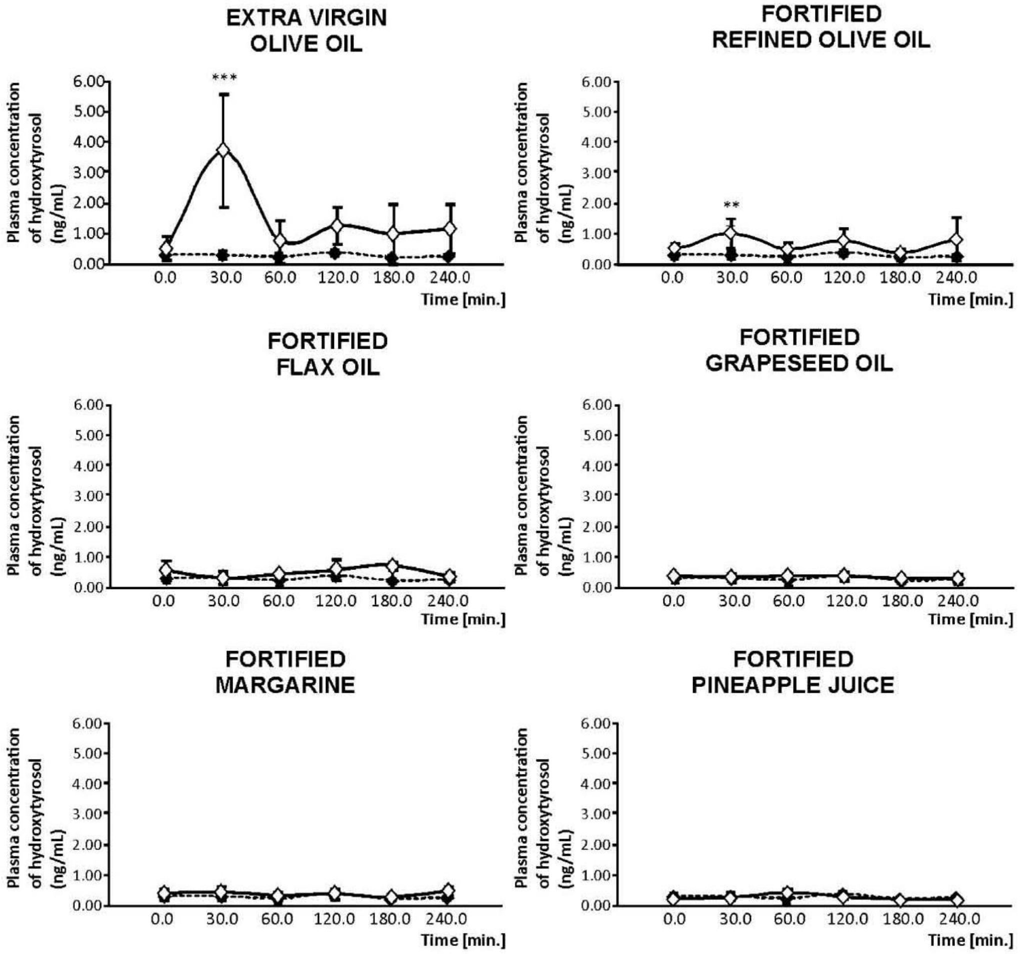

Fig. 2 Pharmacokinetics of HT presented as an overlap of the non-fortified refined olive oil (dotted line) and the specific food matrix at different sampling time-points (0 (Baseline control), 30, 60, 120, 180, and 240 min). Results are expressed as ng/mL. $^{**}p < 0.01$ and $^{***}p < 0.001$

matrices (bioavailability), regardless of the sex of the volunteers, evidenced that the highest concentration was reached when ingesting the 5 mg of HT through the natural source, extra virgin olive oil, which showed a significantly higher urinary content (0.86 µg/mg creatinine in urine at 24 h) with respect to basal urine (Fig. 3). Significant increases of the HT concentration were also found after the ingestion of fortified refined olive oil (0.63 µg/mg creatinine), fortified flax oil (0.55 µg/mg creatinine), and fortified grapeseed oil (0.33 µg/mg creatinine). As for the remaining matrices analysed, refined olive oil (negative control), fortified margarine, and fortified pineapple juice, although the ingestion of HT through these matrices caused an increase in its urine concentrations relative to the basal level, the differences recorded were not statistically significant ($p > 0.05$).

The bioavailability of HT metabolites (HTA, DOPAC, HValc, and Tyr) was analysed by determining their concentrations in urine (reflecting the amount of compound absorbed at the intestinal level or as a result of the metabolism of HT absorbed) (Fig. 3). The results of the urine analysis of volunteers showed no significant modifications of the basal level of the above referred metabolites.

When analysing the increase of the urine concentration of HT, HTA, DOPAC, HValc, and Tyr, considering the sex of the volunteers, it was observed that the highest concentration of HT (34.32 µg/mg creatinine) and HTA (9.58 µg/mg creatinine) in 24-h urine corresponded to men, who surpassed the concentrations recorded in women by 28.2 and 81.4%, respectively, although these differences, because of the inter-individual variations, were not statistically significant (Fig. 4a). With respect to the maximum concentrations

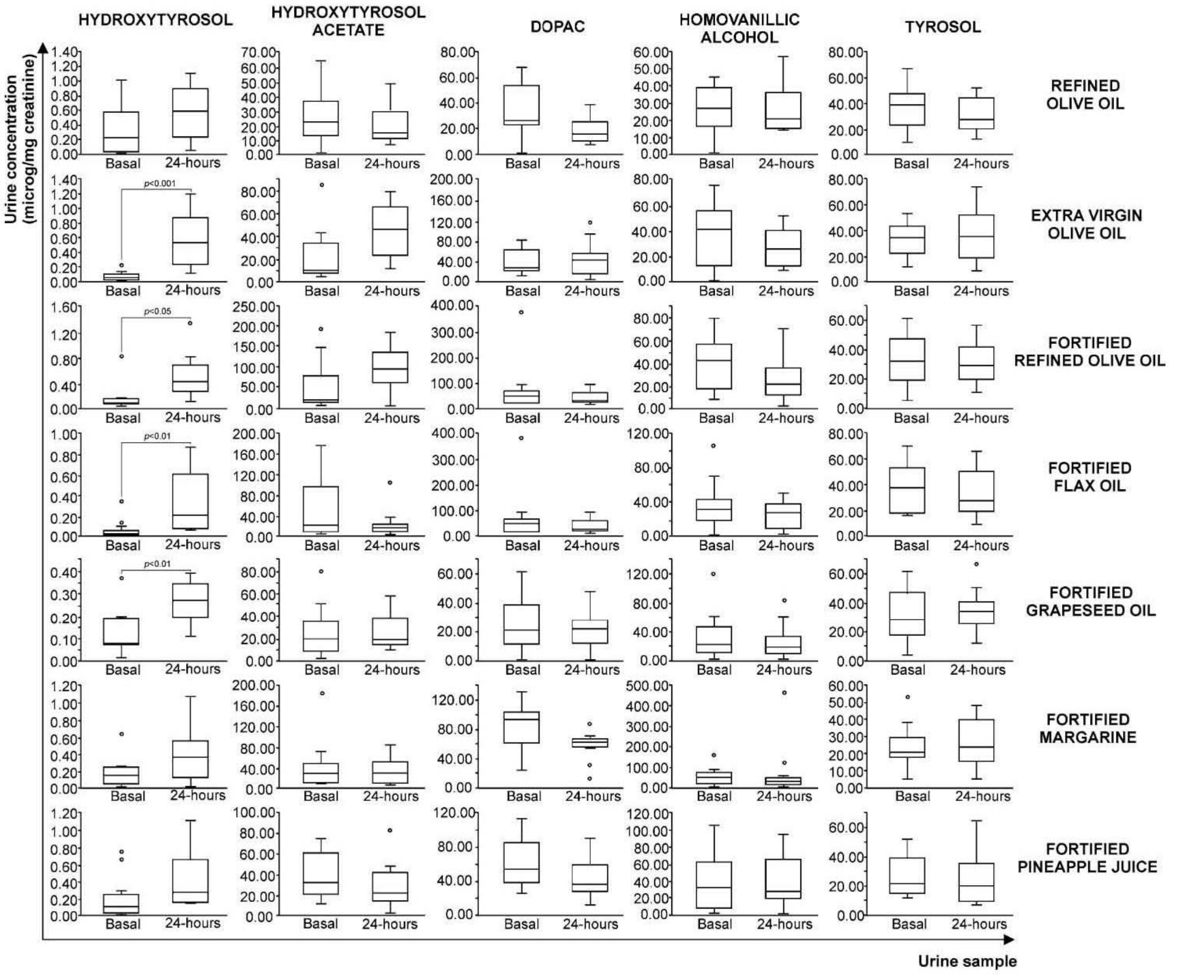

Fig. 3 Bioavailability (µg/mg creatinine) of hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), tyrosol (Tyr), homovanillic alcohol (HValc), and 2,3-dihydroxyphenyl acetaldehyde (DOPAC) was determined in basal and 24-h urine of 20 healthy human volunteers following the administration of 5 mg of HT by diverse matrices: extra virgin olive oil and five fortified food matrices (refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice). Statistically significant differences between the concentration of the separate HT derivatives in basal and 24-h urine samples were set up at $p < 0.05$, according to unpaired *t*-Student tests

of HValc (3.30 µg/mg creatinine) and Tyr (2.86 µg/mg creatinine), the highest values corresponded to women relative to men (32.2 and 15.9% lower, respectively). As for HT and HTA, the differences in bioavailability of HValc and Tyr between women and men were not statistically significant due to the dispersion of the results. Lastly, DOPAC was found in non-statistically different concentrations in both men and women (4.57 µg/mg creatinine, on average) (Fig. 4a).

When comparing the diverse concentrations of HT in 24-h urine, depending on the intake of the array of fortified and non-fortified food matrices (Fig. 4a), the highest level was obtained when administering HT in extra virgin olive oil (34.20 µg/mg creatinine), which was significantly higher than the rest of the matrices ($p < 0.01$), with the exception of the concentration obtained when ingesting fortified flax oil (10.09 µg/mg creatinine). In this regard, relative to extra virgin olive oil, the ingestion of fortified grapeseed oil and margarine gave rise to concentrations of HT in urine that were 87.5 and 78.9% lower on average, respectively. Moreover, statistically significant lower concentrations of HT in urine were observed when comparing extra virgin olive oil with fortified refined olive oil (9.14 µg/mg creatinine, on average) and fortified pineapple juice (8.95 µg/mg creatinine, on average).

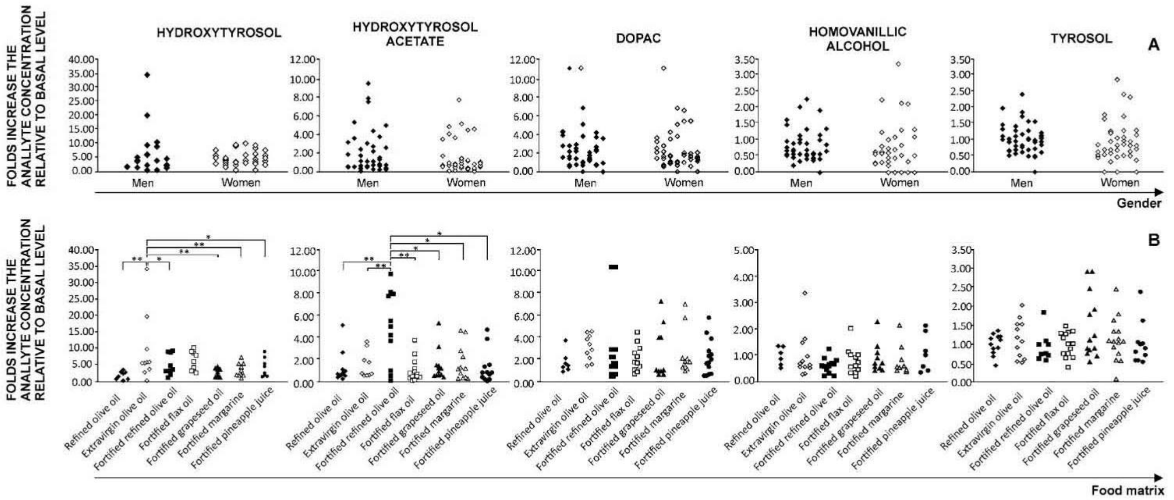

Fig. 4 Increase of the analyte concentration in urine with respect to basal levels according to the sex of volunteers (**a**) and food matrices ingested (**b**)

With respect to metabolites of HT (HTA, DOPAC, HValc, and Tyr) in 24-h urine, after the intake of the different matrices with 5 mg of HT, the only significant difference found corresponded to HTA (Fig. 4b). The highest urine concentration was observed after the intake of fortified refined olive oil (9.58 μg/mg creatinine, on average). When comparing the results of ingesting this matrix with the remaining ones, there were significant differences ($p < 0.01$) with respect to refined olive oil (a 47.9% lower, on average), extra virgin olive oil (a 61.2% lower, on average), and fortified flax oil (a 61.6% lower, on average). As for fortified grapeseed oil, fortified margarine, and fortified pineapple juice, statistically significant lower concentrations were also recorded (5.19, 4.53, and 4.62 μg/mg creatinine, respectively) (Fig. 4b). The analysis of the other metabolites excreted in 24-h urine according to the matrices evaluated, although a trend of increasing levels of DOPAC, HValc, and Tyr was observed after ingesting fortified refined olive oil, extra virgin olive oil, and fortified grapeseed oil, respectively, the differences found were not statistically significant (Fig. 4b).

Discussion

The purpose of this work was to uncover the bioavailability of HT, a phenolic compound with recognized health benefits, especially regarding cardiovascular pathologies, according to scientific opinions published by EFSA and the European Commission [7]. In connection with the recognized interest of HT in the prevention of cardiovascular disorders and its intake through diet, which has given rise to the development of a number of functional foods based on said biological activity, there is a gap of information on the influence of the food matrix on the intestinal absorption and bioavailability of HT and its metabolites. Hence, upon the present work, the differential pharmacokinetic and bioavailability of equal amounts of HT were characterized, after administration through extra virgin olive oil, and an array of food matrices which included refined olive oil, flax oil, grapeseed oil, margarine, and pineapple juice. These matrices feature different physico-chemical characteristics, which were normalized in relation to their concentration of HT to reach the amount recommended by EFSA (5 mg of HT per day) for the achievement of health benefits.

Pharmacokinetics is referred to the study of a given molecule and/or its metabolites' kinetics in the body. It refers to the temporary evolution of a compound and its metabolites in biofluids, tissues, and organs over time [19], while bioavailability informs on the fraction of the ingested nutrient or non-nutrient that is absorbed and available to participate in the various physiological processes or for storage. To the present day, bioavailability has been evaluated by resorting to a plethora of in vitro and in vivo (pre-clinical and clinical) models that provide complementary information [20, 21]. The selection of the model is closely linked to the final objective of the study and strongly conditioned by the availability of previous data on the safety of the compound/s of interest [22]. In addition, it is essential to take into consideration the influence of the diverse in vivo factors such as individual variability, the specific pathophysiological status of volunteers, and the interference of additional components in the food matrix (that could act as competitors or enhancers of intestinal absorption) which may make difficult the in vivo evaluation of bioavailability of bioactive constituents of foods [23]. Once the safety, biological interest, and bioavailability of bioactive compounds upon in vitro models are demonstrated, the application of in vivo approaches cannot be avoided.

At present, in this regard, information is readily available that supports the close linkage of the bioavailability of phenolic compounds with the physicochemical characteristics of the food matrix upon which they are administered. Thus, in connection with the experimental design implemented in the present work, the administration of HT as an ingredient in various food matrices (extra virgin olive oil, refined oil, pineapple juice, flax oil, margarine, seed oil grape) showed significant differences in pharmacokinetics and bioavailability depending on the matrix considered, which is of high interest for understanding the relevance of selecting the most beneficial diets to obtain the highest benefits of this phenolic compound. Thus, the results retrieved support the high interest in extra virgin olive oil to achieve a dietary source of HT in terms of plasma concentration. However, interestingly, a significant increase of urine concentration of HT was also obtained when ingesting this phenolic HT in diverse matrices (fortified refined olive oil, flax oil, and grapeseed oil). This result is in agreement with previous descriptions, which suggest that oily matrices favour the absorption of HT, although in most cases, the influence of the food matrix was restricted to high *versus* low polyphenolic content in olive oil [24–26], diet supplements either in aqueous solution or in capsules [25] or HT-enriched biscuits (5.25 mg HT/day (30 g biscuits)) [15]. This higher bioavailability has been tentatively attributed to the contribution of additional olive oil components to the absorption of HT [27], and dopamine metabolism [16, 25], although the extent to which the chemical diversity in the food matrix affects the absorption of HT by the intestinal epithelium remains to be elucidated. On the other hand, previous reports have revealed an intense enzymatic activity in the intestinal epithelium which affects HT to a greater extent than its derivatives, thus causing their differential transport through the intestinal lumen to the different organs and tissues [2], a phenomenon that was described early through ^{14}C-isotopic labelling [28].

Previous descriptions available in the literature have also suggested that bioavailability of HT could be conditioned by sex [10, 18, 28]. Taking into consideration this background, and to address the influence of sex in the bioavailability of HT when ingested through the different food matrices, a parity in the distribution of sex of the volunteers was considered in the experimental design of the present study to draw rational conclusions. In this sense, the main absorption events occur in the intestine with the participation of passive diffusion and sodium-glucose co-transporter-1 [29]. Regarding the latter, to the present day, divergent levels in some tissues such as the renal tissue in male and female rats have been described [30]. Moreover, the participation of a diversity of transmembrane proteins (such as breast cancer resistant protein, among others) in the absorption of phenolic compounds has also been observed. This transmembrane protein is also differentially expressed in the liver depending on sex, being responsible for the intestinal absorption of specific chemical forms of xenobiotics. However, the efficiency of the intestinal absorption is not the only factor to be considered when evaluating the bioavailability of phenolic compounds. As stated in previous reports, this could also be influenced by recycling processes found in enterohepatic circulation [10, 31]. Nevertheless, the comparison of the levels of bioavailable HT after oral intake of foods fortified with HT did not show significant differences in relation to the sex of the volunteers, although a higher (non-significant) absorption and bioavailability was observed in men compared with women.

In addition to the study of the levels of HT excreted in urine, for a better understanding of the absorption of HT and, therefore, its bioavailability, it is of great interest to study the metabolites derived from this phenolic compound, despite the remarkable difficulty of identifying these metabolites in biological samples due to their low concentrations and the occurrence in plasma and urine of numerous compounds that could interfere with the ionization of the analytes of interest [17]. Hence, in the present work, this constraint was overcome by the application of liquid–liquid and solid-phase-based clean-up methods that allowed removing unspecific molecules that could interfere with the identification and quantification of the analytes of interest. After using this approach, the results of the assessment of plasma and urine of volunteers after the ingestion of the HT through diverse food matrices, showed that no significant differences were found for HTA, DOPAC, HValc, and Tyr regarding sex. These results, which may seem contradictory relative to other studies characterizing the bioavailability of HT and its metabolites [10, 32], could be attributable to inter-individual variations (not present when analysing bioavailability through pre-clinical models with inbreed animals), although a trend towards increasing and decreasing concentrations of HT acetate and DOPAC, respectively, was observed.

Despite the trend observed regarding the evolution of the concentration of HT metabolites, the interpretation of the results retrieved related to previous works is not a simple task, since no clear pattern was observed between the arrays of matrices evaluated. In this aspect, a study by Domínguez-Perles et al. (2017) [10], after measuring the plasma and urine levels, showed that the increase in the amounts of HT metabolites did not follow linear correlations with its oral administration. The authors of this study concluded that although the diverse metabolites were efficiently absorbed and followed a similar metabolism, their pharmacokinetic and bioavailability are conditioned by a complex network of interactions of the intestinal microbiota with the intestinal transporters in the first phase of the metabolism of these compounds [10]. Therefore, additional knowledge on such interactions during their phase I metabolism are required by characterizing the occurrence of identified (target metabolomics) and non-identified (untargeted metabolomics) metabolites.

In summary, the results obtained evidence that the absorption and metabolic profile is highly influenced by the food matrix in which HT is incorporated, with the oily nature of the food matrix being especially relevant for the final bioavailability of this phenolic compound, and extra virgin olive oil being highlighted as the best dietary source of this compound. Alternatively, aside from identifying the most relevant source of HT in terms of pharmacokinetics and bioavailability, the results described in the present work would contribute to a better understanding of the relevance of the nature of the food matrices for the final absorption of HT and thus, their potential use as a component for the development of nutraceutical supplements, with a positive impact on the beneficial effects on cardiovascular health, according to the health claims presently recognized for HT.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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ARTÍCULO II

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Fatty Acid Hydroxytyrosyl Esters of Olive Oils Are Bioaccessible According to Simulated *In Vitro* Gastrointestinal Digestion: Unraveling the Role of Digestive Enzymes on Their Stability

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ABSTRACT: Recently, new bioactive compounds were identified in olive oil, lipophenols, which are composed of a fatty acid (FA) and a phenolic core, such as HT (HT-FA). However, their bioaccessibility remains unknown. Thus, the present study uncovers the impact of the separate phases of gastrointestinal digestion on the release and stability of HT-FAs from oily matrices under *in vitro* simulated conditions. Accordingly, it was found that the bioaccessibility of HT derivatives is largely dependent on the type of FA that esterifies HT, as well as the food matrix. Also, the generation of HT-FAs during intestinal digestion was observed, with pancreatin being the enzyme responsible, to a higher extent, for the *de novo* formation of lipophenolic derivatives. These findings prompt us to identify new applications to oily matrices and their byproducts as potential functional ingredients for the promotion of health, where the possible formation of new lipophenols during digestion should be taken into consideration.

KEYWORDS: *olive oil, hydroxytyrosol, lipophenols, in vitro gastrointestinal digestion, bioaccessibility, lipases*

■ INTRODUCTION

Olive drupes and derived products are considered part of the Mediterranean diet and co-contribute to the healthy characteristics of this diet, especially because of their content of mono- and polyunsaturated fatty acids (FAs) and (poly)phenols such as hydroxytyrosol (HT).[1,2] Moreover, oleic acid is the most abundant FA in olive oil, and HT is its principal phenolic compound.[3] Also, the presence of lipophenol structures, composed of an FA and a phenolic core, has been reported in edible oils. These molecules result from the esterification of HT with fatty acids (*e.g.*, α-linolenic, linoleic, and oleic acids (HT-ALA, HT-LA, and HT-OA, correspondingly)).[4,5] To date, extra virgin and virgin olive oils (EVOO and VOO, respectively) are the oily matrices with the highest concentration of these compounds.[5]

Lipophenols offer functional advantages relative to their molecular constituents (HT and FAs). Specifically, these compounds are characterized by a higher lipophilicity, cell membrane affinity, and improved antioxidant activities.[6−8] However, despite the suggestion of lipophenol's biological power ,[9,10] the actual functions remain almost unexplored. Nevertheless, regarding their antioxidant property, it may progressively be augmented along with the extension of the FA chain length up to a critical point, when additional extension could lower the radical scavenging power. Because of this phenomenon, the so-called cutoff effect, short- to medium-chain lipophilic esters of phenolic compounds (C8−C12) are more effective antioxidants relative to long-chain esters.[11] In contrast, other studies have associated equal or higher activity with long-chain esters (C18−C20).[12,13]

Aside from these considerations, the bioaccessibility of lipophenols remains to be assessed. In this regard, according to previous studies, intense hydrolysis of HT acetate after pancreatin and bile salt digestion has been reported,[14] while tyrosol acyl esters are hydrolyzed by pancreatic lipase to produce free tyrosol, showing a certain sustained-release behavior.[15,16] These results suggest that the time frame for retrieving biological activities from phenolic compounds could be extended due to lipophenol's bioactivity, thus allowing us to obtain additional health benefits.[15] These authors reported that the stability of tyrosol esters under the physicochemical conditions associated with the *in vitro* simulation of gastrointestinal (GI) digestion was correlated both with the chain length and with the number of unsaturations.[16] Nonetheless, the effect of the digestive process on the release of HT-FAs and their stability remains underexplored. Also, the experimental approach implemented thus far, based on the analysis of authentic standards instead of food matrices, is negligible because it ignores the effect of the matrix throughout the digestion.[14] To overcome this limitation, a simulated GI

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Table 1. Preparation of Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

	constituent[a] (mmol/L)					
simulated fluids	KCl	KH_2PO_4	$NaHCO_3$	NaCl	$MgCl_2(H_2O)_6$	$(NH_4)_2CO_3$
simulated gastric fluid (SGF; pH 3)	6.90	0.90	25.00	47.20	0.10	0.50
simulated intestinal fluid (SIF; pH 8)	6.80	0.80	85.00	38.40	0.13	

[a] KCl, potassium chloride; KH_2PO_4, potassium phosphate monobasic; $NaHCO_3$, sodium bicarbonate; NaCl, sodium chloride; $MgCl_2(H_2O)_6$, magnesium chloride hexahydrate; and $(NH_4)_2CO_3$, ammonium carbonate.

digestion by *in vitro* models could be a valuable, time-saving tool, as it requires fewer resources for obtaining preliminary but extremely important information for the evaluation of the structural and chemical changes under GI conditions.[17]

According to this background information and previous findings, the present work aims to gain further insight in the results of the GI digestion on HT-ALA, HT-LA, and HT-OA found in EVOO and VOO to identify the lipophenols released from the food matrix that remain stable during GI digestion and that are available for absorption. Also, the evaluation of the effect of pepsin, pancreatin, and pancreatic lipase allowed us to discover the influence of the digestive enzymes on the stability of lipophenols. The major outcomes retrieved contributed to setting up the bioaccessibility of HT-FAs in the small intestine compared with unesterified HT and to understanding the enzymatic mechanisms responsible for them, providing the theoretical basis of the transformation of lipophenols during the digestive process.

■ MATERIALS AND METHODS

Chemicals and Reagents. The authentic standards of high-purity HT-FAs (HT-ALA, HT-LA, and HT-OA) were synthesized and fully characterized by nuclear magnetic resonance (NMR)-based analysis by the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France) according to previously published procedures.[4] Acetone and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and all LC−MS-grade solvents (deionized water, methanol, and acetonitrile) were from J.T. Baker (Phillipsburg, NJ, USA). A certified reference of FA standards from Sigma-Aldrich was used for their identification and quantification. Hydroxytyrosol, with the following specifications: purity 99.6%, moisture 3.7%, and pH 4.26 (1 M aqueous solution), was provided by Seprox BIOTECH S.L. (Murcia, Spain). All lipophenols were dissolved in dimethyl sulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO, USA) to obtain a stock solution (at the mM range of concentration), and then successive dilutions were prepared in methanol/deionized Milli-Q water (50:50, v/v). All stock solutions of lipophenols were stored at −20 °C in the dark. Porcine pepsin (P6887), pancreatin from porcine pancreas (P7545, 8 × USP), and lipase from porcine pancreas (L3126) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

Fatty Acid Composition Analysis of Edible Oils. Fatty acids of EVOO, VOO, and flaxseed oil (FO) were extracted according to the methodology employed previously[5] and published by the FAO (FAOLEX No. LEX-FAOC141241, http://www.fao.org/faolex/results/details/es/c/%20LEX-FAOC141241/) for the analysis of vegetable oils. Briefly, samples (1 g) were weighed and dried at 70 ± 10 °C for 6 h. The fatty acid content was obtained by the Soxhlet extraction with diethyl ether and heptane and by esterification with methanolic NaOH (1 M). Fatty acids were determined by gas chromatography coupled to a flame ionization detector (GC-FID) (6890 GC Agilent Technologies, Waldronn, Germany). The absolute concentration (g/100 g fw) of fatty acids in the vegetable oils ($n = 3$) was calculated according to the formula individual fatty acid (g/100 g fw) = individual fatty acid (% of fat)/100 × total fat (g/100 g fw), which was used according to FAO/INFOODS guidelines for converting units, denominators, and expression (2012).[18]

Vegetable Oil Samples and Preparation of Analytical Extracts of Hydroxytyrosol Fatty Acid Esters. Extra virgin olive oil (Picual monovarietal; Olimendros S.L. (Murcia, Spain)), VOO (Picual monovarietal; Salvador Gallego El Jota S.L. (Albacete, Spain)), and FO (Laboratorios Almond S.L. (Murcia, Spain)) were chosen due to their dissimilar concentration of HT esterified with ALA, LA, and OA, with FO used as the negative control sample due to its lack of HT-FA ester content.[5] All oil samples were kept in dark glass bottles, closed with screw caps, and stored at 4 °C to avoid oil oxidation to the greatest possible extent. The HT-FAs were extracted from vegetable oils according to the methodology described previously.[5]

***In Vitro* Simulated Gastrointestinal Digestion.** To reproduce the separate digestion phases (gastric, intestinal, and GI), the edible oils (EVOO, VOO, and FO) were processed based on the harmonized *in vitro* digestion protocol described in the literature,[19,20] using the simulated gastric and intestinal fluids (SGF and SIF, respectively) stock electrolyte solution, developed according to the information provided in Table 1.

After digestion, the samples were centrifuged at 1600*g* for 5 min at 4 °C to separate the bioaccessible fraction (BF) and the upper oily phase or residual fraction (RF); the concentration of HT and HT-FAs in each fraction was analyzed. Blanks (negative controls) without enzymes were processed and analyzed in parallel under equal conditions. All samples were protected from light over the entire process. Both BF and RF fractions were frozen immediately at −80 °C and lyophilized. For the HT and HT-FA extraction, the lyophilized samples were then dissolved in 3 mL of an acetone/BHA (99.995:0.005, v/w) solution, vortexed for 1 min, sonicated for 30 min at 40 kHz, and centrifuged at 8750*g* for 5 min at 4 °C according to the procedure described.[5] The samples were dried using a SpeedVac concentrator, and the dry extracts were reconstituted with 500 μL of MeOH, sonicated for 10 min, centrifuged at 8750*g* for 5 min, and filtered through a 0.45 μm filter (Millipore, Burlington, MA, USA).

UHPLC-ESI-QqQ-MS/MS Analysis. The separation and identification of HT and HT-FAs were performed using a UHPLC coupled with a triple quadrupole MS/MS (Agilent Technologies, Waldbronn, Germany) according to the methodology reported in the literature.[5] The retention times recorded for unesterified HT, HT-ALA, HT-LA, and HT-OA were 0.6, 3.8, 4.0, and 4.4 min, respectively. The analyses were performed by multiple reaction monitoring (MRM) in the negative mode, and the quantification and confirmation MRM transitions were as follows: m/z 153 > 123 arbitrary mass units (amu) and m/z 153 > 95 amu, respectively, for HT; m/z 413 > 277 amu and m/z 413 > 260 amu, correspondingly, for HT-ALA; m/z 415 > 279 amu and m/z 415 > 262 amu, respectively, for HT-LA; and m/z 417 > 281 amu and m/z 417 > 264 amu, correspondingly, for HT-OA. Data acquisition and processing were done using the MassHunter software version B.08.00 (Agilent Technologies, Waldbronn, Germany). Both HT and esters of HT were quantified using reference standards, and the concentrations were expressed as nanograms per gram of fresh weight (ng/g fw).

Statistical Analyses. All the analytical extractions of the oily matrices considered in the present work, as well as the products of the gastric, intestinal, and GI digestions, were analyzed in triplicate (n = 3), and the data were expressed as the mean ± SD. Statistical analyses were performed at 5% of the significance level using the SPSS 27.0

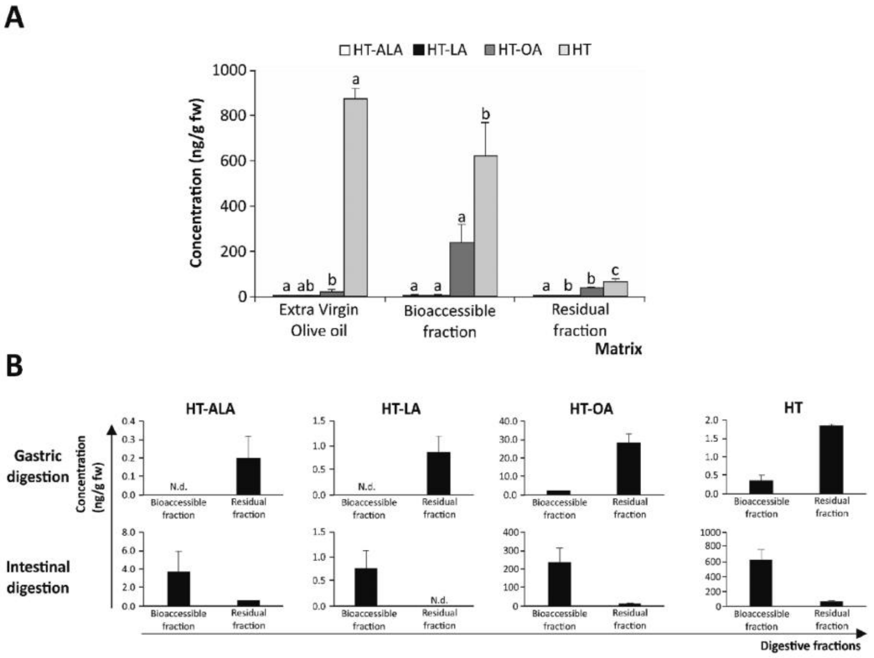

Figure 1. Concentration (ng/g fw) of HT and its lipophenolic derivatives in extra virgin olive oil (EVOO) and its bioaccessible and residual fractions (A) and bioaccessibility resulting from the individual gastric and intestinal digestions (B). N.d., not detected. Bars with a different lowercase letter indicate statistically significant differences among matrices at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

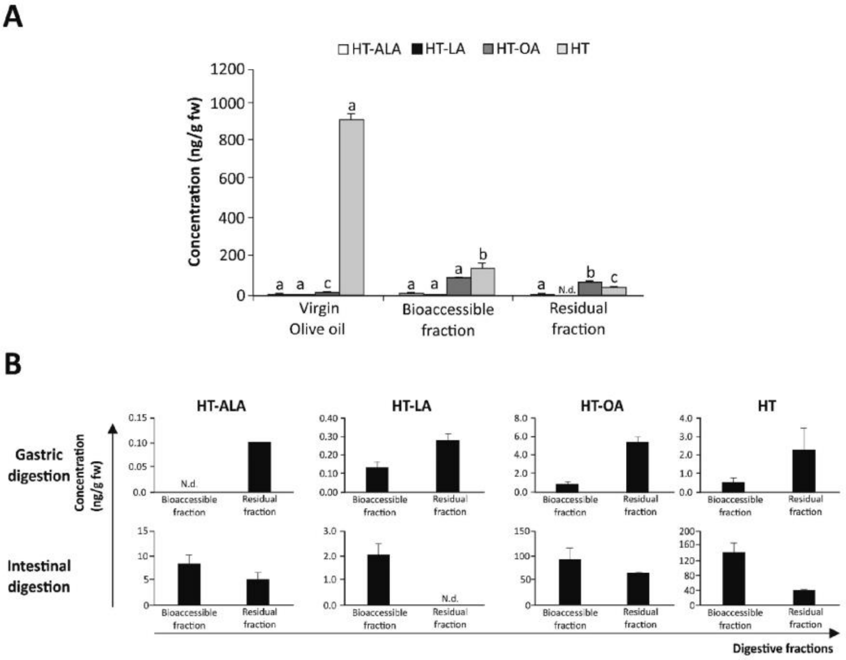

Figure 2. Concentration (ng/g fw) of HT and its lipophenolic derivatives in virgin olive oil (VOO) and its bioaccessible and residual fractions (A) and bioaccessibility resulting from the individual gastric and intestinal digestions (B). N.d., not detected. Bars with a different lowercase letter among matrices indicate statistically significant differences among matrices at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

software package (LEAD Technologies, Inc., Charlotte, NC, USA). Data were subjected to a one-way analysis of variance (ANOVA). The normal distribution of the residuals and the homogeneity of variance were tested with the Kolmogorov−Smirnov and Levene tests, respectively. When statistical differences were identified, the variables were compared using post hoc analysis with Tukey's multiple range test.

■ RESULTS AND DISCUSSION

Quantitative Profile of Hydroxytyrosol-Fatty Acids in Extra Virgin Olive Oil and Virgin Olive Oil. To understand the bioaccessibility of HT-FAs, first, the quantitative profile of unesterified and esterified HT in EVOO and VOO edible oils was set up. As expected, the retention time of HT-FAs displayed a reverse correlation with the degree of unsaturation of its lipid part. Thus, HT-ALA, HT-LA, and HT-OA were eluted at 3.8, 4.0, and 4.4 min, respectively, which are consistent with previous experimental results[5] and other lipophenolic derivatives such as tyrosol esters.[16] After this task, it was observed that the amount of unesterified and esterified HT of both olive oils was similar (Figures 1 and 2) but different from that corresponding to FO, which exhibited an absence of HT FAs (data not shown).

In this way, the unesterified HT was present as 874.74 and 934.09 ng/g fw in EVOO and VOO, respectively. These concentrations were in line with those available in the Phenol-Explorer database (http://phenol-explorer.eu/) on the HT content of EVOO (0.01−3.47 mg/100 g fw) and VOO (<0.01−7.43 mg/100 g fw). Regarding the HT-FAs, values of 2.80, 1.54, and 20.00 ng/g were recorded for HT-ALA, HT-LA, and HT-OA, respectively, in EVOO and 4.56, 2.59, and 11.90 ng/g in VOO (Figures 1 and 2, correspondingly), which are in agreement with previous reports on matching food matrices and analytes,[5] although important factors such as variety, geographical origin, ripening stage, and production and extraction processes may play an important role in lipophenol content.[21]

Fatty Acid Composition of the Edible Oils Assessed. As referred to before, it is worth noting that the bioaccessibility of specific bioactive compounds may be enhanced by the content of FAs in the food matrix through dispersing food components in the digestive tract or by promoting the secretion of pancreatic juice.[22] In this scenario, as one of the objectives of the current study was to describe the bioaccessibility of the of HT-FAs, the quantitative profile of fatty acids of the edible oils under consideration (EVOO, VOO, and FO) was created, given its central importance to the formation of the target bioactive compounds, the HT-FAs. As expected and according to previous descriptions in the literature, the FA composition of the vegetable oils considered in the present work (EVOO, VOO, and FO) involved saturated, monounsaturated, and polyunsaturated fatty acids (SFAs, MUFAs, and PUFAs, correspondingly).[23] Specifically, the oily matrices studied contained a total of 13 individual FAs (Table 2).

The SFAs detected were palmitic (C16:0) and stearic (C18:0) acids, which showed statistically significant differences between samples ($p < 0.05$), with EVOO being the plant oil with the highest content of palmitic acid (12.07 g/100 g fw) in comparison with VOO and FO (10.97 and 5.77 g/100 g fw, respectively). On the other hand, FO provided the greatest concentration of stearic acid (4.40 g/100 g fw) that surpassed by 41.7%, on average, the contents recorded in EVOO and VOO, which remained in similar but lower levels.

Table 2. Concentration of Fatty Acids (g/100 g fw) in the Extra Virgin Olive Oil (EVOO), Virgin Olive Oil (VO), and Flaxseed Oil (FO) Analyzed in the Present Work

	edible oils			
fatty acids[a]	EVOO	VOO	FO	LSD ($p < 0.05$)
C14:0	0.03a[b]	0.03a	0.03a	<0.01
C16:0	12.07a	10.97b	5.77c	0.17
C16:1 n7	0.93a	0.83a	0.10b	0.09
C17:0	0.10a	0.10a	0.10a	<0.01
C17:1 n8	0.10a	0.10a	0.10a	<0.01
C18:0	2.50c	2.63b	4.40a	0.06
C18:1 n9	77.53b	80.10a	19.97c	0.17
C18:2 n6	4.43b	4.10c	15.27a	0.09
C18:3 n3	0.63b	0.60b	53.53a	0.09
C20:0	0.37a	0.60a	0.33a	0.09
C20:1	0.30a	0.30a	0.10b	<0.01
C22:0	0.10a	0.10a	0.10a	<0.01
C24:0	0.47a	0.10b	0.10b	0.06

[a] C14:0: myristic acid; C16:0: palmitic acid; C16:1 n7: palmitoleic acid; C17:0: heptadecanoic acid; C17:1 n8: heptadecenoic acid; C18:0: stearic acid; C18:1 n9: oleic acid; C18:2 n6: linoleic acid; C18:3 n3: alpha-linolenic acid; C20:0: arachidic acid; C20:1: eicosenoic acid; C22:0: behenic acid; and C24:0: lignoceric acid. [b] Means (n = 3) within a row with different lowercase letters are significantly different at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

Moreover, significant differences were recorded regarding the content of palmitoleic and oleic acids (C16:1 n7 and C18:1 n9, respectively) between the diverse edible oils analyzed ($p < 0.05$), with oleic acid underlined as having the most abundant MUFA in all vegetable oils considered (in the range of 19.97−80.10 g/100 g fw). The highest concentration of oleic acid was found in VOO (80.10 g/100 g fw) followed by EVOO (3.2% lower) and FO (75.1% lower) (Table 2).

Finally, when analyzing the content of PUFAs, linoleic (C18:2 n-6) and α-linolenic (C18:3 n-3) acids were found at the highest concentrations in FO (15.27 and 53.53 g/100 g fw, respectively), whereas EVOO and VOO displayed values in a similar lower level (4.27 and 0.62 g/100 g fw, on average, respectively) (Table 2).

The composition results regarding SFAs, MUFAs, and PUFAs are in accordance with information previously reported in the frame of studies comparing the FA composition of several types of edible oils.[5,24] In this regard, the FA profile, together jointly with the presence of HT in the matrices referred to in the study of HT lipophenolic derivatives, would provide a very helpful choice for assessing the effect of GI digestion on the lipophenolic profile of vegetable oils, which is essential for discovering the biological interest of these compounds.

Bioaccessibility of Fatty Acid Esters of Hydroxytyrosol from Extra Virgin Olive Oil and Virgin Olive Oil. For this first description of the GI bioaccessibility of the HT lipophenols, an optimized and standardized static method was applied for the *in vitro* simulation of GI digestion, which allows mimicking the physicochemical conditions in the GI tract, *in vivo*, as closely as possible.[19,20] The lipophenols of HT, HT-ALA, HT-LA, and HT-OA were analyzed in the three vegetable oils utilized in the present study. FO was selected as a negative control as it does not contain either HT or its lipophenolic derivatives according to our previous work.[5]

These molecules were also assessed in the digestates obtained after *in vitro* gastric, intestinal, and GI digestion of the vegetable oils mentioned above. In this regard, both the upper oily or residual phase (RF) and the lower micellar or bioaccessible fraction (BF) resulting from digestion were collected and analyzed to retrieve accurate information on the theoretical and potential bioavailable fractions of these new lipophilic phenolic compounds, and also interconversions between unesterified HT and its esters derivatives, which would condition future *in vivo* research aimed at addressing the actual bioavailability of HT (free and esterified).

The *in vitro* GI digestion performed on EVOO (Figure 1A) and VOO (Figure 2A) revealed that ALA-, LA-, and OA-based lipophenols of esterified HT were present in the BF. The concentrations of HT-FAs corresponding to EVOO were 3.68, 4.04, and 239.41 ng/g fw, while for the VOO samples, these were 8.22, 1.80, and 92.00 ng/g fw for HT-ALA, HT-LA, and HT-OA, respectively, evidencing that the esterified forms of HT were bioaccessible. However, the rate of bioaccessibility depended on both the specific FA-based esterification and the compositional features of the food matrix, as already reported for free HT pharmacokinetics and bioavailability, *in vivo*, after ingestion of different oily matrices.[25]

These results are in good agreement with previous reports on the bioaccessibility of other esters of HT, namely, phosphatidyl-hydroxytyrosol (PHT, phospholipid derivatives of HT with phosphatidylcholine).[17] In their work, Martin et al. described that the portion of HT within the BF, regarding the esterified form (PHT), was significantly higher relative to unesterified HT.[17] In addition, although at lower concentrations than in BF, both unesterified HT and esterified HT were detected in RF (Figures 1A and 2A). Just as with PHT, the amphiphilic properties of HT esterified with FAs could cause its dispersion in the aqueous media, which in turn indirectly enabled the dispersion of the vehiculated HT.[17]

When analyzing the bioaccessibility rate of HT and its lipophenol derivatives, it was found that the unesterified form presented a bioaccessibility of 70.9 and 15.4% for EVOO and VOO, respectively. These results are in line with previous descriptions concerning an important loss of HT in the duodenal compartment (~50%, on average) retrieved from alperujo (olive-mill waste) digestions.[26] These authors described that when digesting only the target bioactive compounds in analytical solvents (without the presence of additional food constituents, such as starch, casein, and fiber), the bioaccessibility of HT was enhanced by ~20%. This effect could be due to the presence of the referred macromolecules, which can bind polyphenols and retain them within the food matrix. Hence, several authors have emphasized the significance and complexity of phenolic compound interactions within the food matrices associated to their bioaccessibility.[26,27]

In relation to the concentrations of lipophenols in EVOO, the concentration of HT-FAs in BF increased 1.3-, 3.0-, and 11.9-fold for HT-ALA, HT-LA, and HT-OA, respectively (Figure 1A). This fact may be due to the interactions between free HT and FAs, where HT could act as a nucleophilic compound able to trap ALA, LA, and OA present in the oily matrix to form the corresponding esters via enzymatic or chemical means under GI conditions,[28] therefore exerting "negative" effects on free HT bioaccessibility but ameliorating the concentration of the esterified forms of HT in the digestate. A similar behavior was observed for VOO as a result of the GI digestion, which resulted in a 1.8- and 7.7-fold rise in concentration for HT-ALA and HT-OA, respectively, in the BF when compared to those found in VOO, with the exception of HT-LA, whose bioaccessibility decreased up to ~70%, on average (Figure 2A). In addition, it should also be stressed that the most abundant HT-FA in the RF was HT-OA, which increased 2.0- and 5.6-fold in comparison with the amounts found in EVOO and VOO, respectively (Figures 1A and 2 and Table 2). This differential occurrence of HT-FAs from EVOO and VOO after GI digestion could be owed to the interactions of these compounds with the food matrix components such as amino acids and proteins, in spite of their low content (0.07–2.4 mg/kg). The presence of these components depends on the ripening stage or olive cultivar, among other factors,[29,30] and may generate fatty acid-binding protein reactions[31,32] that can hamper the bioaccessibility of these compounds.

Conventionally, the RF has not been considered for the evaluation of the bioaccessibility of phenolic compounds after the digestions. However, this fraction is of high relevance because of its high concentration of polyphenols that are not absorbed in the small intestine, which can be metabolized by the gut microbiota,[33] giving rise to additional bioactive derivatives that could also contribute to the healthy attributes of foods. Thus, both fractions (BF and RF) resulting from GI digestion may be responsible for the positive health effects attributed to plant-based foods, including edible oils.[34] In this matter, the capacity of the intestinal microbiota to modify the quantitative phytochemical profile of the digestion products and thereby the bioavailability/absorption capacity of bioactive compounds present in olive and olive oils that have not been absorbed in the small intestine is remarkable, and as a result, the substrate for these metabolic reactions (nonabsorbed phenolics) should not be disregarded. In fact, the gut microbiota plays a prominent role in the biotransformation of both esterified and aglycone forms of phenolic compounds, but the metabolism of lipophenols by the gut microbiota is still unclear. To date, there is only one study that evaluated the fecal microbial metabolism of lipophenols utilizing an *in vitro* fermentation model as well as the *in vivo* absorption and plasma pharmacokinetics of tyrosol esters in rats, where standards of tyrosol and tyrosol esters were administrated in the drinking water.[35] The authors concluded that tyrosol ester derivatives acted longer *in vivo* than native tyrosol by observing a second absorption peak in pharmacokinetic profiles, possibly due to microbiota degradation, with the esterified molecules exhibiting an improved bioavailability as compared to that of free tyrosol. The incubation of tyrosol esters in the fermentation solution liberated free tyrosol in a time-dependent manner, indicating the occurrence of hydrolysis.[35] However, this finding should be tested with rats fed with natural sources of these lipophenols, not with standards, and in the natural concentrations in which they are found.

Contribution of the Gastric and Intestinal Digestion Phases to the Bioaccessibility of HT-FAs. To understand the impact of the digestion process on the bioaccessibility of HT-FAs, the gastric digestates from EVOO and VOO were also analyzed (Figures 1B and 2), which provided valuable information on the release of the target lipophenols on each digestion stage. Upon this analysis, it was observed that unesterified HT and its lipophenol derivatives were present at

Table 3. Concentration (ng/g fw) of the Bioaccessible and Residual Fractions (BF and RF, Respectively) of HT and Its Lipophenolic Derivatives as a Result of the Only Intestinal Digestion on the Different Substrates (Extra Virgin Olive Oil, Virgin Olive Oil, and Their Gastric Digestates)

		concentration (ng/g fw)				
			substrate of the intestinal digestion			
			gastric digestate		olive oil	
oil matrix	analyte[a]	olive oil	BF	RF	BF	RF
EVOO[a]	HT	874.47 ± 41.88a[b]	619.88 ± 144.42b	64.46 ± 12.70d	279.05 ± 63.95c	542.00 ± 34.15b
	HT-ALA	2.80 ± 0.95b	3.68 ± 2.30b	0.61 ± 0.06b	235.96 ± 23.77a	0.64 ± 0.02b
	HT-LA	1.54 ± 0.49bc	4.04 ± 1.97b	N.d.	34.19 ± 1.61a	N.d.
	HT-OA	20.00 ± 8.11c	237.64 ± 76.94b	12.68 ± 2.72c	3599.94 ± 107.20a	102.35 ± 0.41bc
VOO	HT	934.09 ± 29.53a	143.76 ± 25.08bc	39.58 ± 1.27c	207.75 ± 131.67b	251.66 ± 3.38b
	HT-ALA	4.56 ± 1.52b	8.22 ± 1.79b	5.12 ± 1.25b	32.44 ± 5.17a	4.59 ± 0.54b
	HT-LA	2.59 ± 0.46b	1.98 ± 0.44b	N.d.	5.87 ± 1.51a	N.d.
	HT-OA	11.90 ± 4.90b	91.18 ± 2.97b	63.90 ± 4.21b	1205.44 ± 160.57a	85.81 ± 7.79b

[a] HT, hydroxytyrosol; HT-ALA, hydroxytyrosol esterified with α-linolenic acid; HT-LA, hydroxytyrosol esterified with linoleic acid; and HT-OA, hydroxytyrosol esterified with oleic acid. [b] Data are shown as means ± SD ($n = 3$) within the same row followed by the same lowercase letter are not significantly different at $p < 0.001$ according to the analysis of variance (ANOVA) and Tukey's multiple range test. N.d., not detected.

high concentrations in the RF obtained from the gastric digestion for both EVOO and VOO.

When evaluating the impact of gastric digestion on the EVOO lipophenols, low concentrations of HT and HT-FAs were observed in the BF for all individual analytes (not detected, not detected, and 1.77 and 0.33 ng/g for HT-ALA, HT-LA, HT-OA, and HT) (Figure 1B). Similar results were obtained regarding VOO (not detected and 0.13, 0.82, and 0.52 ng/g for HT-ALA, HT-LA, HT-OA, and HT) (Figure 2B).

The low concentrations recorded could be due to the lability of lipophenols against the physicochemical conditions and the enzymatic activity proper of the gastric digestion, which would be associated with a low release or stability of the target analytes. This would be consistent with previous reports on the bioaccessibility of HT in olive leaves,[36] further supporting the influence of the food matrix, the gastric enzymes, or acidic conditions previously described.[37] Nonetheless, the development of intestinal digestion on the chyme provided a high concentration of free HT in the BF. This could be due to the hydrolysis of oleuropein and its aglycone during intestinal digestion that depends on the lipase activity, thus leading to the appearance of HT, as previously stated by Rocchetti et al.[38] Interestingly, as a result of intestinal digestion, the concentration of HT-FAs significantly increased in the BF over the entire GI process, tentatively due to the putative enzymatic activity of pancreatic lipase that may catalyze both the hydrolysis or synthesis of esters.[39]

As far as we know, to date, most research on the bioaccessibility of lipophenols has been performed on solutions of authentic standards exposed to the GI physicochemical conditions. Therefore, despite the referred attributes of the synthesis of lipophenols as a result of the digestive enzymatic activity, additional works have evidenced that HT derivatives, such as hydroxytyrosol acetate, undergo intense hydrolysis during intestinal digestion mainly due to the enzymatic activity of pancreatin.[14] Similarly, it has been reported that tyrosol acyl esters are hydrolyzed by pancreatic lipase to produce free tyrosol.[15,16] Moreover, the stability of resveratrol esters with caprylic acid during digestion is negatively correlated with the degree of substitution of the FA moiety, given that after 120 min of incubation at 37 °C, ~54 and ~11% of monoesters and diesters are hydrolyzed, respectively, while no hydrolysis of the triesters has been noticed.[40] However, these results have been associated with an "artificially" obtained (only with synthesized standards chemical or enzymatically) higher bioaccessibility relative to the effects that occur during the GI digestion of foods because these models do not allow monitoring the extractive capacity of the process, the interconversion between molecules, or the *de novo* synthesis of the target analytes (from the complex pool of molecules present in the food matrix). This gap in knowledge has been overwhelmed by a study on the bioaccessibility of HT with alperujo (solid byproduct of olive oil extraction). This study described a lower bioaccessibility of HT when applying GI digestion on a vegetable matrix due to the presence of fibers and sugars that could interact with HT and the digestive enzymes, modulating the bioaccessibility rate.[26] Accordingly, the assessment of the bioaccessibility of HT or HT-FAs needs to include a food matrix to obtain robust and nonspeculative conclusions.

The relevance of the intestinal digestion phase for the presence of HT lipophenols in the GI digestates prompted us to explore the only effect of intestinal digestion on the intact food matrix, avoiding the uncertainly detrimental effect that could be occurring during the gastric stage (Table 3). Thus, when the intestinal digestion was performed on both EVOO and VOO (without the previous gastric phase), the concentration of HT lipophenols observed in the BF was higher relative to that obtained following the physiological workflow (Table 3). The content of HT-ALA, HT-LA, and HT-OA in BF of the intestinally digested EVOO was ~64-, ~8-, and ~ 15-fold higher, respectively, in comparison with the BF obtained after a complete GI digestion (gastric plus intestinal digestions). This trend was similar to VOO, where the content of lipophenols in the BF increased ~4-, ~3-, and ~13-fold for HT-ALA, HT-LA, and HT-OA, respectively, relative to BF obtained from the sequential gastric and intestinal digestions (Table 3).

On the contrary, the highest concentration of unesterified HT was found in the BF from intestinal digestions developed on gastric digestates of EVOO (619.88 ng/g fw) in comparison with the direct intestinal digestion of the intact food matrix (55.0% lower, $p < 0.001$) (Table 3), while regarding VOO, no statistically significant differences were found, reinforcing the

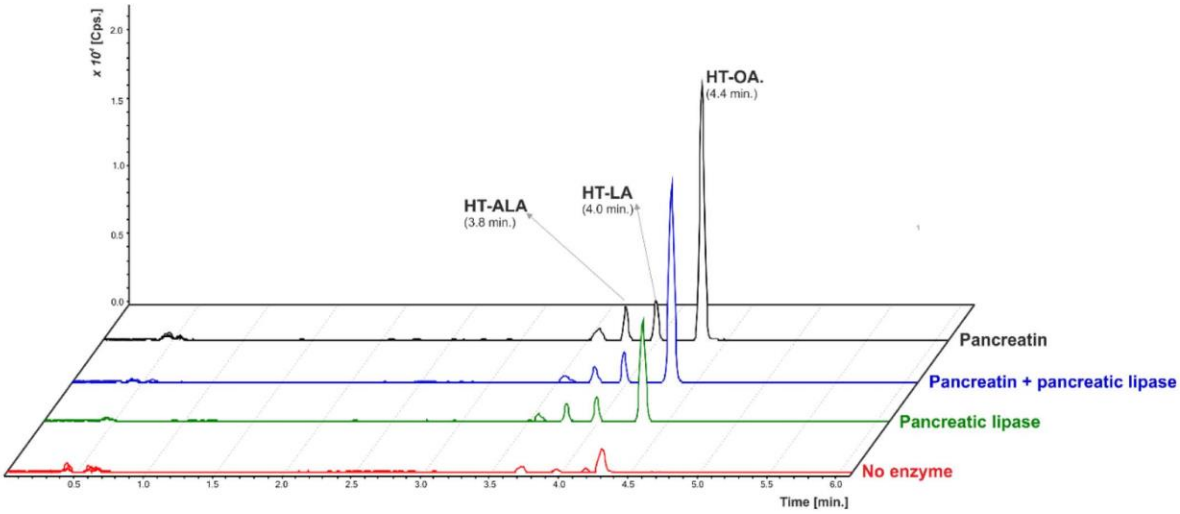

Figure 3. Representative UHPLC-ESI-QqQ-MS/MS-extracted ion chromatograms corresponding to the bioaccessible lipophenols of hydroxytyrosol (HT-ALA, hydroxytyrosol-α-linolenic acid; HT-LA, hydroxytyrosol-linoleic acid; and HT-OA, hydroxytyrosol-oleic acid) in digestates obtained after applying different enzyme combinations on extra virgin olive oil during simulated *in vitro* intestinal digestion using the quantification MRM transitions. CPS, charges per second.

Table 4. Concentration (ng/g fw) of Hydroxytyrosol Esterified with Fatty Acids after Intestinal Digestion of Extra Virgin Olive Oil with Different Combinations of Digestive Enzymes

analyte[a]	control (no enzyme)	pancreatin	pancreatic lipase	pancreatin + pancreatic lipase
HT-ALA	4.58 ± 1.44b[b]	228.85 ± 14.87a	193.94 ± 27.88a	173.40 ± 4.55[a]
HT-LA	0.68 ± 0.17c	25.77 ± 2.25a	21.33 ± 7.50ab	14.54 ± 0.42b
HT-OA	31.83 ± 2.02c	4853.34 ± 69.57a	2235.37 ± 244.55b	2876.17 ± 315.30b

[a] HT-ALA: hydroxytyrosol esterified with α-linolenic acid; HT-LA: hydroxytyrosol esterified with linoleic acid; and HT-OA: hydroxytyrosol esterified with oleic acid. [b] Mean ± standard deviation ($n = 3$) followed by the same lowercase letter are not statistically significant at $p < 0.05$ according to the analysis of variance (ANOVA) and Tukey's multiple range test.

relevance of the food matrix composition for the final bioaccessibility results. The distinct behavior in the digestive hallmark of HT and its lipophenol derivatives could be due to the sparing solubility of HT in olive oil and its consequent location in the aqueous phase (>99%), while, boosted by the amphiphilic nature, HT-FAs are distributed between both the oily and aqueous phases.[41]

Thereby, as expected, HT-OA was the most abundant lipophenol in the food matrix and the GI digestates (Table 3). This lipophenol should be formed through the enzymatic esterification of HT with OA, which is the most abundant FA in the oily matrices considered in the present work (EVOO and VOO) (Table 2). Based on the findings from the formation of HT-FAs under intestinal digestive conditions, the use of protectors of HT against the gastric conditions might be of interest to take advantage of the bioactivities of HT-FAs from biological or technological points of view.

Relative Influence of the Intestinal Enzymes on the Generation of Hydroxytyrosol Lipophenols. Given the relevance of the intestinal stage for the bioaccessibility of HT and its lipophenolic derivatives, the separate and joint effects of intestinal enzymes (pancreatin and pancreatic lipase) on these compounds were further investigated to gain further understanding of their role on the release of HT-FAs into the intestinal lumen and their stability. With this objective, EVOO was digested using SIF containing individual and combined intestinal enzymes at 37 °C for 120 min. The incubation of EVOO in the SIF without any digestive enzyme was considered as a negative control. This approach allowed retrieving critical information on their relative influence on the bioaccessibility of HT-FAs.

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3.) are biocatalysts with a high selectivity and stability that catalyze hydrolysis, esterification, transesterification, and alcoholysis reactions.[42] To date, lipases have been applied to the production of FA derivatives by the agro-food and nutraceutical industries because of their potential uses as flavoring esters, fatty acid esters of antioxidants, and structured lipids with high regio- and stereo-selectivities.[43] Up to now, the biosynthesis of HT-FAs has been done using commercial enzymes on pure standard compounds.[39] These reactions have been reported as being closely dependent on the acyl chain length and the degree of unsaturation of FAs that affect the conversion yield of the esterification process due to differences in their spatial configuration.[44,45]

In the current work, the generation of HT esters with an equivalent carbon alkyl chain (C18) but with different degrees of unsaturation, C18:1, C18:2, and C18:3 corresponding to HT esterified with OA, LA, and ALA, respectively, was monitored, for the first time, after the intestinal digestion of EVOO with individual and combined intestinal enzymes (pancreatin and pancreatic lipase). The conversion yield of HT-FAs was more pronounced when the reaction was catalyzed only by pancreatin than when pancreatic lipase was present (alone or in combination with pancreatin) (Figure 3). HT-OA was the lipophenol with the highest concentration in the intestinal digestate, independently of the enzymes or their combination applied (4853.34, 2235.37, and 2876.17 ng/g with pancreatin, pancreatic lipase, and combinations of the two enzymes, respectively), and showed no statistical differences between pancreatic lipase and the combination of both enzymes ($p > 0.05$) (Table 4). Regarding HT-LA and HT-ALA, the final concentrations achieved in the intestinal digestate, as well as the differences between enzymatic conditions, varied to a lesser extent relative to HT-OA (Figure 3 and Table 4). This may be due to the degree of unsaturation of the FAs involved in the esterification of HT, which may affect their conformation and thereby the reaction time, as stated by previous research.[44]

The generation of HT-FAs has already been described in oily food matrices after fortifying food matrices with HT,[5] as well as in olive oil byproducts during processing and storage, while this was not detectable in intact olives.[46] A hypothesis supporting these findings suggests that HT esters could be formed independently as a result of the esterase activity from lipase-positive yeasts, which are present in plant-based foods. However, the molecular mechanisms responsible for the *de novo* formation of lipophenols, as well as the optimal conditions needed for the successful development of such reactions, remain unclear. Likewise, recently, the formation of tyrosol esters, particularly with OA, has been described, identifying crushing and kneading processes when enzymes are activated and the reactions triggered.[47] Within this frame, the present study contributes to the understanding of the generation of lipophilic hydroxytyrosyl esters during GI digestion in oily matrices, with a discussion of some possible scenarios being necessary.

The different efficiency regarding the formation of the separate lipophenols (HT-ALA, HT-LA, and HT-OA) may be associated with the degree of unsaturation, which in turn modifies the FA spatial configuration and the lipase selectivity. Moreover, the concentration of the precursors in the food matrix (both unesterified HT and FAs) seems to be critical for the synthesis of HT-FAs during digestion as well since the content of free OA in EVOO and VOO was, on average, ~99 and ~95% in comparison with the ALA and LA concentration, respectively (Table 2). This is in agreement with recent research, which utilized nanoparticles with immobilized lipase from *Thermomyces lanuginosus* and employed olive leaf aqueous extract for the synthesis of bioactive hydroxytyrosyl FA esters. The study reported that the conversion yields (%) for OA (C18:1), LA (C18:2), and myristic acid (C14:0) were 55.0, 50.4, and 64.3%, respectively, establishing that the number of double bonds had a direct correlation with the molecular structure of the FA, which, as a result, would affect the advance of the acylation reactions.[45] On the contrary, additional studies working with the pure standard of long-chain unsaturated FAs indicated that there was no relationship between chain length and conversion, as the highest conversion was obtained with eicosapentaenoic acid (EPA) (68.0%) relative to OA (46.0%). The variation in these findings may be caused by the selectivity of lipase for the FAs, with the degree of unsaturation being a more relevant factor rather than FA chain length.[39] In addition, lipase performs heterogeneous reactions, and its catalytic activity is maximum when the enzyme is adsorbed at an oil—water interface. Generally, long-chain esters are mainly dispersed in the oil droplet, while short-chain esters are found mostly in the water droplet.[48] For lipophenols, a cutoff effect that involves a too-short or a too-long hydrophobic chain does not ensure a proper interfacial location, which in turn does not result to an optimal lipase activity,[49] and the cutoff effect is simply a natural consequence of the differential solubility of antioxidants in the aqueous, interfacial, and oil regions of an emulsion.[50]

Furthermore, other phenolic acyl esters, such as tyrosol-FAs, have shown a sustained-release behavior to free tyrosol molecules during the time-consuming digestion process[16] that could improve the bioactivity scope of polyphenols because of extended actions. In this sense, the current work brings new knowledge and strategies for the design of slow-release formulations of HT-FAs that could enhance the phenolic loading cycle time by prolonging the terminal half-life and thereby lengthening their bioactivity. This is particularly important, as several biological properties have been recently suggested for these compounds, namely, antidiabetic, proliferative, and antioxidant capacities.[47,51] In addition, it has been demonstrated that the esterification of phenolic compounds did not compromise the bioactivity of the native molecule but instead may enhance its bioavailability and expand its application domains.[52]

As concluding remarks, the present study provides new evidence on the bioaccessibility of lipophenolic derivatives of HT from diverse olive oils (EVOO and VOO), showing that they are bioaccessible after performing a simulation of GI digestion under *in vitro* conditions, depending largely on the type of FA esterifying HT as well as the food matrix. More importantly, the generation of HT-FAs during intestinal digestion was reported in the present work, as well as the identification of the enzymes responsible for most of the synthesis identified (pancreatin). These findings are of critical relevance from a clinical and technological point of view because of the potential biological effects that could be expected from *de novo* synthesized lipophenols, which could have a critical impact on both the half-life of plant-based foods and the biological benefits already described on those foods and foodstuffs containing the components of lipophenols (*e.g.*, HT and FAs). Indeed, the major outcomes retrieved suggest that the use of oily matrices and their byproducts as potential functional ingredients, foods, supplements, or cosmetics, for health promotion and disease risk reduction, should take into consideration the formation of new lipophenols during digestion and, consequently, the transformation of the final bioactive scope and the effects within the frame of specific pathophysiological processes. Ultimately, further assays concerning the bioavailability of HT-FAs are still needed, with a high importance placed on validating whether lipophenol molecules would migrate across the epithelial barrier, their metabolization circulating reaction, the transporters for these compounds, and the metabolic events that affect their final shelf-life in the organism and bioactivity in the separate tissues and cell types.

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Notes

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■ ABBREVIATIONS

BF, bioaccessible fraction; BHA, butylated hydroxyanisole; DMSO, dimethyl sulfoxide; EVOO, extra virgin olive oil; FAs, fatty acids; FO, flaxseed oil; HT, hydroxytyrosol; HT-ALA, hydroxytyrosol esterified with α-linolenic acid; HT-FA, hydroxytyrosol esterified with fatty acid; HT-LA, hydroxytyrosol esterified with linoleic acid; HT-OA, hydroxytyrosol esterified with oleic acid; MRM, multiple reaction monitoring; MUFAs, monounsaturated fatty acids; NMR, nuclear magnetic resonance; PUFAs, polyunsaturated fatty acids; RF, residual fraction; SFAs, saturated fatty acids; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; VOO, virgin olive oil

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ARTÍCULO III

Unravelling the capacity of hydroxytyrosol and its lipophenolic derivates to modulate the H_2O_2-induced isoprostanoid profile of THP-1 monocytes by UHPLC-QqQ-MS/MS lipidomic workflow

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Unravelling the capacity of hydroxytyrosol and its lipophenolic derivates to modulate the H_2O_2-induced isoprostanoid profile of THP-1 monocytes by UHPLC-QqQ-MS/MS lipidomic workflow

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ABSTRACT

Presently, the attention given to natural substances to counteract damage produced by oxidative stress (OS) has risen sharply. In this scenario, hydroxytyrosol (HT) derivatives, formed as a result of HT conjugation with fatty acids (FAs) (lipophenols), have been recently described in foodstuffs such as extra virgin olive oil, as being powerful bioactive compounds with a higher activity than the unesterified phenolic compound. The present work describes the capacity of HT lipophenols to act on the course of OS and secondary inflammatory processes, based on their capacity to modulate the isoprostanoid profile induced by H_2O_2 in THP-1 monocytic cells. A UHPLC-QqQ-ESI-MS/MS-based lipidomics workflow was applied over a range of 37 human oxylipins. The main outcomes retrieved suggest both HT and HT-lipophenols as regulators of the cellular redox balance, acting as pro-oxidants *in vitro*, which is highly dependent on the experimental conditions. Our outcomes suggest the anti-inflammatory potential of both HT and HT-lipophenols, where the type of the FAs on the HT core appears to be critical for defining the bioactivity of lipophenols, highlighting that a lipidomic approach, with the simultaneous analysis of multiple oxylipins, is critical for the understanding of the bioactivity of lipophenols on isoprostanoid generation and hence, on pathophysiological processes.

1. Introduction

Lipids are involved in numerous biological processes and are key elements for the proper development of essential cell functions. Perturbations in lipid homeostasis, along with micro-environmental conditions that favor oxidative stress (OS), are closely associated with an array of pathophysiological conditions, namely obesity, diabetes, cancer, neurodegenerative disorders, and autoimmune diseases [1–3]. As for lipid oxidation, a clear-cut distinction may be made between enzymatic and non-enzymatic mechanisms, with both oxidative pathways related to the human pathophysiological conditions mentioned previously, for which dietary habits and patterns of nutrition are key aspects

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; BHA, butylated hydroxyanisole; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; ECACC, European Collection of Cell Culture; ESI, electrospray ionization; EVOO, extra virgin olive oil; FAs, fatty acids; FBS, fetal bovine serum; HT, hydroxytyrosol; IL-12, interleukine-12; LA, linoleic acid; LC-MS, liquid chromatography-mass spectrometry; LOX, lipoxygenase; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; OA, oleci acid; OS, oxidative stress; PMS, phenazine methosulfate; PLA2, phospholipase A2; PG, prostaglandin; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; SPE, solid-phase extraction; THP-1, human monocytic cell line; UHPLC, ultra-high pressure liquid chromatography; XTT, sodium 30-[1-(phenylaminocarbony)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate.

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of special interest because of their capacity to modulate cell metabolism [4]. The non-enzymatic oxidation of lipids can be produced by reactions triggered by a high concentration of reactive oxygen species (ROS), such as singlet O_2, hypochlorous acid (HOCl), and ozone (O_3) or by inorganic free radical species derived from nitric oxide (NO), superoxide ion (O_2^{-}), and hydrogen peroxide (H_2O_2) [5]. On the other hand, enzymatic lipid oxidation reactions are catalyzed by peroxidases, which are a group of proteins capable of oxidizing lipids by using H_2O_2 as a source of oxidizing equivalents. This group of enzymes includes the so-called cyclooxygenase (COX), also known as prostaglandin H synthase, which is mainly involved in the oxidation of "free" polyunsaturated fatty acids (PUFA), particularly arachidonic acid (AA), giving rise to prostaglandins and other lipid mediators [6].

Oxidative environments can trigger a complex cascade of molecular reactions within cells to protect critical biomolecules from oxidative modifications. Diverse studies on these mechanisms suggest that phenolic antioxidants present in plant-based foods, and especially in extra virgin olive oil (EVOO), such as hydroxytyrosol (HT), possess specific biological capacities that may contribute to protecting the structure of the lipids from oxidative damage [7–10], thus helping to preserve their functions and ensure normal cell metabolism.

Within the so-called bioactive compounds present in plant-based foods, lipophenols (or phenolipids) have been described in the last decade, which are (poly)phenolic compounds esterified with fatty acids (FAs). Several advantages have been attributed to these molecules relative to the native molecule (unesterified phenolics or FAs) regarding their antioxidant, anti-carbonyl stress potency, radical scavenging, and anti-inflammatory activities [11–14], which have attracted considerable attention in the fields of food science, nutrition, and health [12,15]. Particularly related to the antioxidant activity of lipophenols, to date, a parabolic behavior ("cut-off" effect) has been demonstrated resorting to *in vitro* models. This behavior is characterized by an initial enhancement of antioxidant efficiency, in parallel to the increase in the alkyl chain length, until a critical point is reached when the molecule experiences a slight drop in its biological power [16]. Nevertheless, the scientific literature about the improved antioxidant capacity conferred to (poly) phenols as a result of conjugation with PUFAs is still very scarce. The limited information available on this issue indicates that two quercetin lipophenols (esterified with α-linolenic acid (ALA) and linoleic acid (LA)), display a potent antioxidant activity, being even capable of reducing A2E-induced cell death more efficiently than unbound quercetin [11,12] and a more recent article described that quercetin esterified with FAs showed much-improved lipophilicity, higher cell membrane affinity, and enhanced cellular antioxidant activity than the parent quercetin [17]. Moreover, both the effect of the fatty acid chain length and the degree of unsaturation in the lipid part, on the modulation of the generation of oxidized lipid mediators by lipidomics workflows, remain poorly explored.

Recently, lipophenols of HT, esterified with ALA, LA, and oleic acid (OA) (HT-ALA, HT-LA, and HT-OA, respectively) have been identified in an array of foods and foodstuffs such as EVOO, refined olive oil, flaxseed oil, grapeseed oil, and margarine, in a wide range of concentrations [15,18]. These lipophenols have also been suggested as powerful bioactive compounds with higher activity than the native molecule (HT), presumably due to the combination of the bioactivities of both phenolic compounds and FAs, and the modification of their lipophilicity [15]. However, their actual capacity to modulate molecular changes in pro-oxidative and inflammatory environments in cells and tissues, under oxidant conditions, according to their ability to modulate the quantitative isoprostanoid profile representative of oxidative and inflammatory damage, has not been addressed. Thereby, in this scenario, the present study elucidates the capacity of HT lipophenols to modulate the oxidative response triggered by H_2O_2 in the THP-1 human monocytic cell line, in comparison with unesterified HT *in vitro*, by the application of a lipidomic approach for the high-throughput identification of down- and up-regulated oxidized lipids.

2. Experimental section

2.1. Chemicals and materials

The authentic standards of high purity HT-lipophenols (HT-ALA, HT-LA, and HT-OA) were synthesized and fully characterized (using NMR analysis) by Durand's team at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France), according to previously published procedures [15]. All lipophenols were dissolved in dimethyl sulfoxide (DMSO) from Sigma Aldrich (St. Louis, MO, USA). Hydroxytyrosol with a purity of 99.6% was provided by Seprox BIOTECH S.L. (Murcia, Spain). Acetone, butylated hydroxyanisole (BHA), β-glucuronidase, type H2 from *Helix pomatia*, and BIS-TRIS (Bis-(2-hydroxyethyl)-amino-tris (hydroxymethyl)-methane) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and all LC-MS grade solvents, such as H_2O, methanol, and acetonitrile were from J.T. Baker (Phillipsburg, NJ, USA). Hydrochloric acid was purchased from Panreac (Castellar del Vallés, Barcelona, Spain), and the Strata X-AW, 100 mg/3 mL solid-phase extraction (SPE) cartridges from Phenomenex (Torrance, CA, USA). A total of 37 oxylipins (21 prostaglandins (PGs), 15 isoprostanes (IsoPs), and one thromboxane (TX)) generated from different polyunsaturated FAs (arachidonic acid (AA), dihomo-gamma-linolenic acid (DGLA), and eicosapentaenoic acid (EPA), respectively) in the frame of diverse enzymatic and non-enzymatic synthesis pathways, were assessed in the current study (Table 1). Some of them were purchased from Cayman Chemicals (Ann Arbor, Michigan, USA), including 15-F$_{2t}$-IsoP-d$_4$ (8-*iso*-PGF$_{2\alpha}$-d$_4$ as internal standard (IS)) and others (2,3-dinor-15-F$_{2t}$-IsoP, 2,3-dinor-15-*epi*-15-F$_{2t}$-IsoP, 5-F$_{2t}$-IsoP, 5-*epi*-5-F$_{2t}$-IsoP, 15-*epi*-15-D$_{2t}$-IsoP, 8-F$_{3t}$-IsoP, and 8-*epi*-8-F$_{3t}$-IsoP) were synthesized by Durand's team at the Institut des Biomolecules Max Mousseron (IBMM) (Montpellier, France). The THP-1 cell line and the RPMI-1640 were purchased from the European Collection of Cell Culture (ECACC, Public Health England, Porton Down, Salisbury, UK). L-glutamine, sodium 30-[1-(phenylaminocarbony)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), and phenazine methosulfate (PMS) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and esterified and unesterified hydroxytyrosol treatment

The monocytic human (THP-1) cell line (ECACC® General Cell Collection-88081201) was purchased from the European Collection of Cell Culture (ECACC, Public Health England, Porton Down, Salisbury, UK). The THP-1 cells were grown according to the previously described methodology [18,19]. The passage number of the cells used in this study was between 17 and 20. Cells were treated with 20 μM of individual HT lipophenols (HT-ALA, HT-LA, and HT-OA) and 20 μM of the unesterified molecule (HT) in triplicate ($n = 3$) for 24-h according to the cytotoxicity results of HT lipophenols described by Medina et al. on THP-1 cells [18]. Afterward, the cells were exposed to a pro-oxidative stimulus (50 μM H_2O_2) and maintained under these conditions at 37 °C and 5% CO_2, for another 24-h. Due to the physicochemical stability of compounds is associated with their biological properties, culture media spiked with unesterified and esterified HT was assessed on the quantitative profile of the target analytes by mimicking the experimental conditions, thus ensuring the stability of compounds and discarding auto-oxidation processes of FAs (Fig. S1). The concentration of HT-ALA, HT-LA, and HT-OA assessed on the capacity to modulate the level of isoprostanoids, was selected based on previous studies, which detected HT lipophenols in EVOO [15,18]. This concentration allowed for setting their capacity to prevent oxidative and inflammatory damages in THP-1 cells, under oxidative conditions. In this regard, although the concentration tested could exceed the theoretical level achieved in target cells (which has not been previously described), especially because of the expected breakdown of dietary lipophenols during gastrointestinal digestion, the experimental design established in the present work was utilized to understand the biological potential of the HT lipophenols in comparison

Table 1
Oxylipins assessed in THP-1 cells pre-exposed to 20 μM of the unesterified hydroxytyrosol (HT) and molecules of HT esterified with fatty acids (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) for 24 h followed by 24 h co-exposure with 50 μM of oxidizing agent (H_2O_2).

Prostanoids generated from AA				
Pathway I	Pathway D	Pathway E	Pathway F	Thromboxane
6-keto-PGF$_{1\alpha}$	PGD$_2$ (*)	PGE$_2$ (*)	PGF$_{2\alpha}$ (*)	11-dehydro-TXB$_2$
2,3-dinor-6-keto-PGF$_{1\alpha}$	PGDM (*)	20-OH-PGE$_2$	20-OH-PGF$_{2\alpha}$ (*)	
	Tetranor-PGDM	Tetranor-PGEM	19(R)-OH-PGF$_{2\alpha}$	
	Tetranor-PGDM lactone	Tetranor-PGAM (*)	Tetranor-PGFM	
	11-β-PGF$_{2\alpha}$ (*)		15-keto-PGF$_{2\alpha}$ (*)	
	2,3-dinor-11β-PGF$_{2\alpha}$ (*)			
	Tetranor-PGJM			
PGs generated from DGLA		PGs generated from EPA		
PGE$_1$ PGF$_{1\alpha}$ (*)		17-*trans*-PGF$_{3\alpha}$		
Isoprostanes				
IsoPs generated from AA	IsoPs generated from DGLA	IsoPs generated from EPA		
15-F$_{2t}$-IsoP	15-E$_{1t}$-IsoP	8-*epi*-8-F$_{3t}$-IsoP		
15-keto-15-F$_{2t}$-IsoP (*)	15-F$_{1t}$-IsoP	8-F$_{3t}$-IsoP		
15-*epi*-15-F$_{2t}$-IsoP				
ent-15-*epi*-15-F$_{2t}$-IsoP				
2,3-dinor-15-F$_{2t}$-IsoP				
9-*epi*-15-F$_{2t}$-IsoP				
2,3-dinor-15-*epi*-15-F$_{2t}$-IsoP				
5-F$_{2t}$-IsoP				
5-*epi*-5-F$_{2t}$-IsoP (*)				
15-keto-15-E$_{2t}$-IsoP				
15-*epi*-15-D$_{2t}$-IsoP(*)				

AA: arachidonic acid; DGLA: dihomo- γ-linolenic acid; EPA: eicosapentaenoic acid; IsoPs: isoprostanes; PGs: prostaglandins; TX: Thromboxane. (*) These oxylipins were quantified in the current assay.

with the native unesterified molecule to prevent changes in the quantitative profile of isoprostanoids after exposure to an oxidative environment.

2.3. Extraction of isoprostanoids and analysis by UHPLC-ESI-QqQ-MS/MS

The quantitative isoprostanoid profile was determined in THP-1 cells as the sum of the intracellular concentration (compounds taken by the cells) and the concentration of the compounds secreted into the growth medium to obtain comprehensive information about the metabolism of oxylipins after exposure to 50 μM H_2O_2 with HT and HT-lipophenols treatments in contrast to untreated cells. The cells and supernatants were collected after a 24-h exposure to H_2O_2 and processed according to the procedure described by Campillo et al. [19]. Briefly, cells were lysed using a specific lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, containing 0.005% BHA). MeOH/HCl 200 mM (0.5 mL) was added to the cell lysate and growth medium, and the extracts obtained were centrifuged at 1000×g for 5 min, at 4 °C. The supernatants were collected and stored at −80 °C until oxylipin extraction and analysis.

For the extraction of oxylipins, both the pre-processed growth medium and cell lysate extracts were first enzymatically hydrolyzed (β-glucuronidase Type HP-2 from *Helix pomatia*) to remove glucuronide and sulfate conjugates, according to the method described by Medina et al. [20]. The hydrolyzed extracts were cleaned up by SPE using Strata X-AW cartridges (100 mg/3 mL), and the eluents were analyzed by UHPLC-ESI-QqQ-MS/MS, following the previously published methodology from our research team [20,21]. The 15-F$_{2t}$- IsoP-d$_4$ was used as an internal standard due to its similar fragmentation pattern relative to the target oxylipins considered in the present work. Data acquisition and processing were performed using the MassHunter software version B.08.00 (Agilent Technologies, Walbronn, Germany). The concentration of the isoprostanoids was calculated according to standard curves that were freshly prepared each day of analysis. Additionally, the limit of quantification (LOQ), calculated as a signal/noise ratio of 10, was set at 8 pM, in agreement with the LOQ previously reported by several authors for quantifying lipid peroxidation in cellular systems [19,22].

2.4. Statistical analyses

All treatments and extractions were performed in triplicate ($n = 3$), and the data were expressed as the mean ± SD. Statistical tests were performed at a 5% significance level using the SPSS 27.0 software package (LEAD Technologies, Inc., Chicago, USA). Data were subjected to a one-way analysis of variance (ANOVA), and the fulfillment of the one-way ANOVA requirements, especially the normal distribution of the residuals and the homogeneity of variance, were tested with the Kolmogorov–Smirnov and Levene's tests, respectively. When statistical differences were identified, the variables were compared using the Tukey's HSD post-hoc test. Significant differences among means were considered at $p < 0.05$.

3. Results and discussion

As referred to before, this study aimed to evaluate unesterified HT and esterified with ALA, LA, and OA, as bioactive compounds present in olive oil (EVOO and virgin olive oil (VOO)) on their ability to modulate the isoprostanoids response triggered by the exposure of THP-1 cells to oxidative environments (H_2O_2). The THP-1 is a human leukemia monocytic cell line, characterized by a strong correspondence with the monocytic fraction of peripheral blood mononuclear cells. Although THP-1 cells may not express matching features relative to primary monocytes and their regulatory mechanisms, they are characterized by physiological properties characteristic of primary monocytes, *in vivo* [23]. Because of this, the THP-1 cell line has been used as a model to study the molecular pathways involved in the oxidative and inflammatory responses, as well as the capacity of food compounds to modulate such pathways and the molecular mechanisms responsible for, which are strongly assoicated with the course of an array of pathophysiological situations [19,24].

For this purpose, the physicochemical stability of lipophenols is imperative for research their biological properties, the outcomes from the assays performed in this matter proved the stability of esterified molecules at *in vitro* cell growth conditions (Fig. S1). At this juncture, the present work contributes to elucidate how specific lipophenols can modulate, *in vitro*, the OS and inflammation-dependent isoprostanoid profile induced by the exposure to oxidizing agents. The effect of oxidizing H_2O_2 on the generation of OS and pro-/anti-inflammatory markers (IsoPs and PGs, respectively) [25,26] has already been described in THP-1 cells, following a similar experimental design [19]. The most relevant strength of the current assay is represented by the lipidomic workflow applied, which allows shedding light on the bioactivity of lipophenols *in vitro* through a range of OS and inflammation-related isoprostanoids. This approach allows overcoming the constraints associated with the monitoring of single IsoP or PG that provides incomplete information on the pathophysiological model, as several isoprostanoid markers are rapidly metabolized and their rates of metabolism may vary significantly [27].

3.1. Modulation of the quantitative isoprostane profile of THP-1 monocytic cells by unbound and esterified hydroxytyrosol under oxidative conditions

The capacity of HT and its lipophenolic derivatives to modulate OS was monitored by assessing 15 IsoPs derived from AA, DGLA, and EPA (Table 1), although only three IsoPs from AA (15-keto-15-F_{2t}-IsoP, 5-*epi*-5-F_{2t}-IsoP, and 15-*epi*-15-D_{2t}-IsoP) were found at levels higher than the limit of quantification (LOQ) of the method utilized in the current study (Fig. 1). All the oxylipins were expressed as the sum of the intra- and extra-cellular concentrations recorded, as both are secreted by cells and provide a complete picture of the IsoPs profile and metabolism, under particular conditions. As expected, the exposure of THP-1 cells to 50 μM H_2O_2 caused an increase in the IsoP level in comparison with basal conditions (untreated cells) (Fig. 1), although this trend was not statistically significant for 5-*epi*-5-F_{2t}-IsoP or 15-*epi*-15-D_{2t}-IsoP. This lack of significance regarding the increase of specific OS markers under oxidative growing conditions for most of them may occur because of the concentration of H_2O_2 used in the current assay to trigger OS in THP-1 cells (50 μM), as concentrations of the agent used to generate oxidative damage in cellular models are in the high micromolar range (10–1000 μM) [28]. The nominal concentrations of H_2O_2 used to condition the growth medium, as well as its cytotoxicity strongly, depends on diverse factors such as incubation time or the cell concentrations of different supplements featured by the radical scavenging capacity of the culture medium [29]. However, concentrations above 100 μM are disproportionally high concerning the physiological oxygen concentration (low micromolar) and may affect the viability of THP-1 cells. The concentration of 50 μM was selected based on previous reports which described no statistical differences between the 50 and 100 μM treatments used to induce OS in cells with H_2O_2, whilst 200 μM of the oxidizing agent significantly reduced the viability of cells [30]. Moreover, to the present date, it has been reported that both IsoPs and PGs can be identified and quantified in the supernatant of human THP-1 macrophages, where they are highly produced after a 12 h oxLDL treatment (50 μg/mL) as compared to native LDL [24].

Regarding the 15-keto-15-F_{2t}-IsoP, a metabolite of 15-F_{2t}-IsoP (8-*iso*-$PGF_{2\alpha}$), it was not detected in the untreated controls, whilst after the exposure to 50 μM H_2O_2, it was found at a concentration of 0.097 ng/mL. When monitoring the preventive effect of HT and its lipophenolic derivatives present in VOO and EVOO, it was observed that none of them (HT-ALA, HT-LA, and HT-OA) were competent enough to smooth the

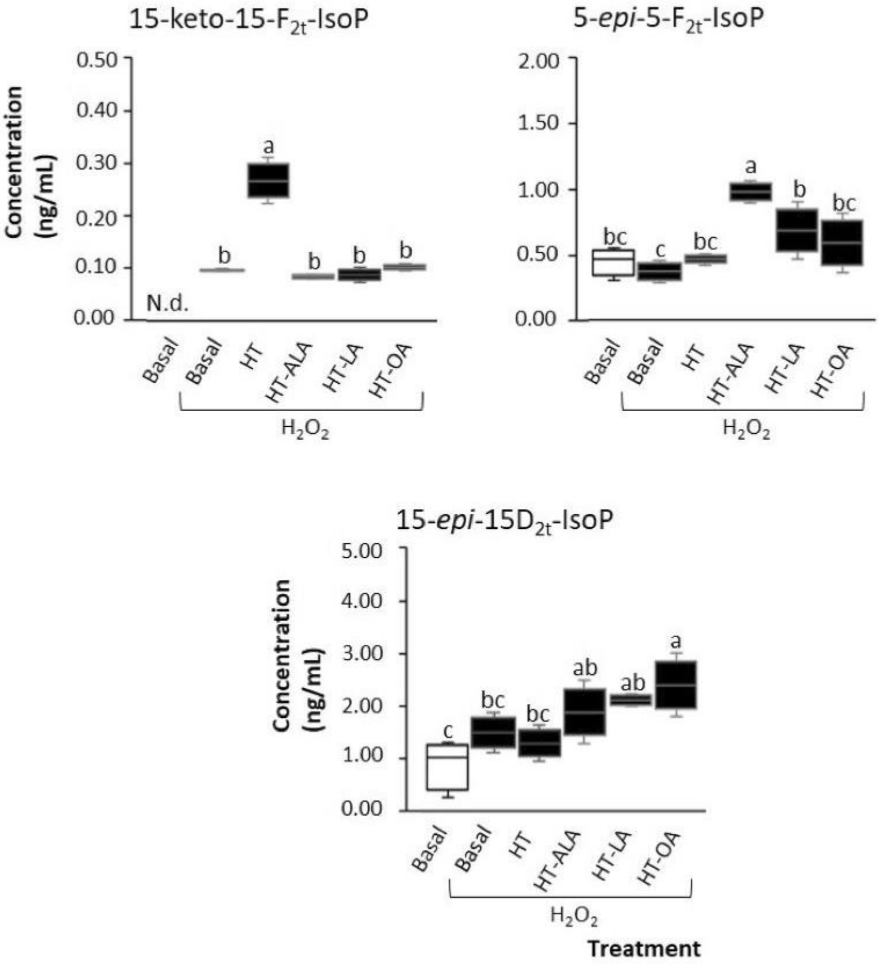

Fig. 1. Modulatory effect of the hydroxytyrosol (HT) and lipophenols of hydroxytyrosol (HT-ALA, HT-LA, and HT-OA) treatments on the quantitative isoprostane profile of H_2O_2-stimulated THP-1 monocytic cells. Isoprostanes were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to a one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

effect of the oxidative stimulus. However, the treatment with unbound HT caused a significant increase in the concentration of this OS marker up to 0.267 ng/mL (Fig. 1), thus evidencing a pro-oxidant effect at the concentration tested. Concerning 5-*epi*-5-F_{2t}-IsoP, the different concentrations induced by the array of treatments applied were not statistically significant relative to that recorded for basal and H_2O_2-treated cells, with HT-ALA being the only compound which induced a significant increase ($p < 0.05$) of 5-*epi*-5-F_{2t}-IsoP, up to the average concentration of 0.979 ng/mL (2.2-fold higher than controls).

To date, the major differences described in humans between the 5- and the 15-series of F_2-IsoPs are referred to as their concentration. Thus, the level of 5-F_{2t}-IsoP has been reported to reach concentrations up to 4 times higher than 15-F_{2t}-IsoP [31]. According to this previous information, the 5-series was found at a concentration 3.6 times higher than the 15-series within the F_2-IsoPs. This finding may provide valuable information about the pathophysiological meaning of OS and its relationship with an array of diseases. In this sense, to date, research on the further differences between these two series of IsoPs noted that 15-F_{2t}-IsoP is a vasoconstrictor and may be involved in the pathogenesis of coronary disorder, whilst 5-F_{2t}-IsoP is not able to develop vasomotor effects and as such, was not likely to be involved in the pathogenesis of vascular diseases or inflammatory processes, which account for the high involvement of vascular events [31]. This is very valuable information due to the close association between olive oil consumption and low mortality within the frame of cardiovascular disease [32].

Regarding the 15-series of D2-IsoPs, 15-*epi*-15-D_{2t}-IsoP displayed concentrations ranging from 0.904 ng/mL in untreated cells, to 2.405 ng/mL in cells exposed to HT-OA. Therefore, the outcomes in the present work suggest a possible pro-oxidant effect of HT-OA as compared with unesterified HT and control samples, tentatively due to a significant rise of the 15-*epi*-15-D_{2t}-IsoP concentration (Fig. 1).

Thus, according to the sum of the individual IsoPs, it is stressed that the level of total IsoPs in THP-1 basal cells (1.353 ng/mL) significantly increased ($p < 0.05$) in both H_2O_2-stimulated and HT-treated cells by 1.5-fold, on average. Also, the treatment with HT-esterified with FAs resulted in a significant rise in the IsoP concentration in comparison with basal level, to reach concentration values of 2.956, 2.892, and 3.098 ng/mL in cells pre-treated with HT-ALA, HT-LA, and HT-OA, respectively ($p < 0.05$, data not shown). These results would indicate a pro-oxidant effect that contrasts with the recognized antioxidant activity of unbound HT. Nevertheless, it may not be forgotten that under certain conditions, HT (as well as other (poly)phenols and their derivatives) may behave as 'pro-oxidant' agents, in a highly dependent fashion on the experimental conditions. This controversial situation, the so-called '(poly)phenols oxidative paradox' indicates that the same molecule may offer protection against highly reactive free radicals or act as a potentially toxic (oxidizer) compound [33]. In this regard, and concerning *in vitro* models, depending on the concentration, HT can generate H_2O_2 as a result of its auto-oxidation by O_2, thus influencing its biological activities [34,35]. Similarly, high HT doses (10–200 µM), in *in vitro* models have been shown to increase ROS generation within tumor cells, leading to their apoptosis [36,37]. On the other hand, research studies which resorted to preclinical models have provided evidence suggesting that high HT doses (300 mg/kg/d) could also induce a systemic pro-oxidant effect when it is supplemented during exercise training [38]. Also, a very recent study by Romana-Souza et al. described that the short-term treatment of neonatal murine dermal fibroblast (72-h) with olive oil extracts contributes to increasing the production of ROS-induced oxidative damage in lipids (as evidenced by the increase in the 15-F_{2t}-IsoP level secondary to the increase in NF-kB and COX-2 expression), which positively correlated with the content of OA [39]. These findings, although more research is necessary, could contribute to elucidate the mechanism of action of bioactive compounds, such as HT and its lipophenols, and their dual biological activity.

3.2. Modulation of the quantitative prostaglandin profile of THP-1 monocytic cells by unbound and esterified hydroxytyrosol under oxidative conditions

Previously, OS had been demonstrated as a powerful activator of molecular pathways responsible for the up-regulated expression of OS-related genes, as well as as a modulator of the production of PGs through the Mitogen-Activated Protein Kinases (MAPKs) pathway [40]. According to this background, the current study aimed at elucidating the capacity of HT and HT lipophenols (HT-ALA, HT-LA, and HT-OA) to modulate the quantitative prostanoid profile of THP-1 monocytic cells exposed to oxidative (50 µM H_2O_2) growing conditions. With this objective in mind, aside from monitoring changes in the IsoP profile (OS markers) referred to in the previous section, we assessed THP-1 lysates and growth media on the concentration of 21 PGs synthesized from diverse FAs through specific synthetic pathways (Table 1), thus providing a complete picture of the PG response.

3.2.1. Modulatory capacity of the prostaglandin D-pathway of hydroxytyrosol and its lipophenolic derivatives

Regarding the PGs synthesized within the frame of the D-pathway from AA, four compounds were identified and quantified in THP-1 cells (PGD_2, PGDM, 11-β-$PGF_{2\alpha}$, and 2,3-dinor-11-β-$PGF_{2\alpha}$), out of the seven monitored (Table 1 and Fig. 2). The exposure to 50 µM H_2O_2, as an oxidizing agent, gave rise to an increase that was only statistically significant ($p < 0.05$) for PGDM and 2,3-dinor-11-β-$PGF_{2\alpha}$ (up to 0.020 and 0.081 ng/mL, respectively). Also, when assessing the effect of HT and HT-lipophenols on the modulation of the PGD_2 concentration induced by H_2O_2, it was observed that all the compounds (esterified and unesterified) significantly enhanced the level of this PG, providing values ranging from 1.213 to 1.880 ng/mL. As for PGDM (a metabolite of PGD_2), its concentration increased to a higher extent in THP-1 samples treated with HT lipophenols in comparison with cells exposed only to the H_2O_2 stimulus and untreated control samples (not detected). Hence, HT-LA induced the highest concentration of PGDM (0.036 ng/mL), although the broad dispersion of the results did not allow identifying statistically significant differences with the increase induced by HT-ALA (Fig. 2). This finding agrees with a previous study, where PGDM was detected in macrophages after oxLDL stimulation, while PGDM was almost absent in control samples [24].

The primary metabolite of PGD_2, 11β-$PGF_{2\alpha}$, showed a rising trend that was represented by a non-significant higher concentration in THP-1 cells exposed to pro-oxidant growing conditions (3.679 ng/mL, 1.4-fold higher than untreated control cells). Similarly, THP-1 cells treated with 20 µM HT and HT-lipophenols (HT-ALA, HT-LA, HT-OA) significantly enhanced the concentration of 11β-$PGF_{2\alpha}$ (6.185, 7.827, 7.019, and 7.025 ng/mL, respectively).

Finally, in THP-1 cells, the presence of a metabolite of 11β-$PGF_{2\alpha}$, the so-called 2,3-dinor-11-β-$PGF_{2\alpha}$, was observed. Again, the concentration of this PG climbed up to 0.081 ng/mL under H_2O_2-induced oxidant conditions, whereas this metabolite was not detected in untreated control cells. Concerning the impact of pre-treating THP-1 cells with HT and its lipophenolic derivatives, only HT-ALA induced a significant increase, thus giving rise to the final concentration of 0.356 ng/mL (Fig. 2).

PGD_2 is the main PG produced by activated mast cells, and its activity is triggered by resorting to two main receptors (DP1 and DP2 (also called CRTH2)). By interacting with these receptors PGD_2 activates multiple signalling pathways that would depend on the activation of a downstream signalling cascade, as a result of the up- or down-regulation of secondary messengers, such as cAMP and Ca^{2+}. Thus, this PG seems to be responsible for putative biological functions, including anti-tumorigenic activity [41,42], vasodilation or antithrombotic properties [43], and both pro- and anti-inflammatory capacities [44]. In this scenario, the present work provides evidence on the capacity of HT lipophenols to boost the PGs D-pathway more efficiently than unesterified HT, perhaps due to the up-regulation of COX-2, which can shed

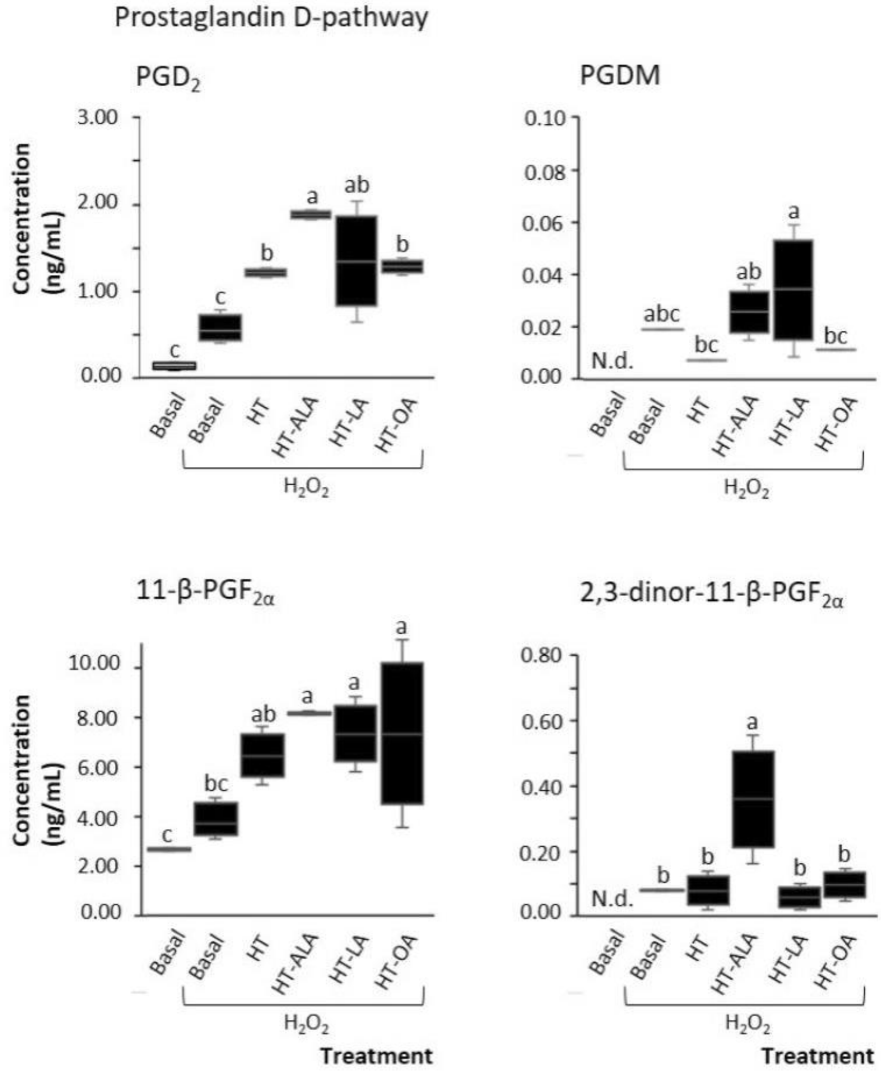

Fig. 2. Modulatory effect of the hydroxytyrosol (HT) and lipophenols of hydroxytyrosol (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin D-pathway from arachidonic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

some light on the diversity of biological activities that are specifically attributable to HT esterified with different FAs. Also, based on our outcomes referred to before, the type of FAs and the degree of unsaturation of lipophenols may be a critical feature of this class of bioactive compound, closely linked to their bioactivity, which merits further investigation. This is of special relevance, given that HT-OA provided a similar modulatory capacity of the PGs D-pathways as HT, although it was different as compared to HT-ALA and HT-LA. Accordingly, there is a need for additional experimental inputs for a sound comparison of the biological activities of the HT-FAs. This would allow selecting those plant-based foods (mainly, oleaginous matrices) with the most appropriate quantitative profile of HT lipophenols, in agreement with the diverse pathophysiological conditions related to OS and inflammation.

3.2.2. Modulatory capacity of the prostaglandin E-pathway of hydroxytyrosol and its lipophenolic derivatives

As mentioned above, ROS have been demonstrated to initiate a variety of molecular mechanisms in *in vitro* and *in vivo* eukaryotic cell models. Among them, ROS modulates the production of PGE_2 through the MAPKs pathway [40]. Moreover, PGE_2 is one of the most widely investigated PGs, which has been promoted as a biomarker of inflammation, informing about the state of a disease or therapeutic effectiveness. However, due to its rapid metabolism, the direct measurement of PGE_2 in biological samples is difficult, and is sometimes analyzed through its metabolites. In this regard, the major metabolite of PGE_2 is tetranor-PGEM. This serves as an indirect marker of PGE_2 biosynthesis, but again, it is also chemically unstable. When PGE_2 and tetranor-PGEM are not found, tetranor-PGAM, a dehydration product of tetranor-PGEM, can be measured as a surrogate PG to set the level of tetranor-PGEM in biological samples [45]. Therefore, a lipidomics workflow with the simultaneous analysis of multiple oxylipins is critical for understanding the activity of compounds (*e.g.*, lipophenols) on the generation of isoprostanoid and hence, on the curse of diverse pathophysiological processes [46]. Concerning the PG E-pathway from AA, in the current study, four PGs (PGE_2, 20-OH-PGE_2, tetranor PGEM, and tetranor-PGAM) were analyzed, but only two of them (PGE_2 and tetranor PGAM) were detected in THP-1 cells (Fig. 3). The exposure to 50 μM H_2O_2 increased the concentration of PGE_2 in THP-1 cells 2.2-fold as compared to control (untreated) cells, whereas for tetranor-PGAM, it was not detected in neither under basal cells nor oxidative conditions.

The analysis of the effect of HT-ALA, HT-LA, and HT-OA on the concentration of PGE-pathway-based PGs provided evidence of the rising concentrations of both PGE_2 and tetranor-PGAM relative to controls, reaching 0.638, 0.520, and 0.676 ng/mL as the average concentrations of PGE_2, respectively. However, when THP-1 cells were treated with unesterified HT, the concentration of PGE_2 (0.393 ng/mL) was significantly lower than the one induced by HT-ALA and HT-OA (Fig. 3). On the contrary, regarding tetranor-PGAM, unesterified HT provided a significant increase of this metabolite (up to 1.871 ng/mL) as compared with HT lipophenols (1.166, 0.554, and 0.666 ng/mL for HT-ALA, HT-LA, and HT-OA, respectively). Thus, a joint analysis of the results retrieved for PGE_2 and tetranor-PGAM suggested that the decreased concentration of PGE_2 could be due to its metabolization to tetranor-PGAM. In this scenario, a cautious examination of these findings should be made to avoid misinterpretation, as PGE_2 is responsible for various biological activities, in many cases opposed, such as pro-/anti-inflammatory or pro-/anti-thrombotic, depending on their interaction with different E-prostanoid (EP) receptors (EP1, EP2, EP3, and/or EP4) [47].

PGE_2 is responsible for inflammatory symptoms but has also been associated with suppression of pro-inflammatory cytokines production (*e.g.*, IL-6). Moreover, specifically in monocytes, PGE_2 can interact with EP4, contributing to modulate TNF-α production and thus, controlling the progress of the inflammatory response [48]. In this sense, HT-OA has been described as a molecule with anti-inflammatory properties, which are developed in a concentration-dependent manner by suppressing PGE_2 production within 24-h, by inhibiting the COX-2 enzyme, as well as the expression of inducible NO synthase and the synthesis of interleukin-1β [49]. At this point, in the human oxylipin analyses, two main issues should be considered; firstly prostanoids have a very similar chemical structure and so, cross-reactivity may occur, especially with $PGF_{1\alpha}$ and $PGF_{2\alpha}$ when analyzing PGE_2 [45], and secondly, the measurement of a unique PG, particularly referring to PGs with dual biological activity, is not appropriate for the understanding of the inflammatory response, as it is composed of a very complex signaling cascade, including both pro-inflammatory and anti-inflammatory mediators. Consequently, a lipidomic approach plays an essential role in determining the biochemical mechanisms of lipid-related disorder processes through the identification of changes in the concentration of cellular lipid metabolites and their trafficking.

3.2.3. *Modulatory capacity of prostaglandin F-pathway of hydroxytyrosol and its lipophenolic derivatives*

Three out of the four PGs produced in the PG F-pathway were detected in the present work ($PGF_{2\alpha}$, 15-keto-15-$PGF_{2\alpha}$, and 20-OH-$PGF_{2\alpha}$) (Fig. 4). Concerning $PGF_{2\alpha}$, it was either detected in THP-1 cells under control conditions or after exposure to 50 μM H_2O_2, but no statistical differences were found between them. As shown in Fig. 4, the cells treated with HT, HT-LA and HT-OA induced a decrease in $PGF_{2\alpha}$ until undetectable amounts, and only HT-ALA produced an increase in this PG up to concentrations that were not statistically different when compared with untreated controls and H_2O_2-treated cells. There, the type of FA may be critical for the bioactivity of lipophenols. Specifically, $PGF_{2\alpha}$ may be synthesized from PGH_2 via the aldo–keto reductase (AKR) 1C3 enzyme, and may also be synthesized from PGE_2 via the AKR1C1 and AKR1C2 enzymes [50]. Thereby, the synthesis of this PG involves several substrates and different enzymes. The capacity to modulate the concentration of $PGF_{2\alpha}$ is of special relevance, it is a prostanoid with several biological activities, it is involved in vasoconstriction, and it contributes with the creation of the inflammatory environment by influencing the synthesis and secretion of IL1β, IL6, IL8, and TNFα [51]. Accordingly, our outcomes concerning $PGF_{2\alpha}$ strongly suggested the anti-inflammatory potential of both HT and HT lipophenols (mainly HT-ALA and HT-OA) as previously stated by [52,53], which highlighted HT-OA as an effective anti-inflammatory agent. Also, two products from $PGF_{2\alpha}$ were also detected; 15-keto- $PGF_{2\alpha}$, the first metabolite of $PGF_{2\alpha}$ in the 15-hydroxyprostagladin dehydrogenase (PGDH) pathway quantified in all samples, noting that, the exposure to H_2O_2 caused an increased concentration, with up to 3.8-fold higher values than untreated THP-1 cells (0.967 *vs.* 0.251 ng/mL) (Fig. 4). For this $PGF_{2\alpha}$ metabolite, both HT and its lipophenolic derivatives lowered the amount produced in THP-1 cells relative to that achieved when exposing cells only to the oxidative environment, although in any case, the decrease observed was statistically significant. Similarly, as related to 20-OH-$PGF_{2\alpha}$, a product of $PGF_{2\alpha}$ synthesized by cytochrome P450-catalyzed ω-oxidation, it was not detected in neither untreated controls nor H_2O_2-treated cells. HT-ALA gave rise to a smaller increase of this metabolite than HT, HT-LA, and HT-OA. It should also be underlined that the molecules responsible for the decrease in $PGF_{2\alpha}$ (HT, HT-LA, and HT-OA), were the most efficient in increasing the concentration of

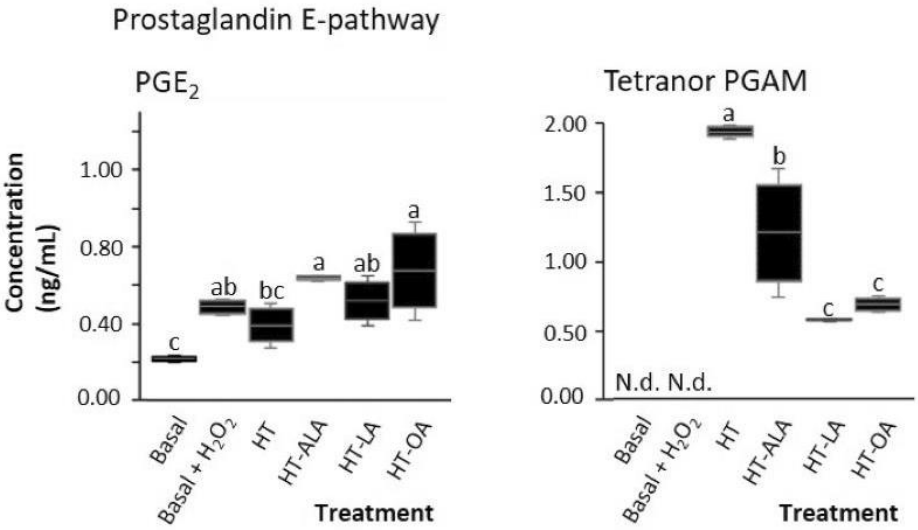

Fig. 3. Modulatory effect of hydroxytyrosol (HT) and HT lipophenols (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin E-pathway from arachidonic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to a one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

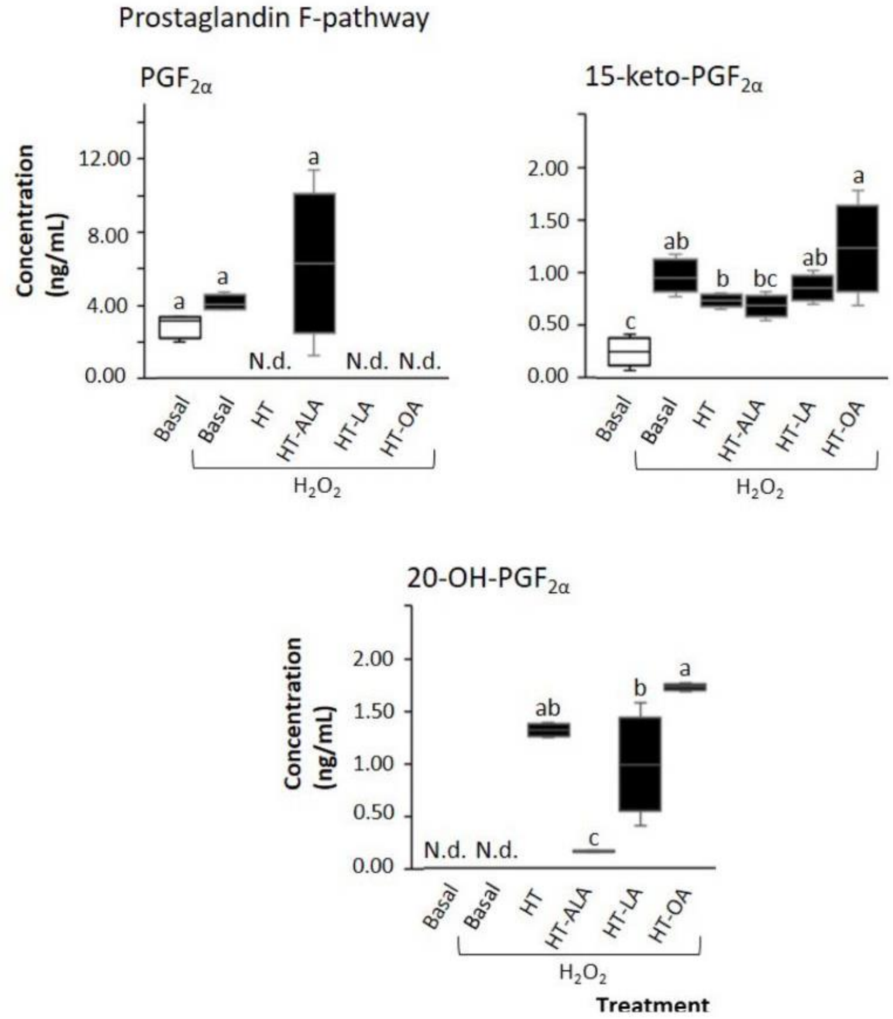

Fig. 4. Modulatory effect of hydroxytyrosol (HT) and HT lipophenols (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin F-pathway from arachidonic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD (n = 3). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to one-way analysis of variance (ANOVA) and Tukey's multiple range test. N.d.: not detected.

$PGF_{2\alpha}$ metabolites (15-keto- $PGF_{2\alpha}$ and 20-OH-$PGF_{2\alpha}$). Therefore, our outcomes reveal the importance of the concentration of the oxidizing agent, the degree of unsaturation of the FAs, and the esterification of the native molecules for the modulation action on the amount of PGs belonging to F-pathway.

3.2.4. *Modulation of the level of prostaglandins from dihomo-gamma-linolenic acid by hydroxytyrosol and its lipophenolic derivatives*

The C_{20} polyunsaturated FA, dihomo-gamma-linolenic acid (DGLA; 20:3 ω-6), is also a substrate for eicosanoid production and yields 1-series PGs (*e.g.*: PGD_1, PGE_1, and $PGF_{1\alpha}$), which are generally viewed as possessing anti-inflammatory and cytoprotective properties, and have been shown to modulate vascular reactivity [54]. However, DGLA may be indirectly converted to 2-series PGs through AA. As shown in Fig. 5, one of the two 1-series PGs from DGLA assessed in the current assay (PGE_1 and $PGF_{1\alpha}$) were quantified in all the samples ($PGF_{1\alpha}$). The treatment with the oxidizing agent caused an increase in the $PGF_{1\alpha}$ concentration, which was not statistically different from untreated samples (Fig. 5). For this $PGF_{1\alpha}$, only unesterified HT (1.012 ng/mL) exhibited the capacity to significantly attenuate the increase triggered by H_2O_2 (1.839 ng/mL), as compared with HT-OA (1.949 ng/mL). As mentioned above, it should be noticed that depending on the level of ROS, different redox-sensitive transcription factors may be activated and thus, cell-specific biological responses could be observed. In this context, both stimuli with H_2O_2 and HT-OA increased the levels of $PGF_{1\alpha}$, a PG with anti-inflammatory properties [54,55], a rise that may be due to a cellular defense mechanism. Nevertheless, to the present date, scarce information about the effects of the 1-series PGs on inflammation has been reported, and there is still an open discussion on their capacity to enhance the status of human health, in this case, regarding the inflammation processes.

4. Conclusions

Based on the main outcomes found for both HT and HT-FAs present in EVOO and VOO, they seem to actively participate in the redox balance of THP-1 cells, acting as pro-oxidants *in vitro*. This could be explained by the high concentration, cell type, and cell culture media, which are highly dependent on the experimental conditions. On the contrary, our outcomes strongly suggested the anti-inflammatory potential of both HT and HT-FAs. Although this activity, in the case of esterified molecules, appeared to depend on the type of FA, other factors such as the concentration of the oxidizing agent, could modulate the quantitative isoprostanoid profile. According to our results, unesterified and esterified HT molecules are defined by a different capacity to modulate the synthesis of isoprostanoids, depending on the pathway and FA involved.

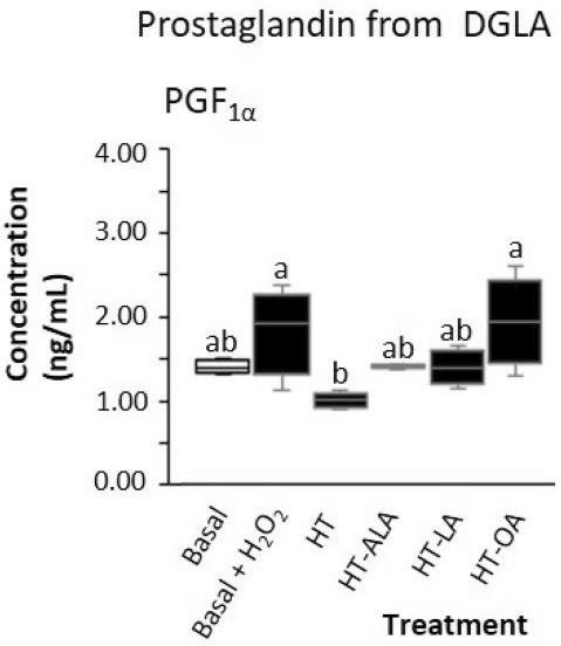

Fig. 5. Modulatory effect of hydroxytyrosol (HT) and HT lipophenols (HT-ALA, HT-LA, and HT-OA) treatments on the prostaglandin from dihomo-gamma-linolenic acid of H_2O_2-stimulated THP-1 monocytic cells. Prostaglandins were determined in THP-1 monocytic cells (cell lysate and growth media) pre-exposed to 20 μM of unesterified HT and lipophenolic derivatives of HT (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA)) and 50 μM H_2O_2. Values are shown as mean ± SD ($n = 3$). Different capital letters within each box and whisker plots indicate significantly different treatments at $p < 0.05$, according to one-way analysis of variance (ANOVA) and Tukey's multiple range test.

Accordingly, there is a need for additional experimental inputs for a sound comparison of the biological activities of esterified HT molecules, which could allow selecting those plant-based foods (mainly, oleaginous matrices, natural sources of HT) that have an adequate quantitative profile for HT-FAs, according to the diverse pathophysiological conditions associated with OS and inflammation. Also, further mechanistic studies are needed to shed light on the effect of gastrointestinal digestion to elucidate the bioaccessible and bioavailable fractions of HT-FAs, as well as the involvement of the diverse cytokines and chemokines as co-modulators of the inflammatory response that is composed of an elaborate cascade of both pro-inflammatory and anti-inflammatory mediators. Therefore, a lipidomics workflow with the simultaneous analysis of multiple oxylipins is of paramount importance for understanding the activity of lipophenols on isoprostanoid generation and hence, on pathophysiological processes.

CRediT authorship contribution statement

Carolina Alemán: Investigation, Formal analysis, Writing – original draft. **Raúl Domínguez-Perles:** Conceptualization, Investigation, Validation, Writing - review & editing. **Federico Fanti:** Formal analysis, Writing – original draft. **Juana I. Gallego-Gómez:** Investigation, Formal analysis, Resources. **Agustín Simonelli-Muñoz:** Investigation, Resources. **Espérance Moine:** Validation, Visualization. **Thierry Durand:** Writing - review & editing, Visualization, Supervision. **Céline Crauste:** Validation, Visualization. **Ángel Gil-Izquierdo:** Investigation, Writing - review & editing, Supervision, Funding acquisition. **Sonia Medina:** Conceptualization, Methodology, Investigation, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2021.106703.

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CAPÍTULO V -

DISCUSIÓN GENERAL

Actualmente, se dispone de evidencias científicas acerca de la estrecha relación existente entre bioaccesibilidad y biodisponibilidad de los compuestos bioactivos presentes en los alimentos y su capacidad de desarrollar funciones biológicas. No obstante, las transformaciones o bioconversiones sufridas por estos compuestos durante el proceso de digestión no han sido descritas en su totalidad, siendo este un factor crítico para la biodisponibilidad y bioactividad finales. En este marco, el propósito de esta Tesis se centra en profundizar en el conocimiento sobre la bioaccesibilidad y biodisponibilidad del HT en su forma libre y esterificado con ácidos grasos (lipofenoles), evidenciando dichas eventuales reacciones de esterificación y/o transesterificación durante el proceso de digestión gastrointestinal y analizando los mecanismos responsables, así como, los posteriores beneficios para la salud.

Actualmente, el desarrollo de alimentos "funcionales" basados en HT se ha impulsado por la información disponible acerca de la capacidad de este alcohol fenólico de disminuir la incidencia de patologías relacionadas con el estrés oxidativo e inflamación (destacando entre ellas las ECVs). Sin embargo, para obtener una imagen completa de la relevancia biológica del HT es necesario esclarecer la farmacocinética y biodisponibilidad del mismo cuando es ingerido a través de distintas fuentes dietéticas (aceite de oliva virgen extra y refinado, aceite de lino, aceite de semilla de uva, margarina y zumo de piña). Por tanto, este constituyó el primer objetivo de la presente Tesis, lo que permitirá diseñar nuevos productos que proporcionen una mayor asimilación y actividad biológica (reconocida de acuerdo con las Opiniones Científicas publicadas por la EFSA y la Comisión Europea).

En este contexto, aplicando un modelo de intervención nutricional en humanos (ensayo clínico – *in vivo* –) se evidenció la estrecha relación entre las diferentes características fisicoquímicas de un panel de matrices alimentarias y la asimilación digestiva del HT. Las mayores concentraciones plasmáticas se obtuvieron al ingerir aceite de oliva virgen extra, en tanto que los valores de asimilación general de HT (mayores concentraciones en orina) se debieron a la ingesta tanto de aceite de oliva virgen extra como de otras matrices de naturaleza oleaginosa (aceite de oliva refinado, aceite de lino y aceite de semilla de uva), todos ellas enriquecidas con el compuesto fenólico a concentraciones de 5 mg de

HT/20 g de matriz alimentaria. En general estos resultados están de acuerdo con la información disponible hasta la fecha que promovía las matrices de naturaleza oleosa como las mejores fuentes dietéticas de HT, sugiriendo las características fisicoquímicas del alimento como factor decisivo independientemente de la naturaleza puramente oleosa de la matriz.

Por otro lado, estudios previos desarrollados mediante la aplicación de modelos pre-clínicos han indicado que la biodisponibilidad del HT podría estar condicionada por el sexo y las particularidades hormonales y fisiológicas de hombres y mujeres. Para esclarecer este aspecto dicho factor fue considerado en el diseño experimental, aun cuando la variabilidad interindividual no permitió obtener datos concluyentes en este sentido.

Los resultados obtenidos confirmaron que la farmacocinética y biodisponibilidad del HT dietético, más allá de las características de la matriz alimentaria, es fuertemente dependiente de una compleja red de interacciones (efecto del proceso digestivo en la estabilidad del compuesto, transportadores intestinales, microbiota, etc.) que hacen necesario ahondar en estos mecanismos para obtener resultados con aplicación práctica.

Entre los distintos factores referidos, en el marco de la presente Tesis se inició la caracterización del efecto de la digestión gastrointestinal sobre la forma química del HT (libre o esterificado con ácidos grasos – lipofenoles –). La identificación y cuantificación de dichos lipofenoles contribuiría a disponer de información específica acerca de las transformaciones ocurridas durante el proceso de digestión gastrointestinal y, por tanto, de la bioaccesibilidad y biodisponibilidad real de estas moléculas. Esta información es de especial relevancia para la determinación final de la biodisponibilidad de HT ya que, de hecho, se ha sugerido que la formación de lipofenoles contribuye a prolongar la liberación de los compuestos fenólicos de la matriz alimentaria y su capacidad de atravesar la barrera intestinal y ejercer sus funciones biológicas (*per se* o a través de sus derivados esterificados). En base a la literatura científica existente en la actualidad, estas ventajas parecen ser dependientes de la longitud de la cadena alquílica y el grado de insaturación de la parte lipídica de la molécula.

El objetivo de establecer el perfil cualitativo del HT (formas libres y esterificadas) en el extracto digerido se abordó mediante la aplicación de un modelo de digestión gastrointestinal estático *in vitro*. Este permitió modelizar los diversos factores implicados en este proceso y así entender los mecanismos responsables de las transformaciones químicas observadas. Así, la formación de los derivados esterificados de HT con los ácidos grasos linolénico, linoleico y oleico (HT-ALA, HT-LA y HT-OA, respectivamente) durante la digestión gastrointestinal dio lugar a su presencia en la fracción bioaccesible en una concentración superior a la registrada en la matriz sin digerir. No obstante, es importante destacar que no todos los derivados presentaron la misma bioaccesibilidad, que resultó dependiente de las características químicas de los ácidos grasos participantes en dicha esterificación (longitud de la cadena alquílica y número de dobles enlaces) y, por supuesto, de las propiedades intrínsecas de la matriz alimentaria. Estos resultados son coincidentes con caracterizaciones previas en relación con la mayor bioaccesibilidad de derivados esterificados de HT con fosfatidilcolina en relación con la molécula de HT libre (sin esterificar). Esta circunstancia sugiere la formación de esterificaciones durante la digestión gastrointestinal como resultado de reacciones enzimáticas o no enzimáticas, lo que tendría implicaciones en la funcionalidad de las distintas fuentes dietéticas de HT.

Para entender la contribución de las diferentes fases de la digestión gastrointestinal en la formación de derivados lipofenólicos y su bioaccesibilidad, se analizaron las fracciones correspondientes a las digestiones gástrica e intestinal de forma aislada. Este abordaje evidenció que tanto el HT libre como sus derivados esterificados son escasamente bioaccesibles tras la fase gástrica, lo que podría deberse a una capacidad insuficiente para extraer estos compuestos o la labilidad de los mismos en las condiciones enzimáticas y de pH de la misma. Por otro lado, en relación con la digestión intestinal, esta dio lugar a un aumento de concentración de HT libre y esterificado en la fracción bioaccesible, en comparación con la digestión gástrica, probablemente como resultado de la hidrólisis de oleuropeina que depende en gran medida de la actividad enzimática lipasa. Por otro lado, esta fase intestinal aumenta significativamente la concentración de derivados esterificados de HT en la fracción bioaccesible. Este resultado permite confirmar que la doble actividad enzimática de la lipasa pancreática que puede catalizar tanto reacciones hidrolíticas como reacciones de esterificación es decisiva en la formación de lipofenoles. El papel relevante de la fase de digestión intestinal se pudo confirmar mediante la aplicación de un modelo teórico, directamente sobre la matriz alimentaria intacta (no sometida a una digestión gástrica previa), proporcionando resultados de bioaccesibilidad de derivados esterificados superiores en relación con el efecto de la digestión intestinal en un contexto fisiológico (digestión intestinal sobre el extracto procedente de la digestión gástrica). Así pues, la constatación de la mayor bioaccesibilidad correspondía a la molécula de HT esterificada con ácido oleico (monoinsaturado), resultado esperable dado que el ácido oleico es el ácido graso más abundante en las matrices consideradas. Este aumento en la generación de esterificaciones con ácido oleico podría estar condicionado a su vez por la conformación espacial conferida por una única insaturación y la diferente polaridad/solubilidad de los ácidos considerados, lo que modularía la reactividad.

Finalmente, se analizó la participación en la generación *de novo* de los diversos derivados esterificados de HT de diversas enzimas presentes en la fase intestinal de la digestión, fundamentalmente, pancreatina y lipasa pancreática. Así, la formación de los lipofenoles fue más eficiente cuando las reacciones estaban catalizadas solo por pancreatina que cuando estaba presente en el medio la lipasa pancreática (sola o en combinación con la pancreatina). Nuevamente, la distinta eficiencia de las diferentes enzimas podría estar condicionada por su conformación espacial o por su sitio activo en relación con la configuración tridimensional de los sustratos (HT y ácidos grasos) que puede polarizar la selectividad de las enzimas por los ácidos grasos.

Las esterificaciones descritas durante la digestión gastrointestinal son de especial relevancia en relación con la biodisponibilidad y actividad biológica diferencial atribuida a estos compuestos en relación con la molécula de HT libre. Así, una vez estudiada la bioaccesibilidad de los lipofenoles (HT-ALA, HT-LA e HT-OA) las propiedades biológicas preventivas del estrés oxidativo (actividad biológica paradigmática atribuida a los compuestos fenólicos) y el inicio de la cascada inflamatoria se testaron en un modelo *in vitro*, monitorizando la modulación de los niveles de isoprostanoides –isoprostanos y prostaglandinas-desarrollada en respuesta a un estímulo pro-oxidante en una línea celular monocítica.

En relación con los marcadores de estrés oxidativo, se observó un efecto pro-oxidante a las concentraciones testadas para algunos de los lipofenoles evaluados, siendo evidenciada la dependencia entre el ácido graso formando parte de la esterificación, su concentración y el efecto biológico observado. Este resultado contrasta con la reconocida actividad antioxidante del HT libre, y participaría de la conocida como paradoja oxidativa de los polifenoles (del inglés "*polyphenols oxidative paradox*"), que indica que un mismo compuesto puede actuar ofreciendo protección frente a radicales libres o actuar como pro-oxidante dependiendo de las condiciones ambientales.

Adicionalmente y dada la demostrada relación del estrés oxidativo con la modificación del patrón de expresión de prostaglandinas a través de la vía de las MAPKs, se caracterizó la capacidad de los derivados esterificados de modular el perfil prostanoide en el modelo descrito. Así, se observó una capacidad diferencial de modular el nivel de las distintas clases de prostaglandinas.

Tanto HT libre como todos sus derivados esterificados ensayados aumentaron los niveles de prostaglandina D_2 (PGD_2) y sus metabolitos, aun cuando el metabolito-respuesta específico y la dimensión de la respuesta observada varió dependiendo del ácido graso que formaba parte del lipofenol, la longitud de su cadena y el grado de insaturación. En todo caso, la capacidad moduladora es más eficiente para los lipofenoles en relación con el HT libre por una modulación más eficaz de la ciclooxigenasa-2, enzima responsable del metabolismo del ácido araquidónico y su conversión a prostaglandina H_2 (PGH_2), precursor de los prostanoides. La relevancia de este resultado está asociada con las funciones biológicas de las prostaglandinas de la clase "D", asociadas a actividades antitumorales, vasomotoras, antitrombóticas y pro/anti-inflamatorias.

En relación con su efecto sobre los metabolitos de las prostaglandinas E_2 (PGE_2) y F_2 (PGF_2), se observó un aumento y una reducción de su concentración en presencia de HT y sus derivados esterificados, respectivamente. Estos hallazgos reforzaron la idea previa de que la actividad biológica de los lipofenoles está estrechamente relacionada con el tipo de ácido graso que participa en la formación de la molécula esterificada. En este marco, los resultados observados permiten sugerir un potencial anti-inflamatorio de los derivados esterificados evaluados, destacando la potencialidad de HT-OA en este sentido. En cualquier caso, la modulación de los niveles de PGF_2 y sus metabolitos es de especial relevancia dado que estos se han asociado con una amplia diversidad de respuestas biológicas, incluyendo actividades vasomotoras y moduladoras de la inflamación.

Por lo tanto, la caracterización *in vitro* de la capacidad moduladora del perfil de oxilipinas en un ambiente pro-oxidante permite proponer que los derivados esterificados podrían desarrollar una actividad pro-oxidante, en tanto que contribuirían a atenuar la respuesta inflamatoria secundaria, siendo estas capacidades biológicas fuertemente dependientes de la intensidad del estímulo pro-oxidante, el tipo de ácido graso al que se encuentra esterificado el HT y la concentración de los lipofenoles en el medio. Este abordaje permitirá proporcionar evidencias más robustas acerca de su actividad biológica, especialmente dado que los isoprostanoides modulados han sido asociados a un importante componente vasomotor, fuertemente relacionado a su vez con las ECVs, lo que podría relacionar los efectos saludables atribuidos al consumo de aceite de oliva en relación con estas patologías. Así mismo, es necesario ahondar en los mecanismos responsables de estas actividades biológicas (participación de citoquinas y quimoquinas), especialmente en relación con las ejercidas por las concentraciones fisiológicas alcanzadas en los distintos tejidos tras la ingesta de una fuente dietética.

CAPÍTULO VI - CONCLUSIONES

1. La máxima concentración plasmática de hidroxitirosol y sus derivados metabólicos (hidroxitirosol acetato y ácido 3,4-dihidroxifenilacético) se alcanza a los 30 minutos tras su ingesta, independientemente de la matriz alimentaria (normalizadas respecto de su contenido en hidroxitirosol). Dicha información es muy útil para poder relacionar la dosis administrada con la actividad biológica esperada.
2. El aceite de oliva virgen extra constituye la mejor fuente dietética de hidroxitirosol en términos de farmacocinética (concentración plasmática) y el aceite de oliva virgen extra y aceite de oliva virgen en términos de biodisponibilidad en relación con la excreción urinaria de hidroxitirosol libre y acetato de hidroxitirosol, respectivamente, no siendo detectadas diferencias atribuibles al sexo.
3. Las moléculas de hidroxitirosol esterificadas con ácidos grasos – lipofenoles – presentes en los aceites de oliva virgen y virgen extra son bioaccesibles, aumentando su concentración en la luz intestinal más allá del contenido registrado en la matriz alimentaria, siendo dicha bioaccesibilidad dependiente del tipo de ácido graso y las características fisicoquímicas de la matriz alimentaria en la que se encuentran.
4. Las condiciones fisicoquímicas y la actividad enzimática durante la digestión gastrointestinal favorecen la formación de derivados esterificados de hidroxitirosol, destacando la relevancia de las condiciones básicas y la actividad enzimática presentes en la fase de digestión intestinal frente a las condiciones de la digestión gástrica.
5. La generación de moléculas esterificadas de hidroxitirosol durante la digestión intestinal está fuertemente influenciada por la actividad lipasa de la pancreatina como el factor más relevante en su síntesis durante el proceso digestivo.
6. Altas concentraciones de hidroxitirosol libre y esterificado muestran un efecto pro-oxidante *in vitro,* modulando los niveles de marcadores de estrés oxidativo – isoprostanos – con diferente eficacia dependiendo tanto de la longitud de la cadena alquílica, como del número de insaturaciones del ácido graso. Este hecho es de especial relevancia en el diseño de futuros ensayos de bioactividad.

7. Los derivados esterificados de hidroxitirosol con ácido oleico y linolénico tras un estímulo oxidativo previenen la formación de prostaglandinas proinflamatorias ejerciendo un efecto antiinflamatorio. Estos hallazgos abren la puerta a la identificación de nuevas aplicaciones para matrices alimentarias oleaginosas y sus subproductos como ingredientes bioactivos coadyuvantes en la terapia antiinflamatoria para la enfermedad cardiovascular.

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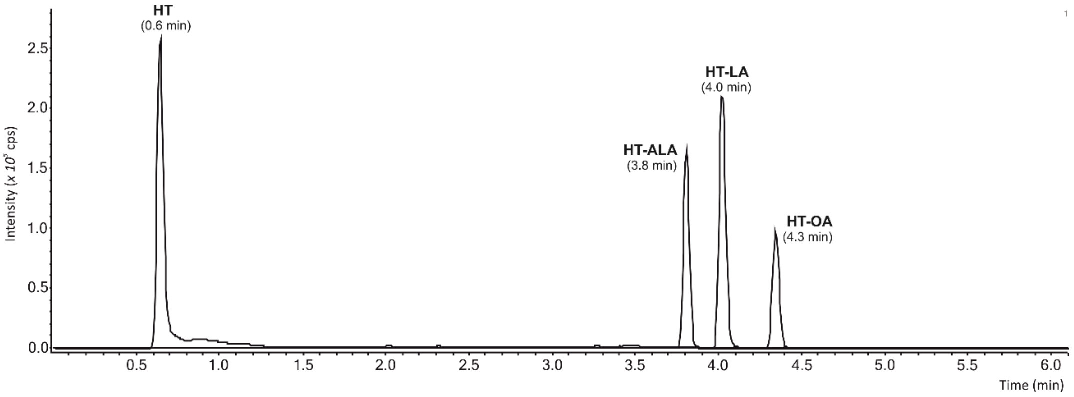
CAPÍTULO VIII - ANEXOS

ANEXO 1: UHPLC chromatogram of hydroxytyrosol and hydroxytyrosol esterified with fatty acids (α-linolenic acid (ALA), linoleic acid (LA), and oleic acid (OA) from in vitro culture of THP-1 cells under experimental conditions.