UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE FARMACIA
DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR II

TESIS DOCTORAL

Estudio *in vitro* de la capacidad antiglicante y mecanismo de acción de subproductos agroalimentarios. Obtención de un extracto vegetal antiglicante y su evaluación en una matriz alimentaria y modelo celular

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Marta Navarro Gómez

DIRECTOR

Francisco José Morales Navas

Madrid, 2017

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ESTUDIO *IN VITRO* DE LA CAPACIDAD ANTIGLICANTE Y MECANISMO DE ACCIÓN DE SUBPRODUCTOS AGROALIMENTARIOS. OBTENCIÓN DE UN EXTRACTO VEGETAL ANTIGLICANTE Y SU EVALUACIÓN EN UNA MATRIZ ALIMENTARIA Y MODELO CELULAR.

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Memoria presentada por Marta Navarro Gómez para obtar al grado de Doctor por la Universidad Complutense de Madrid (UCM)

Vº Bueno del director **Vº Bueno del doctorando**

Dr. Francisco José Morales Navas Marta Navarro Gómez

Madrid, 2016

FRANCISCO JOSÉ MORALES NAVAS, Investigador Científico del Departamento de Caracterización, Seguridad y Calidad del Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN) de la Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC),

INFORMA: que **Dª MARTA NAVARRO GOMEZ**, Licenciada en Ciencia y Tecnología de Alimentos por la Universidad Complutense de Madrid y Máster en Bioquímica, Biología Molecular y Biomedicina por la misma Universidad, ha realizado su memoria de tesis doctoral con el título "**ESTUDIO *IN VITRO* DE LA CAPACIDAD ANTIGLICANTE Y MECANISMO DE ACCIÓN DE SUBPRODUCTOS AGROALIMENTARIOS. OBTENCIÓN DE UN EXTRACTO VEGETAL ANTIGLICANTE Y SU EVALUACIÓN EN UNA MATRIZ ALIMENTARIA Y MODELO CELULAR"** bajo mi tutela y dirección para optar al grado de Doctor por la Universidad Complutense de Madrid, dando mi conformidad para que sea presentada y defendida. El trabajo realizado es original y ha dado lugar, hasta el momento, a ocho artículos científicos en revistas con un amplio reconocimiento internacional, y otras dos publicaciones en distinta fase de revisión.

Madrid, a 24 de Octubre de 2016

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La empresa Cafés Fortaleza (Bilbao, España) ha suministrado las muestras de cascarilla de café.

La empresa TIKTA.CO (*Agricultural Climatization and Technological Research Corporation*, Ankara, Turquía) ha suministrado los subproductos agroalimentarios a partir de semillas de pimiento verde, albaricoque, melocotón, avellana, almendra, guinda, sésamo y granada.

La empresa *Azienda Agricola Fangiano* (Calabria, Italia) ha suministrado alpechín desecado del resultado de la producción de aceite de oliva virgen de la variedad *Carolea*. Las hojas de olivo de la variedad Picual pertenecen al paraje Miragalanes del término municipal Doña Mencía (denominación de origen Baena).

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"Un objetivo, una meta, un blanco, sirven para determinar la acción de hoy y obtener el resultado de mañana"

[Peter Drucker]

"No puedo cambiar la dirección del viento, pero si ajustar mis velas para llegar siempre a mi destino"

[*James Deam*]

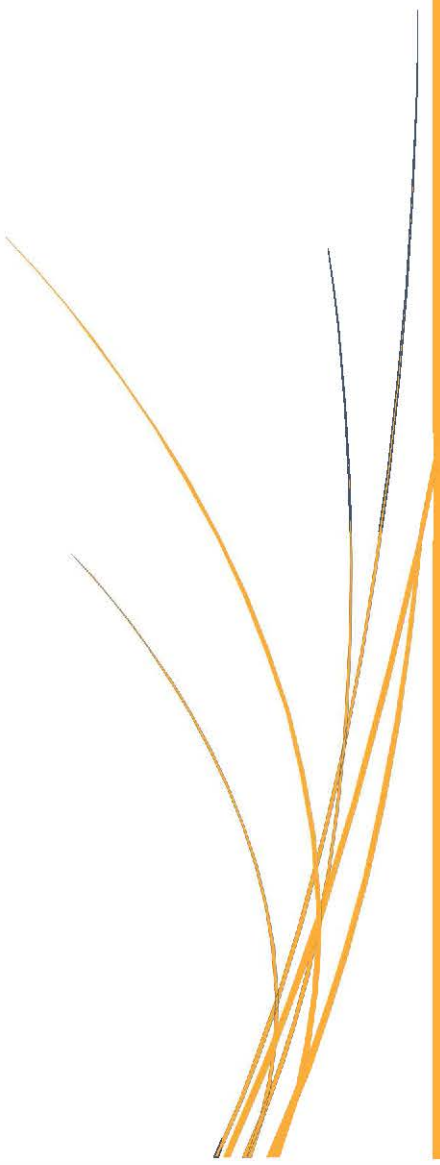
A mis padres

ÍNDICE

3,4-DGE:	3,4-deoxiglucosona-3-ene
3-DG:	3-deoxiglucosona
3-DGal:	3-deoxigalactosona
AG:	Aminoguanidina
AGE-R1:	Receptor 1 de productos de glicación avanzada
AGEs:	Productos de glicación avanzada ("*Advanced glycation end products*")
ALEs:	Productos de lipoxidación avanzada ("*Advanced lipoxidation end products*")
Arg:	Arginina
BSA:	Albúmina sérica bovina
CAS:	Chemical Abstracts Service
CEL:	Nε-carboxietil-lisina
CML:	Nε-carboximetil-lisina
DOPAC:	Ácido 3,4-dihidroxifenil acético
DOPAL:	3,4-dihidroxifenil acetaldehído
GC:	Cromatografía de gases
GLC:	Glucosa
GLUCOS:	Glucosona
GO:	Glioxal
HDL:	Lipoproteína de alta densidad
HMF:	5-hidroximetilfurfural
HT:	Hidroxitirosol
HTA:	Hidroxitirosol acetato
IC_{50}:	Concentración requerida de una sustancia para ejercer una inhibición del 50 % en un ensayo *in vitro*
IL-6:	Interleuquina-6
LC:	Cromatografía líquida
LDL:	Lipoproteína de baja densidad

Lys:	Lisina
MAPKs:	Proteínas quinasas activadas por mitógenos
MGO-H1:	Metilglioxal-imidazolona
MGO:	Metilglioxal
NADPH:	Nicotinamida adenina dinucleótido fosfato reducida
NF-κB:	Factor de transcripción nuclear kappa B
OLE:	Extracto de hoja de olivo
OLEU:	Oleuropeína
PEPT1:	Péptido transportador 1
PI3K:	Fosfatidil-inositol-3-quinasa
Pm:	Peso molecular
PRM:	Productos de la Reacción de Maillard
RAGE:	Receptor de productos de glicación avanzada
RCS:	Especies Carbonílicas Reactivas
RL:	Radical libre
RM:	Reacción de Maillard
ROS:	Especies de Oxígeno Reactivas
sRAGE:	Receptor de AGEs soluble
***t*-BOOH:**	*tert*-butil hidroperóxido
TNF-α:	Factor de necrosis tumoral-alfa
UV:	Ultravioleta
VB:	Verbascósido
VCAM-1:	Molécula de adhesión de células vasculares-1

I. RESUMEN



Introducción:

La Reacción de Maillard, también conocida como glicación cuando tiene lugar en sistemas biológicos, es un complejo entramado de reacciones químicas iniciadas generalmente por la condensación entre un azúcar reductor o compuesto carbonílico reactivo y un resto amino de una proteína. Los productos de glicación avanzada, conocidos por sus siglas en inglés "AGEs", gozan de especial relevancia en la actualidad puesto que su acumulación en el organismo se relaciona con el proceso biológico del envejecimiento y con el avance y las complicaciones de enfermedades multifactoriales de alta prevalencia en nuestra sociedad como la diabetes, Alzheimer y enfermedades cardiovasculares. En este sentido, la inhibición total o parcial de la glicación proteica ha sido un objetivo perseguido desde diferentes disciplinas científicas. Las primeras aproximaciones surgieron desde el ámbito farmacológico con el diseño de compuestos de síntesis, como la aminoguanidina. Sin embargo los efectos secundarios adversos manifestados en los ensayos clínicos posteriores dirigieron las investigaciones hacia la búsqueda de compuestos bioactivos de origen natural. Los compuestos fenólicos se situaron en el centro de atención de las investigaciones debido principalmente a la relación entre la capacidad antioxidante de los mismos y los beneficios frente diversas patologías.

Los subproductos vegetales generados en la industria agroalimentaria pueden suponer un problema de gestión medioambiental, además de limitar el rendimiento económico del proceso. Sin embargo, estos mismos subproductos son una fuente rica de compuestos bioactivos beneficiosos para la salud que podrían ser aprovechados como suplementos alimentarios, nutracéuticos, cosméticos e incluso destinados a usos farmacológicos. En este sentido, los subproductos del cultivo del olivo y generados durante el procesado para la obtención del aceite de oliva son considerados una fuente importante de compuestos fenólicos de gran interés.

El hidroxitirosol es uno de los compuestos fenólicos más característicos del aceite de oliva y de los subproductos del olivar. Investigaciones *in vivo* e *in vitro* le han atribuido numerosos efectos saludables, destacando la actividad antidiabética, cardioprotectora, antiinflamatoria o neuroprotectora. El mecanismo por el cual el hidroxitirosol desempeña dichos efectos se ha relacionado directamente con su capacidad antioxidante ejercida a través de la quelación de metales o el bloqueo de radicales libres. Sin embargo, no han sido explorados otros mecanismos de acción que puedan actuar sobre la glicación y con ello aliviar o prevenir las patologías asociadas.

Objetivos:

El objetivo general fue identificar y valorizar un subproducto de la industria agroalimentaria con elevada capacidad antiglicante. Para ello se realizó un cribaje previo entre diferentes subproductos

de semillas de frutos, de café y del olivo así como una identificación de los principales compuestos bioactivos potencialmente responsables del efecto. Posteriormente, se desarrollaron sistemas modelo *in vitro* para profundizar en los mecanismos de acción del compuesto potencialmente responsable de la capacidad antiglicante del subproducto seleccionado. Finalmente, se evaluó la eficacia del extracto natural sobre el desarrollo de la Reacción de Maillard en un modelo de alimento y su actividad inhibitoria en la glicación de proteínas en un modelo celular de estrés carbonílico.

Resultados:

Capítulo 1: *Estudio y selección de subproductos agroalimentarios con potencial capacidad antiglicante in vitro e identificación de los compuestos responsables del efecto.*

En un cribado inicial sobre una selección de extractos hidrosolubles de subproductos de origen vegetal se realizó una evaluación previa de la capacidad antioxidante y antiglicante mediante el empleo de diferentes sistemas *in vitro* en condiciones fisiológicas simuladas. Se evidenció que la granada ejerció la mayor capacidad antioxidante comparada con el resto de semillas evaluadas y un notorio efecto antiglicante en los dos modelos de glicación *in vitro* empleados. El efecto anti-AGEs de la semilla de granada fue relacionado con su contenido fenólico y concretamente con su destacado contenido en ácido gálico. Si bien el estrés oxidativo se asocia generalmente con el estrés carbonílico, no se halló una correlación significativa entre la capacidad antioxidante y antiglicante de los extractos estudiados y se propuso el atrapamiento de α-dicarbonilos como uno de los mecanismos antiglicantes más probable (*Artículo 1*).

Tras concluir que el extracto hidrosoluble de granada ejerce un destacado efecto anti-AGEs se examinó su posible toxicidad, dado los escasos y controvertidos resultados de los estudios realizados hasta el momento. El extracto no sólo mostró una ausencia de toxicidad en el rango de dosis estudiado (1-100 µg/mL) en un modelo celular de hepatoma humano (HepG2) sino que presentó un efecto protector frente al estrés oxidativo inducido por *tert*-butil hidroperóxido (*Artículo 2*).

Paralelamente al estudio con semillas, los extractos hidrosolubles de cascarilla de café y alpechín se propusieron para su valorización como potenciales extractos antiglicantes por su preciada composición fenólica. La capacidad antioxidante y antiglicante de la cascarilla de café se evaluó en función de la variedad botánica del grano (*Coffea arabica, Coffea canephora*). La variedad robusta presentó una mayor capacidad antioxidante acorde con su mayor contenido en compuestos fenólicos, principalmente ácidos clorogénicos, siendo su capacidad antiglicante similar a la arábica. Sorprendentemente, la capacidad de atrapamiento de α-dicarbonilos fue determinante para

discriminar entre ambas variedades puesto que la variedad robusta presentó un efecto 11 veces - superior a la arábica debido posiblemente a su mayor contenido en ácidos clorogénicos (*Artículo 3*).

Igualmente se investigó la capacidad antiglicante de dos extractos hidrosolubles de alpechín obtenidos por dos procesos de filtración diferentes (nano y ultrafiltración). En la etapa inicial de la glicación, el extracto inhibió la formación del producto de Amadori, mientras que en la etapa intermedia actuó a través del atrapamiento de compuestos α-dicarbonilos, como el metilglioxal (MGO) y el glioxal (GO). Como consecuencia directa del efecto inhibitorio en ambas etapas se produjo una reducción de la formación de AGEs en la etapa avanzada de la glicación. El análisis del efecto antiglicante de dos de sus componentes bioactivos mayoritarios, el hidroxitirosol (HT) y el verbascósido y el posterior fraccionamiento del extracto confirmó que estos dos compuestos tuvieron un papel decisivo en dicho efecto. El extracto hidrosoluble de alpechín obtenido por ultrafiltración presentó la mayor actividad antiglicante, siendo la metodología de separación aplicada un factor a tener en cuenta (*Artículo 4*).

Capítulo 2: Estudio del mecanismo de acción antiglicante del hidroxitirosol en modelos in vitro. Obtención de un extracto de hoja de olivo como fuente de hidroxitirosol y evaluación de su capacidad antiglicante.

Con la intención de obtener una visión más amplia del mecanismo de acción del HT, se planteó un estudio pormenorizado de su acción antiglicante en modelos de glicación *in vitro*. Los resultados revelaron que el HT inhibió de manera eficaz la glicación en varias etapas del proceso, desde la etapa temprana hasta la intermedia con una repercusión directa en la reducción de la formación de AGEs específicos como la carboximetil-lisina (CML), carboxietil-lisina (CEL) y argipirimidina (ArgP) en la etapa avanzada. En este sentido, el HT bloqueó la formación de productos de Amadori probablemente a través de la interacción con los grupos amino de la lisina reactiva de la proteína y de su capacidad de bloquear radicales libres para mitigar la degradación oxidativa asociada a la primera etapa de la glicación. En etapas intermedias, el HT bloqueó la acción glicante de los compuestos α-dicarbonilos a través de su atrapamiento, impidiendo así su reacción con el grupo amino de la proteína (*Artículo 5*).

En un estudio más exhaustivo del mecanismo a través del cual el HT ejerce su efecto antiglicante, se evidenció una afinidad hacia el atrapamiento de α-dicarbonilos superior a la presentada por los propios aminoácidos precursores de la glicación, como son la lisina, arginina e histidina. Se constató que para ejercer la inhibición de la glicación, el HT se oxidaba previamente a ácido 3,4-dihidroxifenil acético (DOPAC), siendo, presumiblemente, el responsable final de dicha actividad. En la

confirmación de esta hipótesis se monitorizaron la formación de aductos por adición nucleofílica entre el DOPAC y el MGO. Adicionalmente se constató una relación actividad-estructura entre la capacidad de atrapamiento de MGO y la estructura de compuestos fenólicos similares al HT, resultando clave la presencia de un grupo hidroxilo en posición 2 (*Artículo 6*).

Una vez constatada la relevancia del mecanismo de atrapamiento de GO y MGO, se planteó la duda de si el comportamiento sería extensible a compuestos α-dicarbonilos C6. Hasta la fecha, los únicos estudios de glicación reseñados en la bibliografía hacían referencia a compuestos α-dicarbonilos C2 (GO) y C3 (MGO). Sin embargo cada vez hay más evidencias de la relevancia biológica de los C6 en la glicación. En este sentido, se evaluó el atrapamiento de compuestos α-dicarbonilos C6 como la 3-deoxiglucosona (3-DG), 3-deoxigalactosona (3-DGal), 3,4-dideoxiglucosona-3-ene y la glucosona. El HT y sus derivados, como el acetato de hidroxitirosol y el DOPAC mostraron una capacidad de atrapamiento de los compuestos α-dicarbonilos C6 más estables, como el 3-DG o el 3-DGal, aunque es relevante constatar que tanto en sistemas simples como competitivos de reacción, el HT mostró una preferencia significativa hacia el atrapamiento de compuestos α-dicarbonilos C3. Por su parte, el DOPAC ejerció la mayor actividad (*Artículo 7*).

Con objeto de llevar este conocimiento hacia una aplicación práctica, se obtuvo un extracto a partir de la hoja de olivo como fuente natural de HT. Tras una previa caracterización del perfil fenólico y un estudio de su capacidad antioxidante y antiglicante, se confirmó que el HT previa oxidación a DOPAC, contribuía al efecto antiglicante, aunque en este caso, más centrado en las etapas intermedias-avanzadas del proceso. Por otro lado, la contribución de los efectos anti-AGEs de otros compuestos fenólicos presentes, la degradación de la oleuropeína en HT así como los posibles sinergismos establecidos, explicarían la potente capacidad de inhibición de la glicación del extracto en su conjunto (*Artículo 8*).

Capítulo 3: Evaluación del hidroxitirosol y del extracto de hoja de olivo como potenciales ingredientes antiglicantes en un modelo de galleta.

Los prometedores resultados anti-AGEs obtenidos en el estudio *in vitro* del extracto de hoja de olivo y específicamente del HT como su principal principio activo hacen de ellos unos buenos candidatos para ser propuestos como ingredientes anti-AGEs. Con dicho objetivo, tanto el extracto como un estándar de HT fueron incluidos como ingredientes en un modelo de galleta para el análisis de su influencia sobre el desarrollo de la Reacción de Maillard. Los resultados obtenidos concluyeron que el HT inhibió la formación de 3-DG y 5-hidroximetilfurfural de forma correlacionada, así como la formación de AGEs fluorescentes libres y de pentosidina, principal AGE fluorescente ligado a proteínas. La adición del extracto de hoja de olivo destacó por reducir la formación tanto de CEL,

AGE cuantitativamente mayoritario en el modelo de galleta estudiado, como de pentosidina (*Artículo 9*).

Capítulo 4: Evaluación in vitro de la capacidad antiglicante de dos extractos de hoja de olivo con diferente composición fenólica. Estudio de la actividad antiglicante de un extracto de hoja de olivo concentrado en hidroxitirosol en un modelo celular de estrés carbonílico.

En un estudio *in vitro* previo se confirmó que el extracto de hoja de olivo obtenido durante una etapa adicional de maceración en medio levemente ácido incrementó su capacidad antiglicante posiblemente debido al mayor contenido en estructuras fenólicas más sencillas y concretamente a su contenido en HT procedente de la ruptura de la oleuropeína y otros derivados. Con objeto de investigar la posible inhibición ejercida por el extracto de hoja de olivo concentrado en HT sobre la glicación de las proteínas celulares y la consiguiente generación de AGEs, se desarrolló un modelo celular de estrés carbonílico. Se observó que el extracto de hoja de olivo inhibió significativamente la carbonilación de las proteínas intracelulares producida por el MGO. Además, se determinó que CEL y ArgP fueron los AGEs que se generaron mayoritariamente tras el estrés carbonílico, siendo la ArgP inhibida significativamente por el extracto. Finalmente se concluyó que la actividad antiglicante del extracto podría atribuirse a los posibles sinergismos establecidos entre el HT y compuestos con similar polaridad ya que el estándar de HT no ejerció por si solo tal efecto a diferencia del extracto de hoja de olivo concentrado en HT (*Artículo 10*).

Conclusiones:

La evaluación de diferentes subproductos de la industria agroalimentaria llevada a cabo en la presente Tesis Doctoral apoya la idea de una posible valorización de dichos subproductos en base a su capacidad antiglicante, si bien, diferentes factores como la variedad, tipo de extracción o perfil fenólico influyen en los resultados obtenidos. La investigación en mayor profundidad de la capacidad antiglicante del HT concluye que este compuesto fenólico ejerce un efecto antiglicante en todas las etapas de la glicación, resultando en una inhibición de AGEs específicos como la CML, CEL y ArgP. La capacidad de atrapamiento de compuestos α-dicarbonilos C2, C3 y C6 por adición nucleofílica se propuso como principal mecanismo antiglicante en condiciones fisiológicas simuladas, requiriendo la previa oxidación del HT a DOPAC. En base a los resultados obtenidos, se seleccionó un extracto antiglicante a partir de la hoja de olivo como fuente natural de HT. El extracto se valorizó como ingrediente alimentario al reducir la formación de CEL y pentosidina en un modelo de galleta y como nutracéutico al inhibir la formación de ArgP en la línea celular HepG2.

INTRODUCTION:

The Maillard Reaction, which is called glycation in biological systems, is a complex framework of chemical reactions usually initiated by the condensation of a reducing sugar or reactive carbonyl compound with a protein. Currently, advanced glycation end products, named “AGEs”, have a special relevance since their accumulation in the body is related to aging process and the progress and complications of multifactorial diseases such as diabetes mellitus, Alzheimer and cardiovascular diseases.

In this sense, the total or partial inhibition of the protein glycation has been a target for different scientific disciplines. The first approaches emerged from the pharmacological field with the design of synthetic compounds, as aminoguanidine. However, the side effects observed in the clinical trials led the investigations to search of naturally occurring bioactive compounds. Investigations were focused on phenolic compounds since exert a high antioxidant capacity and promote beneficial effects against a number of human pathologies.

The vegetable by-products generated in the food industry can be an environmental problem besides the limitation of the economic yield of the process. However, in several cases, by-products are considered a source rich of bioactive compounds with beneficial health properties and they could be proposed as food supplements, nutraceuticals, cosmetics and even intended pharmacological uses. The olive by-products generated during the extraction of olive oil are considered an important source of interesting phenolic compounds.

The hydroxytyrosol (HT), a characteristic phenolic compound from olive oil and its by-products, has been associated with antidiabetic, cardio protective, anti-inflammatory or neuroprotective activity by *in vivo* and *in vitro* investigations. It has been suggested that the mechanism responsible for these effects is the antioxidant activity of HT that is exerted through metal chelations or its radical scavenging capacity. However, other mechanisms of action that could act on the glycation, and then preventing the progression of associated diseases have not been explored.

OBJETIVES

In order to select and recover a vegetable by-product of the food industry with high antiglycative capacity, a previous screening of different by-products and an identification of the main bioactive compounds responsible for this effect was performed. Subsequently, *in vitro* model systems were developed to get more insight the mechanisms of action of the compounds responsible for the antiglycative capacity. Finally, the effect on the development of the Maillard Reaction in a food

model and its inhibitory activity on the glycation of intracellular proteins in a cellular carbonyl stress model were evaluated.

RESULTS

Chapter 1: *Study and selection of vegetable by-products with in vitro potential antiglycative capacity and identification of the compounds responsible for this effect.*

A selection of water-soluble extracts from vegetable by-products were initially screened for their antioxidant and antiglycative capacities. Antiglycative capacity was performed by different *in vitro* systems carried out under simulated physiological conditions. The pomegranate extract showed the highest antioxidant capacity compared with other tested seed extracts, together with a notorious antiglycative effect on the two models *in vitro* glycation used. The anti-AGEs effect of the pomegranate seed was related to its phenolic content and specifically to its outstanding gallic acid content. Although oxidative stress is generally associated with the carbonyl stress, a significant correlation between antioxidant and antiglycative capacities of extracts was not found and the α-dicarbonyl trapping capacity was proposed as one of the most likely antiglycative mechanism (*Paper 1*).

After concluding that the water-soluble extract of pomegranate has a prominent anti-AGEs effect, its potential toxicity was examined, due to the limited studies conducted until now and some controversial results arisen. The extract not only showed an absence of toxicity in the dose range studied (1-100 μg/mL) in the HepG2 cell model, but it showed a protective effect against an oxidative stress induced by *tert*-butyl hydroperoxide (*Paper 2*).

In parallel to the study with seeds, water-soluble extracts of coffee silverskin and olive oil mill wastewater were proposed as potential antiglycative extracts due to their phenolic profile. The antioxidant and antiglycative capacity of coffee silverskin depends on the variety of beans (*Coffea arabica, Coffea canephora*). The robusta variety showed a major antioxidant capacity and the highest phenolic compounds content, mainly clorogenic acids, but its antiglycative capacity was similar to this from the Arabica variety. Surprisingly, the α-dicarbonyl trapping capacity was determinant to discriminate between the two varieties since robusta variety presented an effect 11-fold higher than this from arabica variety possibly due to their higher content of chlorogenic acid (*Paper 3*).

Likewise the antiglycative capacity of two vegetable water-soluble extracts obtained by two different filtration processes (nanofiltration and ultrafiltration) was investigated. In the early stage of the glycation, the extract inhibited the formation of Amadori products while in the intermediate

stage acted through the trapping of α-dicarbonyl compounds such as methylglyoxal (MGO) and -glyoxal (GO). As a direct result of the inhibitory effect in both steps there was a reduction of the AGEs formation in the advanced stage of the glycation. The analysis of the antiglycative effect of two of its main bioactive components, hydroxytyrosol and verbascoside, and the subsequent fractionation of the extract confirmed that these two compounds had a decisive role in this effect. The water-soluble olive oil mil wastewater extract obtained by ultrafiltration had the highest antiglycative activity, the separation methodology being applied a factor to consider (*Paper 4*).

Chapter 2: *Study of the antiglycative mechanism of action of hydroxytyrosol in in vitro models. Obtention of an olive leaf extract as a source of hydroxytyrosol and evaluation of its antiglycative capacity.*

Intending to get a wider view of the mechanism of action of hydroxytyrosol (HT), a detailed study of its antiglycative action was proposed in different *in vitro* glycation models. The results revealed that HT effectively inhibited the glycation in various stages of the process from the early stage to the intermediate stage with a direct impact on reducing the formation of specific AGEs such as CML, CEL and ArgP in the advanced stage. In this sense, the HT blocked the formation of Amadori products probably through the interaction with amino groups of lysine reactive protein and because of its ability to capture free radicals to mitigate the oxidative degradation associated with the progress of the glycation process. In intermediate stages, the HT blocked the glycative action of α-dicarbonyl compounds through its trapping capacity thereby preventing their reaction with the amino group of the protein (*Paper 5*).

The mechanism through which the HT exerts its antiglycative effect was studied in detail. HT showed higher affinity than the precursor amino acids such as lysine, arginine and histidine by the α-dicarbonyl trapping. It was also observed that the HT was previously oxidized to 3,4-dihydroxyphenylacetic acid (DOPAC) to exert the inhibition of the glycation, being presumably the responsible for such activity. In the confirmation of this hypothesis, the adducts MGO-DOPAC obtained from the condensation by nucleophilic addition between DOPAC and MGO were monitored. Additionally, a relationship between structure and activity between the MGO-trapping capacity and the phenolic compound structure similar to HT was revealed. For this fact, the presence of a hydroxyl group in position 2 was important (*Paper 6*).

After checking the relevance of the trapping mechanism of GO and MGO, the question whether the behavior could be extended to α-dicarbonyl C6 was raised. To date, the glycation studies reported in the literature have been related to α-dicarbonyl compounds C2 and C3. However, there is evidence of a biological relevance of α-dicarbonyl C6 in the glycation process. In this regard, the α-

dicarbonyl C6 compounds such as 3-deoxyglucosone (3-DG), 3-deoxygalactosone (3-DGal), 3,4-dideoxyglucosone-3-ene and glucosone were evaluated. The HT and its derivatives such as hydroxytyrosol acetate and DOPAC, showed a trapping capacity of the more stable α-dicarbonyl compounds C6, such as 3-DG or 3-DGal. However, it is relevant to note that the HT and its derivatives showed a significant preference for the α-dicarbonyl C3-trapping in both simple and competitive reaction systems (*Paper 7*).

In order to lead this knowledge to a practical application, an olive leaf extract as a natural source of HT was obtained. After a preliminary characterization of the phenolic compundsand a study of its antioxidant and antiglycative capacity, it was confirmed that the previous HT oxidation to DOPAC contributed to the antiglycative effect. In this case, the effect was more focused on the intermediate-advanced stages of process. On the other hand, the contribution of the anti-AGEs effects of other phenolic compounds present, the degradation of oleuropein in HT as well as the possible synergisms amoung the compounds could explain the potent ability to inhibit the glycation of the whole extract (*Paper 8*).

Chapter 3: *Evaluation of the hydroxytyrosol and the olive leaf extract as potential antiglycative ingredients in a biscuit model*

The anti-AGEs promising results obtained in the *in vitro* study of the olive leaf extract and specifically the HT as their main active principle make them good candidates to be proposed as anti-AGEs ingredients. With this objective, both the extract and a the HT standard were included as ingredients in a biscuit model for the analysis of their influence on the development of MR. Results concluded that the HT inhibited both the formation of 3-DG and 5-hydroxymethylfurfural in a correlated form and the formation of free fluorescent AGEs and pentosidine as main fluorescent AGEs linked to protein. The addition of the olive leaf extract was characterized by reducing the formation of both CEL, the predominant AGE present in the biscuit model, and pentosidine (*Paper 9*).

Chapter 4: *In vitro evaluation of the antiglycative capacity of two olive leaf extracts with different phenolic composition. Study of the antiglycative capacity of an olive leaf extract concentrated in hydroxytyrosol in a carbonyl stress cellular model.*

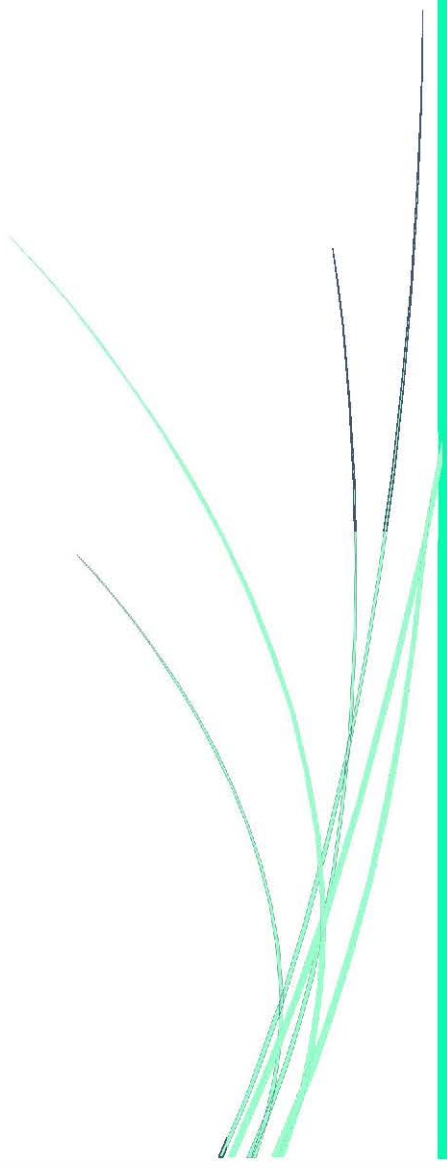
In a previous *in vitro* study it was confirmed that the olive leaf extract obtained during an additional step of maceration slightly acid increased its antiglycative capacity. This fact was possibly due to the higher content in simple phenolic structures and specifically due to its HT content generated from the rupture of oleuropein and other derivatives. In order to investigate the possible inhibition exerted by the olive leaf extract concentrated in HT on the protein cellular glycation and the

consequent generation of AGEs, a carbonyl stress cellular model was developed. It was observed that the olive leaf extract significantly inhibited the carbonylation of intracellular proteins produced by MGO. Furthermore, it was determined that CEL and ArgP were AGEs mainly generated after the carbonyl stress, ArgP being significantly inhibited by the extract. Finally it was concluded that the antiglycative activity of the extract could be attributed to possible synergisms established between the HT and compounds with similar polarity, since the HT standard did not exert this effect by itself unlike the olive leaf extract concentrated in HT (*Paper 10*).

CONCLUSIONS:

The evaluation of different by-products of the food industry conducted in this Doctoral Thesis supports the idea of the potential valorization of these by-products based on their antiglycative ability, although different factors such as the variety, the type of extraction or the phenolic profile can influence the results. A deeper research on the antiglycative capacity of the HT shows that this phenolic compound has an antiglycative effect at all the stages of the glycation process, resulting in the inhibition of specific AGEs such as CML, CEL and ArgP. The trapping capacity of α-dicarbonyl C2, C3 and C6 compounds by a nucleophilic addition was proposed as the main antiglycative mechanism of the HT after oxidation to DOPAC. From these results, an olive leaf extract was selected as a source of HT with an antiglycative capacity. The reduction of the formation of CEL and pentosidine in a biscuit model makes the extract to be valued as a food ingredient and as a nutraceutical compound by inhibiting the formation of ArgP in a cellular model.

II. REVISIÓN BIBLIOGRÁFICA



1. LA REACCIÓN DE MAILLARD

1.1. Aspectos generales de la Reacción de Maillard

El tratamiento térmico es el proceso tecnológico más ampliamente utilizado para preservar los alimentos y extender su vida útil. La aplicación de calor al alimento actúa como un potente catalizador de una serie de reacciones químicas entre sus constituyentes donde la generación de compuestos pardos y aromas característicos son quizás las consecuencias más evidentes para el consumidor. Las reacciones de pardeamiento no enzimático, y en concreto la Reacción de Maillard (RM), son los principales responsables de esos cambios. Es por ello que la RM se considera parte fundamental del proceso tecnológico y culinario de los alimentos como por ejemplo ocurre durante la elaboración de la cerveza, el tostado del café o el cacao y la gran variedad de productos de panadería, galletería, confitería, etc. Sin embargo, la importancia del desarrollo de esta reacción en los alimentos no sólo radica en una mejora de la palatabilidad del alimento, sino que también son objeto de debate sus múltiples implicaciones a nivel nutricional y toxicológico (O´Brien y Morrissey, 1989).

La RM está cobrando especial relevancia en las sociedades occidentales donde el ritmo de vida, en especial en zonas urbanas y la gran oferta de alimentos preparados, han perfilado nuestros hábitos alimentarios. Asimismo se ha experimentado un notable cambio caracterizado por el incremento del consumo de productos procesados en detrimento de la dieta mediterránea asociada a un alto consumo de frutas, hortalizas, cereales, leguminosas y aceite de oliva. Una de las consecuencias de este cambio de hábitos, independientemente de la repercusión sobre la calidad nutricional, es el progresivo aumento de la ingesta de Productos de la Reacción de Maillard (PRM) que ya forman parte, y de manera destacada, de nuestra dieta habitual (Martins y col. 2001).

En definitiva, la gran complejidad de la reacción, la presencia de PRM en la dieta y las connotaciones tanto positivas como negativas que suscita la RM refuerzan la prioridad de la investigación en este área, siendo de interés obtener un equilibrio entre tres características fundamentales que se buscan en un alimento: la calidad nutricional, la aceptabilidad organoléptica y la seguridad de su consumo.

1.2. Fundamentos químicos de la Reacción de Maillard

La RM debe su nombre al químico francés Louis Camille Maillard que describió en 1912 la formación de compuestos fuertemente coloreados e insolubles durante el calentamiento de aminoácidos y azúcares reductores (Maillard, 1912). Esta reacción suscitó un gran interés entre los químicos de los alimentos pero pasó inadvertida entre los diabetólogos que años más tarde, tras el

descubrimiento de la insulina, establecerían los primeros estudios en sistemas biológicos (Billaud y Adrian, 2003).

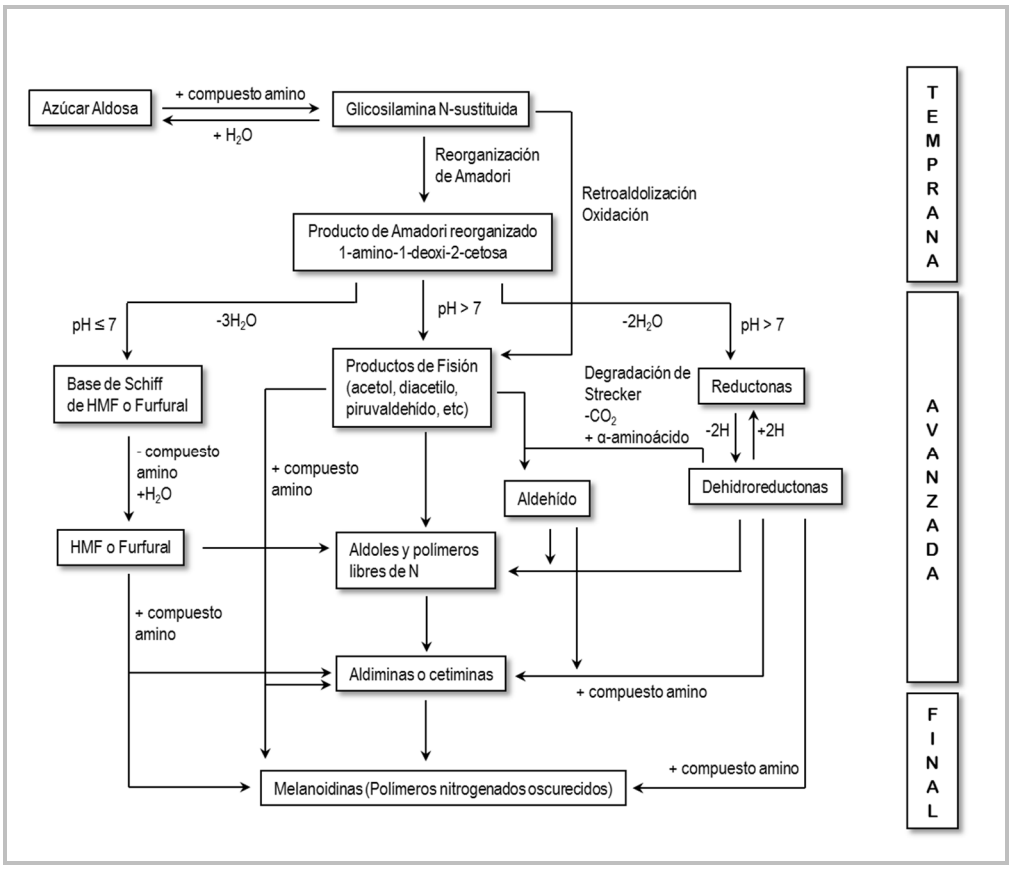


Figura 1: Esquema general de la Reacción de Maillard
(Hodge, 1953; Mauron, 1981; Nursten, 1986)

Un gran avance en la comprensión de la RM fueron los trabajos del Dr. John Hodge (*USDA Northern Regional Research Center*, USA) que por primera vez en 1953 esquematizó de una manera coherente el desarrollo de la reacción en tres etapas denominadas inicial, intermedia y final (**Figura 1**). Posteriormente, el Dr. Jean Mauron (*Nestlé Research Centre*, CH) renombró las etapas de la reacción como temprana, avanzada y final (Mauron, 1981) mientras que el Prof. Harry E. Nursten (Reading University, UK) propuso la inclusión de un nuevo paso concerniente a la degradación de compuestos intermediarios de Maillard por acción de radicales (Nursten, 1986). En definitiva, el diagrama de Hodge aportó una clarividencia tal que a día de hoy es aún referente en la comprensión de la química de esta reacción.

La RM podría considerarse como un complejo entramado de reacciones de condensación, ciclación, oxidación, escisión, deshidratación, etc., que pueden estar desarrollándose de manera paralela y en cascada, y que darán lugar a un amplio espectro de nuevos compuestos denominados

genéricamente PRM. La RM es extraordinariamente compleja y por consiguiente difícil de controlar una vez iniciada, puesto que muchos de los compuestos que se generan pueden retroalimentar la reacción, siendo complicado identificar en qué etapa de reacción se encuentra un determinado alimento. Otros factores dependientes de las características del alimento y de las condiciones de procesado intervienen activamente en la modulación de la reacción siendo los más relevantes la temperatura, el tiempo de procesado, el tipo y concentración de los reactantes, la actividad de agua, el pH o la presencia de metales, entre otros. Aunque la RM está habitualmente asociada a procesos térmicos, es importante resaltar que también puede tener lugar a temperatura ambiente, aunque a tiempos más prolongados, siendo especialmente relevante durante el almacenamiento de determinados alimentos (O´Brien y Morrissey, 1989; Rufián-Henares y col., 2009).

1.2.1. Etapa temprana

De manera general, la reacción es iniciada por la condensación entre un grupo amino libre de un aminoácido, péptido o proteína y un grupo carbonilo de un azúcar reductor o compuesto carbonílico generado en la propia RM, en la oxidación lipídica o en la degradación del ácido ascórbico (Ledl y Schleicher, 1990; Friedman, 1996). Tras la pérdida de una molécula de agua, el compuesto de adición resultante genera una base de Schiff que rápidamente se ciclará para dar lugar a la correspondiente glicosilamina-N-sustituida, producto inestable que experimenta un reordenamiento irreversible (Ames, 1992). Si el azúcar de partida es una aldosa tendrá lugar el reordenamiento de Amadori formando el correspondiente compuesto de Amadori (1-amino-1-deoxi-2-cetosa). Del mismo modo, si el azúcar de partida es una cetosa, se formará el compuesto de Heyns (2-amino-2-deoxi-aldosa) mediante el reordenamiento de Heyns (Ames, 1992). La etapa temprana de la RM ha sido ampliamente estudiada y posiblemente sea la mejor descrita. En esta etapa no se producen compuestos coloreados ni fluorescentes, y las proteínas no sufren alteraciones estructurales importantes. Sin embargo, el bloqueo del grupo amino de la proteína al formarse los productos de Amadori o de Heyns disminuye la disponibilidad de los aminoácidos y, en consecuencia, el valor nutricional (Nursten, 2005).

1.2.2. Etapa avanzada

La etapa avanzada implica la degradación del compuesto de Amadori/Heyns hacia diversos PRM, que en muchos casos serán responsables de aportar al alimento aromas y sabores únicos (Ledl and Schleicher, 1990; Friedman, 1996; Henle, 2005). En función del pH, la RM puede proceder de manera diferente a través de dos vías de degradación principales, la enolización 1,2 y la enolización 2,3 (Hodge, 1967). Cuando el pH del sistema es neutro o ligeramente ácido se ve favorecida la vía de enolización 1,2 que implica una enolización en el C1 y C2, la pérdida del grupo carboxilo y amino

en posición 3 y 1, respectivamente, y sucesivas deshidrataciones. Los furfurales, como el 5-hidroximetilfurfural (HMF), son los productos característicos de esta vía de degradación del compuesto de Amadori. Por el contrario, si el pH del sistema es básico se favorece la vía de enolización 2,3 que, además de la correspondiente enolización en el C2 y C3, implica una desaminación en C1 y una deshidratación moderada generando una variedad de productos de fisión de bajo peso molecular tales como reductonas, α-dicarbonilos y aldehídos. Pese a que el acetol, piruvaldehído, diacetilo, metilglioxal o glioxal se consideran compuestos relevantes en la generación de sabores y aromas típicos, estos compuestos son altamente reactivos, lo que implica que participen en otras reacciones con otros productos intermediarios de la reacción (Nursten, 2005).

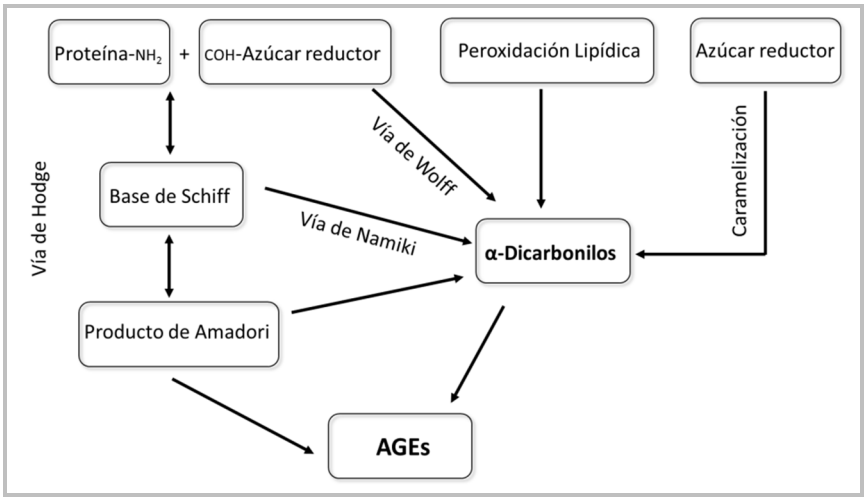


Figura 2. Rutas implicadas en la formación de compuestos α-dicarbonilos (Peng y col., 2011)

Los compuestos α-dicarbonilos requieren de una mención especial ya que son intermediarios con un papel muy relevante en la propagación de la reacción debido a su elevada reactividad. Estos compuestos pueden reaccionar con aminoácidos a través de la reacción de Strecker y formar aldehídos con un carbono menos y con la liberación de dióxido de carbono, contribuyendo a diversificar los compuestos que intervienen en el aroma de los alimentos calentados (Ghiron y col., 1988). Sin embargo, es importante resaltar que los α-dicarbonilos tienen otras vías de generación paralelas a la RM y que se esquematizan en la **Figura 2**. Los α-dicarbonilos como el metilglioxal o el glioxal pueden ser generados a partir de la fragmentación oxidativa de la base de Schiff (ruta de Namiki) (Semchyshyn y Lushchak, 2012), la autoxidación de azúcares (ruta de Wolff) (Wolff y col. 1991), la oxidación de lípidos y a partir de otros procesos como la caramelización, que a elevadas temperaturas podría generar compuestos α-dicarbonilos a partir de carbohidratos y sin participación de grupos aminos (Degen y col., 2012; Semchyshyn y Lushchak, 2012).

Si bien existe una elevada diversidad estructural de α-dicarbonilos y una escasa información sobre su presencia y cuantificación en los alimentos, el glioxal (GO), el metilglioxal (MGO) y la 3-deoxiglucosona (3-DG) se han considerado los más representativos. En la **Tabla 1** se describen los niveles de α-dicarbonilos en una serie de alimentos y bebidas.

Alimento/Bebida	3-DG	MGO	GO	Referencias
Galletas	8.5-385	1.8-68	17.9-27	Degen y col., 2012; Kocadagli y col., 2016
		3.7-78	4.8-20	Arribas-Lorenzo y Morales, 2010
Pan	13-619	nd-47	nd-28	Degen y col., 2012
		0.8	0.3	Nagao y col.1986
Miel	76-808	0.2-2.9	0.1-11	Arena y col. 2011
Salsa de soja	32-832	nd-12		Degen y col., 2012
		8.7	4.9	Nagao y col.1986
Refresco	nd-28	nd		Degen y col., 2012
	Tr-87	Tr-0.63		Gensberger y col. 2013
		0.1-1.4	Tr-1.7	Lo y col. 2008
Vino	2.2-95	Tr-4.5		Degen y col., 2012
		1.0-2.6	2.2-4.4	De Revel y col. 2000
Cerveza	18-54	nd-1.0		Degen y col., 2012
		0.1-0.2	Tr	Barros y col. 1999

Tabla 1. Contenido de 3-deoxiglucosona (3-DG), metilglioxal (MGO) y glioxal (GO) en alimentos y bebidas. Los contenidos están expresados en mg/kg de alimento o mg/L de bebida. nd: no detectado; Tr: trazas

1.2.3. Etapa final

Si las condiciones del procesado térmico se mantienen, la RM evoluciona hacia una etapa final que se caracteriza por la formación de polímeros pardos de alto peso molecular, denominados genéricamente melanoidinas, a partir de la condensación de los compuestos generados en la etapa avanzada. Las melanoidinas constituyen una amalgama de compuestos poliméricos de muy diversa tipología y funcionalidad química, física y biológica que principalmente depende de sus precursores, el mecanismo de formación, su complejidad estructural y presencia de otros constituyentes en el alimento (Rufián-Henares y Morales, 2007a; Morales y col., 2012). Básicamente se pueden agrupar en melanoproteinas cuando el esqueleto de la estructura está mayoritariamente constituido por proteínas, y melanoidinas cuando el esqueleto es mayoritariamente un carbohidrato (Fogliano y

Morales, 2011). El café, los productos de panadería, la pasta, la cerveza y el cacao son ejemplos de las principales fuentes dietéticas de estos compuestos. Las melanoidinas contribuyen a las propiedades organolépticas y de textura del alimento, sin embargo debido a su heterogeneidad se hace difícil su aislamiento e identificación (Rufián-Henares y Morales, 2007b; Fogliano y Morales, 2011).

1.3. Variables implicadas en el desarrollo de la Reacción de Maillard

Factores	Condiciones favorables
Naturaleza y concentración de reactantes	Carbohidratos: • Bajo peso molecular (O´Brien y Morrissey, 1989) • Forma acíclica (Labuza y Baisier, 1992) • Presencia de grupos cargados (Bunn y Higgins, 1981) • Aldosa > Cetosas (Yeboah y col., 1999) • Aumento concentración (Warmbier y col., 1976) Compuesto amino: • Aminoácidos. Grupos amino básicos, siendo la lisina la más reactiva (O´Brien y Morrissey, 1989) • Péptidos: mayor longitud de cadena (De Kok y Rosing, 1994) • Proteínas: amino terminal > aminoácidos básicos > aminoácidos azufrados (O´Brien y Morrissey, 1989)
pH	• 6-8 (Alais y Linden, 1990)
Temperatura y tiempo de calentamiento **Tratamiento culinario**	• Aumento de Temperatura (Martins y col., 2001) • Aumento de tiempo de almacenamiento (Ryu y col., 2003) • Fritura (Delgado-Andrade y col., 2010)
Actividad de agua	• 0.5-0.8 (Mathlouthi, 2001)
Otros factores	• Sales de Fosfatos (Reynolds, 1959) • Metales: cobre, hierro y cobalto en medio ácido (Ellis, 1959) • Productos de oxidación de lípidos (Pokorny, 1981)

Tabla 2: Factores que inciden en el desarrollo de la Reacción de Maillard

Como se ha comentado anteriormente, determinadas características físico-químicas del alimento como son la naturaleza y concentración de reactantes, pH, actividad de agua o concentración salina así como las condiciones de su procesamiento como la temperatura, el tiempo o tratamiento culinario son parámetros críticos para el progreso de la RM. En la **Tabla 2** se describen las condiciones más favorables para el avance de la RM en el alimento.

1.4. Consecuencias de la Reacción de Maillard en el alimento

1.4.1. Aspectos tecnológicos y organolépticos

El desarrollo de la RM en los alimentos implica la formación de una diversidad de compuestos que dotan al alimento de un aroma, sabor y color característico (Cerny, 2008). Tradicionalmente la industria alimentaria ha perseguido el desarrollo de la RM ya que aumenta la aceptabilidad general del producto por el consumidor, aunque en determinados grupos de alimentos, como por ejemplo en la leche y los de base mayoritariamente láctea, el objetivo es minimizar su extensión (Baltes, 1982). Se han identificado cuatro precursores principales del aroma y color: α-hidroxicarbonilos, α-dicarbonilos, α-aminocarbonilos y 1-aminoácido-2-carbonilo (Yaylayan, 2003). Mientras que los α-hidroxicarbonilos podrían ser considerados precursores de los otros tres, los α-dicarbonilos son intermediarios implicados en el desarrollo de color. Por otro lado, los α-aminocarbonilos se asocian con el aroma del alimento mediante la formación de compuestos aromáticos heterocíclicos que contienen nitrógeno como pirazinas, pirroles y oxazoles. La importancia del 1-aminoácido-2-carbonilo radica en su implicación en el color ya que son intermediarios de la formación de polímeros coloreados que contienen nitrógeno. Otro importante intermediario en la formación de compuestos coloreados es la acetilformoina que depende de la presencia de aminoácidos primarios o secundarios como reactantes (Yaylayan 2005). En definitiva, diversos intermediarios de la RM podrían tomar parte en la generación de las características organolépticas típicas del alimento.

1.4.2. Aspectos nutricionales

El bloqueo del grupo ε-amino de la lisina y su consiguiente transformación en el compuesto de Amadori es la consecuencia nutricional más significativa del desarrollo de la RM, reduciendo el valor biológico de la proteína del alimento. Dependiendo de la intensidad del tratamiento térmico, otros restos aminoacídicos en la proteína pueden verse afectados como son la arginina, histidina, triptófano y aminoácidos azufrados (O´Brien y Morrissey, 1989; Somoza, 2005). No obstante, hay discrepancias si la microflora intestinal podría regenerar los aminoácidos libres a partir de los compuestos de Amadori pero a muy bajos niveles (Moughan, 2003). Paralelamente, la digestibilidad de las proteínas se ve comprometida al verse reducida la actividad hidrolítica de las proteasas o peptidasas aumentando el nitrógeno fecal (Moughan, 2003). Se ha descrito que determinados PRM de bajo peso molecular podrían ejercer un efecto inhibitorio sobre enzimas digestivas. Ejemplo de ello son las premelanoidinas que inhiben la tripsina y la aminopeptidasa N (Martins y col., 2001; Malec y col., 2002) así como el HMF inhibe la carboxipeptidasa A (Seiquer y col. 2006). Sin embargo, algunos autores como Yeboah y col. (2004) apuntan a un aumento de actividad de algunas enzimas digestivas como la tripsina sobre la lisozima glicada, ya que al ser

modificada su conformación se aumenta la exposición de sus sitios activos y con ello su digestibilidad.

Tradicionalmente se ha asociado el desarrollo de la RM con la generación de sustancias antinutritivas. Las melanoidinas son estructuras polianiónicas que tienen un efecto quelante sobre determinados minerales pudiendo formar complejos solubles e insolubles interfiriendo así en su absorción, metabolismo, excreción y, en definitiva, en su biodisponibilidad (Delgado-Andrade y col. 2004a). A este respecto, la biodisponibilidad de minerales como el zinc, cobre, hierro, calcio, magnesio, fósforo y sodio podría verse comprometida (O´Brien y col. 1988; Delgado-Andrade y col. 2008; Mesías y col., 2009).

Sin embargo, en los últimos años se han puesto de manifiesto diversas propiedades beneficiosas derivadas del desarrollo de la RM como la actividad antimicrobiana (Rufián-Henares y Morales, 2007), el efecto prebiótico (Ames y col. 1999) y la capacidad antioxidante de determinados productos avanzados de la reacción (Lee y Shibamoto, 2002; Rufián-Henares y Morales, 2007b). A este respecto, numerosas investigaciones se han centrado en caracterizar las propiedades antioxidantes de las melanoidinas. Básicamente, su capacidad antirradicalaria y de quelación de iones metálicos confieren un papel relevante en la mitigación de la rancidez oxidativa en alimentos como cereales horneados (ej. galletas) y productos cárnicos procesados (ej., patés, hamburguesas) (Morales y col., 2012). Sin embargo, pese a los prometedores resultados obtenidos *in vitro* y a nivel celular, son escasos los estudios realizados en humanos (Somoza, 2005).

1.4.3. Aspectos toxicológicos

El desarrollo de la RM puede dar lugar a su vez a compuestos potencialmente nocivos con efectos mutagénicos, cancerígenos y alergénicos que pueden comprometer la seguridad del alimento (Lee y Shibamoto, 2002). En este sentido, se han descrito algunos compuestos con diverso potencial carcinógeno como son las aminas aromáticas heterocíclicas en carne y pescado (Felton y col. 2000), la acrilamida en patatas fritas (EFSA, 2015) o el furano en café (EFSA, 2010). En referencia a la alergenicidad de los PRM existen discrepancias entre distintos autores. Mientras que algunos defienden la idea de que los PRM se asocian con un aumento de la alerginicidad de proteínas de alimentos como es el caso de la proteína del cacahuete (Maleki y col. 2000), otros afirman que la glicación de proteínas puede reducir su carácter alergénico como en el caso de la soja (Babiker y col. 1998). En contraposición a los aspectos toxicológicos mencionados, algunas investigaciones han descrito actividades antimutagénicas (Wagner y col., 2007), antihipertensivas (Rufián-Henares y Morales, 2007b) o anticancerígenas (Somoza, 2005) de determinados PRM en sistemas modelo.

2. REACCIÓN DE MAILLARD *IN VIVO:* GLICACIÓN

2.1. Aspectos generales

Los organismos vivos homeotermos reúnen las condiciones fisiológicas adecuadas para que se desarrolle la RM. Por otra parte, la presencia y aporte constante de los principales reactantes de la reacción (glucosa, proteínas y lípidos) se conjugan con una temperatura que permanecerá alrededor de los 37 ^{o}C y que incidirá en la velocidad de reacción (Somoza, 2005). La RM cuando tiene lugar en sistemas biológicos (células, fluidos, tejidos y órganos) se denominada glicación o proceso de glicosilación no enzimático. A diferencia de los alimentos donde la combinación temperatura-tiempo es quizás el principal catalizador de la reacción, en los sistemas biológicos es la concentración del azúcar reductor (glucemia) el factor limitante, así como el tiempo de exposición (la edad). De este modo, el aumento de glucosa circulante o su deficiente clarificación promueven el proceso de glicación en el que se ven involucradas proteínas y, en ocasiones, lípidos endógenos para formar como fin último productos de glicación avanzada ("advanced glycation end products", AGEs) o productos de lipoxidación avanzada ("advanced lipoxidation end-products", ALEs) (Vistoli y col., 2013).

A principios de los años 80, los investigadores Monnier y Cerami, pioneros de la "teoría de la glicosilación no enzimática del envejecimiento", plantearon que durante el envejecimiento los entrecruzamientos mediados por AGEs sobre proteínas de larga vida contribuyen a la disminución de su función a nivel celular y de tejidos (Monnier y Ceremi, 1981). Es un hecho irrefutable la acumulación de AGEs en diferentes tejidos y órganos, acumulación que está directamente relacionada con la edad, el recambio de proteínas y el estado glucémico del individuo. El estudio del proceso de glicación *in vivo* ha recibido una atención creciente desde el ámbito médico y farmacológico en los últimos veinte años debido a la notoria asociación de los AGEs con ciertas enfermedades crónicas como enfermedades cardiovasculares, renales (Zieman y Kass, 2004; Semba y col., 2009a), neurodegenerativas (Münch, 1997), diabetes mellitus y sus complicaciones a largo plazo (Gould y col., 2011; Milne y Brownstein, 2013; Vlassara y Uribarri, 2014), así como durante el normal proceso de envejecimiento (Verzijl y col., 2000; Avery y Bailey, 2006).

2.2. Especificidades de la Glicación y formación de AGEs

La complejidad del proceso de glicación, al igual que ocurre en los alimentos, hace necesaria la esquematización del proceso para una mayor comprensión. Asimismo, se han descrito tres etapas: la etapa inicial, la etapa intermedia y la etapa avanzada tal y como se representan en la **Figura 3**.

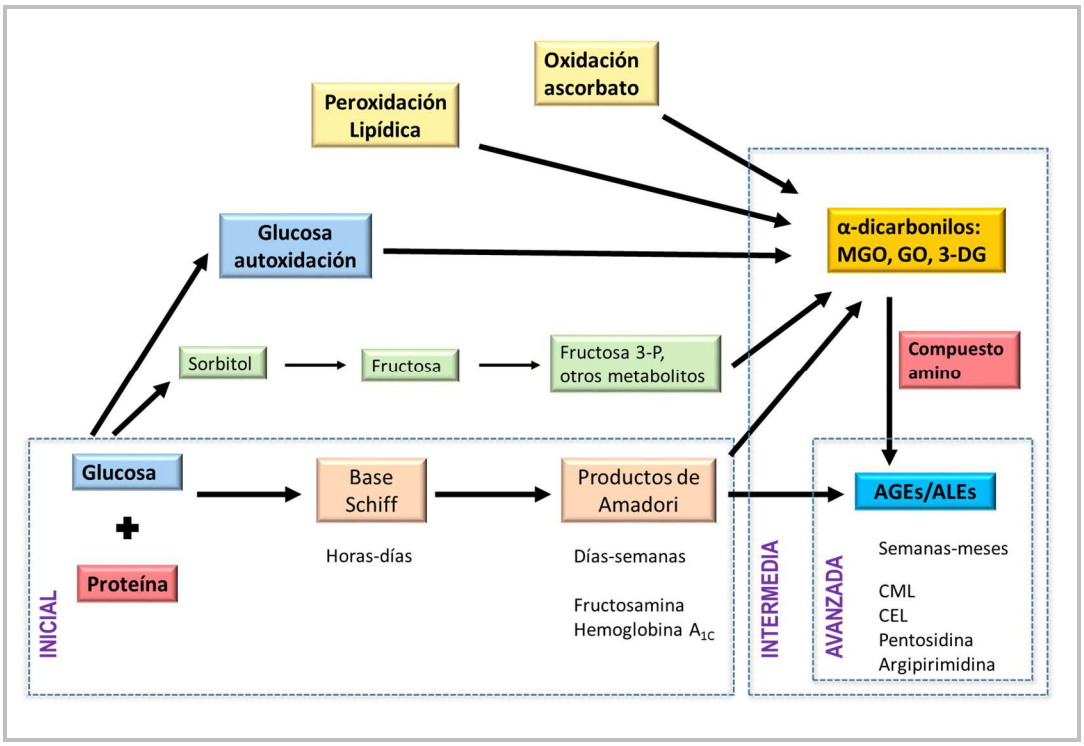


Figura 3: Esquema del proceso de formación de AGEs/ALEs in vivo (modificado de Luevano-Contreras y Chapman-Novakofski, 2010).

La ***etapa inicial*** de la glicación comienza cuando la glucosa se condensa con un aminoácido libre, proteína, lípido o DNA para dar lugar a la correspondiente base de Schiff. Esta etapa puede prolongarse durante horas y depende del tiempo de exposición a la glucosa y de su concentración, de tal modo que si la concentración de glucosa disminuye, el proceso puede ser revertido (Edeas y col., 2010). La base de Schiff puede sufrir una serie de reordenamientos que darán lugar a la formación de productos de la glicación inicial más estables, como es el producto de Amadori Nε-fructosil-lisina (fructosamina) cuya generación puede durar días (Peyroux y Sternberg, 2006) o bien puede oxidarse y generar directamente compuestos α-dicarbonilos. La proteína glicada más conocida es la hemoglobina glicada (HbA_{1c}) donde la glicación se produce sobre el resto N-terminal de la valina de su cadena β, siendo aún utilizada como indicador de la extensión de la diabetes (Tessier, 2010). La acumulación de productos de Amadori por un mantenimiento elevado de la concentración de glucosa como ocurre en la diabetes, puede llevar a que estos productos sufran oxidaciones, reducciones y deshidrataciones y guíen al proceso hacia la ***etapa intermedia***, en la que se generarán compuestos α-dicarbonilos y en su extensión hacia la ***etapa avanzada*** en la que se formarán los AGEs de manera irreversible durante semanas e incluso meses (Brownlee y col., 1984; Ahmed, 2005). De nuevo, los niveles de formación de AGEs dependerán de la duración de la hiperglucemia y de la velocidad de recambio de las proteínas más que de la concentración de glucosa (Ahmed, 2005).

Pese a que la glicación es la principal ruta de formación de AGEs, hay otras rutas que pueden ser consideradas también como precursoras, entre las que destacan i) la vía de los polioles, ii) la oxidación de la glucosa, y iii) la peroxidación lipídica. Estas vías alternativas al proceso de glicación serán capaces de generar compuestos α-dicarbonilos como el MGO, GO o 3-DG, que son increíblemente reactivos frente a ciertos aminoácidos de la cadena peptídica (preferiblemente lisina y arginina) (Thornalley, 2005; Uribarri y Tuttle, 2006) y que tendrán un papel fundamental en la formación de AGEs/ALEs como se describirá más adelante.

2.3. Formación endógena de α-dicarbonilos. Estrés carbonílico y glicoxidación

Pese a que el nivel circulante de glucosa es el principal desencadenante del proceso de glicación proteico, la glucosa presenta un menor ratio de glicación en comparación con otros azúcares reductores procedentes del metabolismo celular (Bierhaus y col., 1998). Las múltiples reacciones de fragmentación que sufren los restos de azúcares contribuyen de manera activa a la complejidad del proceso de glicación, puesto que constituyen destacados puntos de ramificación del proceso. De este modo se establecen nuevas reacciones que pueden desarrollarse paralelamente o incluso retroalimentar el proceso al reaccionar con compuestos de la etapa inicial (Lertittikul y col. 2007). Entre la amalgama de compuestos químicos que se generan, los compuestos α-dicarbonilos son clave en el proceso de glicación ya que pueden formar AGEs directamente, es decir, sin requerir precursores como los productos de Amadori al reaccionar directamente con proteínas, nucleótidos y fosfolípidos. Además, estos intermediarios son capaces de potenciar el ratio de glicación de proteínas ya que son 20000 veces más reactivos que la glucosa (Thornalley, 2005). *In vivo*, se han descrito cinco fuentes endógenas para la formación de los compuestos α-dicarbonilos: tres de ellas por acción enzimática, como la glicolisis, la vía de los polioles o la oxidación de aminoácidos y dos de ellas con un origen no enzimático, como la peroxidación lipídica y la glicación (Semchyshyn, y Lushchak, 2012). La contribución de la dieta como fuente exógena de compuestos α-dicarbonilos se ha puesto de manifiesto en algunas investigaciones que relacionan el consumo de estos compuestos a través de los alimentos con el incremento de los niveles circulantes (Uribarri y Tuttle, 2006) aunque esta aseveración no está del todo esclarecida. En la **Figura 4** se incluyen los principales α-dicarbonilos descritos en el proceso de glicación *in vivo* e identificados en su mayoría en alimentos que forman parte de la dieta occidental.

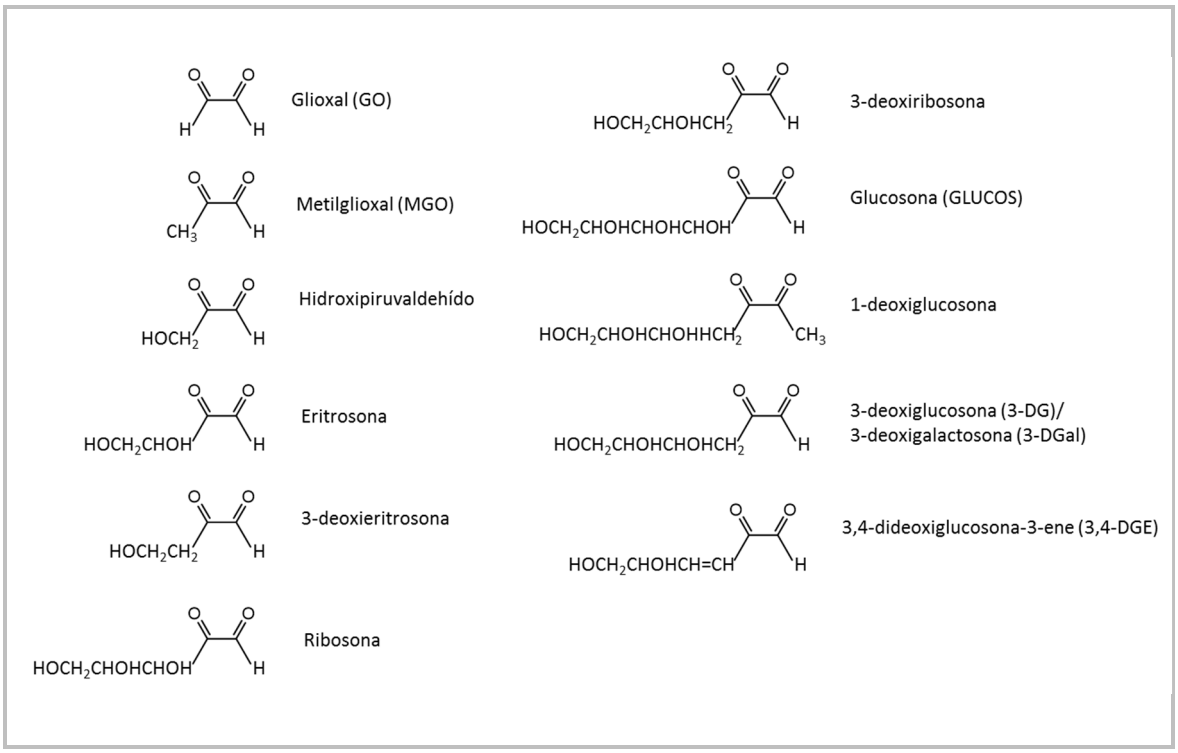
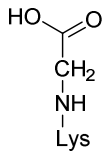
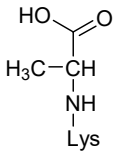
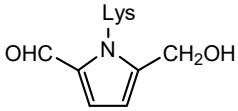
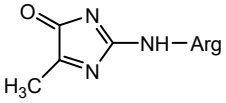
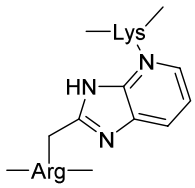
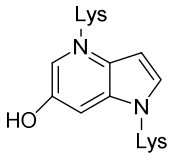


Figura 4: Principales compuestos α-dicarbonilos (Thornalley, 2005)

El término de estrés carbonílico fue descrito por Miyata y col. (1999) como la situación que se deriva del desequilibrio entre el ratio de formación de especies carbonílicas reactivas (RCS), procedentes de la oxidación de carbohidratos y lípidos, y el ratio de eliminación o inactivación de los mismos. Acorde con esta definición, numerosas investigaciones han relacionado estrechamente el estrés oxidativo producido por altas concentraciones de especies reactivas de oxígeno (ROS) con el incremento del estrés carbonílico y viceversa. Pese a que no es una condición *sine qua non* para que la glicación tenga lugar, cuando se entrecruzan ambos procesos forman los denominados productos de glicoxidación. Un claro ejemplo de producto de glicoxidación es la pentosidina, un AGE en cuya generación existe una asociación del estrés oxidativo con el proceso de glicación, ya que se ha demostrado que no se forma en ausencia de oxígeno (Sell y col., 1991; Bierhaus y col., 1998).

2.4. Tipología y análisis de AGEs

Los AGEs constituyen un grupo complejo y heterogéneo de compuestos que pueden ser clasificados en función de las propiedades de fluorescencia y de la capacidad entrecruzante de proteínas. En la **Tabla 3** se describen las estructuras y principales características químicas y biológicas de los AGEs más conocidos.

AGE	Propiedades	Precursores principales	Estructuras dianas	Referencias
AGEs No fluorescentes/No entrecruzantes				
Nε-carboximetil-lisina (CML) - 	$C_8H_{16}N_2O_4$ Pm: 204.22 g/mol CAS: 5746-04-3 AGE/ALE	GO Lys	- Colágeno - Cristalino - Suero - Riñón - Neuronas	Dunn y col., 1991 Nerlich y Schleicher 1999 Pamplona, 2011 Delgado-Andrade, 2016
Nε-carboxietil-lisina (CEL) 	$C_9H_{18}N_2O_4$ Pm: 218.25 g/mol CAS: 5746-03-2	MGO Lys	- Cristalino - Suero - Cartílago	Ahmed y col., 1997 Verzijl y col., 2000 Nagai y col., 2003
Pirralina 	$C_{12}H_{18}N_2O_4$ Pm: 254.28 g/mol CAS: 74509-14-1	3-DG Lys	- Suero - Glomérulo renal - Arterias renales	Miyata y Monnier, 1992 Monnier y col., 1996 Hellwig y col., 2015
MGO-hidroimidazolona isómero 1 (MGO-H1) 	$C_9H_{16}N_4O_3$ Pm: 228.25 g/mol CAS: 149204-50-2	MGO Arg	- Cristalino - Suero - Hígado - Corazón - Cerebro - Glomérulo renal - Colágeno	Ahmed y col., 2003 Thornalley, 2005
AGEs fluorescentes/ entrecruzantes				
Pentosidina - 	$C_{17}H_{26}N_6O_4$ Pm: 378.43 g/mol CAS: 124505-87-9 Fluorescencia: Em/Ext. 335nm/382nm	Lys Arg Ribosa	- Colágeno - Astrocitos - Hueso - Riñón - Cristalino	Nakamura y col., 1997 Tessier y col., 1999 Odetti y col. 2005 Salman y col., 2009
Vesperlisina - 	$C_{19}H_{28}N_4O_5$ Pm: 392.45 g/mol CAS: 188985-17-3 Fluorescencia: Ext./Em: - 370nm/440nm -	Glucosa Lys	- Lente - Riñón - Colágeno	Salahuddin y col., 2014

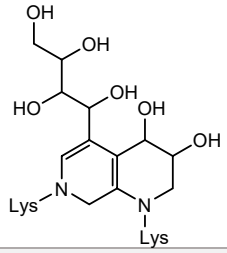
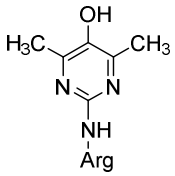
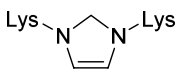
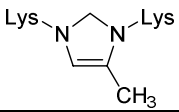
Croslina 	$C_{24}H_{40}N_4O_8$ Pm:512.60 g/mol CAS: 857058-48-1 Fluorescencia: Ext./Em: 370nm/440nm	Glucosa Lys	- Riñón - Cristalino - Suero	Ienaga y col., 1996 Nemet y col., 2011 Salahuddin y col., 2014
AGEs fluorescentes/ no entrecruzantes				
Argpirimidina (ArgP) 	$C_{11}H_{18}N_4O_3$ Pm: 254.29 g/mol CAS: 195143-52-3 Fluorescencia: Ext./Emm: 335nm/380nm	MGO Arg	- Suero - Cristalino - Riñón - Proteína amiloide -Tumores laringe y mama	Shipanova y col., 1997 Wilker y col., 2001 Van Heijst y col., 2005 Sreejayan y Yang, 2008
AGEs no fluorescentes/ entrecruzantes				
Dímero de glioxal-lisina (GOLD) 	$C_{15}H_{26}N_4O_4$ Pm: 327.40 g/mol CAS: 209267-39-0 AGE/ALE	GO 2 x Lys	- Suero - Cristalino	Frye y col., 1998 Durán-Jiménez y col., 1999 Pamplona, 2011
Dímero de metilglioxal-lisina (MOLD) 	$C_{16}H_{29}N_4O_4$ Pm: 341.43 g/mol	MGO 2 x Lys	Suero Cristalino	Frye y col., 1998 Durán-Jiménez y col., 1999

Tabla 3: Descripción y ubicación fisiológica de los AGEs más conocidos

Lys: lisina; Arg: arginina; Go: glioxal; MGO: metilglioxal; Ext.: longitud de onda de excitación; Emm.: longitud de onda de emisión

Entre los AGEs más relevantes se hará especial hincapié en la Nε-carboximetil-lisina, Nε-carboxietil-lisina, pentosidina y argipirimidina por ser los AGEs más estudiados y de mayor distribución en el organismo (Miyata y Monnier, 1992; Thornalley y col., 2003; Gomes y col., 2005):

- Nε-Carboximetil-lisina (CML)

  La CML se caracteriza por ser un AGE no fluorescente ni entrecruzante que puede generarse a partir de la interacción del grupo ε-amino de un resto de lisina con el GO, precursor generado en diferentes rutas como la oxidación de productos de Amadori, ácido ascórbico o lípidos, considerándose la CML en este último caso un ALE (Dunn y col., 1990; Ahmed y col., 1997;

Pamplona, 2011). Bajo esta premisa y en función de la ruta de obtención, la CML puede ser considerada como un producto de glicoxidación ya que en determinadas ocasiones es necesario un proceso de oxidación previo (Nguyen y col., 2014). La formación de CML se ha evaluado en alimentos procesados térmicamente y a nivel *in vivo,* en estudios con animales y humanos donde ha sido propuesto como un biomarcador de envejecimiento y procesos patológicos crónicos como la diabetes (Delgado-Andrade, 2016). Asimismo se han cuantificado niveles más elevados de CML en el colágeno de la piel, en la pared de los vasos sanguíneos y en la proteína del cristalino durante el envejecimiento (Dunn y col., 1991; Xu y col., 2003), en el riñón de pacientes diabéticos con un proceso de nefropatía (Zheng y col., 2002) o en neuronas en la enfermedad de Alzheimer (Münch y col., 1997).

- N^{ε}-Carboxietil-lisina (CEL)

  La CEL ha sido clasificada como un AGE no entrecruzante y sin características de fluorescencia, producto de la interacción entre un grupo amino libre de la lisina con el MGO. Se ha identificado en el plasma (Degenhardt y col., 1998), cartílago (Verzijl y col., 2000) y en el cristalino en una concentración similar a la CML. Sus niveles han sido correlacionados con la edad (Ahmed y col., 1997; Degenhardt y col., 1998).

- Pentosidina

  La pentosidina es considerada uno de los AGEs mejor caracterizados y más estudiados en el organismo. Se forma mediante una glicoxidación entre pentosas como la ribosa y grupos amino libres de lisina y arginina aunque también ha sido sugerida su formación a través de otros sustratos como la glucosa, fructosa o ascorbato (Wilker y col., 2001). Se caracteriza por su capacidad para formar entrecruzamientos y por sus propiedades fluorescentes. Los niveles de pentosidina en el organismo se han visto incrementados en pacientes con diálisis peritoneal y en enfermedades ligadas a la hiperglucemia, al estrés oxidativo y procesos inflamatorios como la retinopatía (Salman y col., 2009), nefropatía (Miyata y col., 1996), neuropatía (Salahuddin y col., 2014), lesión vascular (Yoshida y col., 2005), afectación ósea (Odetti y col., 2005) o artritis reumatoide (Miyata y col., 1998).

- Argipirimidina (ArgP)

  La ArgP es un AGE obtenido a partir de la interacción de residuos de arginina de proteínas con dos moléculas de MGO (Shipanova y col., 1997) y se caracteriza por sus propiedades fluorescentes y no entrecruzantes (Sreejayan y col., 2008). La ArgP ha sido detectada en proteínas del riñón y del cristalino, en tejidos tumorales y en proteínas amiloides de sujetos con

polineuropatía amiloide familiar (Gomes y col., 2005; van Heijst y col., 2005). Wilker y col. (2001) estimaron que los niveles de ArgP en proteínas del cristalino podían elevarse hasta 10 veces en personas de edad avanzada con respecto a jóvenes, e incluso 10 veces en procesos de cataratas con respecto a personas de edad avanzada. En este sentido, se estimó que la ArgP tanto en proteínas del suero como del cristalino, se hallaba en unos niveles 10-25 veces superiores que la pentosidina, AGE que no se deriva exclusivamente del MGO como la ArgP proponiendo que, la reacción del MGO con la arginina podría ser predominante con respecto a la reacción con lisina bajo condiciones fisiológicas (Shipanova y col., 1997; Wilker y col. 2001).

Técnica	Características	Ventajas	Inconvenientes
Espectrofluorometría	- Método inespecífico	- Rápido, sencillo, económico - Cribado inicial	- Determinación de AGEs fluorescentes - No específico - Interferencias
Inmunoensayo (ELISA, "*Enzyme-Linked ImmunoSorbent Assay*")	- Método específico - Uso de anticuerpos monoclonales que pueden ser específicos para un AGE o grupos de AGEs	- Rápido, sencillo - Ampliamente utilizado en matrices biológicas	- Especificidad del anticuerpo - Interferencias con la matriz que requiere validación - Coste elevado - Expresión de resultados en unidades arbitrarias (Kilounidades de AGEs)
Espectrometría de masas (ms/ms) acoplada a cromatografía de gases (GC) o líquida (LC)	- Método específico - Monitorización de ion molecular y fragmentos	- Alta especificidad y sensibilidad - Posibilidad de derivatización - Reproducible - No dependiente de la matriz - Comparable. Base de datos de niveles de AGEs en alimentos (Universidad de Dresden, DE)	- En GC, necesidad de obtener productos volátiles para la cuantificación - Hidrolisis ácida puede destruir determinados AGEs - Hidrólisis enzimática menos eficiente que hidrólisis ácida

Tabla 4: Métodos de análisis directos e indirectos para la cuantificación de AGEs (Poulsen y col., 2013; Nguyen y col., 2014; Kellow y Coughlan, 2015).

Los AGEs cuentan con propiedades físicas y polaridades muy diversas y este hecho, junto con el gran desconocimiento que existe aún de un gran número de ellos, hace de su cuantificación y detección un reto analítico (Poulsen y col., 2013). Actualmente existen diversos métodos para la determinación de AGEs y son clasificados básicamente como métodos específicos o inespecíficos. Mientras que los métodos específicos son capaces de monitorizar los niveles de un determinado AGE, los inespecíficos evalúan un grupo de AGEs que comparten alguna propiedad, como es el caso de la fluorescencia. La **Tabla 4** aborda brevemente los métodos de detección más destacados.

2.5. Mecanismos de acción de AGEs

Los efectos negativos de los AGEs en los sistemas biológicos son atribuidos fundamentalmente a su capacidad entrecruzante y a su acción inflamatoria y pro-oxidante (Brownlee y col., 1984; Ahmed, 2005). Así, estos efectos pueden ser ejercidos fundamentalmente a través de dos mecanismos de actuación, dependiente de receptor o independiente de receptor, tal y como muestra la **Figura 5:**

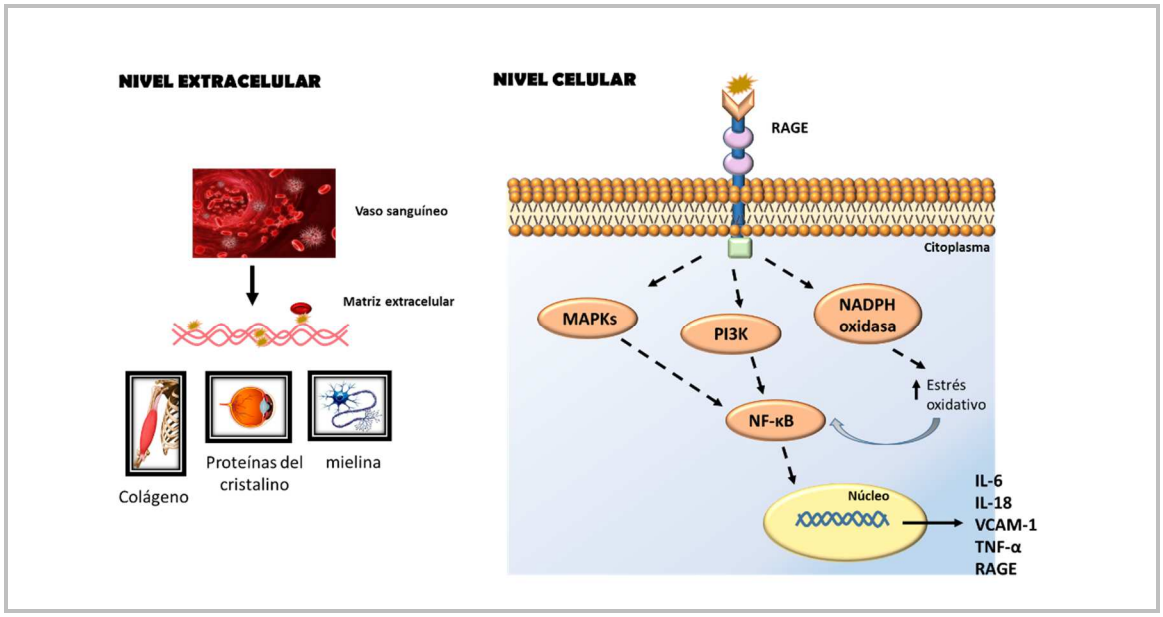


Figura 5: Mecanismos de acción de AGEs a nivel celular (dependiente de receptor) y a nivel extracelular (independiente de receptor). RAGE: Receptor de AGEs; MAPKs: proteínas quinasas activadas por mitógenos; PI3K: fosfatidil-inositol-3-quinasa; NADPH oxidasa: nicotinamida-adenina-dinucleótido-fosfato reducida oxidasa; NF-κB: factor de transcripción nuclear kappa-B; IL-6: interleuquina-6; IL-18 = interleuquina 18; VCAM-1: molécula 1 de adhesión vascular; TNF-α: Factor de necrosis tumoral-alfa

- Dependiente de Receptor

RAGE es un receptor que pertenece a la superfamilia de las Inmunoglobulinas y está ampliamente localizado en algunos tipos celulares como células del músculo liso, hepatocitos, neuronas, células endoteliales y monocitos (Xie y col., 2013). Presenta tres porciones diferenciadas: una porción extracelular, donde se localizan dos dominios constantes (C1 y C2) y uno variable (V) al cual se unirán los ligandos (Mosquera, 2010); una porción transmembrana, que lo mantendrá ubicado en la membrana plasmática celular y una última porción citosólica, mediante la cual se hace la transducción de la señal al interior celular (Kalousova y col., 2002). Pese a que los AGEs no son ligandos exclusivos de este receptor, la interacción de AGE-RAGE induce una serie de señalizaciones en las que pueden estar involucradas las vías de PI3K o MAPKs. La inducción de estas cascadas de señalización celular desencadenan la activación del factor de transcripción NF-κB, que tras ser translocado al núcleo, produce la transcripción de citoquinas proinflamatorias, factores de crecimiento y moléculas de adhesión como TNF-α, IL-6 o VCAM-1 (Mosquera, 2010; Xie y col., 2013). La interacción AGE-RAGE, además, podría activar la NADPH oxidasa que, aparte de activar el NF-κB y producir la transcripción de factores proinflamatorios, incrementa el estrés oxidativo intracelular. Asimismo, la activación de NF-κB puede producir un incremento de la expresión de RAGE, provocando una retroalimentación positiva que aumenta consecuentemente la producción de promotores de la inflamación (Wautier y col., 2001; Lin y col., 2009).

- Independiente de Receptor

Las consecuencias de la unión de AGEs a proteínas de bajo recambio como el colágeno y el entrecruzamiento AGE-AGE entre diferentes zonas de la misma proteína o entre dos proteínas distintas a través de enlaces covalentes, produce un aumento de la rigidez de las proteínas de la matriz. Esta rigidez compromete la función de las proteínas estructurales generando un incremento de la resistencia a su eliminación por mecanismos proteolíticos y afectando al proceso de remodelación tisular (Singh y col., 2001; Cárdenas-León y col., 2009). Cuando en el entrecruzamiento intervienen proteínas del plasma de vida media corta como lipoproteínas de baja densidad, albúmina o inmunoglobulinas y proteínas de la matriz extracelular, se generan una serie de aglomerados insolubles y resistentes a la acción proteolítica que se asocian a engrosamientos de la membrana basal de vasos sanguíneos e hipertrofia de la matriz extracelular, con las consecuencias patológicas que ello conlleva (Bucala y col., 1994; Monnier y col., 1996).

2.6. Implicaciones para la salud

2.6.1. Enfermedades cardiovasculares

Numerosos estudios han establecido una relación directa entre la acumulación de AGEs en los vasos sanguíneos y la aterosclerosis, una patología cardiovascular caracterizada por el engrosamiento y pérdida de elasticidad de las paredes arteriales como consecuencia de un proceso patológico que incluye la formación de una placa de ateroma (Nerlich y Schleicher, 1999; Zieman y Kass, 2004). Un estudio de la rigidez aórtica en diabéticos post-morten probó que los entrecruzamientos de AGEs con el colágeno jugaban un papel muy importante en la rigidez de los vasos sanguíneos. Se apreció que las LDL glicadas eran menos susceptibles de ser captadas por receptores celulares contribuyendo al entrecruzamiento del colágeno de la pared arterial. Asimismo, la acumulación de LDL modificadas por AGEs provocó su captación por parte de los macrófagos que condujeron a la formación de células espumosas y finalmente a la formación de la placa de ateroma (Bucala y col., 1994; Zieman y Kass, 2004). Por otro lado, la presencia de AGEs en tejido cardíaco ha sido relacionado con una alteración en la relajación del miocardio debido a la disminución de la vida media de la óxido nítrico sintasa endotelial, involucrada en la vasodilatación (Xu y col., 2003).

2.6.2. Diabetes

La Federación Internacional de Diabetes (*International Diabetes Federation*, IDF) estimó en 415 millones de personas afectadas por esta enfermedad en el año 2015 a nivel mundial suponiendo la diabetes mellitus tipo 2 el 85-95% de los casos en países desarrollados. La diabetes tiene una etiología multifactorial aunque cada vez cobra mayor relevancia el estilo de vida como factor crítico en su desarrollo. Se caracteriza por una inflamación crónica, una resistencia a la insulina y un incremento crónico del estrés oxidativo y, pese a que la hiperglucemia mantenida está asociada con sus complicaciones a largo plazo (Vlassara y Uribarri, 2014), otros factores como la acumulación de AGEs están siendo objeto de estudio en pacientes diabéticos (Duckworth y col., 2009; Skyler y col., 2009). Es un hecho conocido que la hiperglucemia está estrechamente relacionada con el proceso de glicación en proteínas de tejidos insulino dependientes, donde las células no poseen un mecanismo eficaz de control de entrada de glucosa como ocurre en células del tejido nervioso periférico, endoteliales, renales o del cristalino, que en estas condiciones incrementan la concentración de la glucosa o de otros compuestos más reactivos como los α-dicarbonilos.

Existen fuertes evidencias que relacionan la acumulación de AGEs en diabéticos con el desarrollo y/o agravamiento de las complicaciones a largo plazo que presenta esta enfermedad crónica. La **retinopatía diabética** se produce cuando los pequeños vasos sanguíneos de la retina se dañan y se

considera una de las principales causas de la ceguera. La acumulación de AGEs promueve la muerte de pericitos, el engrosamiento de la membrana basal de capilares, la mejora de la permeabilidad y consecuentemente las fugas vasculares, hechos que llevan a la expresión clínica de la retinopatía (Milne y Brownstein, 2013). Otra complicación importante en pacientes diabéticos es el proceso de **cicatrización de heridas** que puede llegar a ocasionar graves infecciones. En ratones diabéticos, la interacción AGE-RAGE ocasionó la transducción de señales que llevó a un incremento de TNF-α y MMP, factores proinflamatorios relacionados con el retraso de la cicatrización de heridas y deterioro de colágeno implicado en el proceso (Gould y col., 2011).

Sin embargo, aunque parece clara la relación entre una situación de hiperglucemia mantenida y la formación y acumulación de AGEs, en los últimos años el debate se ha centrado en cómo los AGEs dietéticos pueden contribuir como posible causa de esa situación patológica. Algunos autores mantienen que el consumo crónico de alimentos altamente procesados y con un elevado contenido de AGEs dietéticos podría causar una depleción y sobrecarga de los sistemas naturales de defensa antioxidante creando un desequilibrio que mantendría una situación de estrés oxidativo y de inflamación. Esta situación sostenida en el tiempo, ocasionaría un deterioro de la producción y sensibilidad de la insulina contribuyendo a la progresión de una situación prediabética a diabética (Vlassara y col., 2002; Uribarri y col., 2007 y 2011).

2.6.3. Enfermedades renales

Numerosos estudios han establecido que una mayor concentración de AGEs circulantes podrían ser predictores de una disminución de la función renal (Semba y col., 2009a). Los niveles elevados de AGEs activarán la cascada de señalización mediada por RAGE que llevará a la transducción de factores de crecimiento y citoquinas responsables de la promoción de la permeabilidad vascular y de la reducción de las actividades de barrera (Yamamoto y col., 2001) y por consiguiente, de una alteración en el funcionamiento renal. Por otra parte, la filtración glomerular se puede ver afectada por la acumulación de AGEs en el colágeno de la membrana basal provocando un engrosamiento de la misma (Ahmed, 2005). A este respecto, el estudio InCHIANTI, en Italia, en el que participaron hombres y mujeres de más de 64 años, demostró una relación directa entre la concentración plasmática de CML, la tasa de filtración glomerular y la enfermedad renal crónica durante un seguimiento de 6 años (Semba y col., 2009a). En el mismo sentido, otro macroestudio en Baltimore mostró que el 51.6% de los participantes disminuyeron la tasa de filtración glomerular al aumentar los niveles séricos de CML (Semba y col., 2009b).

2.6.4. Enfermedades neurodegenerativas

Las enfermedades neurodegenerativas, como la enfermedad de Alzheimer, Parkinson o esclerosis lateral amiotrófica, se caracterizan por la pérdida gradual y progresiva de tejido neuronal o neuronas, así como por una deposición de proteínas mal plegadas y/o agregados de proteínas. La enfermedad de **Alzheimer** es una de las enfermedades neurodegenerativas más comunes. Se caracteriza por la formación de dos tipos de agregados proteicos: las placas seniles en el espacio extracelular donde se localizan las proteínas modificadas β-amiloide y los ovillos neurofibrilares que se localizan en el interior de la neurona donde se encuentra la proteína tau. Estudios recientes han relacionado la acumulación de AGEs con la formación y agregación de proteína β-amiloide modificada y con la formación de ovillos neurofibrilares (Münch, 1997; Salahuddin y col., 2014). Así, la presencia de AGEs se ha vinculado con la regulación de la expresión de la proteína precursora amiloide y con la aceleración de la polimerización de β-amiloide (Ko y col., 2010). Otros estudios en ratas relacionan a los AGEs con una mayor fosforilación de tau afectando a las sinapsis neuronales y por tanto a la memoria del animal (Li y col., 2012). Además se ha observado que la proteína tau puede ser glicada en su sitio de unión a la tubulina e inducir estrés oxidativo (Ledesma y col., 1995).

2.6.5. Enfermedades osteoarticulares

El colágeno es el componente principal del tejido conectivo y como tal, la afectación del mismo por el proceso de glicación puede llevar a cambios estructurales e incluso biomecánicos del hueso, cartílago o tendón. Mesías y col. (2009) evidenciaron que dietas ricas en PRM consumidas por adolescentes produjeron una reducción significativa de la deoxipiridinolina, indicativo de una menor eficiencia de recambio óseo. Otros estudios en pacientes diabéticos de edad avanzada y mujeres menopáusicas relacionaron el acúmulo de AGEs a nivel del hueso con el deterioro de sus propiedades mecánicas. Se observó que un incremento del nivel de AGEs en hueso redujo la densidad y mineralización ósea, incrementando la porosidad y el riesgo de fracturas (Odetti y col., 2005; Delgado-Andrade y col., 2008; Yamamoto y col., 2008).

2.6.6. Envejecimiento

El envejecimiento es un proceso complejo y multifactorial donde la acumulación progresiva de daños con el paso del tiempo conduce a un trastorno funcional de células, órganos y tejidos y finalmente a la enfermedad y muerte (Viña y col., 2007). Actualmente hay descritas 300 posibles teorías del envejecimiento, sin embargo, ninguna de ellas puede explicar todos los cambios observados en dicho proceso (Gkogkolou y Böhm, 2012).

La acumulación cutánea de AGEs es una característica del envejecimiento de la piel, puesto que proteínas como la fibronectina, elastina y el colágeno de tipo I y IV que poseen una tasa de recambio lenta pueden ser modificadas por el proceso de glicación contribuyendo a la disfunción dermal (Mizutari y col., 1997; Verzijl y col., 2000). El colágeno glicado puede producir rigidez, disminución de la flexibilidad y cambio de la suceptibilidad a los estímulos mecánicos (Avery y Bailey, 2006) contando con una tasa de acumulación anual aproximadamente del 3.7% y con un incremento de 30-50% a los 80 años de edad (Dunn y col., 1991).

Las implicaciones en la salud de la acumulación de AGEs se resumen en la **Figura 6**.

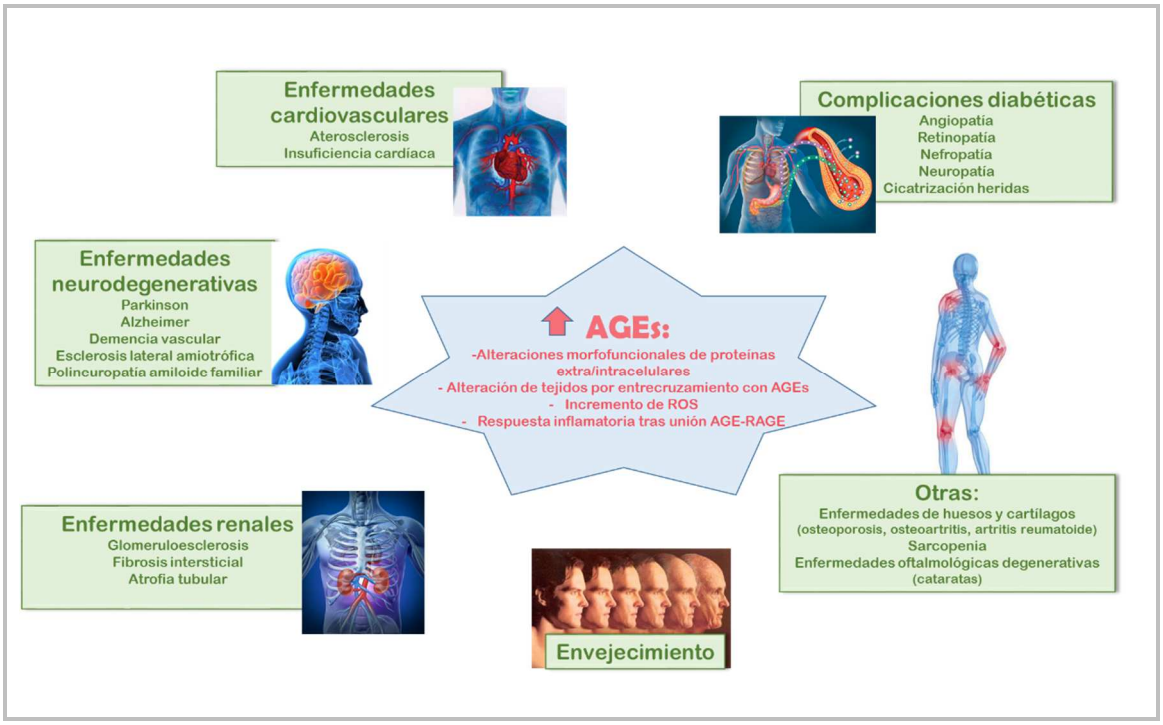


Figura 6: Acumulación de AGEs y sus implicaciones en la salud

2.7. AGEs dietéticos. Absorción y metabolismo

Los estudios sobre la absorción, biodisponibilidad, destino metabólico y excreción de los AGEs dietéticos han sido investigados en ratas y humanos pero el conocimiento es muy limitado. Pese a que existe una gran controversia, numerosos estudios indican que una dieta con un contenido elevado de AGEs dietéticos incrementa la carga de AGEs endógenos, ya que podrían ser absorbidos aumentando la circulación total de AGEs (Koschinsky y col., 1997) y promoviendo el incremento de factores proinflamatorios (López-García y col., 2004; Uribarri y col., 2005). Son muchos los autores que sustentan que los AGEs totales circulantes corresponden a la suma de AGEs formados endógenamente, como consecuencia de los niveles de azúcar en sangre, o por el consumo de

intermediarios reactivos y AGEs exógenos que puedan ser absorbidos a nivel intestinal (Degen y col., 2012). En este sentido, Delgado-Andrade y col. (2012) llevaron a cabo una investigación con adolescentes alimentados con dietas ricas y bajas en AGEs que confirmó que la absorción y excreción fecal de AGEs estuvo fuertemente influida por la dieta consumida aunque su eliminación urinaria alcanzó un límite o saturación.

Estudios en ratas han estimado que los AGEs dietéticos pueden llegar a ser absorbidos en un 10-30% a nivel intestinal (Faist y Erbersdobler, 2001) y tras pasar al torrente sanguíneo pueden ser distribuidos de forma generalizada por todo el organismo, aunque se muestra una preferencia por el tejido renal y hepático y en menor medida, corazón, pulmón y bazo (He y col., 1999). No obstante, el destino del 70-90% de los AGEs dietéticos restantes es objeto de numerosas investigaciones ya que, dado que los aminoácidos modificados por el calor de los alimentos escapan a esta digestión intestinal (Verzijl y col., 2000), se cree que una proporción significativa podría alcanzar el colon donde podría modular el crecimiento de la flora intestinal (Faist y Erbersdobler, 2001; Rai y col., 2012). Los AGEs con mayor peso molecular tienen unas tasas de absorción más lentas y menos eficientes, ya que necesitan ser degradados por proteasas para ser absorbidos. Por otro lado, estos AGEs pasarán por el intestino grueso para ser excretados por heces y/o actuar como un sustrato de fermentación de microorganismos colónicos (Tuohy y col., 2006). La unión de AGEs a proteínas o péptidos es otro factor a considerar en la posible absorción de los mismos. De acuerdo a esta afirmación, Grunwald y col. (2006) manifestaron que la CML libre podría ser absorbida por difusión simple mientras que Hellwig y col. (2011) constataron que la CML unida a dipéptidos sería absorbida por transportadores de péptidos como PEPT1, transportadores también utilizados por CEL, MGO-H1 o pirralina (Geissler y col., 2010; Hellwig y col., 2011).

Henle (2003) estimó que los humanos consumen alrededor de 500-1200 mg de productos de Amadori y 25-75 mg de AGEs procedentes de la dieta diaria. Sin embargo, pese al destacado consumo de estos productos, no existen resultados unánimes que relacionen la ingesta dietética de AGEs con los niveles circulantes (Cai y col., 2004; Uribarri y col., 2007; Piroddi y col., 2011; Semba y col., 2012). Se considera que los resultados contradictorios probablemente sean debidos a los distintos métodos de análisis utilizados, la inconsistencia en la duración y tipo de estudios aplicados, presencia de agentes interferentes y distractores, la preparación de alimentos y la diversidad de sujetos. En definitiva, se estima necesaria la realización de estudios epidemiológicos con un considerable número de participantes y con una duración a largo plazo, puesto que las consecuencias del consumo de AGEs no son debidas a ingestas puntuales sino a un consumo crónico y prolongado en el tiempo.

3. MECANISMOS DE ANTIGLICACIÓN

3.1. Sistemas fisiológicos de detoxificación

Con objeto de mantener estacionarios los niveles de RCS o reducirlos lo más rápidamente posible en un sistema biológico, existen una serie de mecanismos que pueden jugar un papel importante.

Por una parte, existen una serie de **sistemas enzimáticos** que metabolizan los RCS a compuestos menos tóxicos para ser posteriormente excretados de la célula (Atanasiu y col., 2006; Ellis y col., 2007). Una de las principales vías de catabolización de α-dicarbonilos a α-hidroxiácidos es la dirigida por el sistema glioxalasa que comprende las enzimas citosólicas glioxalasa I y II y el glutation. (Atanasiu y col., 2006; Ellis, 2007; Xue y col., 2011). El MGO es uno de los α-dicarbonilos con mayor relevancia en la formación de AGEs, por ello sus rutas de formación y degradación han suscitado un gran interés. El sistema de detoxificación mayoritario para el MGO incluye su transformación a D-lactato a través del sistema de las glioxalasas (Xue y col., 2011). Existen otras vías secundarias para la detoxificación del MGO como son su trasformación a acetol con la participación de la aldosa reductasa así como su oxidación a piruvato con la implicación de la betaína aldehído deshidrogenasa (ALDH9) o la 2-oxoaldehído deshidrogenasa (Vander Jagt y Hunsaker, 2003).

Por otra parte, los **inhibidores fisiológicos** de formación de AGEs son otros sistemas de detoxificación a considerar. Un claro ejemplo es la carnosina que está ampliamente distribuida en tejidos de mamíferos como el músculo y el cerebro. En sistemas modelo se ha observado que la carnosina podría ejercer una protección frente a la glicación y entrecruzamiento de AGEs a través de su efecto inhibitorio en los sitios de glicación proteica preferentes, como es acetil-lisina-histidina-amida (Münch y col., 1997).

Los **receptores de AGEs** podrían también estar involucrados en la detoxificación de AGEs. El AGE-R1 se ha descrito en células hepáticas y renales y su sobreexpresión se ha relacionado con la reducción de los niveles circulantes de AGEs y del estrés oxidativo, mejorando la resistencia a la hiperglucemia y protegiendo contra la inflamación *in vivo* (Lu y col., 2004). sRAGE es otro receptor destacable por ejercer como "señuelo" y competir por la unión de los ligandos de RAGE y de este modo mitigar la transducción de señales dependientes de RAGE (Lin y col., 2012).

3.2. Estrategias terapéuticas contra el estrés carbonílico y oxidativo

Las estrategias llevadas a cabo para prevenir o combatir el estrés carbonílico que frecuentemente viene asociado a un estrés oxidativo se centran fundamentalmente en los puntos críticos de las distintas etapas del proceso de glicación, así como en las posibles dianas o daños mediados por

AGEs (Peyroux y Sternberg; 2006; Peng y col., 2011) (**Figura 7**). A este respecto, las posibles estrategias terapéuticas pueden ser clasificadas en tres grandes bloques:

3.2.1. Terapia sobre el control del índice glucémico.

3.2.2. Terapia antioxidante.

3.2.3. Terapia antiglicante.

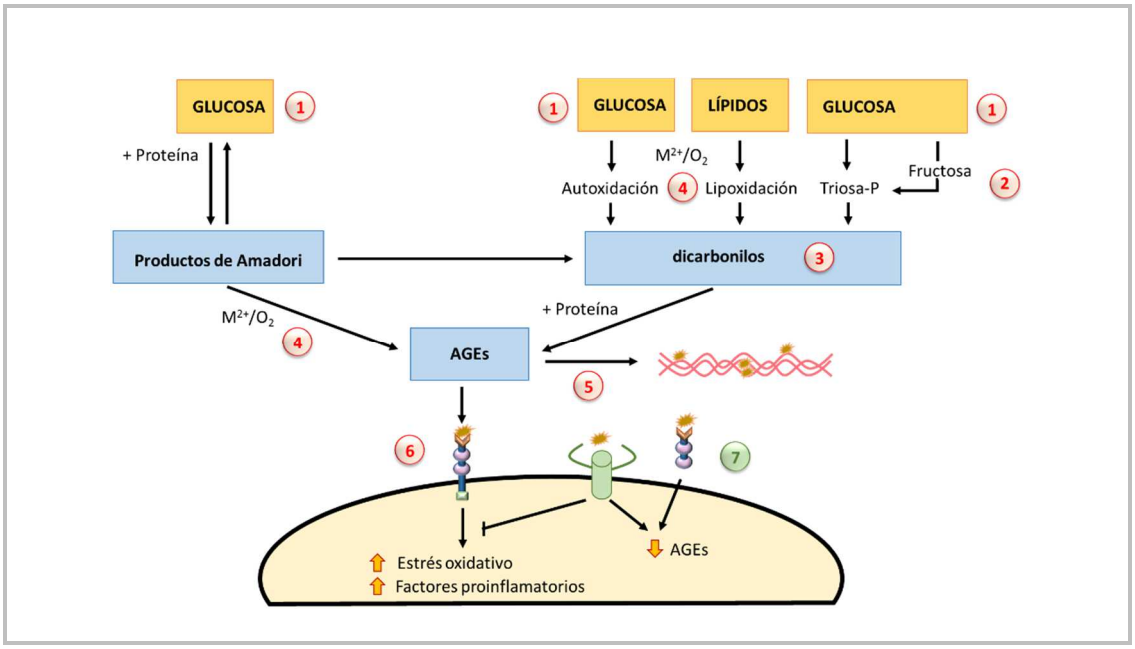


Figura 7: potenciales sitios de acción de compuestos antiglicantes. 1: hipoglucemiantes; 2: inhibidores de enzimas del metabolismo de la glucosa; 3: atrapantes de α-dicarbonilos; 4: quelantes de metales y secuestrantes de RL; 5: compuestos de escisión de entrecruzamientos; 6: bloqueantes AGE-RAGE; 7: potenciadores de sRAGE y AGE-R1 (modificado de Peyroux y Sternberg, 2006). M: metal.

3.2.1. Terapia sobre el control del índice glucémico

El nivel de glucosa en sangre es uno de los principales factores limitantes del desarrollo de la glicación y es por ello por lo que la normalización de los niveles de glucosa en condiciones de **hiperglucemia** es considerado un punto importante en las estrategias terapéuticas. La insulina, una hormona glucorreguladora, actúa simultáneamente en la captación de glucosa celular, aumenta la glucogénesis e inhibe la secreción de glucagón. Algunos fitoquímicos son capaces de mimetizar las actividades de la insulina para reducir los niveles de glucosa en sangre y mejorar la resistencia a la insulina. En este sentido, polifenoles como la rutina pueden actuar sobre el control metabólico de la glucosa mejorando su homeostasis por incremento de la insulina y del contenido de glucógeno en músculo e hígado (Stanley Mainzen Prince y Kamalakkannan, 2006). La **α-amilasa** y **α-glucosidasa** contribuyen al incremento de la glucosa en sangre, ya que son enzimas de ruptura de

polisacáridos en monosacáridos. Se ha descrito que el uso de fitoquímicos como la epigalocatequina, epicatequina galato, isoflavonas, ácido tánico, saponinas, ácido clorogénico, proantocianidinas y glicirricina son capaces de reducir la actividad de estas enzimas y el transporte de la glucosa a nivel intestinal (Gayova y col., 1999; Katsuya y col., 2003).

Otra enzima implicada en la reducción de la glucosa en sangre es la aldosa reductasa. La **aldosa reductasa** es una enzima de la vía de los polioles que reduce la glucosa a sorbitol, consumiendo NADPH, para que éste posteriormente se oxide a fructosa mediante la enzima sorbitol deshidrogenasa. Estudios en ratas diabéticas han sugerido que una inhibición de la aldosa reductasa podría reducir la formación de MGO y por consiguiente la formación de AGEs (Hallam y col., 2010). No obstante, esta vía de inhibición es aún controvertida, ya que en individuos normoglucémicos su activación constituye un mecanismo de disminución de los niveles de glucosa en sangre (Khangholi y col., 2016).

3.2.2. Terapia antioxidante

Existen dos focos fundamentales de actuación dentro de la terapia antioxidante, la quelación de metales y la captación de radicales libres. En un estado hiperglucémico, los metales de transición como hierro y cobre en presencia de oxígeno catalizan la autoxidación de la glucosa o la peroxidación lipídica generando productos de Amadori que tras reaccionar con proteínas forman AGEs. Con resultados similares, en la reacción de Fenton los metales de transición catalizan el peróxido de hidrógeno generando radicales hidroxilo altamente reactivos y potenciales responsables de la glicoxidación. La **quelación de metales** puede ocurrir a través de la unión de metales de transición a los grupos hidroxilo o carbonilo de polifenoles, destacando como ejemplo de dicho mecanismo los clorogénicos presentes en manzanas, fresas, piña, arándanos o café (Gugliucci y col., 2009). En este sentido, cabe destacar que numerosos estudios han establecido relaciones diversas entre la actividad quelante y la estructura molecular del polifenol. Más concretamente, en el caso de los flavonoides existe un consenso en cuanto a sus características estructurales y su actividad antioxidante. La presencia de la estructura catecol en el anillo B, un doble enlace en posición 2,3 y grupos hidroxilo en posición 3 y 5 son características que denotarán una potente actividad antioxidante, como es el caso de la quercetina (Martínez-Flórez y col., 2002; Khangholi y col., 2016).

Existen evidencias que señalan que el estrés oxidativo mantenido en el tiempo puede modular la génesis de las proteínas glicadas (Edeas y col., 2010). A su vez, una situación de hiperglucemia está asociada a un incremento de la autoxidación de la glucosa, glicación de proteínas y activación del metabolismo de polioles, procesos que incrementan el estrés oxidativo al llevar asociados un

incremento de ROS y una disminución de actividad de enzimas antioxidantes (Gil y Bengmark, 2007; Castelao y Gago-Domínguez, 2008). En este círculo vicioso creado entre el fenómeno de glicación y de oxidación, la mitocondria juega un papel central (Rosca y col., 2005). Las mitocondrias dañadas generan un mayor número de ROS intracelulares y un déficit de energía en forma de adenosín trifosfato (ATP) presentando una mayor dificultad para consumir glucosa y lípidos que son acumulados fuera de la mitocondria (Edeas y col., 2010). Ejemplos conocidos son los compuestos extraídos del ajo que han mostrado una actividad inhibitoria en la formación de AGEs/ALEs atribuido fundamentalmente a sus potentes propiedades antioxidantes y concretamente a su capacidad de **captar radicales libres** (Ramkissoon y col., 2012). Entre ellos, es reseñable la N-acetilcisteína cuyos grupos sulfidrilo libres tienen la capacidad de reaccionar con compuestos electrofílicos como los ROS para impedir que éstos puedan provocar la oxidación de moléculas y a su vez contribuya a una mejora del estatus del glutation celular, pieza fundamental del sistema antioxidante más importante del organismo (Khangholi y col., 2016). Además se ha descrito que polifenoles como flavonoides, carotenoides y tocoferoles son capaces de limitar la interacción de enzimas responsables de la generación de ROS con sus sustratos a través de la quelación o neutralización de metales de transición y donación de un electrón o hidrógeno a radicales libres como superóxido, peroxilo e hidroxilo (Dembinska-Kiec y col., 2008).

3.2.3. Terapia antiglicante

I. *Captación de especies carbonílicas reactivas*

En los últimos años ha cobrado especial relevancia la búsqueda de compuestos bioactivos capaces de captar α-dicarbonilos como mecanismo de inhibición del proceso de glicación ya que, como se ha comentado anteriormente, estos compuestos intermediarios son altamente activos en la formación de AGEs. A este respecto, Lo y col. (2011) concluyeron que los compuestos fenólicos con tres sustituyentes OH en el anillo benceno mostraban una mayor eficacia de atrapamiento de MGO tras una incubación de una hora en condiciones fisiológicas simuladas con respecto a los que contaban únicamente con uno o dos sustituyentes que apenas mostraban actividad. Adicionalmente, la presencia de un grupo carboxilo en el bencenotriol produjo impedimentos estéricos y cambios de carga en los carbonos, por lo que influyó negativamente en su reactividad con el α-dicarbonilo (Lo y col., 2011).

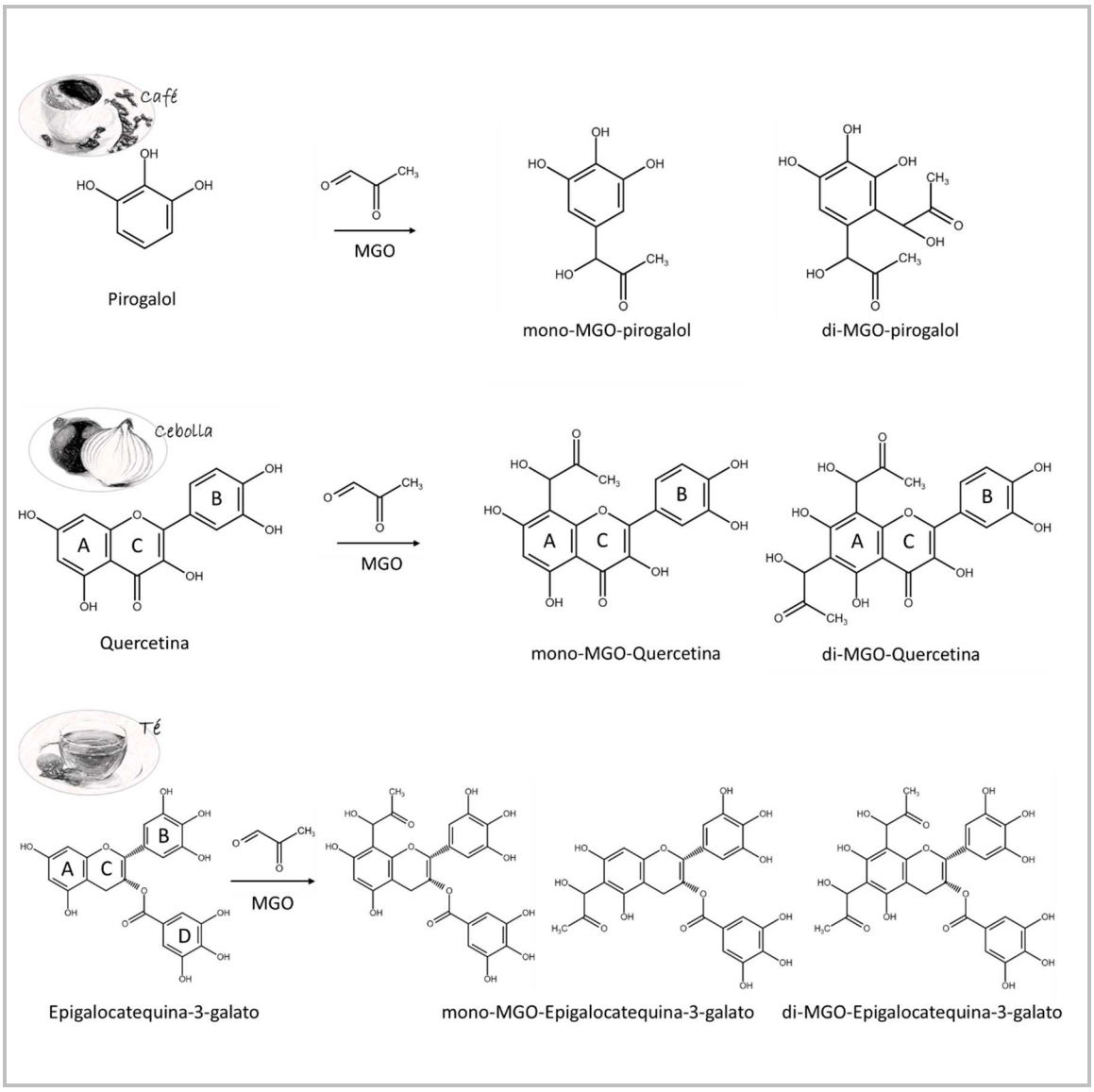


Figura 8: Atrapamiento de MGO por adición nucleofílica a compuestos fenólicos (Sang y col., 2007; Li y col., 2014; Shao y col., 2014)

Otro estudio similar llevado a cabo por Shao y col. (2014) mostró que el anillo A de los flavonoides era el responsable de la captación de MGO mediante una reacción nucleofílica. Concretamente, se describió que el pirogalol tiene su sitio activo en posición 5, donde el MGO se conjuga con dicho flavonoide para dar lugar al aducto pirogalol-MGO (**Figura 8**). Las posiciones orto mostraron ser más favorecedoras para el desarrollo de la reacción con el α-dicarbonilo que las posiciones meta, aunque el número de hidroxilos presentes en el anillo B o C no influyó.

A pesar de las conclusiones obtenidas, la mayoría de las investigaciones se han desarrollado a nivel *in vitro* por lo que existe un gran desconocimiento de cómo influyen ciertos factores que entran en juego *in vivo* como la biodisponibilidad de los flavonoides. Recientemente Wang y col. (2016) propusieron que la genisteína, la isoflavona más representativa de la soja, podría tener una

actividad antiglicante *in vivo*. Para ello y considerando un estudio previo que mostraba el atrapamiento de α-dicarbonilos *in vitro* (Lv y col., 2011), estudiaron su capacidad de captar MGO en ratones tras una administración por sonda oral de 400 mg/kg peso corporal. La confirmación de la detección en orina de la presencia de aductos formados por la reacción del MGO con el anillo A de la genisteína y seis metabolitos de la misma, supone un avance en el entendimiento de la posible actividad antiglicante *in vivo* de compuestos bioactivos presentes en la dieta.

II. *Nivel extracelular. Escisión de entrecruzamientos de AGEs*

Como se ha comentado previamente, el desarrollo de la glicación en el organismo es un proceso lento y condiciona que los entrecruzamientos de AGEs tengan lugar en proteínas con vida media larga. Las consecuencias de la formación de los entrecruzamientos de AGEs con proteínas estructurales se manifiestan con una evidente rigidez en tejidos, correlacionado con la gravedad de las complicaciones de la diabetes como neuropatías, nefropatías, cataratas y enfermedades cardiovasculares (Shikata y col., 1995; Milne y Brownstein, 2013; Salahuddin y col., 2014). A este nivel cabe destacar las investigaciones focalizadas en compuestos de síntesis y la carencia de datos relativos a la actuación de compuestos naturales. Asimismo, un estudio realizado en ratas diabéticas con el fármaco *ALT-711* (3-fenacil-4,5-dimetiltiazolium cloruro) postuló su capacidad de prevenir y escindir entrecruzamientos de AGEs establecidos con el colágeno e incluso con la albúmina y colágeno conjuntamente, refiriendo consecuentemente, una mejora de la hidratación de la piel y de la función diastólica ventricular (Vasan y col., 2003). Sin embargo, pese a los resultados obtenidos en ratas, en humanos los efectos de ALT-711 han sido de escasa relevancia o muy limitados.

III. *Nivel celular. Receptores y señalización celular*

La búsqueda de compuestos que incidan en la interacción de AGEs con su receptor RAGE y por tanto interfieran en las cascadas de señalización responsables del incremento del estrés oxidativo celular y la expresión de genes inflamatorios ha sido el blanco de numerosas investigaciones. Muestra de estos compuestos es la luteolina, implicada en la reducción de la expresión de RAGE y por consiguiente la expresión de genes inflamatorios de TNF-α, Interleuquina-1b o ciclooxigenasa-2 en cultivos celulares (Wu y col., 2009; Suh y col., 2016). Igualmente, se ha descrito que el ácido gálico podría mejorar la sensibilidad al receptor de insulina (Khangholi y col., 2016) y la curcumina estimularía la expresión de AGE-R1 en células hepáticas, receptor de AGEs responsable de su detoxificación (Lin y col., 2012).

3.3. Compuestos con actividad antiglicante

3.3.1. Productos de síntesis

Numerosos estudios han mostrado que la mayoría de los compuestos antiglicantes podrían estar actuando a distintos niveles en el proceso de glicación. A nivel farmacológico se han estudiado compuestos de síntesis que actúan principalmente en la inhibición de la formación de AGEs, sustancias con capacidad deglicante y bloqueantes de la unión AGE-RAGE y por consiguiente del progreso de la correspondiente señalización (Chinchansure y col., 2015).

La **aminoguanidina (AG),** uno de los primeros inhibidores de AGEs estudiados *in vitro* e *in vivo*, es una hidracina capaz de atrapar compuestos α-dicarbonilos como MGO, GO o 3-DG (Thornalley y col., 2000) y ejercer una actividad antioxidante al quelar radicales hidroxilos libres (Giardino y col., 1998). La AG se ha mostrado químicamente más reactiva que el grupo ε-amino de la lisina. Estudios iniciales establecieron una asociación de la AG con la inhibición de la formación de CML, CEL, entrecruzamientos y fluorescencia del colágeno de la piel de ratas diabéticas y por consiguiente con el retraso observado de complicaciones diabéticas como la nefropatía, neuropatía y vasculopatía (Brownlee, 1994), aunque sin demostrar la eficacia sobre la hiperglucemia. Sin embargo las investigaciones se vieron limitadas por los graves efectos secundarios observados en la fase clínica del compuesto en pacientes diabéticos tipo I, ya que durante el tratamiento se constató una inhibición de la óxido nítrico sintasa (Corbett y col., 1992), el desarrollo de anemias perniciosas y de anticuerpos anti-nucleares (Nilsson, 1999) así como la aparición de glomerulonefritis (Bolton y col., 2004) por lo que la fase clínica se vio suspendida. Independientemente de los efectos adversos observados, la AG sigue siendo, a día de hoy, un prototipo de compuesto antiglicante en la búsqueda de nuevas moléculas para dicho fin (Choi y col., 2008). En este sentido, la industria farmacéutica se ha centrado en la búsqueda de otros fármacos análogos a la AG, capaces de reproducir la potente acción antiglicante salvando los efectos secundarios. Actualmente algunos compuestos se encuentran en una fase preclínica donde cobra especial relevancia la acción que puedan ejercer a nivel de la interacción AGE-RAGE, rompiendo AGEs ya preformados o interfiriendo en la formación y acumulación de los mismos (Peyroux y col., 2006). Entre los potentes antiglicantes farmacológicos propuestos detacan la metformina, pioglitazona, pentoxifilina (Rahbar y col., 2000), fármacos antiinflamatorios como el ibuprofeno, el ácido acetilsalicílico (Sobal y Menzel, 2000) o el diclorofenaco (Van Boekel y col, 1992).

3.3.2. Fuentes naturales

Tras el estudio de productos de síntesis con potente actividad antiglicante pero con considerables efectos secundarios, la piridoxamina emergió como un potente anti-AGE de origen natural y digno sustituto de los mismos. La piridoxamina es un vitámero de B6 que puede ser introducido a través de la dieta en forma de piridoxamina-5´-fosfato aunque sucesivas modificaciones tienen lugar a su paso por el tracto intestinal para finalmente en el hígado transformarse en piridoxal-5´-fosfato (Voziyan y Hudson, 2005). Sus mecanismos antiglicantes han sido justificados principalmente por su capacidad de bloqueo de la degradación oxidativa de productos de Amadori o por el secuestro de ROS y productos carbonílicos reactivos procedentes de azúcares y lípidos.

Actualmente la FDA (Food and Drug Administration) no ha aprobado ningún medicamento para la inhibición de la glicación de proteínas que retrase o evite la progresión de las complicaciones diabéticas (Dorsey y Greenspan, 2014). Por esta razón y ante la ventaja de sustituir los compuestos de síntesis por compuestos naturales con similar actividad y con la posibilidad de introducirlos como parte de la dieta diaria, las fuentes naturales con alto contenido en fitoquímicos bioactivos han constituido un enfoque de gran interés para combatir el desarrollo de enfermedades crónicas. En la **Tabla 5** se incluye una selección de ejemplos de extractos naturales de diferente procedencia que han sido descritos como potenciales agentes antiglicantes. Entre los más destacados se encuentran las especias, frutas, hortalizas, hierbas y semillas cuya riqueza en fitoquímicos bioactivos les confiere la actividad buscada.

Las *especias* han sido utilizadas durante miles de años como agentes aromatizantes, colorantes y conservantes de alimentos aunque destacan especialmente por ser utilizadas como remedio natural para el tratamiento de ciertas enfermedades crónicas por su alto contenido en compuestos bioactivos (Srinivasan, 2005). Recientemente Moniruzzaman y col. (2015) determinaron la actividad antiglicante de 40 especias en diferentes modelos de glicación *in vitro* donde el clavo (*Syzygium aromaticum*) mostró la mayor actividad antiglicante correlacionada directamente con su mayor contenido en **compuestos fenólicos**. Otro grupo con un alto contenido en compuestos fenólicos son las *frutas y hortalizas*. Estudios llevados a cabo *in vitro* en la *granada* (*Punica granatum*) destacan sus propiedades antioxidante, antidiabética, hipolipemiante, antiinflamatoria, antitumoral y cardiosaludable atribuidas a su contenido fenólico y especialmente a su contenido en taninos hidrolizables, punicalagina, antocianinas y derivados del ácido elágico (Viuda-Martos y col., 2010). En este sentido, Chao y col. (2010) concretaron que una suplementación dietética de un 5% de ácido elágico durante 12 meses en ratones diabéticos redujo la HbA_{1c}, la glicación de la albúmina y la formación de AGEs como la CML y pentosidina, actividad atribuida *in vitro* a su

capacidad de atrapar α-dicarbonilos (Muthenna y col., 2012). El *té*, una bebida popular ampliamente consumida en todo el mundo es otro ejemplo de extracto natural con actividad antioxidante y antiglicante correlacionadas con su composición en polifenoles. Concretamente, la (-)-epigalocatequina-3-O-galato (EGCG) y (-)-epicatequina-3-O-galato (ECG) han mostrado ser las responsables de la actividad antiglicante *in vitro* (Nakagawa y col., 2002) y en cultivos celulares donde se ha descrito el atrapamiento de α-dicarbonilos como principal vía de actuación. Asimismo, en estudios con ratas se ha observado la inhibición de entrecruzamientos con el colágeno, la activación de RAGE y la acumulación de ciertos AGEs como la CML y CEL (Rutter y col., 2003; Yamabe y col., 2009).

Fuente natural	Componentes bioactivos	Mecanismo antiglicante	Referencias
Especias			
Ajo (*Allium sativum*) 	S-alilcisteina S-etilcisteína N-acetilcisteína	Antioxidante (⊘ RL) ↓ AGEs y HbA_{1c} ↓ LDL glicado	Ahmad y Ahmed, 2006 Lin y Yin, 2008
Romero (*Rosmarinus officinalis*) 	Ác. Rosmarínico Carnosol y derivados	Antioxidante ⊖ LDL oxidadas ⊘ α-dicarbonilos	Kim y Kim, 2003 Hsieh y col., 2007
Canela (*Cinnamomum zeylanicum*) 	Catequina Epicatequina Procianidina B_2	Antioxidante Imitación insulina ⊘ α-dicarbonilos	Peng y col., 2008 y 2010 Qin y col., 2010 Zhang y col., 2015
Cúrcuma (*Curcuma long*) 	Cúrcuma	↓ Glucosa ↓ Sorbitol deshidrogenasa	Jain y col., 2009

Frutas, Vegetales y Hierbas

Arándanos (*Vaccinium myrtillus*) 	Antocianidinas Procianidinas Quercetina	Inactivación NF-kB ⊘ α-dicarbonilos ↓ Productos de Amadori	Wang y col., 2011 Nair y col., 2006
Grosella negra (*Ribes nigrum*) 	Antocianidinas Delfinidina-3-rutinósido Cianidina-3-rutinósido	Antioxidante ⊘ α-dicarbonilos	Chen y col., 2014
Granada (*Punica granatum*) 	Polisacáridos Ác. elágico, ác. gálico Punicalagina Antocianinas Taninos hidrolizables	Antioxidante ⊘ α-dicarbonilos ↓ AGEs y HbA_{1C}	Kokila y col., 2010 Chao y col., 2010 Muthenna y col., 2012 Liu y col., 2014
Uva tinta (*Vitis vinifera*) 	Antocianinas Resveratrol	Antioxidante ⊖ LDL oxidadas ⊘ α-dicarbonilos	Harsha y col., 2016 Wenzel y col., 2005
Brócoli (*Brassica oleracea*) 	Sulforafano Kaempferol	Antioxidante; ⊖ LDL oxidasa ↓ Resistencia insulina ⊘ α-dicarbonilos ↓ Expresión RAGE Inactivación NF-κB	Bahadoran y col., 2013 Maeda y col., 2014
Calabaza (*Curcubita pepo*) 	Polisacáridos Trigonelina Ácido nicotínico	⊖ Aldosa reductasa ↓ HbA_{1C}	Yoshinari y col., 2009 Wang y col., 2012
Tomate (*Solanum Lycopersicum*) 	Rutina Quercetina Licopeno Kaempferol	Antioxidante ↓ Resistencia insulina ⊘ α-dicarbonilos Bloqueo AGE-RAGE	Kiho y col., 2004 Tabrez y col., 2015 Cervantes-Laurean y col., 2006 Abo-Salem y col., 2014

Yerba mate (*Ilex paraguariensis*) 	Ác. clorogénico Ác. caféico	Antioxidante ↓ AGEs	Gugliucci y col., 2009 Lunceford y Gugliucci, 2005
Té (*Camellia sinensis*) 	Epigalocatequina-3-o-galato Epicatequina	Antioxidante ⊘ α-dicarbonilos ⊖ Entrecruzamientos ⊖ AGE-RAGE	Rutter y col., 2003 Nakagawa y col., 2002 Sang y col., 2007
Soja (*Glycine max*) 	Vitexina Isovitexina Isoflavonas (Genisteína)	Antioxidante: ⊘ RL quelación metales ⊘ α-dicarbonilos	Wang y col., 2016 Peng y col., 2008

Hongos, Carnes, Pescado y Algas

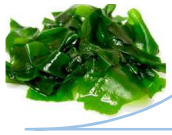
Hongos: Cordyceps Sinensis militaris 	Ergosterol	Antioxidante ↓ Productos de Amadori, AGEs, α-dicarbonilos ⊖ Señalización vía RAGE	Tai y col., 2016
Aceite de pescado 	Omega-3: Eicosapentaenoico docosahexaenoico	Antioxidante Antiinflamatorio ⊘ Inactivación NF-kB ↓ α-dicarbonilos, HbA_{1C} Productos de Amadori, AGEs	Jangale y col., 2013
Carne 	Creatina Carnosina	Antioxidante ↓ LDL glicadas ⊘ α-dicarbonilos	Löbner y col., 2015 Wu y col., 2011
Alga Chlorella 	Astaxantina Luteína Ác. araquidónico Ác. linoleico Ác. eicosapentanoico	Antioxidante: ⊘ RL ↓ quelación metales Antiinflamatorio ↓ Productos de Amadori	Sun y col., 2010 y 2011 Jangale y col., 2013

Tabla 5: Fuentes naturales de compuestos con potencial actividad antiglicante.

↓disminución; ⊖ inhibición; ⊘ atrapamiento/bloqueo

Actualmente, fuentes de compuestos antiglicantes alternativas a los compuestos fenólicos - provenientes de fuentes vegetales están siendo también objeto de estudio. Recientemente Löbner y col. (2015) en un estudio con voluntarios sanos siguiendo una dieta no vegetariana y vegetariana, postularon que la **creatina** procedente del consumo de carne podía interaccionar hidrofílicamente con el MGO formando aductos MGO-creatina identificados en orina (Löbner y col., 2015). Las microalgas como *Chlorella* podrían ser consideradas una fuente emergente de componentes antiglicantes como la luteína, astaxantina o determinados **ácidos grasos insaturados** de los que se cree que pudieran tener dicha actividad y que actualmente están en estudio (Sun y col., 2010). Otro compuesto a destacar es el **ergosterol**, esterol característico de los hongos que ha sido estudiado *in vitro* como posible agente anti-AGEs. Tai y col. (2016) evidenciaron que el ergosterol tenía efectos inhibitorios en la transcripción de genes proinflamatorios vía RAGE y en la generación de fructosamina, AGEs y α-dicarbonilos al reducir el estrés oxidativo. Es de prever que ciertas **vitaminas** con actividad antioxidante puedan mostrar cierta capacidad antiglicante como la vitamina C o E. Aoki y col. (1992) observaron una reducción del estrés oxidativo y consecuentemente una reducción de AGEs en ratas diabéticas suplementadas con vitamina E. En el mismo sentido, una dieta suplementada con 1000 mg de ácido ascórbico durante 4 semanas en individuos redujo la prevalencia de proteína glicada en un 46.8% (Vinson y Howard, 1996).

4. PRODUCTOS Y SUBPRODUCTOS DEL OLIVO

4.1. Producción y consumo

El olivo (*Olea europaea L.*), perteneciente a la familia botánica Oleaceae, es un árbol de origen milenario muy valorado por su fruto, la aceituna, una pequeña drupa ovoide de sabor amargo que es utilizada para la obtención del alimento base de la dieta mediterránea, el aceite de oliva. Actualmente en España existen más de 260 variedades cultivables aunque poco más de veinte son las más cultivadas suponiendo la picual el 50% de los olivos de España y el 20% a nivel mundial (Martínez y Villarino, 2005). España es el país con mayor superficie y producción mundial de aceite de oliva seguido a gran distancia por Italia y Grecia. Sus más de 300 millones de olivos ocupan una superficie superior a los 2.5 millones de hectáreas y aunque se extiende por 34 provincias españolas predomina en la mitad meridional. En la cosecha 2013/2014 la producción española de aceite de oliva supuso el 72% de la producción de la Unión Europea y el 55% de la mundial (MAGRAMA, 2016).

El aceite de oliva supone uno de los mejores ejemplos de alimento que concilia la gastronomía y la salud. Numerosos estudios epidemiológicos han puesto de manifiesto que el consumo de aceite de oliva reduce el riesgo de enfermedad coronaria, obesidad, síndrome metabólico, diabetes mellitus tipo 2 y ciertos tipos de cáncer (Tuck y Hayball, 2002; Lee y col., 2009; Lockyer y col., 2012), reconocimientos estudiados paralelamente a las virtudes atribuidas a la dieta mediterránea.

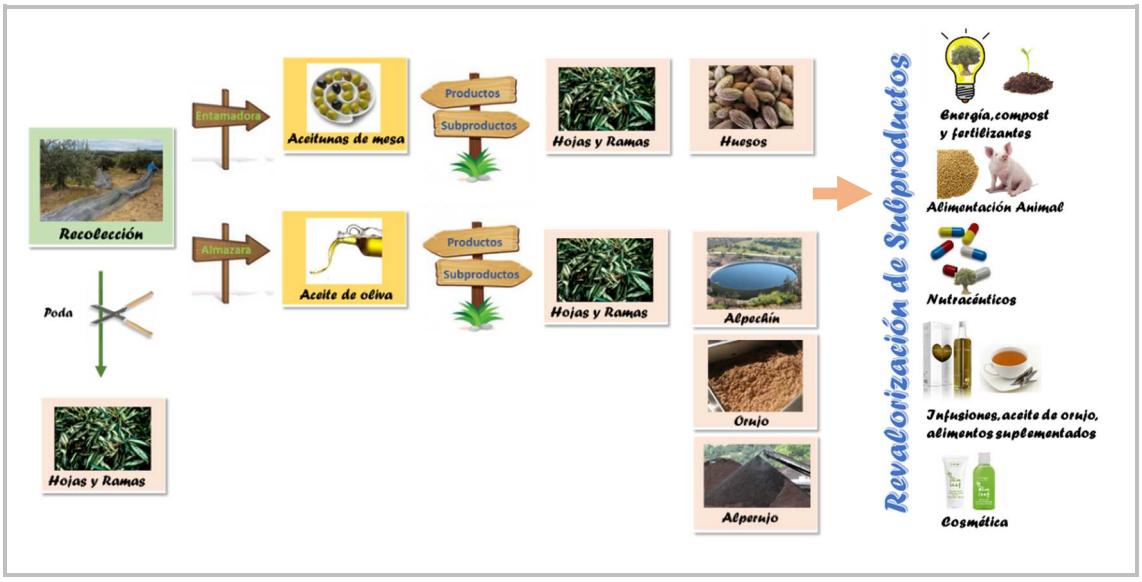


Figura 9: Obtención de subproducto del olivo y posible valorización

Sin embargo, durante la recogida y procesado del fruto del olivo para obtener el aceite de oliva virgen se generan una serie de subproductos de gran relevancia a día de hoy ya sea por su valor energético o nutricional como por el problema medioambiental que pudieran ocasionar, ya que para obtener un litro de aceite de oliva son necesarios de 4 a 5 kilos de aceitunas (Delgado y Estrella, 2009). La **Figura 9** muestra un esquema de los subproductos derivados de la obtención del aceite de oliva y de aceitunas de mesa, así como las propuestas de valorización y aprovechamiento de los mismos.

Entre los subproductos del olivo cabe destacar el **orujo**, **huesos de aceituna**, **hojas** y **ramas de poda** que pueden ser utilizados como combustible de biomasa para la obtención de energía térmica, eléctrica o bioetanol. Los orujos además, pueden ser utilizados para alimentación animal al mezclarlo con salvado o para la obtención de aceites de orujo. Por su parte, las hojas de olivo se han utilizado tradicionalmente como ungüento para el tratamiento de estados febriles y tratamiento de enfermedades como la malaria (El y Karakaya, 2009) y actualmente gozan de una creciente reputación. Otros subproductos característicos que suponen un gran problema medioambiental por su elevada carga orgánica son el **alpechín**, agua de vegetación obtenida tras la extracción y decantación del aceite y el **alperujo**, mezcla de alpechín y orujo. Actualmente la reutilización de estos subproductos mediante una purificación y modificación orgánica para la obtención de fertilizantes o recuperación de compuestos fenólicos es objeto de estudio (Giuffrè y col., 2012).

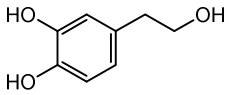
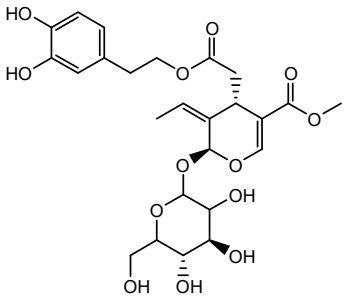
4.2. Fracción fenólica y efectos sobre la salud

Históricamente, los beneficios obtenidos por el consumo de aceite de oliva virgen se han atribuido a su fracción saponificable, que constituye el 95-99% del producto y más concretamente a su perfil lipídico monoinsaturado destacado por su contenido en ácido oleico (Tripoli y col., 2005). El ácido oleico representa el 70-80% de la fracción saponificable y se ha relacionado con la disminución de la incidencia de aterosclerosis al producir un incremento en los niveles de lipoproteínas de alta densidad, una disminución de los niveles de lipoproteínas de baja densidad y específicamente una reducción de la peroxidación de éstas (Tripoli y col., 2005). Sin embargo, en posteriores estudios con aceites de otras semillas con alto contenido en ácidos grasos monoinsaturados como el aceite de girasol, de colza o soja, no se constataron mejoras en determinados biomarcadores fisiológicos relacionados con el riesgo de enfermedades crónicas que sí se habían confirmado con el aceite de oliva (Aguilera y col., 2004). Tras estos resultados, estudios *in vivo* e *in vitro* ratificaron que la fracción insaponificable que representa el 0.4-5% del aceite de oliva, contiene una serie de compuestos fenólicos con destacada actividad antioxidante responsables de los efectos

beneficiosos que marcaban la diferencia con respecto al resto de aceites de semillas (Cicerale y col., 2010).

Considerando los conocimientos existentes hasta el momento de los beneficios del consumo de aceite de oliva, la Food and Drug Administration (FDA, EEUU) relacionó el consumo diario de 23 gramos de aceite de oliva con una reducción de riesgo coronario (López-Miranda y col., 2010). Asimismo, la Autoridad Europea de Seguridad Alimentaria (EFSA) en 2011 estableció que "el consumo de polifenoles del aceite de oliva contribuye a la protección de los lípidos sanguíneos frente al daño oxidativo", por ello recomienda el consumo de 5 mg de hidroxitirosol y sus derivados al día a través del consumo moderado de aceite de oliva dentro de una dieta equilibrada.

El contenido en compuestos fenólicos del olivo depende de varios factores como el clima, el grado de maduración, parte de la planta y procesamiento (Ryan y col., 2003). Por ejemplo, las hojas tienen un mayor contenido en polifenoles que el fruto (1450 mg/100 g de hoja fresca frente a los 110 mg/100 g de fruto (Lockyer y col., 2016)) mientras que el contenido fenólico del aceite de oliva virgen (23 mg/100 mL) (Owen y col., 2000) se verá intensamente mermado al someter al aceite a un refinado. Si se concreta en función del compuesto fenólico, la oleuropeína se encontrará como fenol mayoritario en hojas con respecto al fruto mientras que el contenido en hidroxitirosol aumenta en los frutos durante la maduración (Ryan y col., 2003).

Polifenol	Características	Referencias
Hidroxitirosol $C_8H_{10}O_3$ Pm: 154.16 g/mol CAS: 10597-60-1		Cornwell y col., 2008 Fernández-Mar y col., 2012 Hu y col., 2014 Vilaplana-Pérez y col., 2014
Propiedades: antioxidante (captación RL, quelación de metales), antiinflamatoria, antiaterosclerótica, cardioprotectora, neuroprotectora, antitumoral, antibacteriana, antidiabética		
Oleuropeína $C_{25}H_{32}O_{13}$ Pm: 540.51 g/mol CAS: 32619-42-4		Hamdi y Castellón, 2005 El y Karakaya, 2009 Omar, 2010 Ahmadvand y col. 2014
Propiedades: antioxidante (captación RL), antitumoral, hipoglucemiante, hipolipemiante, antiaterogénico, neuroprotector, antiviral		

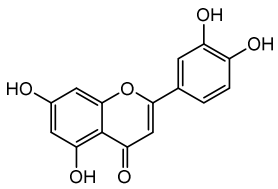
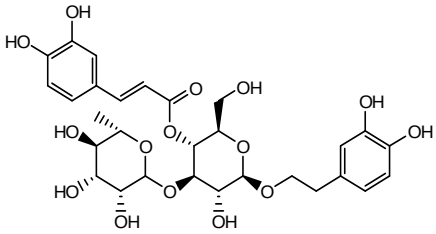
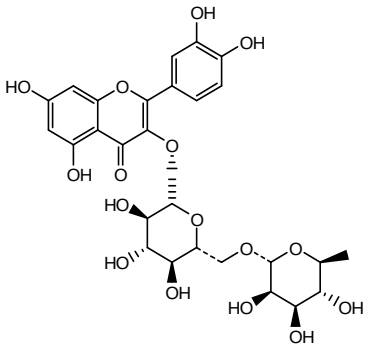
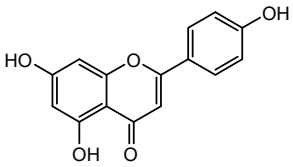
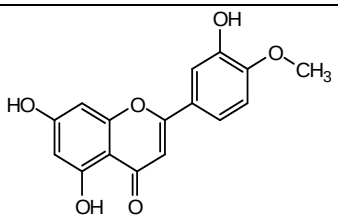
Luteína $C_{15}H_{10}O_6$ Pm: 286.24 g/mol CAS: 491-70-3		Cheng y col., 2010 Zhu y col., 2011 Chen y col., 2012
Propiedades: antioxidante (quelación de metales), antitumoral, antiinflamatorio, neuroprotector		
Verbascósido $C_{29}H_{36}O_{15}$ Pm: 624.59 g/mol CAS: 61276-17-3		Paola y col., 2011 Alipieva y col., 2014
Propiedades: antioxidante (captación RL), antiinflamatorio (disminución NF-κB, óxido nítrico sintasa inducible), antitumoral, neuroprotector		
Rutina $C_{27}H_{30}O_{16}$ Pm: 610.52 CAS: 153-18-4		Guardia y col., 2001; Dall´Agnol y col., 2003; Chua, 2013
Propiedades: antioxidante (captación RL), antiinflamatorio, antimicrobiano, antiasmático, antitumoral		
Apigenina CAS: 520-36-5 Pm: 270.24 g/mol $C_{15}H_{10}O_5$		Gupta y col., 2001 Liu y col., 2011 Chen y col., 2012
Propiedades: antioxidante (quelación de metales, captación RL), antiinflamatorio, antitumoral, neuroprotector, antimutagénico		
Diosmetina $C_{16}H_{12}O_6$ Pm: 300.26 g/mol CAS: 520-34-3		Morel y col., 1993 Patel y col., 2013
Propiedades: antioxidante (quelación de metales), antiglicante, antitumoral, antiinflamatorio, antimicrobiano		

Tabla 6: Compuestos fenólicos característicos de productos y subproductos del olivo

De forma general el contenido en compuestos fenólicos de productos y subproductos del olivo podría dividirse en cinco grupos: oleuropeósidos (oleuropeína y verbascósido), flavonas (luteolina-4-glucósido, apigenina-7-glucósido, diosmetina-7-glucósido, luteolina y diosmetina); flavonoles (rutina); flavan-3-ols (catequina) y fenoles sustituidos (tirosol, hidroxitirosol, vanillina, ácido vaníllico, ácido caféico) (El y Karakaya, 2009; Kiritsakis y col., 2010). La **Tabla 6** muestra los compuestos fenólicos más frecuentes y característicos de los productos del olivo y el efecto saludable atribuido a cada uno de ellos.

Pese a la riqueza del perfil fenólico de los productos del olivo, el hidroxitirosol y la oleuropeína son considerados los fenoles de mayor relevancia ya sea por ser cuantitativamente mayoritarios como por su presencia exclusiva en estos productos.

4.2.1. Hidroxitirosol

El hidroxitirosol es un orto-difenol también conocido como 3,4-dihidroxifenil etanol (HT). Es el compuesto fenólico principal del aceite de oliva y puede hallarse en forma libre, en su forma acetato o formando parte de la oleuropeína, secoiridoide principal en los productos del olivo. El contenido en HT del aceite de oliva virgen se ve incrementado por la hidrólisis que sufre la oleuropeína durante el proceso de maduración de las aceitunas, por el tratamiento que sufren las aceitunas de mesa para su elaboración o durante el almacenamiento del aceite. En los últimos años, el HT ha sido el blanco de numerosas investigaciones que le han atribuido una acción preventiva y atenuante de enfermedades como diabetes, enfermedad coronaria, neurodegenerativa o cáncer (Fernández-Mar y col., 2012) al ser considerado un potente antioxidante natural. Su efecto protector puede ser ejercido de manera directa, principalmente a través de su capacidad de captar radicales responsables del estrés oxidativo e indirecta, a través de su posible implicación en el incremento de la expresión y actividad de enzimas antioxidantes responsables de mantener el estatus redox intracelular (Martín y col., 2010; Fernández-Mar y col., 2012). Esta actividad antioxidante ha sido atribuida a su estructura orto-dihidroxilo y se ha considerado más potente que la presentada por compuestos como el ácido ascórbico o el α-tocoferol (Mateos y col., 2003). Ejemplos de los efectos beneficiosos atribuidos al HT son la prevención de tumores de próstata y colon mediante una actividad antiproliferativa, proapoptótica y antiinflamatoria (Lipworth y col., 1997), prevención de enfermedades cardiovasculares por inhibición de la agregación plaquetaria, reducción de la presión arterial y oxidación de LDL (Visioli y col., 1995; Covas, 2007) así como prevención y reducción de complicaciones diabéticas por su efecto en la mejora de la sensibilidad a la insulina y en la capacidad secretora de células β pancreáticas (de Bock y col., 2013).

En un estudio en humanos, Vissers y col. (2002) estimaron que al menos el 55-66% de los polifenoles ingeridos a partir del aceite de oliva se absorbieron y que el 5-16% se excretaron como tirosol e hidroxitirosol en orina. Durante la metabolización del HT se describió un proceso de o-metilación, principalmente en hígado y de conjugación con el ácido glucurónico en enterocitos e hígado considerándose los derivados glucurónidos los principales metabolitos excretados (Spencer y col., 1999; Vissers y col., 2002). Un estudio en ratas elucidó que el HT podría sufrir una serie de conversiones enzimáticas y generar derivados oxidados como el MOPET (4-hidroxi-3-metoxifeniletanol, alcohol homovanílico), HVA (ácido 4-hidroxi-3-metoxifenilacético, ácido homovanílico), DOPAC (ácido 3,4-dihidroxifenil acético) y DOPAL (3,4-dihidroxifenil acetaldehído) así como sulfoconjugados todos ellos distribuidos principalmente en plasma, cerebro, corazón, riñón, hígado y pulmón (D´Angelos y col., 2001).

En términos de seguridad alimentaria, los estudios realizados en ratas no mostraron toxicidad con una administración oral de 2-5 g/kg peso corporal/día (D´Angelos y col., 2001; Soni y col., 2006) mientras que en humanos se ha llegado a establecer un NOAEL (*Non Observed Adverse Effects Leve*") de 500 mg/kg peso corporal/día.

4.2.2. Oleuropeína

La oleuropeína pertenece a la familia de los secoiridoides y se caracteriza químicamente por ser un éster de hidroxitirosol y ácido elenólico con un residuo glucosídico. Tanto la oleuropeína como su aglicona son compuestos responsables del amargor de las aceitunas que son sometidas a un tratamiento alcalino para romper el enlace éster de estos compuestos generando HT y ácido elenólico glucosilado, compuestos fenólicos no amargos. En el fruto, la oleuropeína se va acumulando durante la fase de crecimiento, llegando a alcanzar en el fruto joven un contenido del 14% en materia seca. Este contenido se ve mermado durante las fases posteriores de maduración verde o negra (Omar, 2010) donde se hidroliza por acción de esterasas en hidroxitirosol y ácido elenólico glucósido (Esti y col., 1998) considerados indicadores de maduración.

En cuanto a su metabolismo, se cree que la oleuropeína es metabolizada a HT tras observar un aumento en la excreción urinaria de HT después de un consumo de oleuropeína-glucósido en un estudio en humanos (Vissers y col., 2002). Además otros estudios han mostrado que la oleuropeína fue rápidamente absorbida después de una administración oral y alcanzó su máxima concentración en plasma a las dos horas (Boccio y col., 2003; Tan y col., 2003). Aunque el mecanismo de absorción de los compuestos fenólicos del olivo no es del todo claro si se sabe que el HT es el principal metabolito de la oleuropeína y que ambos son excretados en orina principalmente como glucorónidos y en menor medida en forma libre (Boccio y col., 2003; Tan y col., 2003).

La oleuropeína está comercialmente disponible como suplemento y se han descrito una serie de propiedades beneficiosas atribuidas a su capacidad antioxidante (Visioli y col., 2002), antiinflamatoria (Visioli y col., 1998), antiaterogénica (Ahmadvand y col., 2014), antitumoral (Owen y col., 2000), antimicrobiana, antiviral (Omar, 2010), cardioprotectora (Andreadou y col., 2007), neuroprotectora (Khalatbary y Ahmadvand, 2012), antidiabética e hipolipemiante (El y Karakaya, 2009; Ahmadvand y col., 2014). En este sentido la oleuropeína destaca por ser considerado un antioxidante capaz de atenuar el estrés oxidativo, mejorar los sistemas de defensa antioxidante y a su vez potenciar la liberación de insulina y aumentar la absorción periférica (Omar, 2010).

4.3. Subproductos del olivo

4.3.1. Obtención de extractos y perfil fenólico

Actualmente, los extractos naturales con alto contenido en compuestos bioactivos son considerados por la industria como una excelente opción en la formulación de suplementos dietéticos o nutracéuticos, ingredientes alimentarios e incluso como productos farmacéuticos y cosméticos (Omar, 2010). Como ya se ha comentado, los productos del olivo contienen un perfil fenólico variado haciendo que sea complicada la obtención de un método simple y óptimo para su completa extracción. Sus diferencias de polaridad han hecho que diferentes medios de extracción como el agua, el metanol, el etanol o mezclas de alcohol y agua hayan sido utilizados para la obtención de estos compuestos. No obstante, recientes trabajos ya introducen otras técnicas de extracción como fluidos supercríticos, líquidos presurizados, microondas o ultrasonidos (Herrero y col., 2011). Por otro lado, para la determinación de la composición fenólica se emplean muy diversas técnicas analíticas. El ensayo colorimétrico *Folin-Ciocalteu* es la técnica más rápida y ampliamente extendida para la determinación del total de compuestos fenólicos polares presentes en el extracto, sin embargo, para una determinación más exacta e individual de compuestos se emplea la *cromatografía líquida acoplada a espectrometría visible/UV* o *espectrometría de masas* (MS/MS, QTof) (Briante y col., 2002). La espectrometría de masas se considera una herramienta muy eficaz para completar la caracterización y aportar más información de forma selectiva y con alta resolución sobre compuestos relacionados estructuralmente.

4.3.2. Valorización

Pese a que el aceite de oliva es el producto del olivo más conocido por los beneficios saludables de su fracción fenólica, otros subproductos del olivo se han destacado como fuentes importantes de polifenoles. De hecho el **alperujo**, una fuente barata de estos compuestos bioactivos, contiene una concentración de hasta 100 veces superior a la presentada por el aceite de oliva, mientras que las

hojas de olivo se caracterizan por tener la mayor actividad antioxidante atribuida a su perfil fenólico (El y Karakaya, 2009).

En los últimos diez años, los estudios científicos con extractos de **hoja de olivo** se han intensificado notablemente. Su alto contenido en compuestos fenólicos hace de las hojas de olivo una buena alternativa para múltiples usos que van más allá de considerarse un simple subproducto del olivo. Las **hojas de olivo** se introdujeron en la dieta humana en forma de infusión o como nutracéutico por su alto contenido en compuestos fenólicos a los que se les han atribuido numerosos beneficios para la salud. En la hoja, la oleuropeína representa el compuesto fenólico mayoritario (1-14%) siendo la parte del olivo más rica en este compuesto. Un ejemplo de ello es el contenido en oleuropeína en el aceite de oliva virgen y en alperujo que representaría un 0.005-0.12% y un 0.87% respectivamente mientras que el contenido en las hojas representaría un 1-14% (Japon-Lujan y col., 2006). Las tendencias futuras apuntan a que las hojas de olivo serán utilizadas para la fortificación o incremento de la estabilidad de alimentos dada su riqueza de compuestos bioactivos antioxidantes, su elevada biodisponibilidad y la ausencia de efectos tóxicos. Además de los beneficios saludables arriba descritos para la oleuropeína y el HT, se han valorado potenciales usos a nivel tecnológico y en la producción de alimentos. Un claro ejemplo es el enriquecimiento de aceites y encapsulación con extractos de hojas de olivo, así como la extracción de oleuropeína para la producción de HT (Erbay y Icier, 2010). Aplicaciones médicas, farmacológicas y cosméticas han sido propuestas para futuros usos (Visioli y Bernardini, 2011).

Durante la extracción del aceite tienen lugar una serie de reacciones enzimáticas y químicas que modifican el perfil de compuestos fenólicos. En concreto, la hidrólisis de las uniones glucosídicas y la apertura del anillo secoiridoideo conducen a la obtención de compuestos más hidrofílicos donde aproximadamente el 98% permanecen en el residuo acuoso (Caruso y col., 2000; Rodis y col., 2002; Obied y col., 2008) provocando que los subproductos que se obtienen del procesamiento de la aceituna se concentren en estos compuestos.

El **alpechín** es un subproducto que se obtiene del proceso de obtención del aceite de oliva en el que se incluye una decantación a tres fases que exige la adición de agua. El alpechín contiene más de 60 compuestos fenólicos entre los que destaca el HT cuantificado en 24.63-64.9 mg/L, seguido de otros compuestos como la forma aldehídica de la oleuropeína aglicona y ligstrósido aglicona, tirosol, ácido vanílico, ácido ferúlico o ácido p-cumárico (Giuffrè y col., 2012). Al igual que ocurre con otros subproductos, se están proponiendo diversas aplicaciones industriales como la producción de nutracéuticos, formulación de piensos para peces y la conservación de alimentos (Troise y col., 2014).

El **alperujo** es otro subproducto obtenido del proceso de obtención del aceite de oliva con una decantación de dos fases que puede ser considerado para su potencial valorizacióncomo alternativa de obtención de compuestos bioactivos, principalmente hidroxitirosol.

III. OBJETIVOS

Actualmente, una gran parte de los subproductos generados por la industria agroalimentaria son valorizados por su alto contenido en compuestos bioactivos con efectos beneficiosos para la salud, como los polifenoles. Sin embargo, existe un conocimiento muy limitado sobre su aplicación como agentes antiglicantes, y prácticamente es desconocido su mecanismo de acción. En este sentido, la búsqueda de extractos naturales con propiedades antiglicantes se plantea como una estrategia que contribuirá a combatir las posibles causas del desarrollo de enfermedades tan recurrentes en las sociedades occidentales como el Alzheimer, la diabetes y sus complicaciones, así como las repercusiones que conlleva el envejecimiento.

El desarrollo de la presente Tesis Doctoral tiene como *objetivo general*:

- La identificación de subproductos de la industria alimentaria con capacidad antiglicante. Selección y estudio pormenorizado de un extracto obtenido a partir de un subproducto agroalimentario en la inhibición de la glicación en sus diferentes etapas, la repercusión en la formación de productos de glicación avanzada y su propuesta de valorización como ingrediente alimentario funcional o nutracéutico.

Para lograr este objetivo se abordaron los siguientes objetivos específicos:

1. Puesta a punto y validación de una batería de procedimientos analíticos en modelos de reacción proteína-carbonilo, en un modelo de alimento y en un modelo de estrés carbonílico celular para caracterizar las etapas inicial, intermedia y avanzada de la glicación. Desarrollo y validación de un procedimiento analítico para evaluar la capacidad de atrapamiento de α-dicarbonilos C6, C3 y C2 de compuestos antiglicantes.
2. Examinar la capacidad antiglicante *in vitro* de diferentes extractos naturales procedentes de subproductos de la industria alimentaria validando las metodologías analíticas que evidencien las diferentes etapas de la glicación e identifiquen los posibles compuestos bioactivos responsables del efecto antiglicante.
3. Seleccionar un compuesto bioactivo con destacada capacidad antiglicante *in vitro* para el estudio pormenorizado de su mecanismo de acción antiglicante. A continuación obtener un extracto a partir de un subproducto de la industria alimentaria concentrado en dicho compuesto.
4. Evaluar el efecto del extracto natural seleccionado y de su principio activo sobre el desarrollo de la Reacción de Maillard en un modelo de alimento y con especial atención en la mitigación de la formación de AGEs dietéticos.
5. Analizar la actividad inhibitoria del extracto y de su compuesto bioactivo en la glicación de proteínas intracelulares y particularmente en la generación de AGEs a partir de un modelo celular de estrés carbonílico.

IV. RESULTADOS

CAPÍTULO 1: *Estudio y selección de subproductos agroalimentarios con potencial capacidad antiglicante in vitro e identificación de los compuestos responsables del efecto*

Antecedentes: La formación y acumulación de AGEs en el organismo está relacionada con el proceso de envejecimiento y con el desarrollo y evolución de enfermedades crónicas como la diabetes mellitus. La alta prevalencia de estas enfermedades conduce hacia la búsqueda de extractos naturales como fuente de compuestos bioactivos capaces de mitigar la glicación y constituir, a su vez, una alternativa eficaz a los compuestos antiglicantes de síntesis con efectos adversos demostrados.

Objetivos: Evaluación del potencial efecto antiglicante de extractos vegetales hidrosolubles obtenidos a partir de diferentes subproductos agroalimentarios con un elevado y variado contenido en compuestos fenólicos.

Metodología: Una selección de extractos de semillas de frutas y vegetales, cascarilla de café y alpechín obtenidos mediante extracción acuosa, se caracterizaron en función de diferentes parámetros. Para ello, se evaluaron parámetros fisicoquímicos básicos, la presencia de inductores de la glicación como proteínas solubles y azúcares reductores y la presencia de potenciales agentes antiglicantes mediante la determinación total y específica de compuestos fenólicos. En base a la estrecha relación establecida en la bibliografía entre el proceso oxidativo y la glicación, la capacidad antioxidante de los extractos se evaluó mediante los métodos ORAC, ABTS, DPPH y FRAP.

El estudio de la capacidad antiglicante de los extractos propuestos se llevó a cabo en sistemas *in vitro* previamente validados y en condiciones fisiológicas simuladas, donde la solución salina del tampón fosfato sódico (100 mM, pH 7.4) así como una temperatura de incubación de 37 ^{o}C aportaron una evolución controlada de la reacción. Mediante una serie de métodos directos e indirectos se evaluó la actividad inhibitoria de las muestras ensayadas en las diferentes etapas del proceso de glicación. De manera diferenciada, la inhibición de AGEs fluorescentes, correlacionados previamente con el contenido de AGEs totales, se estudió en dos modelos de reacción, BSA-GLC y BSA-MGO. El sistema BSA-GLC se utilizó como indicativo del avance global de la glicación a partir de la formación previa de los productos de Amadori en la etapa inicial. En este sentido, se evaluó la fructosamina con el objeto de estimar la etapa en la cual el inhibidor ejerce su actividad. El sistema BSA-MGO se empleó para determinar la inhibición de los AGEs fluorescentes formados a partir de los α-dicarbonilos procedentes de la etapa intermedia de la glicación o de otras vías como la peroxidación lipídica o la oxidación de la glucosa. Dada la relevancia de los compuestos α-dicarbonílicos y su contribución al proceso de glicación se determinó la capacidad de atrapamiento de MGO o GO como posible mecanismo antiglicante.

Un aspecto importante a considerar antes de proponer un nuevo extracto como ingrediente alimentario o principio de uso farmacológico, es conocer los aspectos toxicológicos del mismo. Para ello, se evaluó el efecto del extracto de granada, con potencial capacidad anti-AGEs, sobre la viabilidad de las células HepG2, así como su posible citotoxicidad e inducción de ROS intracelulares. Adicionalmente, se determinó el posible efecto protector en las células previa exposición a *t*-BOOH como agente inductor de citotoxicidad y estrés celular.

Resultados: En un cribado previo de ocho extractos hidrosolubles de semillas, el extracto de granada destacó por su capacidad antioxidante y su alto contenido en ácidos fenólicos donde el ácido gálico representó el 39%. En cuanto a la actividad antiglicante, el extracto de pimiento verde, sésamo y granada presentaron las mayores actividades inhibitorias de formación de AGEs en el sistema BSA-GLC, mientras que en el sistema BSA-MGO destacó la actividad del extracto de melocotón, granada y albaricoque. Paralelamente el melocotón y el albaricoque ejercieron una potente capacidad de atrapamiento de MGO (IC_{50} efectiva de 0.014 mg/mL) frente a la granada (IC_{50} efectiva de 0.16 mg/mL). Tras concluir que el extracto hidrosoluble de granada podría ser propuesto como ingrediente antiglicante, se evaluó la citotoxicidad y cambios en el estatus redox en un cultivo de células HepG2, donde el extracto en un rango de concentración 1-100 µg/mL no ocasionó ningún efecto citotóxico o de estrés oxidativo, si bien produjo una reducción significativa de ROS y citotoxicidad inducidos por *t*-BOOH.

Por otra parte, la cascarilla de café de la variedad robusta presentó una capacidad antioxidante superior a la variedad arábica, que a su vez presentó un menor contenido en fibra, melanoidinas, clorogénicos y compuestos fenólicos totales. La capacidad antiglicante de ambas variedades fue similar y se observó una mayor eficacia antiglicativa en el sistema BSA-GLC (IC_{50} de 0.6 mg/mL) que en el sistema BSA-MGO (IC_{50} de 1.3 mg/mL). La diferencia entre las dos variedades vino marcada por su capacidad de atrapamiento de MGO donde la variedad robusta presentó un efecto 11 veces superior a la arábica. Dicho efecto, en parte, pudo ser atribuido a su mayor contenido en clorogénicos, ya que éstos presentaron de forma aislada una potente actividad de atrapamiento de MGO.

Por último, el extracto hidrosoluble de alpechín presentó una potente capacidad antioxidante dependiente de su método de obtención por nano (NOMW) o ultrafiltración (UOMW). La capacidad de inhibir la formación de AGEs por parte de UOMW y NOMW en los sistemas de BSA-GLC fue similar (IC_{50} de 0.4 mg/mL) así como su actividad inhibitoria de fructosamina (IC_{50} de 2.4 mg/mL), indicando que los extractos podrían estar ejerciendo su actividad en etapas iniciales de la glicación. Sin embargo, la actividad inhibitoria difirió significativamente en los sistemas de BSA-

MGO, donde alcanzaron un IC_{50} de 0.89 y 1.03 mg/mL respectivamente. Estos resultados estuvieron en línea con los obtenidos en el atrapamiento de MGO y GO, donde nuevamente UOMW presentó una mayor actividad (IC_{50} de 0.29 y 0.56 mg/mL, respectivamente). Finalmente, tras el fraccionamiento del extracto en función de la elución de los compuestos fenólicos en metanol, se observó que la fracción que contenía el verbascósido y parte del HT mostró la mayor actividad antioxidante y antiglicante, mientras que la fracción con mayor contenido en HT presentó un mayor atrapamiento de MGO. Estos resultados fueron confirmados con los obtenidos en el estudio de patrones de HT y verbascósido, que exhibieron una destacada actividad inhibitoria en el sistema BSA-GLC (IC_{50} de 0.40 y 0.24 mg/mL respectivamente) y BSA-MGO (IC_{50} de 0.09 y 0.05 mg/mL respectivamente).

Conclusión: Tras un cribado previo de diversos extractos hidrosolubles de subproductos de la industria alimentaria no se estableció una correlación entre su capacidad antioxidante y antiglicante por lo que se dedujo que otras vías no oxidativas debían ser consideradas en el proceso de glicación. Este hecho, sumado al tipo de extracción utilizado para la obtención del extracto así como su perfil fenólico, influyó notablemente en los resultados obtenidos. El extracto obtenido a partir de alpechín ejerció el mayor efecto antiglicante en las diferentes etapas de la reacción de glicación, siendo plausible que su actividad sea debida a su contenido en HT y verbascósido.

Research Article

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Antiglycative effect of fruit and vegetable seed extracts: inhibition of AGE formation and carbonyl-trapping abilities

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Abstract

BACKGROUND: Advanced glycation end-products (AGEs) are the final products derived from the non-enzymatic glycation process. AGEs are involved in the development of several health complications associated with diabetes and aging. Searching for anti- AGE extracts is necessary to mitigate the effects of age-related pathologies.

RESULTS: The antioxidant and antiglycative activities of eight aqueous extracts of fruit and vegetable seeds were evaluated. All seed extracts (3.6 mg mL^{-1}) exhibited anti-AGE activity in protein–glucose assay, ranging from 20 to 92% inhibition compared with aminoguanidine (4.87 mmol L^{-1}). Green pepper extract exerted the highest anti-AGE activity. However, peach and pomegranate extracts exhibited the highest anti-AGE activity in protein–methylglyoxal assay, ranging from 0 to 79% inhibition. Hazelnut, almond and sesame extracts were not effective when methylglyoxal was the promoter. Apricot and peach extracts appeared to inhibit the formation of AGEs through their capacity for direct trapping of 1,2-dicarbonyls (IC_{50}=0.14 mg mL^{-1}). No relationship between antioxidant and phenolic compound content and antiglycative activity was found. Therefore other hydrophilic constituents in addition to phenolic acids must be involved in the antiglycative activity of the extracts.

CONCLUSION: Aqueous extracts of fruits and vegetables can be considered in the prevention of glycation-associated complications of age-related pathologies.

Keywords: advanced glycation end-products (AGEs); seed extract; glycation; glycation inhibitors; phenolic compounds

INTRODUCTION

The Maillard reaction or non-enzymatic glycation process is produced by the interaction between reducing sugars and free amino groups of proteins, lipids and nucleic acids. In the human body the final products that derive from this reaction are called advanced glycation end-products (AGEs). AGEs are a group of complex and heterogeneous products that can be classified according to their structures and characteristics as fluorescent crosslinking AGEs such as pentosidine, non-fluorescent crosslinking AGEs such as methylglyoxal–lysine dimer (MOLD) and non-crosslinking AGEs such as carboxymethyllysine (CML) and pyrraline.[1]

It is known that AGEs are involved in the development of several health disorders such as diabetes and its complications,[2] atherosclerosis,[3] Alzheimer's disease and normal aging.[4] For this reason, the search for AGE formation inhibitors has recently received much attention.[5–8] Various AGE inhibitors have been developed in the last few years, which can be divided into three groups: (1) inhibitors that prevent glycoxidation through metal ion chelation; (2) 1,2-dicarbonyl-trapping agents; (3) crosslink breakers.[9] Several AGE inhibitors have been described, a few of them exerting their effects at the early stage of glycation but most of them preventing the formation of AGEs at the late stage of glycation. Inhibition can occur through interference with the initial attachment between reducing sugars and amino groups, through trapping of carbonyls and radicals formed during glycation or through blocking the formation of intermediate Amadori products.[10] Aminoguanidine (AG) and pyridoxamine (PM) are well-known AGE inhibitors, both being considered as potent carbonyl scavengers. However, although such synthetic compounds have proved to be strong AGE inhibitors, they have also been associated with several adverse effects in *in vivo* assays.[11,12] Therefore the search for natural products with the ability to inhibit AGE formation is currently being widely pursued.

Many plant extracts have been evaluated for their inhibitory effects on the formation of AGEs, both through preventing glycoxidation and by scavenging reactive 1,2-dicarbonyls such as methylglyoxal (MGO), which are important precursors of AGEs.[6,13,14] Most studies have been carried out in methanolic, ethanolic or other organic solvents,[7,13,15] since the inhibitory effects are mainly attributed to polyphenols owing to their potent antioxidant activities.[16] It is known that phenolic compounds possess strong antioxidant abilities as a result of their redox

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properties; moreover, it has been reported that such antioxidant effects might contribute to the inhibition of protein modifications in the glycation process.[17] Among these studies, only a few have evaluated aqueous extracts of samples.[18,19]

In this regard, the aim of the present study was to investigate the inhibitory effects on AGE formation of aqueous extracts from eight different fruit and vegetable seeds considered as secondary by-products in the industry. For this purpose, different *in vitro* models of AGEs induced by glucose and MGO were evaluated. Samples were extracted in water, since, despite the fact that the antiglycative effect of aqueous extracts might be lower than that of organic solvent extracts, the extraction procedure is both more economical and environmentally friendly and therefore would be of greater interest for industrial applications. The extracts were also tested for their capacity in direct trapping of MGO. Moreover, antioxidant activity and phenolic compound content were examined in order to study their possible relationship with AGE-inhibitory activity.

MATERIALS AND METHODS

Materials

Commercially available fruit and vegetable seeds (green pepper, apricot, hazelnut, peach, sour cherry, sesame, almond and pomegranate) were provided by TIKTA (Ankara, Turkey). Detailed information on the different samples is listed in Table 1. D(+)-Glucose, bovine serum albumin (BSA), 400 g L^{-1} methylglyoxal solution (MGO), sodium azide, aminoguanidine (AG), 5-methylquinoxaline (5-MQ), *o*-phenyldiamine (OPD), gallic acid, Trolox, fluorescein and phenolic acid standards were purchased from Sigma (St Louis, MO, USA). Sodium dihydrogen phosphate monohydrate, glacial acetic acid, formic acid and high-performance liquid chromatography (HPLC)-grade methanol were acquired from Merck (Darmstadt, Germany). Folin–Ciocalteu reagent and sodium carbonate were obtained from Panreac Quimica (Barcelona, Spain). 2,2-Azobis(methylpropionamidine)dihydro (AAPH) and pyridoxamine (PM) were purchased from Fluka Chemical (Madrid, Spain). A Pierce bicinchoninic acid (BCA) protein assay kit was obtained from Thermo Scientific (Rockford, IL, USA). The Milli-Q water used was produced using an Elix3 water purification system coupled to an Advantage10 Milli-Q module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

Preparation of seed extracts

Powder (500 mg) of each seed sample was extracted in water (25 mL × 2) at 50 °C by agitation for 10 min each time. Pellets of the extracts were removed by centrifugation (1400 × *g*) and supernatants were collected, lyophilised and weighed. Soluble extracts were coded as described in Table 1.

Measurement of pH

Each lyophilised extract (250 mg) was mixed with 10 mL of water and vortexed for 3 min. The mixture was held at room temperature for 1 h to separate solid and liquid phases. After carefully removing the supernatant layer, the pH was measured using a CG-837 pH meter (Schott, Mainz, Germany).

Determination of soluble protein

Soluble protein measurements were performed using a modified BCA protein assay.[20] According to Thermo Scientific, BCA protein assay reagent A contains sodium carbonate, sodium bicarbonate, BCA and sodium tartrate in 0.1 mL L^{-1} sodium hydroxide, while reagent B contains 40 g L^{-1} cupric sulfate. BCA working reagent was prepared by mixing 50 parts of reagent A and one part of reagent B. For sample analysis, 10 mg of lyophilised extract was dissolved in 1 mL of phosphate buffer (0.1 mol L^{-1}, pH 7.4) and vortexed for 10 min. The mixture was held at room temperature for 1 h and centrifuged at 8000 × *g* for 10 min. A 50 µL aliquot of the supernatant was mixed with 500 µL of BCA working reagent. After incubation for 90 min at 37 °C, the absorbance at 562 nm was recorded using a Synergy™ HT-multimode microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) and quantified using BSA as a standard. BioTek Gen5™ data analysis software was used. The limit of quantification was set at 5.15 mg g^{-1} sample.

Determination of reducing sugars

Reducing sugars were determined according to the method described by Miller.[21] A calibration curve was constructed using standard glucose solutions in the concentration range 0.25–2 mg mL^{-1}. Each lyophilised extract (50 mg) was suspended in 5 mL of distilled water at 50 °C, vortexed for 20 min and centrifuged at 1400 × *g* for 15 min at 4 °C. The supernatant was collected. The extraction was repeated twice and the supernatants were pooled. Following treatment with Carrez-I and Carrez-II solutions, the supernatant was used to determine reducing sugars after blank correction. Results were expressed as mg glucose equivalent g^{-1} sample. The limit of quantification was set at 25.2 mg glucose equivalent g^{-1} sample.

Determination of total phenolic content

Total phenolic content (TPC) in the extracts was determined by the Folin–Ciocalteu method as described by Singleton *et al.*[22] and adapted to a plate reader. Each lyophilised extract was dissolved in water to obtain a 10 mg mL^{-1} solution. In a 1.5 mL Eppendorf microtube, 100 µL of sample (appropriately diluted if necessary) and 250 µL of Folin–Ciocalteu reagent (diluted 1:1 (v/v) in methanol) were mixed and vortexed. After exactly 3 min, 500 µL of 75 g L^{-1} sodium carbonate solution and 4 mL of methanol/water (50:50 v/v) were added, then the mixture was vortexed for a further 10 min and allowed to stand at room temperature in darkness for 60 min. The absorbance at 750 nm was recorded using a BioTek microplate spectrophotometer as described above and quantified using gallic acid as a standard. Results were expressed as mg gallic acid equivalent (GAE) g^{-1} sample. The limit of quantification was set at 4.5 mg GAE g^{-1} sample.

Determination of total extracted phenolic acids

Total extracted phenolic acids were determined according to the method described by Kim *et al.*[23] and Ross *et al.*[24] First, 1 mL of 2 mol L^{-1} sodium hydroxide containing 13.4 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) and 20 mL L^{-1} ascorbic acid was added to 30 mg of each lyophilised extract. The mixture was flushed with nitrogen and allowed to hydrolyse under agitation for 16 h at room temperature. After hydrolysis, the sample was centrifuged at 2370 × *g* for 10 min at 4 °C and the supernatant was acidified by adding 0.3 mL of acetic acid. The liberated phenolic acids were extracted with ethyl acetate (2 × 2 mL). The organic layer containing the phenolic acids liberated by base hydrolysis was collected by pipetting off the upper organic (supernatant) layer from the bottom aqueous residue layer. The two organic layers

Table 1. Description of tested seed samples and their solubility in water[a]

Sample extract ID	Common name	Scientific name	Family	Soluble extract (mg g^{-1})
Gp	Green pepper	*Capsicum annuum*	Solanaceae	194.9 ± 0.64b
Ap	Apricot	*Prunus armeniaca*	Rosaceae	445.8 ± 22.27de
Ha	Hazelnut	*Corylus avellana*	Betulaceae	436.2 ± 17.04d
Pe	Peach	*Prunus persica*	Rosaceae	478.1 ± 16.05e
Sc	Sour cherry	*Prunus cerasus*	Rosaceae	257.1 ± 29.27c
Se	Sesame	*Sesamum indicum*	Pedaliaceae	224.6 ± 8.06bc
Al	Almond	*Prunus dulcis*	Rosaceae	442.8 ± 15.77de
Po	Pomegranate	*Punica granatum*	Punicaceae	129.8 ± 4.31a

[a] Results are expressed as mean ± SD for n=3. Different letters denote significant differences ($P < 0.05$).

were combined and evaporated to dryness in a speed-vac for 1 h at 45 °C. The residue was dissolved in 1 mL of methanol/water (75:25 v/v) and filtered through a 0.45 µm filter, then the sample was analysed by HPLC. Quantification was conducted with a Shimadzu HPLC system (Kyoto, Japan) equipped with an LC-20AD pump, an SIL-10ADvp autosampler, a CTO-10ASVP oven and an SPD-M20A diode array detector. Chromatographic separation was carried out on a Kinetex C-18 100 Å column (100 mm × 4.6 mm, 2.6 µm; Phenomenex, Torrance, CA, USA). The flow rate was 0.6 mL min^{-1} and the injection volume was 5 µL. The mobile phase consisted of 1 mL L^{-1} formic acid in water (solvent A) and methanol (solvent B) and the gradient program was as follows: 0 min, 25% B; 0–5 min, 25–30% B; 5–10 min, 30–60% B; 10–12 min, 60% B; 12–13 min, 60–80% B; 13–14 min, 80% B; 14–15 min, 80–25% B; 15–18 min, 25% B. The total run time was 18 min and chromatograms were analysed at 254, 280 and 325 nm. The following phenolic acids were identified: *p*-hydroxybenzoic acid (PHB), syringic acid (SYN), vanillic acid (VA), *p*-coumaric acid (pCU), caffeic acid (CA), ferulic acid (FA), protocatechuic acid (PCA), gallic acid (GA), gentisic acid (GE), sinapinic acid (SIN) and ellagic acid (EA). The limit of quantification was set at 2 µg g^{-1} sample.

Determination of benzoic acids

The presence of benzoic acids was determined using the HPLC method described by Lamuela-Raventós and Waterhouse.[25] Procedures for sample preparation, quantification and chromatographic separation were the same as those described for total extracted phenolic acid determination. The total run time was 18 min and quantification was made at 280 nm (expressed as gallic acid). The limit of quantification was set at 2.5 µg GAE g^{-1} sample.

Determination of flavonoids

Flavonoid content was determined using the aluminium chloride method described by Abdel-Hameed.[26] Each lyophilised extract was dissolved in water at 25 mg mL^{-1}. Then 80 µL of sample was mixed with 80 µL of aluminium trichloride in ethanol and 100 µL of sodium acetate. The mixture was incubated in darkness for 90 min and centrifuged at 14 926 × g for 3 min. Flavonoids were determined based on the formation of a flavonoid–aluminium complex with absorptivity maximum at 440 nm. Absorbance readings were taken using a BioTek microplate spectrophotometer as described above. Quercetin was used as a reference standard. Results were expressed as µg quercetin equivalent per 100 g sample. The limit of quantification was set at 0.02 µg quercetin equivalent per 100 g sample.

ORAC assay

Alkylperoxyl free radical ($ROO^{\bullet}$)-scavenging activity was measured by monitoring the fluorescence decay due to ROO-induced oxidation of fluorescein, known as the oxygen radical absorbance capacity (ORAC) assay. The water-soluble azo initiator AAPH was applied as a clean and controllable source of thermally produced $ROO^{\bullet}$ in aqueous media. $ROO^{\bullet}$ were generated by AAPH in a microplate reader at 37 °C. The antiradical activity against AAPH was estimated according to the procedure reported by Dávalos *et al.*[27] A BioTek Synergy™ HT-multimode microplate reader with automatic reagent dispense and temperature control was used. All reaction mixtures were prepared in duplicate and four independent assays were performed for each sample. Raw data were processed by the microplate reader, and the area under the curve (AUC) was calculated. ORAC was expressed as Trolox equivalent antioxidant capacity (TEAC) using a standard curve constructed for each assay. Results were expressed as µmol TEAC g^{-1} sample. The limit of quantification was set at 18.1 µmol TEAC g^{-1} sample.

ABTS assay

Antioxidant activity was estimated in terms of the radical-scavenging activity of samples in aqueous media following the procedure described by Delgado-Andrade and Morales[28] with slight modification. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical cations ($ABTS^{\bullet+}$) were produced by reacting 7 mmol L^{-1} ABTS stock solution with 2.45 mmol L^{-1} potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution (stable for 2 days) was diluted with distilled water to an absorbance of 0.70 ± 0.02 at 734 nm. Each lyophilised extract was dissolved in water at 10 mg mL^{-1}. Following the addition of 40 µL of sample (appropriately diluted if necessary) and Trolox standard to 200 µL of water and 40 µL of diluted $ABTS^{\bullet+}$ solution, an absorbance reading was taken after 10 min using a Bio-Tek Synergy™ HT-multimode microplate reader as described above. Aqueous solutions of Trolox at concentrations of 0.016–0.5 mmol L^{-1} were used for calibration. The limit of quantification was set at 1.1 µmol TEAC g^{-1} sample.

In vitro glycation assay with BSA–glucose

The BSA–glucose (Glc) assay was based on Peng *et al.*[6] and was used as an *in vitro* model for comparison of the antiglycation activities of the different seed extracts. First, BSA (35 mg mL^{-1}) and Glc (175 mg mL^{-1}) were dissolved separately in phosphate buffer (0.1 mol L^{-1}, pH 7.4). Then 200 µL of BSA solution containing 0.1 g mL^{-1} sodium azide (to ensure aseptic conditions) was incubated

with 400 µL of Glc solution at 37 °C for 21 days in the absence or presence of seed extract solutions (100 µL, 25 mg mL^{-1}). Blanks containing BSA–Glc but no test sample were kept at −80 °C until measurement. A 4 mg mL^{-1} solution of AG (32.49 mmol L^{-1}) was used as positive control, corresponding to 4.87 mmol L^{-1} in the reaction media. In parallel, seed extracts dissolved in phosphate buffer (0.1 mol L^{-1}, pH 7.4) were incubated at 37 °C for 21 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was 10 mg mL^{-1} for BSA, 100 mg mL^{-1} for Glc, 0.6 mg mL^{-1} for AG and 3.6 mg mL^{-1} for seed extracts.

In vitro glycation assay with BSA–MGO

The BSA–MGO assay was performed according to the method described by Lunceford and Gugliucci[29] with slight modification and was used to evaluate the inhibitory effects of the different seed extracts on protein glycation induced by MGO. First, BSA (35 mg mL^{-1}) and MGO (0.4 mg mL^{-1}) were dissolved separately in phosphate buffer (0.1 mol L^{-1}, pH 7.4). Then 200 µL of BSA solution containing 0.1 g mL^{-1} sodium azide (to ensure aseptic conditions) was incubated with 400 µL of MGO solution at 37 °C for 14 days in the absence or presence of seed extracts solutions (100 µL, 25 mg mL^{-1}). Blanks containing BSA–MGO but no test sample were kept at −80 °C until measurement. A 4 mg mL^{-1} solution of AG (32.49 mmol L^{-1}) was used as positive control, corresponding to 4.87 mmol L^{-1} in the reaction media. In parallel, seed extracts dissolved in phosphate buffer (0.1 mol L^{-1}, pH 7.4) were incubated at 37 °C for 14 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was 10 mg mL^{-1} for BSA, 0.23 mg mL^{-1} for MGO, 0.6 mg mL^{-1} for AG and 3.6 mg mL^{-1} for seed extracts.

AGE fluorescence measurement

Measurements of the fluorescent intensity of total AGEs and the intrinsic fluorescence of the different seed extracts after incubation were performed using a BioTek microplate spectrophotometer as described above. The presence of total AGEs was characterised by typical fluorescence with respective excitation and emission maxima at 360 and 420 nm for the BSA–Glc assay and 340 and 420 nm for the BSA–MGO assay. The percentage inhibition of AGE formation by each extract was calculated using the following equation: inhibition (%)={1 − [(fluorescence of solution with inhibitor − intrinsic fluorescence of sample)/fluorescence of solution without inhibitor]} × 100.

Evaluation of direct MGO -trapping capacity

Direct MGO-trapping capacity was tested using the method described by Peng *et al.*[15] with slight modification. MGO (0.4 mg mL^{-1}) was dissolved in phosphate buffer (0.1 mol L^{-1}, pH 7.4), OPD (derivatisation agent, 10.8 mg mL^{-1}) was dissolved in methanol and 5-MQ (internal standard, 1 mg mL^{-1}) was dissolved in 500 mL L^{-1} methanol. PM solution (1 mg mL^{-1} in 0.1 mol L^{-1} phosphate buffer, pH 7.4) was used as positive control. A 100 µL aliquot of MGO solution was mixed with 750 µL of phosphate-buffered saline (PBS), 50 µL of 5-MQ and 100 µL of either PBS (blank), seed extract solutions (0.005–10 mg mL^{-1}) or PM solution. Therefore the final concentration of each reactant in the reaction medium was 0.04 mg mL^{-1} for MGO, 0.1 mg mL^{-1} for PM and 0.0005–1 mg mL^{-1} for seed extracts. After mixing, samples were incubated at 37 °C for 168 h. Then controls and samples were taken out, 200 µL of OPD was added and each mixture was shaken by vortex for 5 s. After 30 min (when the derivatisation reaction was complete) the residual MGO was quantified on the basis of the amount of the derivatised product 2-methylquinoxaline (2-MQ) formed in each sample. Quantification was conducted using a Shimadzu HPLC system equipped with an LC-20AD pump, an SIL-10ADvp autosampler, a CTO-10ASVP oven and an SPD-M20A diode array detector. Chromatographic separation was carried out on a Mediterranea-Sea-ODS2 column (150 mm × 3 mm, 5 µm; Tecknokroma, Barcelona, Spain). The flow rate was 0.5 mL min^{-1} and the injection volume was 10 µL. Isocratic elution was applied using a mobile phase of 5 mL L^{-1} acetic acid/methanol (50:50 v/v). The total run time was 7 min and chromatograms were recorded at 315 nm. The amount of unreacted MGO in each sample could be determined on the basis of the ratio of peak areas of 2-MQ and 5-MQ. The percentage decrease in MGO was calculated using the following equation: MGO decrease (%)=[(amount of MGO in control − amount of MGO in sample with tested seed extract solution or PM solution)/amount of MGO in control] × 100%. IC_{50} values of samples were evaluated from the dose–response curves of each experiment using Microsoft Excel.

Statistical analysis

Statistical analyses were performed using Statgraphics Centurion XV (Herndon, VA, USA). Data were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) and the least significant difference (LSD) test were applied to determine differences between means. Differences were considered to be significant at $P < 0.05$. Relationships between the different parameters analysed were evaluated by computing Pearson linear correlation coefficients at the $P < 0.05$ confidence level.

RESULTS AND DISCUSSION

As a first step, the solubility of each extract was evaluated. The extracts showed a wide range of solubility varying from from 129.8 to 478.1 mg g^{-1}, with pomegranate and green pepper seeds being least soluble and peach, apricot and almond seeds being most soluble (Table 1). Similarly, a wide variability in the pH of the extracts was observed when the lyophilised samples were reconstituted in water at a concentration of 25 mg mL^{-1}, ranging from pH 4.0 for green pepper extract to pH 8.5 for pomegranate extract (Table 2).

Since sugars and proteins are reactants of the Maillard reaction and may be involved in the glycation process, reducing sugars and soluble proteins were analysed in the aqueous extracts. The highest reducing sugar content was observed in sour cherry extract (201.1 mg g^{-1}), followed by peach (136.1 mg g^{-1}) and sesame (124.0 mg g^{-1}) extracts, while almond and hazelnut extracts exhibited the lowest values (41.5 and 34.4 mg g^{-1} respectively). Soluble protein content varied from 139.7 mg g^{-1} in sesame extract to 383.3 mg g^{-1} in hazelnut extract (Table 2).

The antioxidant activities of the aqueous extracts were assessed by two methods: the ORAC assay and the ABTS assay (free radical-scavenging capacity). Table 2 shows the analytical results. The capacity to scavenge O_2^- radicals ranged from 107.4 µmol TEAC g^{-1} extract for peach to 6378 µmol TEAC g^{-1} extract for pomegranate, a huge difference. Similarly, the ABTS results varied markedly, ranging from 4.6 to 232.7 µmol TEAC g^{-1} extract. Pomegranate extract had the highest free radical-scavenging activity, followed by green pepper extract, while sour cherry and apricot extracts showed the lowest scavenging activity. No

Table 2. Characterisation of aqueous extracts from fruit and vegetable seeds[a]

Sample extract	pH	Soluble protein (mg g^{-1})	Reducing sugars (mg g^{-1})	ORAC (μmol TEAC g^{-1})	ABTS (μmol TEAC g^{-1})	TPC (mg GAE g^{-1})
Gp	4.0	219.0 ± 10.3b	93.3 ± 2.75c	359.6 ± 5.16c	91.6 ± 35.4c	10.7 ± 0.3d
Ap	4.5	282.0 ± 19.1cd	71.5 ± 0.67b	145.6 ± 6.36ab	8.9 ± 2.1a	4.8 ± 0.2a
Ha	5.5	383.3 ± 42.3e	34.4 ± 0.30a	144.7 ± 2.83ab	17.3 ± 2.1ab	10.2 ± 0.8d
Pe	5.5	209.0 ± 2.5b	136.1 ± 3.42e	107.4 ± 14.28a	10.4 ± 2.3a	8.8 ± 0.2c
Sc	5.5	330.3 ± 28.5de	201.1 ± 5.29f	300.4 ± 0.35c	4.6 ± 1.6a	7.3 ± 0.7b
Se	6.5	139.7 ± 8.3a	124.0 ± 0.01d	272.9 ± 2.55bc	42.7 ± 2.6b	7.8 ± 0.3bc
Al	6.5	227.6 ± 0.9bc	41.5 ± 0.29a	124.7 ± 1.56a	24.3 ± 5.7ab	10.9 ± 0.1d
Po	8.5	368.4 ± 39.8e	119.6 ± 0.79d	6378 ± 160.9d	232.7 ± 16.1d	22.2 ± 1.1e

[a] Gp, green pepper; Ap, apricot; Ha, hazelnut; Pe, peach; Sc, sour cherry; Se, sesame; Al, almond; Po, pomegranate. Results are expressed as mean ± SD for n=4. Different letters denote significant differences ($P < 0.05$).

significant correlation was found between ORAC and ABTS, but the capacity of certain extracts such as those of pomegranate and green pepper to scavenge O_2^- radicals was related to their capacity to scavenge ABTS radicals.

Since phenolic compounds have been proposed as major contributors to antiglycative activity,[6] they were also determined (Table 2). TPC varied from 4.8 mg GAE g^{-1} in apricot extract to 22.2 mg GAE g^{-1} in pomegranate extract. The proportion of individual phenolic acids in the total extracted phenolic acids was also evaluated (Table 3). Among these phenolic acids, one can note the significant contributions of caffeic acid in apricot extract (41.3%), ferulic acid in sesame extract (39.9%) and gallic acid in pomegranate extract (39.8%).

With the aim of finding other compounds present in the extracts that may be involved in antiglycative activity, benzoic acids and flavonoids were also determined. Benzoic acid content ranged from 3.5 to 7.9 mg GAE g^{-1} extract, with almond and pomegranate extracts having the lowest and highest content respectively (data not shown). Flavonoid content was lower than the limit of quantification (0.02 μg quercetin equivalent per 100 g sample) for all extracts except that of pomegranate, whose content was 0.29 μg quercetin equivalent per 100 g sample (data not shown).

In order to evaluate the inhibitory effect of seed extracts against AGE formation *in vitro*, the fluorescence intensity was measured using AG as an AGE inhibitor. Figures 1A and 1B display the inhibitory effects at 25 mg mL^{-1} on AGE formation in BSA–Glc and BSA–MGO assays respectively (final concentration in reaction medium 3.6 mg mL^{-1}). The results indicated significant differences in AGE-inhibitory activity among most samples ($P < 0.05$). In BSA–Glc assay the AGE-inhibitory rate ranged from 20.7 to 91.9%. Green pepper exhibited the highest inhibitory capacity with a value close to the effect of AG solution (average inhibitory rate 92.7%), followed by sesame and pomegranate with 66.1 and 61.7% inhibition respectively. The lowest inhibitory activity was observed for peach and apricot with 20.7 and 23.2% inhibition respectively. In contrast, both peach and apricot, together with pomegranate, resulted in more than 60% reduction in the formation of fluorescent AGEs in BSA–MGO assay, whereas sesame, hazelnut and almond had no inhibitory activity. In this assay the AGE-inhibitory rate of seed extracts ranged from 0 to 78.6%, with the highest values being lower than the effect of AG solution (average inhibitory rate 99.2%).

Several inhibitors can suppress AGE formation by scavenging certain precursors such as 1,2-dicarbonyls. An evaluation of direct MGO-trapping capacity was carried out in order to observe whether our tested seed extracts could directly scavenge these compounds. Figure 2 shows the different trapping abilities of the samples. All aqueous extracts trapped MGO in a dose-dependent manner and, with the exception of hazelnut and sesame, their activity at a concentration of 10 mg mL^{-1} was comparable to or higher than the effect of 1 mg mL^{-1} PM solution (99.6%). The values for IC_{50} (mg mL^{-1}) are presented in Table 4. As can be observed, apricot and peach had the lowest IC_{50} (0.14 mg mL^{-1}), followed by sour cherry (0.48 mg mL^{-1}), associated with their high MGO-trapping capacity. In contrast, sesame and hazelnut had the highest IC_{50}, corresponding to their low MGO-trapping capacity (Fig. 2). As expected, IC_{50} from MGO-trapping assay was negatively correlated with antiglycative activity from BSA–MGO assay (r=−0.890, P=0.003). In this sense, peach and apricot exhibited the lowest IC_{50} by MGO-trapping assay and, at the same time, the highest antiglycative activity by BSA–MGO assay together with pomegranate. In contrast, no relation was found for pomegranate between IC_{50} from MGO-trapping assay and antiglycative activity from BSA–MGO assay. Regarding BSA–Glc assay, no relationship was found between IC_{50} and anti-AGE capacity according to this assay ($P > 0.05$).

In the present study it has been demonstrated that aqueous extracts of fruit and vegetable seeds possess antiglycative activity. In addition, the samples displayed concentration-dependent MGO-trapping ability. However, as mentioned above, some of the extracts were found to be inhibitors of AGE formation in BSA–Glc assay, whereas little or no effect was observed when these samples were subjected to BSA–MGO assay (Fig. 1). Since inhibitors of glycation can act in multiple steps, it is important to apply different scenarios such as BSA–Glc and BSA–MGO assays to reach a conclusion on their anti-AGE ability. Consequently, both positive and negative inhibitory effects should not be discounted.

In previous studies, several authors have found correlations between TPC and the inhibitory effect on AGE formation of different extracts.[6,7] The possible association of AGE inhibition and antioxidant activity was analysed in our assays. No correlation between ORAC or ABTS and antiglycative activity was found in either BSA–Glc or BSA–MGO assay. Regarding the phenolic compound content, it was not correlated with anti-AGE activity in either absolute or relative amounts. Similar results have been observed by Povichit *et al.*,[13] who reported that extracts of certain medicinal plants exhibited high antiglycative activity although they had low phenolic content. In accordance with Sun *et al.*,[7] such absence of correlation suggests that phenolic compounds are not the sole antiglycative agents of the selected seed extracts

Table 3. Proportion (%) of phenolic acids in each seed extract[a]

Sample extract	PHB	SYN	VA	pCU	CA	FA	PCA	GA	GE	SIN	EA
Gp	24.0	2.1	5.2	5.2	5.6	18.7	9.6	ND	ND	5.1	24.4
Ap	3.0	1.8	4.8	13.5	41.3	26.7	5.7	0.7	ND	2.5	ND
Ha	5.6	10.4	32.0	4.7	1.2	3.2	22.2	15.4	ND	5.3	ND
Pe	21.5	7.7	1.9	17.5	2.4	17.2	28.0	1.7	ND	2.0	ND
Sc	20.1	3.5	1.1	16.3	20.8	3.3	14.7	4.1	12.2	4.0	ND
Se	7.7	2.6	0.4	30.1	11.3	39.9	4.6	0.1	ND	3.3	ND
Al	9.8	38.4	2.7	10.3	12.3	7.1	12.5	2.7	ND	4.3	ND
Po	11.8	8.8	0.1	8.3	1.6	4.5	4.2	39.8	ND	1.3	19.6

[a] Gp, green pepper; Ap, apricot; Ha, hazelnut; Pe, peach; Sc, sour cherry; Se, sesame; Al, almond; Po, pomegranate; PHB, *p*-hydroxybenzoic acid; SYN, syringic acid; VA, vanillic acid; pCU, *p*-coumaric acid; CA, caffeic acid; FA, ferulic acid; PCA, protocatechuic acid; GA, gallic acid; GE, gentisic acid; SIN, sinapinic acid; EA, ellagic acid; ND, not detected (<2 μg g^{-1} sample).

Table 4. MGO-trapping capacity of different seed extracts[a]

Sample extract	IC_{50} (mg mL^{-1})
Gp	1.70
Ap	0.14
Ha	3.30
Pe	0.14
Sc	0.48
Se	2.65
Al	2.25
Po	1.60
PM	0.06

[a] Gp, green pepper; Ap, apricot; Ha, hazelnut; Pe, peach; Sc, sour cherry; Se, sesame; Al, almond; Po, pomegranate; PM, pyridoxamine.

and therefore other compounds present in the samples must be involved in their anti-AGE ability. It should be taken into account that most authors have investigated the preventive effect on AGE formation through extraction of samples with various organic solvents, which are more efficient than water for extracting phenolic compounds.[30] As mentioned above, we carried out our assays using aqueous extracts, which might justify the lack of relationship between phenolic composition and antiglycative activity of the extracts.

Another factor to consider is the individual phenolic acid composition in the different aqueous extracts. In this sense, Wu *et al*.[31] evaluated the ability of dietary phenolic acids to inhibit glucose-mediated protein glycation. The authors concluded that at a concentration of 1 mmol L^{-1} most phenolic acids inhibited the glycation process, with vanillic, gallic and ferulic acids being the most significant inhibitors. According to these results, the high inhibitory activity of sesame and pomegranate in BSA–Glc assay could be explained, as their major phenolic acids were ferulic acid and gallic acid respectively (Table 3, Fig. 1A). Recently, Muthenna *et al*.[32] reported ellagic acid to be a potent antiglycating agent in a protein–sugar system, which, together with gallic acid, could explain the high antiglycative activity of pomegranate extract. Regarding green pepper, its anti-AGE ability in this system could be associated mainly with its ellagic acid content, but in addition with its ferulic acid and *p*-hydroxybenzoic acid content, which, although at lower intensity, has also been found to be an antiglycative agent.[31] The anti-AGE effect of the compounds, however, depends on the tested concentrations; thus low, high or no antiglycative activity has been described for ferulic acid at different incubation conditions in protein–sugar model systems.[19,33,34] In the BSA–MGO system, Gugliucci *et al*.[35] reported high AGE inhibition by caffeic acid. This may justify apricot extract showing one of the highest antiglycative activities in the present study (Fig. 1B), but it does not justify pomegranate or peach extract activity. It should be highlighted that, in accordance with Chompoo *et al*.,[5] the presence of more than one inhibitor in an extract may have a synergistic effect. In this way, Rice-Evans *et al*.[36] reported that phenolic compounds may have antagonistic or synergistic effects with themselves or with other constituents of a sample, which may justify the diversity found among the results of the present study. To the best of our knowledge, antiglycative effects of sesame, green pepper, apricot and peach seeds have not been reported before. Regarding pomegranate seed, anti-glycative ability associated with its free radical-scavenging property has been reported by Rout and Banerjee.[37]

CONCLUSIONS

Eight aqueous extracts of fruit and vegetable seeds were evaluated for their antiglycative activity using different *in vitro* models. All extracts exhibited positive inhibitory effects on the formation of AGEs in BSA–Glc assay, although to different extents. However, BSA–MGO assay and direct MGO-trapping assay showed that some extracts exerted their activity through a different mechanism of action. Aqueous extracts of peach, pomegranate and apricot seeds appeared to inhibit the formation of AGEs through their capacity for trapping 1,2-dicarbonyls. These findings are relevant for focusing on potential extracts to combat the main promoters of aging in humans. On the other hand, no relationship between antioxidant and phenolic compound content and antiglycative activity of the extracts was found. This finding leads us to conclude that other hydrophilic constituents in addition to phenolic acids must be involved in the antiglycative activity of the aqueous seed extracts. Isolation and characterisation of different target compounds are under current investigation by advanced structural identification methodologies.

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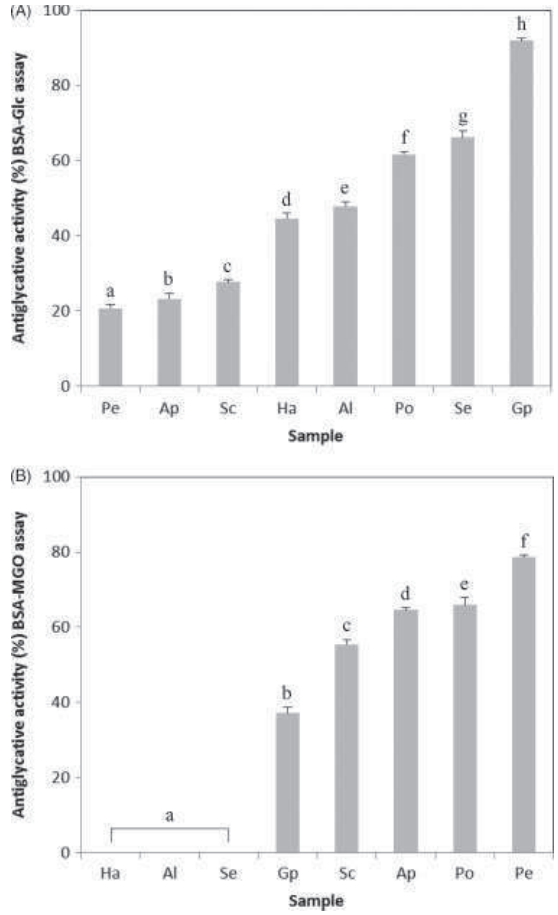

Figure 1. Antiglycative activity of green pepper (Gp), apricot (Ap), hazelnut (Ha), peach (Pe), sour cherry (Sc), sesame (Se), almond (Al) and pomegranate (Po) seed extracts on formation of AGEs in (A) BSA–Glc and (B) BSA–MGO assays. The concentration of each extract was 25 mg mL^{-1} (final concentration in reaction medium 3.6 mg mL^{-1}). Results are expressed as mean ± SD for n=4. Different letters denote significant differences ($P < 0.05$). AG solution (final concentration 0.6 mg mL^{-1}) presented an antiglycative activity of 92.7 and 99.2% in BSA–Glc and BSA–MGO assays respectively.

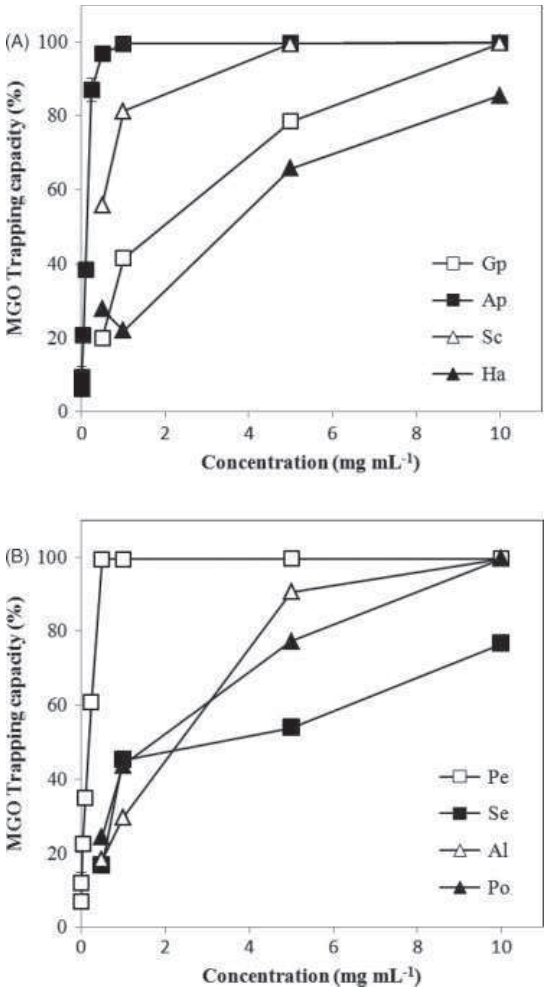

Figure 2. Dose-dependent results for MGO-trapping capacity (168 h) of (A) green pepper (Gp), apricot (Ap), sour cherry (Sc) and hazelnut (Ha) and (B) peach (Pe), sesame (Se), almond (Al) and pomegranate (Po) seed extracts. Results are expressed as mean ± SD for n=4. PM solution (final concentration 0.1 mg mL^{-1}) presented an MGO-trapping capacity of 99.6%.

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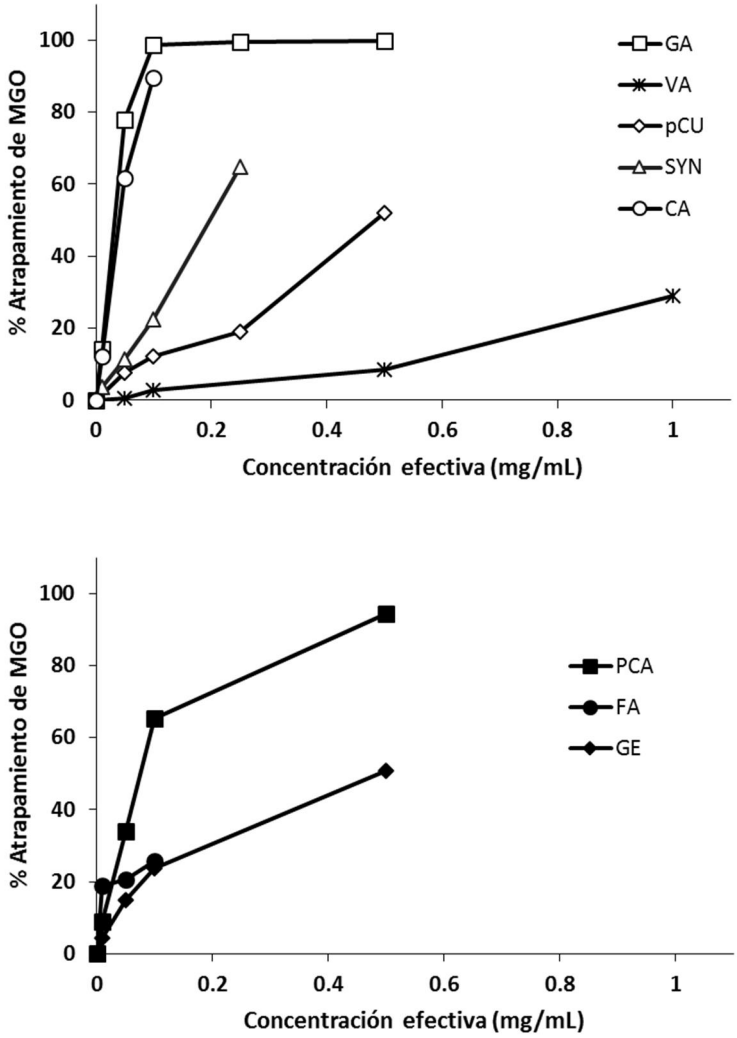

Figura 1: Actividad de atrapamiento de MGO de ácidos fenólicos presentes en los extractos de semillas ensayados tras una incubación en tampón fosfato sódico (100 mM, pH 7.4/ 37 ºC/ 168 h) en un rango de concentración de 0.1-1 mg/mL. Los ácidos fenólicos estudiados corresponden a ácido gálico (GA), ácido vanílico (VA), ácido p-cumárico (p-CU), ácido sinapínico (SIN), ácido caféico (CA), ácido protocatecuico (PCA), ácido ferúlico (FA) y ácido gentísico (GE). El ácido elágico (EA), ácido siríngico (SYR) y ácido p-hidroxibenzóico (pHB) no mostraron actividad a las concentraciones ensayadas. La piridoxamina (0.1 mg/mL) que fue utilizada como control positivo presentó una actividad de atrapamiento de 98.4%.

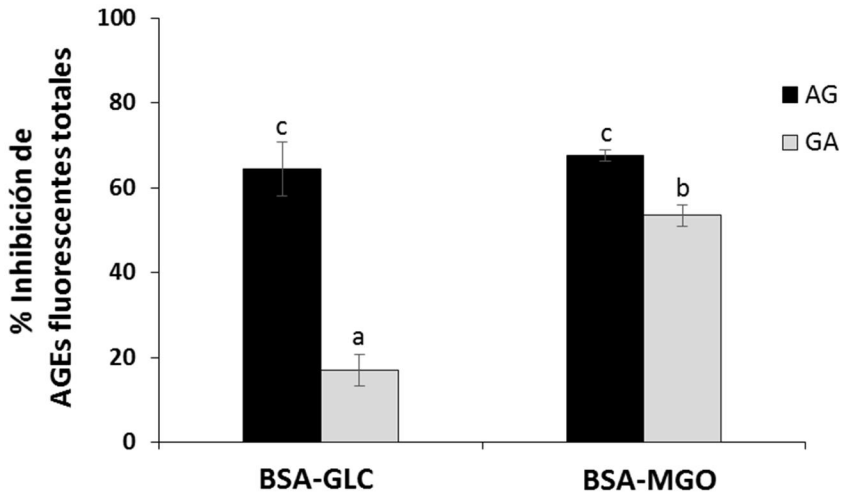

Figura 2: Inhibición de la formación de AGEs fluorescentes totales en los sistemas de glicación *in vitro* de BSA-GLC y BSA-MGO por acción del ácido gálico (GA), ácido fenólico mayoritario del extracto acuoso de granada y la aminoguanidina (AG, Control positivo) a una concentración de 0.14 mg/mL. El GA presentó un IC_{50} en el sistema BSA-MGO de 0.13 mg/mL mientras que en el sistema BSA-GLC alcanzó un máximo de actividad de 17%. La AG presentó un IC_{50} para BSA-GLC y BSA-MGO de 0.11 mg/mL y 0.10 mg/mL respectivamente. Los resultados fueron expresados como una media ± SD (n = 4). Las letras distintas indican diferencias significativas $P < 0.05$

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An aqueous pomegranate seed extract ameliorates oxidative stress of human hepatoma HepG2 cells

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Abstract

BACKGROUND: Aqueous pomegranate seed extract (PSE), a by-product of the pomegranate juice industry, was recently identified as a potential antiglycative ingredient. Ellagic acid was proposed as the major polyphenol responsible for the antiglycative activity as exerted in *in vitro* models. However, there is no information on safety aspects of this extract in biological systems before its application as ingredient. The cytotoxicity of PSE (1–100 μg mL^{-1}) was evaluated by determining its effect on cell viability and redox status of cultured HepG2 cells. The protective effect of the PSE against oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BOOH) was also investigated.

RESULTS: No changes in cell integrity or intrinsic antioxidant status resulted from a direct treatment with aqueous PSE, even at high dosage. In addition, reactive oxygen species (ROS) induced by *t*-BOOH were reduced by 21% when cells were pretreated with 100 μg mL^{-1} of aqueous PSE at 180 min. The range of concentrations investigated was effective in decreasing the ROS formation but not in a dose-dependent manner.

CONCLUSION: Aqueous pomegranate seed extract enhances human hepatoma cells integrity and resistance to cope with a stressful situation at concentration up to 100 μg mL^{-1}.

Keywords: pomegranate seed; glycation; cytotoxicity; HepG2 cell model; reactive oxygen species

INTRODUCTION

Punica granatum (pomegranates), belonging to the *Punicacea* family, has a remarkable content in polyphenols, particularly hydrolysable tannins (ellagitannins and gallotannins) and anthocyanins (delphinidin, cyanidin and pelargonidin). Based on its high content of antioxidants, consumption of pomegranate as fruits or juice has been largely related to chemopreventive and anti-inflammatory effects.[1] Hence several pomegranate-containing products have been marketed around the world in recent years. However, pomegranate fruits contain considerable amounts of seeds (about 20% w/w), which are an important by-product in the elaboration of the pomegranate juice. Recently, our group identified a significant antiglycative activity of an aqueous pomegranate seeds extract (PSE). PSE showed *in vitro* inhibitory effect of the formation of advanced glycation end-products (AGEs) through two mechanisms: (1) preventing glycation and (2) scavenging methylglyoxal, a potent AGE precursor.[2]

AGEs have received great attention because of their association with the ageing process[3] and related chronic diseases such as diabetes mellitus and its complications[4] Alzheimer's disease[5] or atherosclerosis.[6] For this reason, many investigations have been driven by the search for inhibitors of AGE formation. Initial investigations focused on synthetic AGE inhibitors such as aminoguanidine,[7] and other inhibitors (carnosine[5] or pyridoxamine[7]). However, it has been demonstrated that aminoguanidine can exert serious side effects in animal models. In fact, aminoguanidine can inhibit diamine oxidase and nitric oxide synthase enzymes involved in vascular and inflammatory processes.[8] In this sense, efforts have been devoted to the study of natural substances.[7] Plant by-products have been considered as a good alternative to present compounds with antiglycative and antioxidant capacity. These phytochemicals are mostly phenolic compounds, being the most widely investigated ellagic acid, chlorogenic acid, gallic acid, ferulic acid and caffeic acid.[9–12]

Several studies have shown that pomegranate fruit has a positive effect on the prevention and treatment of the diseases mentioned previously. Again, the high content of phenolic compounds plays a pivotal role on the biological properties of the pomegranate, although each compound contributes in a different way.[13] On the other hand, the fruit part, variety, extraction method or industrialisation process influence the content of these biochemical compounds.[1] In this regard, research has been

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carried out with pomegranate juice or peel, while studies with seeds have been focused on the beneficial effects of certain polyphenols, polyunsaturated fatty acids, conjugated linolenic acid, punicic acid and tocopherols,[14] all present in the seed oil. Nevertheless, there are few studies on the potential genotoxicity of whole pomegranate hydroalcoholic extracts tested both *in vitro* and *in vivo*.[15] Basic toxicological information is required before further suggestion of its application as an ingredient in foods or pharmacological preparations.

The aim of this study was to investigate the toxicity, in terms of cell injury, and cellular redox status potential change of a human hepatoma cell line (HepG2) after treatment with different concentrations of PSE. The effect of PSE on cell viability and oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BOOH) was evaluated.

MATERIALS AND METHODS

Materials

Pomegranate seeds were provided by TIKTA (Ankara, Turkey). Human hepatoma HepG2 cells were supplied by Dr Paloma Martín-Sanz (Instituto de Bioquímica, CSIC, Madrid, Spain). Dubelcco's modified Eagle's medium (DMEM-F12) and fetal bovine serum (FBS) were purchased from Biowhitaker Europe (Innogenetics, Madrid, Spain). Gentamicin, penicillin, streptomycin, *t*-BOOH, crystal violet, nicotine adenine dinucleotide reduced form (NADH) and Tris were from Sigma Chemical (Madrid, Spain). Sodium dodecyl sulfate salt, dimethyl sulfoxide (DMSO) and pyruvate were obtained from Panreac Quimica (Barcelona, Spain). The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR, USA) and cell culture dishes were acquired from Falcon (Cajal, Madrid, Spain). All other chemicals and reagents were of analytical grade.

Preparation of seed extracts

A portion (500 mg) of ground pomegranate seeds previously characterised[2] was extracted in water (25 mL, twice) by means of agitation for 10 min at 50°C. Supernatants were pooled, centrifuged (1400 × *g* for 10 min at 4°C) and lyophilised. The extract thus obtained is rich in phenolic compounds, mostly gallic and ellagic acids, as reported elsewhere.[2]

Cell culture and treatment with pomegranate seed extracts

HepG2 cells were grown in DMEM-F12 medium, supplemented with 2.5% FBS and 50 mg L^{-1} of gentamicin, penicillin and streptomycin antibiotics. Cells were incubated in humidified atmosphere of 5% CO_2 at 37°C. Under these conditions, two sets of experiments were designed for this study: (1) experiments of direct treatment of cells with PSE for 20 h to test for an effect of the tested compounds dissolved in FBS-free medium, since FBS might interfere in the performance of the assays and alter the results;[16] and (2) experiments of pretreatment of cells with PSE 20 h [cytotoxicity and reactive oxygen species (ROS) experiments] before submitting cells to an oxidative stress induced by *t*-BOOH (200 μmol L^{-1}, 3 h) to test for a protective effect.

Cell viability assay

The viability of HepG2 cells was determined using the crystal violet assay.[17] Cells were plated in 96-well plates with a density of 10^4 cells per well. Then, these were grown for 20 h with different concentrations of tested extracts (1–100 μg mL^{-1}), washed with PBS and incubated with 50 μL of crystal violet (0.2% in 2% ethanol) for 20 min. Plates were rinsed with water and dried twice. After adding 100 μL of 1% sodium dodecyl sulfate, the absorbance was measured at 570 nm using a Synergy™ HT-multimode microplate spectrophotometer (Biotek Instruments, Inc, Winooski, VT, USA). Results are expressed as percentage of cell viability regarding viable untreated cells.

Cytotoxicity assay

Cell cytotoxicity was determined following the lactate dehydrogenase leakage (LDH) method.[18] Cells were plated at a density of 1.5×10^6 cells per plate in 60-mm diameter plates and pretreated with different concentrations of pomegranate extract (1–100 μg mL^{-1}) for 20 h in FBS-free medium. After that, culture medium was collected and the cells scraped in phosphate-buffered saline (PBS). Cells were sonicated to break down the cell membrane and release the total amount of LDH. Then, they were centrifuged (1000 × *g*, 15 min) and the supernatant was collected. LDH was determined in culture medium and cell lysates, mixing with 5 mmol L^{-1} pyruvate, 0.35 mmol L^{-1} NADH and 84 mmol L^{-1} Tris. LDH leakage was estimated as the ratio between the LDH activity in the culture medium and total LDH cell content.

In parallel, to evaluate the protection of PSE against a cytotoxic agent HepG2 cells were submitted to the procedure described above but, in this case, *t*-BOOH (200 μmol L^{-1}) was added 3 h before the measurement.

Determination of reactive oxygen species

The dichlorofluorescein (DCFH) assay was used to quantify cellular ROS as a marker of the extent of cellular oxidativestress.[19] Cells were plated at a cell density of 2×10^5 cells per well in 24-well plates and changed to FBS-free medium the day after. In direct treatment experiments, 10 μL of DCFH (10 mmol L^{-1} in DMSO) were added to the wells and incubated at 37°C for 30 min. Then, cells were washed twice with PBS and 0.5 mL of PSE dissolved in FBS-free medium (1–100 μg mL^{-1}) were incorporated into the wells. Plates were immediately measured (time 0) using a microplate reader (Synergy™ HT-multimode microplate spectrophotometer) at an excitation/emission wavelength of 485/530 nm. This method is based on the DCFH probe oxidation, by intracellular oxidants or *t*-BOOH, to DCF which emit fluorescence. ROS were determined by quantifying fluorescence at different times (0, 60, 120 and 180 min). In protection experiments, cells were pre-incubated with 500 μL of the PSE (1–100 μg mL^{-1}) for 20 h. After that, 10 μL of DCFH (10 mmol L^{-1} in DMSO) were added to the wells and incubated at 37°C for 30 min. Then, cells were washed twice with PBS and 500 μL of *t*-BOOH in FBS-free medium added. Control samples without *t*-BOOH were used as negative control. Results are expressed as fluorescence units.

Statistical analysis

Statistical analysis of data was performed using the Statgraphics Centurion XV Statistical program (Herndon, VA, USA). Data were tested for homogeneity of variances by using Levene's test. Analysis of variance was performed using ANOVA procedures followed by a Bonferroni test or Tamhane test when the variances were homogeneous or non-homogeneous, respectively. Differences were considered significant at $P < 0.05$. Data from three independent assays were expressed as the mean value ± SD.

RESULTS AND DISCUSSION

After our previous study on the antiglycative and antioxidant capacity of PSE,[2] a question concerning safety issues of the PSE arose. Therefore, potential toxicity of PSE in terms of cell viability and effects on the general oxidative status was assessed in a human cell line. Since liver is the major location for xenobiotic metabolism, research on xenobiotics should be focused first on liver cells. HepG2 cells are a transformed cell line, which is well differentiated and characterised. In addition, HepG2 is a reliable cell model that responds to different test conditions.[18] Several studies have employed this cell line to evaluate the toxicity or protective effects of phenolic compounds against oxidative stress induced by *t*-BOOH.[19–21]

After selecting HepG2 cells as an appropriate model, the crystal violet assay was used to determine potential effects of PSE on HepG2 viability. Cells were pretreated with different concentrations of PSE (1, 10 and 100 μg mL^{-1}) for 20 h. As shown in Table 1, reduction of cell viability determined as the relative density of adherent cells was not observed. Cell viability remained unchanged even at high concentrations of PSE (100 μg mL^{-1}), which represents a high dose of this natural ingredient.

LDH leakage from the cell cytosol to the culture medium was used as an indicator of cytotoxicity, and generally as an indicator of cell integrity. LDH leakage is estimated from the ratio between the LDH activity in the culture medium and the whole cell content. A range of PSE doses between 1 and 100 μg mL^{-1} was tested for a period of 20 h for their potential deleterious effect on HepG2 cells, as depicted in Fig. 1 (empty bar). Treatment with PSE significantly decreased LDH leakage as compared with untreated control cells. However, a dose-dependent effect was not observed at the concentrations tested (1, 10 and 100 μg mL^{-1}) since results only ranged from 9.7% to 9.2% LDH activity in the culture medium after 20 h for PSE concentrations from 1 to 100 μg mL^{-1}. These results confirm the lack of cytotoxicity of PSE on HepG2 cells, even at high concentrations.

The protective effect of PSE against a cytotoxic inductor agent such as *t*-BOOH was also evaluated. It is known that decomposition of *t*-BOOH accelerates lipid peroxidation chain reactions, depletion of cell glutathione, and induction of cell toxicity by damage to DNA and apoptosis.[18] Cells were pretreated with different concentrations of PSE (1, 10 and 100 μg mL^{-1}) for 20 h and then treated with *t*-BOOH. A batch of cells not submitted to PSE was treated with 200 μmol L^{-1} *t*-BOOH for 3 h as a positive control for severe cell damage conditions. As depicted in Fig. 1 (solid bar), PSE efficiently prevented LDH leakage at concentrations of 10 and 100 μg mL^{-1} as compared with the control cells without pretreatment with PSE. A 50.6% of LDH leakage was obtained in control cells whereas in cells pretreated with PSE toxicity was significantly decreased to 37.3 and 34.9% of LDH leakage at the concentrations of 10 and 100 μg mL^{-1}, respectively. However, this protection was not statistically significant at the concentration of 1 μg mL^{-1}.

Table 1. Effect of pomegranate seed extract on HepG2 cells viability after treatment for 20 h

Sample	Cell viability (%)
Control	100.00 ± 6.86
Seed extract (μg mL^{-1})	
1	104.92 ± 10.05
10	105.85 ± 11.21
100	103.22 ± 11.21

Results are expressed as mean ± SD ($n = 4$).
Significant differences were not observed ($P < 0.05$).

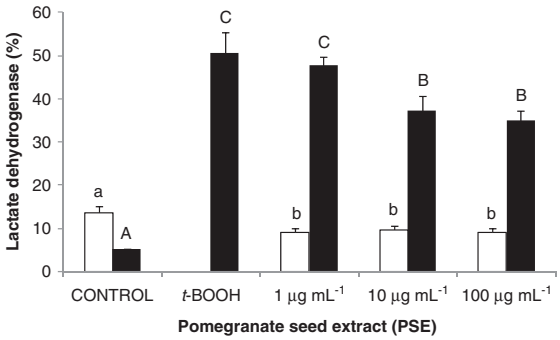

Figure 1. Lactate dehydrogenase (LDH) leakage after treatment with pomegranate seed extract (PSE; 1, 10 and 100 μg mL^{-1}) for 20 h (white bars) and after *t*-BOOH exposure for 3 h (black bars). Data show the mean ± SD ($n = 5$). Different lower-case (white bars) and upper-case (black bars) letters indicate significant differences ($P < 0.05$).

The DCFH assay was used to determine intracellular ROS generation by measuring the fluorescence emitted by DCF after oxidation of DCFH. ROS generation is an important marker of the cell status since it causes damage to cellular components impairing or altering their metabolic functions. Figure 2 shows that PSE was effective in decreasing the formation of intracellular ROS, but its effectiveness varies with the time of exposure and the concentration of the extract. It is important to notice that direct application of PSE on HepG2 cells did not promote intracellular ROS formation at any concentration or time tested. The rate of ROS formation after 60 min of exposure was significantly reduced to 87.1, 86.4, and 84.9% for 1, 10 and 100 μg mL^{-1}, respectively. Nevertheless, effective protection was not observed for the highest concentration of PSE tested at the longest time of exposure. On the other hand, PSE concentration at 1 and 10 μg mL^{-1} for 180 min significantly reduced the generation of intracellular ROS. These results confirm that PSE, even at the highest rate of dosage, did not induce an oxidative damage into the cell model.

In a next assay, cells were treated with *t*-BOOH as a chemical compound that induces oxidative stress. *t*-BOOH can decompose to other alkoxyl and peroxyl radicals in a reaction that generates ROS.[18] Figure 3 shows that PSE significantly decreased the ROS formation for all the concentrations tested as compared with untreated *t*-BOOH control. Although a dose–response effect was not observed, the formation of ROS was significantly decreased with the time of exposure (79.1, 76.9 and 79.3% were achieved for 1, 10 and 100 μg mL^{-1} at 180 min, as compared with 100% of *t*-BOOH). These results confirm the protective effect of PSE against an oxidative insult on HeG2 cell.

Various studies have shown that pomegranate is a fruit with anti-ageing, anti-atherosclerosis and anti-inflammatory effects, with potential protective effect against other cardiovascular diseases and neurodegeration.[22–24] These effects are likely due to its content of bioactive compounds such as phenolics acids, flavonoids, punicalagin and ellagitannins.[23,25] Extraction of the former bioactive compounds is strongly related to the extraction solvent used. Thus a methanolic extract of pomegranate peel presents higher antioxidant activity and phenolic content than

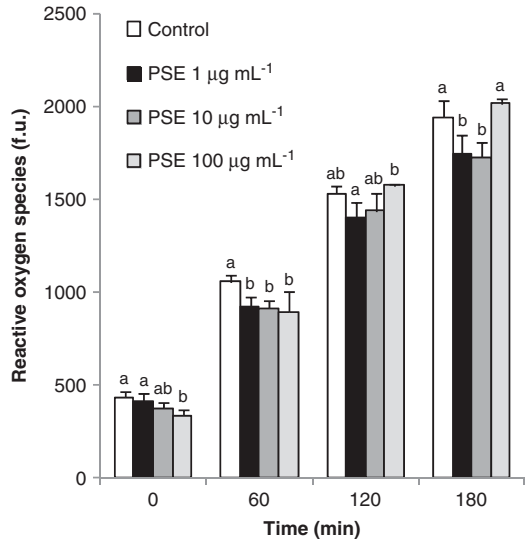

Figure 2. Intracellular generation of reactive oxygen species after treatment with pomegranate seed extract (PSE; 1, 10 and 100 μg mL^{-1}) at 0, 60, 120 and 180 min. Results are expressed as mean ± SD ($n = 4$). Different letters denote significant differences ($P < 0.05$) within the same incubation time. f.u.: fluorescence units.

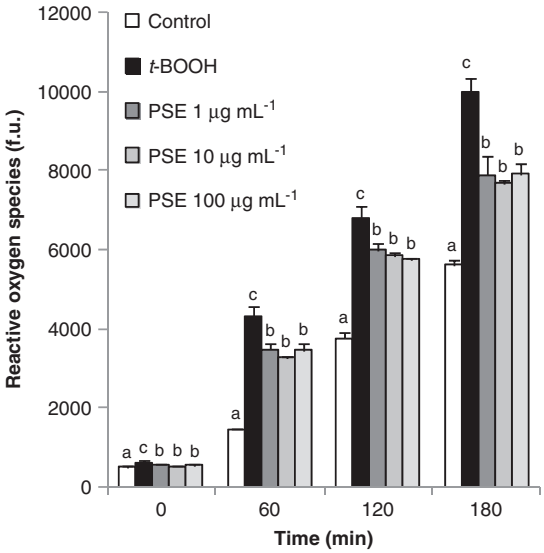

Figure 3. Protective effect of pomegranate seed extract (PSE) pretreatment (1, 10 and 100 μg mL^{-1}) for 20 h against oxidative stress induced by *tert*-butyl hydroperoxide (*t*-BOOH) after 0, 60, 120 and 180 min of exposure. Data show the mean ± SD ($n = 4$). Different letters denote significant differences ($P < 0.05$). f.u.: fluorescence units.

does an aqueous extract and other parts of pomegranate, such as seed or leaf.[26]

In the present study, the seed coat was chosen as a by-product and water was used for extraction as a cost effective and environmentally compatible approach[27] that was safer than if non-polar solvents were used. Under these conditions, the hydrophilic compounds with antioxidant and antiglycative activities, such as gallic acid, ellagic acid and other phenolic compounds, were efficiently extracted.[2,28] Although methanol or ethanol have been largely used as extraction solvents pomegranate,[15,28,29] aqueous extracts of tropical plants (roselle, spring onion, sweet potato, wild lime, or tapioca among other plants) have been proposed as a source of functional ingredients.[30]

A number of researchers have stressed the need to study the possible toxicity of non-edible parts of pomegranate since few studies have shown a certain toxicity of whole fruit extracts both in *in vivo* and *in vitro* assays. Sánchez-Lamar *et al.*[15] reported that a hydroalcoholic pomegranate fruit extract could be genotoxic at various expression levels. According to Vidal *et al.*[29] amounts over 0.2 mg of pomegranate hydroalcoholic extract produced embryotoxicity. In addition, other authors also suggested that roots and barks from pomegranate presented toxic effects probably due to their alkaloids, tannins or flavonoids.[15] Nevertheless, these toxic effects have been observed at higher concentrations than those that are effective as antivirals or are intended for the treatment of respiratory diseases. On the contrary, studies performed with aqueous extracts have not shown toxic effects, although there is some controversy. Settheetham and Ishida[31] reported that aqueous pomegranate peel extract induced apoptosis in human cells in *in vitro* assays. In the present study the safety of the PSE was evaluated employing HepG2 as a cellular model. Cells were pretreated with a range of PSE concentrations (1 to 100 μg mL^{-1}) and it was observed that extracts did not decrease cell viability or produce a cytotoxic effect. Moreover, treatment with PSE showed a significant protection of cells against *t*-BOOH toxicity. Aqueous extracts would be easier to obtain in a juice or seed infusion compared with non-aqueous extracts so it has more advantages for the consumer.

Hiraganahalli *et al.*[32] stated that pomegranate peel extract exerts a significant liver protective effect against *t*-BOOH toxicity and this might be correlated with a radical scavenging effect. Likewise, other studies have shown a hepatoprotective capacity of pomegranate peel extract against CCl_4, a cytotoxic agent studied as a liver toxicant. Studies *in vivo* have shown a protective effect of pomegranate in liver cells by recovery the normal hepatic architecture.[28] In the present study, intracellular ROS production was reduced after undergoing oxidative injury with *t*-BOOH. The literature points out that pomegranate's phenolic content may be related to its antioxidant capacity, which is higher in peels and flowers than in leaves and seeds.[33] Nevertheless, at high concentrations (100 μg mL^{-1}) the effect was significantly reduced regarding the time of exposure compared with lower concentrations of 1 and 10 μg mL^{-1}. This behaviour could be explained by dual antioxidant and pro-oxidant activities of phenolic compounds such as gallic or ellagic acid,[34] which are the most abundant phenolic compounds in PSE.[2,35]

Recent studies have reported several biological functions of ellagic acid, highlighting its anti-inflammatory, antioxidant and antimutagenic activities. In addition, ellagic acid can also exert a hepatoprotective effect against CCl_4 both *in vitro* and *in vivo*, contributing to the recovery of cellular antioxidants.[36] In a similar way, gallic acid may exhibit antimutagenicity and a significant antioxidant effect. Yeh *et al.*[37,38] revealed that this phenolic acid is a potent inducer of phenolsulfotransferase which shows a cytoprotector effect against oxidative injury. However, gallic and ellagic acids may behave as pro-oxidants like other phenolic compounds, although the mechanism of action has not been elucidated yet.[34] Gallic acid has shown a slight metal chelating and high electron donating capacities, which could explain its pro-oxidant effects at high concentrations. Its ability to

induce cytotoxicity and apoptosis in certain cell lines strengthens this dual activity.[11] On the other hand, the possibility that phenolic compounds may have antagonistic or synergistic effects with themselves or with other compounds can influence this duality.[2]

CONCLUSION

The studied aqueous pomegranate seed extract with antiglycative properties did not exert toxicity on HepG2 cell as a human hepatic cell model even at high exposure levels. Hepatic cells should metabolise PSE without detrimental effects on cell viability. Although literature describes that delphinidin, cyanidin and pelargonidin present in hydroalcoholic pomegranate extracts could exert cytotoxic effects,[39] the aqueous extract investigated in this study showed a remarkable positive effect on the prevention and reduction of the oxidative stress of HepG2 cells. The protective effect was particularly accentuated at the lowest PSE concentrations assayed. Although *in vivo* studies are needed in a further step, this investigation points out that aqueous pomegranate extract with antiglycative properties can be safely used, in terms of cell toxicity and protection against oxidative stress, as an ingredient in foods and pharmacological preparations.

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Antiglycative and carbonyl trapping properties of the water soluble fraction of coffee silverskin

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Advanced glycation endproducts (AGEs)
Coffee silverskin extract

ABSTRACT

Carbonyl stress and accumulation of advanced glycation end-products (AGEs) in human tissues are involved in diabetic complications, atherosclerosis, Alzheimer's disease and aging. The objective of this study was to evaluate the *in vitro* protective effect of aqueous extracts of coffee silverskin (CS) in the formation of AGEs and trapping of carbonyl reactive species such as methylglyoxal (MGO). Aqueous extracts of CS from Arabica and Robusta coffee varieties were obtained under environment friendly extraction conditions. CS extracts were characterized by the analysis of dietary fiber, caffeine, chlorogenic acids (CGAs), total phenolic compounds, browning, melanoidins, and antioxidant capacity. CS extracts and CGA exhibited a dose-dependent anti-AGE capacity in the protein–glucose model system (37 °C/21 days) with an IC_{50} of 0.6 mg/mL and 0.4 mg/mL, respectively. Caffeine did not prevent AGE formation under the studied conditions. Regardless to protein–MGO assay (37 °C/14 days), the anti-AGE capacity of CS extracts and CGA was also dose-dependent with an IC_{50} of 1.3 mg/mL and 0.1 mg/mL, respectively. Caffeine weakly inhibited the reaction of protein and MGO. The MGO trapping capacity was established as a model for protection against carbonyl stress. Robusta CS was very effective for the direct trapping of MGO with an IC_{50} of 0.055 mg/mL as compared with Arabica CS (IC_{50} of 0.6 mg/mL). CGA and caffeine showed an IC_{50} for MGO trapping capacity of 0.14 mg/mL and >10 mg/mL, respectively. The highest CGA content in the Robusta CS extract could explain its higher MGO trapping activity as compared with the Arabica CS extract. The anti-AGE and MGO trapping capacities of CS may be associated to other chemical components besides CGA. In conclusion, aqueous CS extract may be considered as a natural source of inhibitors of *in vitro* formation of AGEs and carbonyl stress. The inhibitory effect of the coffee extracts may be associated to their carbonyl trapping capacity.

1. Introduction

AGEs (advanced glycation end-products) are the final products derived from the Maillard reaction or non-enzymatic glycation process produced in the human body. It is known that AGEs are involved in the development of several health disorders such as diabetes and its complications (Vlassara & Palace, 2002), atherosclerosis (Vlassara, 1996), Alzheimer's disease and normal aging (Münch, Thome, Foley, Schinzel, & Riederer, 1997). In addition, the increase in reactive carbonyls in tissues is known as carbonyl stress which leads to directly increase chemical modification of proteins (glycation) and lipids (lipoxidation) in diabetes. Reactive carbonyl species generated from carbohydrate, lipid and amino acid metabolism such as methylglyoxal (MGO), glyoxal, glyoxalaldehyde, dehydroascorbate, 3-deoxyglucosone and malondialdehyde, are potent precursors of AGE formation and protein cross-linking (Thornalley, Langborg, & Minhas, 1999). MGO derived AGE structures, including CEL (N-epsilon-(carboxyethyl)-lysine) and MOLD (methylglyoxal-lysine dimer), are increased in diabetes (Baynes & Thorpe, 1999). Thus, preventing AGE formation/accumulation may control significantly the pathogenesis of diabetes complications.

The inhibition of AGE formation might follow several mechanisms involving, *e.g.*, aldose reductase, antioxidant activity, reactive dicarbonyl trapping, sugar autoxidation inhibition and amino group binding, where the antiglycative activity of phytochemicals has been usually linked to oxidative reactions (Bousová et al., 2005). The inhibition of AGE formation by some synthetic compounds such as aminoguanidine (AG) has been well documented. However, this compound has been associated with several adverse effects in *in vivo* studies (Thornalley, 2003; Williams, 2004) since it is a highly reactive nucleophilic reagent that reacts with many biological molecules (pyridoxal phosphate, pyruvate, glucose, malondialdehyde, and others). Hence, the search for natural products which can inhibit AGE formation has recently been an objective of worldwide research (Peng, Cheng, et al., 2008; Povichit, Phrutivorapongkul, Suttajit, Chaiyasut, & Leelapornpisid, 2010; Wang, Sun, Cao, & Tian, 2009).

Coffee consumption has been associated with reduction in chronic disease risk such as type 2 diabetes (Van Dam & Hu, 2005). Coffee has

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been suggested as a potential natural source of inhibitors of AGEs. Verzelloni, Tagliazucchi, Rio, Calani, and Conte (2011) stated that coffee melanoidins inhibit the formation of AGEs by (i) acting as a radical scavenger and an Fe-chelator in the post-Amadori phase of the glycation reaction and (ii) inhibiting dicarbonyl reactive compound formation during glucose autoxidation. However, only chlorogenic acid (CGA) effectively inhibits protein glycation and dicarbonyl compound formation.

CS is the tegument of green coffee beans (outer layer) and is the major by-product of the roasting procedure (Napolitano, Fogliano, Tafuri, & Ritieni, 2007). CS is characterized by the presence of high amounts of dietary fiber and antioxidant activity (Borrelli, Esposito, Napolitano, Ritieni, & Fogliano, 2004; Napolitano et al., 2007), and contains several bioactive compounds with potential application in food and health (Pourfarzad, Mahdavian-Mehr, & Sedaghat, 2013). Indeed, CS has been proposed as a functional ingredient due to its health-promoting properties (Esquivel & Jiménez, 2012). The use of aqueous extracts of coffee silverskin (CS) as health promoter has been recently proposed (del Castillo et al., 2013; Martinez-Saez et al., 2014). Among other bioactive compounds, the extract contains CGA and its health benefits have been in part associated to the presence of this bioactive phytochemical. No studies on the effect of the aqueous CS extract as a natural source of scavengers of dicarbonyls and its anti-AGE properties have been reported which are of great interest and it is the goal of the present investigation.

2. Materials and methods

2.1. Materials and reagents

Coffee silverskin from Arabica (*Coffea arabica*) and Robusta (*Coffea canephora*) varieties were provided by Fortaleza S.A. (Spain). According to the manufacturer the weight portion of the coffee silverskin represents 0.5% of the green beans and 0.6% of the roasted beans.

Bovine serum albumin (BSA), 40% methylglyoxal solution (MGO), sodium azide, aminoguanidine (AG), 5-methylquinoxaline (5-MQ), o-phenyldiamine (OPD), 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate, ammonium bicarbonate, caffeine (CFF) and chlorogenic acid (CGA) standards were purchased from Sigma (St. Louis, MO, USA). Folin–Ciocalteu reagent, iron(III) chloride, sodium phosphate monobasic, sodium bicarbonate, hydrogen peroxide, sodium chloride, chloroform, and hydrochloric acid were obtained from Panreac (Madrid, Spain). 2,2′-Azobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-striazine (TPTZ), 2,2-azobis (methylpropionamidine) dihidro (AAPH) and piridoxamine were purchased from Fluka Chemical (Madrid, Spain). Fiber kit was from Megazyme International Ireland Ltd. Methanol, acetonitrile, glacial acetic acid, potassium hexacyanoferrate and zinc sulfate were purchased from Merck (Darmstadt, Germany). Milli-Q water used was produced using an Elix3 Millipore water purification system coupled to a Milli-Q module (model Advantage10) (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

2.2. Equipments

Synergy™ HT-multimode microplate reader with an automatic reagent dispense and temperature control from Biotek Instruments (VT, USA), capillary electrophoresis apparatus Agilent G16000A (Agilent, Madrid, Spain) and HPLC Shimadzu (Kyoto, Japan) equipped with a LC-20AD pump, a SIL-10ADvp autosampler, a CTO-10ASVP oven, and a DAD (SPD-M20A).

2.3. Preparation of soluble extracts from coffee silverskin

Arabica and Robusta CS extracts were prepared by aqueous extraction according to the procedure patented by del Castillo et al. (2013b). Briefly, 50 mL of boiling water was added to 2.5 g of coffee silverskin. The mixture was stirred at 250 rpm for 10 min, filtered by Whatman paper no. 4 and the filtrate was freeze dried. The powdered extracts were stored in dark and dry place until analysis.

2.4. Determination of total, soluble and insoluble fiber

Total, soluble and insoluble dietary fibers of CS extracts were determined by an enzymatic–gravimetric method based on the AOAC methods 991.43 and 985.29 (Lee, Prosky, & De Vries, 1992; Prosky, Asp, Schweizer, Devries, & Furda, 1988, 1992) and employing a commercial kit. All measurements were performed in triplicate. Results were expressed as mg fiber/g CS extracts.

2.5. Determination of melanoidins

Melanoidin content of CS extracts (1 mg/mL) was determined according to Silván, Morales, and Saura-Calixto (2010). Extracts were then subjected to ultrafiltration using a Microcon YM-10 regenerated cellulose 10 kDa (Millipore, Bedford, MA) at 12000 *g* for 10 min. Melanoidin content was measured spectrophotometrically at 405 nm. Results were expressed as mg/g CS extracts.

2.6. Determination of CGA and caffeine

The procedure for the determination of CGA and caffeine was performed according to del Castillo, Ames, and Gordon (2002). The separation was carried out in a capillary electrophoresis system provided with an ultraviolet visible detection system. Calibration curves of caffeine and CGA were constructed. The analyses were performed in triplicate. Results were expressed as mg/g CS extracts.

2.7. Browning determination

Browning was measured at 405 nm using a microplate reader. CS extracts were dissolved in water at 10 mg/mL in order to obtain an absorbance reading less than 1.5 arbitrary units. All the measurements were made in triplicate. Results were expressed as absorbance units/g CS extracts.

2.8. Determination of total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method as described by Contini, Baccelloni, Massantini, and Anelli (2008). CGA calibration curve was used for quantification. All measurements were performed in triplicate. Results were expressed as mg CGA equivalent/g CS extract.

2.9. ABTS +. assay

ABTS +. assay estimated in terms of radical scavenging activity was employed for determining the antioxidant capacity of the extracts as described by Mesías, Navarro, Gökmen, and Morales (2013). Absorbance reading was taken using a microplate reader. Aqueous solutions of Trolox at various concentrations were used for calibration. All measurements were performed in triplicate. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC)/g CS extract.

2.10. ORAC assay

ROO• scavenging activity was measured by monitoring the fluorescence decay as result of ROO-induced oxidation of fluorescein, known

as the oxygen radical absorbance capacity (ORAC) assay as described by Dávalos, Gómez-Cordovés, and Bartolomé (2004). ORAC values were expressed as Trolox equivalents by using the standard curve calculated for each assay and the final results were expressed in μmol equivalents of Trolox/g CS extract. All measurements were performed in triplicate.

2.11. FRAP assay

Ferric reducing antioxidant power (FRAP) was determined as described by Morales, Martin, Açar, Arribas-Lorenzo, and Gökmen (2009). Aqueous solutions of Trolox at various concentrations were used for calibration. All measurements were performed in triplicate. Results were expressed as μmol TEAC/g CS extract.

2.12. DPPH assay

DPPH radical-scavenging activity was determined as described by Morales et al. (2009). Aqueous solutions of Trolox at various concentrations were used for calibration. All measurements were performed in triplicate. Results were expressed as μmol TEAC/g CS extract.

2.13. In vitro glycation assay with BSA–glucose

BSA–glucose (BSA–Glc) assay was based on Peng, Cheng, et al. (2008). Briefly, BSA (35 mg/mL) and glucose (175 mg/mL) were dissolved in phosphate buffer (0.1 M, pH 7.4). BSA solution also contained 0.1 g/mL sodium azide. BSA solution (200 μL) was incubated with glucose solution (400 μL) at 37 °C for 21 days in the absence or the presence (100 μL) of soluble CS extracts, caffeine, or CGA (concentration of the stock solutions at 1, 5, and 10 mg/mL). In parallel, CS extracts and standards were incubated at 37 °C for 21 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was: 10 mg/mL for BSA, 100 mg/mL for glucose, 0.6 mg/mL for AG, and 0.14, 0.71 or 1.42 mg/mL for the different solutions of caffeine, CGA, and CS extracts. The IC_{50} values (concentration in mg/mL required to inhibit glycation by 50%) were calculated from the dose–response curves using Microsoft-Excel computer software.

2.14. In vitro glycation assay with BSA–MGO

BSA–MGO assay was performed according to Lunceford and Gugliucci (2005) with minor modifications. Briefly, BSA (35 mg/mL) and MGO (0.4 mg/mL) solutions were prepared in phosphate buffer (0.1 M, pH 7.4). Two hundred microliters of BSA solution, containing 0.1 g/mL sodium azide, was incubated with 400 μL of MGO solution. The incubation was carried out at 37 °C for 14 days in the absence or the presence (100 μL) of soluble CS extracts, caffeine, or CGA (concentration of the stock solutions at 1, 5, and 10 mg/mL). In parallel, CS extracts and standards were incubated at 37 °C for 14 days in order to measure their intrinsic fluorescence. The final concentration of each reactant in the reaction medium was 10 mg/mL for BSA, 0.23 mg/mL for MGO, 0.6 mg/mL for AG and 0.14, 0.71 and 1.42 mg/mL for the different solutions caffeine, CGA and CS extracts. The IC_{50} values were calculated from the dose–response curves using Microsoft-Excel computer software.

2.15. AGE fluorescence measurement

The measurement of fluorescent intensity of total AGEs and the intrinsic fluorescence of the CS extracts and standards after incubation were performed using a microplate spectrophotometer. The presence of total AGEs was characterized by a typical fluorescence with excitation and emission maxima at 360 and 420 nm, for BSA–Glc assay and 340 and 420 nm, for BSA–MGO assay. Percentage inhibition of AGE formation by each sample was calculated using the following equation; % inhibition = [1 − ((fluorescence of the solution with inhibitors − intrinsic fluorescence of the samples) / fluorescence of the solution without inhibitors)] × 100%.

2.16. Evaluation of direct MGO trapping capacity

Direct MGO trapping capacity was as described by Peng, Zheng, et al. (2008) with some modifications (Mesías et al., 2013). Pyridoxamine (PM) was used as the positive control. The final concentration of each reactant in the reaction medium was 0.04 mg/mL for MGO, 0.1 mg/mL for PM and a range of 0.001–0.5 mg/mL for soluble CS extracts, caffeine and CGA. Samples were incubated at 37 °C for 168 h, after that 200 μL of OPD was added. The unreacted MGO was quantified by HPLC (Shimadzu, Kyoto, JP) on the basis of the amount of the derivatized product, 2-methylquinoxaline (2-MQ). The chromatographic separation was carried out on a Mediterranean-Sea-ODS2 column (150 × 3 mm, 5 μm, Tecknokroma, Barcelona, Spain). The amounts of unreacted MGO in the samples could be determined on the basis of the ratios of peak area of 2-MQ and 5-MQ. Percentage decrease in MGO was calculated using the following equation: MGO decrease % = [(amounts of MGO in control − amounts of MGO in sample or PM solution) / amounts of MGO in control] × 100%. The IC_{50} values of samples were calculated from the dose–response curves using Microsoft-Excel computer software.

2.17. Statistical analysis

Statistical analyses were performed using the Statgraphics Centurion XV statistical program (Herndon, VA). Data were expressed as the mean value ± SD. Analysis of variance (ANOVA) and the Duncan test were applied to determine differences between means. Differences were considered to be significant at $p < 0.05$.

3. Results and discussion

Table 1 summarizes the content in dietary fiber (total, soluble and insoluble), melanoidins, chlorogenic acids, caffeine, total phenolic, antioxidant and browning of water soluble CS extracts. TPC was of 31.0 and 35.4 mg equivalents CGA/g for Arabica and Robusta CS extracts, respectively. CS has previously been considered a good source of phenolic compounds (Bresciani, Calani, Bruni, Brighenti, & Del Rio, in press). CGA content was significantly higher in Robusta CS extract (68.52 mg/g) than in Arabica CS extract (11.18 mg/g). Results of CGA are in line with

Table 1
Characterization of Arabica coffee silverskin (ACS) and Robusta coffee silverskin (RCS) extracts.

Parameters	ACS extract	RCS extract
Fiber		
Total (mg/g)	286.89 ± 19.15 a	362.18 ± 13.74b
Soluble (mg/g)	240.15 ± 19.5a	268.04 ± 6.44b
Insoluble (mg/g)	46.75 ± 0.34a	94.14 ± 7.30b
Melanoidins (mg/g)	172.67 ± 3.54a	239.46 ± 8.49b
CGAs (mg/g)	11.18	68.52
3-CGA (%)	13.43	11.97
4-CGA (%)	16.26	13.28
5-CGA (%)	57.75	38.45
Caffeine (mg/g)	30.26	33.98
Browning (A_{405}/g)	94.55 ± 1.51a	106.88 ± 0.83b
Total phenolic content (mg eq GA/g)	31.00 ± 0.24a	35.41 ± 0.42b
ABTS (μmol TEAC/g)	85.20 ± 1.91a	225.8 ± 24.8b
ORAC (μmol TEAC/g)	1194 ± 76.62a	1513 ± 9.99b
FRAP (μmol TEAC/g)	829.8 ± 38.16a	640.1 ± 39.78b
DPPH (μmol TEAC/g)	219.9 ± 4.34a	231.3 ± 4.73b

Results are expressed as mean ± SD for $n = 4$. Different letters mean significant differences.

those reported by Narita and Inouye (2012) and del Castillo et al. (2013). In a similar way, caffeine content was also higher in Robusta CS extract (33.98 mg/g) in comparison with Arabica CS extracts (30.26 mg/g). For browning determination and melanoidin content, moreover, these differences were significant. These results agree with those from Martinez-Saez et al. (2014) who described that levels of CGA, caffeine concentration, melanoidins and browning, expressed as color, were significantly greater ($p < 0.05$) in beverages made with Robusta coffee silverskin extract than Arabica.

The antioxidant capacity (radical scavenging, and hydrogen and electron donating capacities) of the CS extracts was assessed by ABTS, ORAC, DPPH and FRAP assays, showing results of 85.20, 1194, 829.8 and 219.9 μmol TEAC/g sample in the case of Arabica CS extract and 225.8, 1513, 640.1 and 231.3 μmol TEAC/g sample for Robusta CS extract. Arabica CS extract showed higher reducing power than Robusta CS extract. In contrast, Robusta CS extract showed significantly higher scavenging properties against the ABTS radical than Arabica CS extract. The results agree with those described by Napolitano et al. (2007), del Castillo et al. (2013) and Martinez-Saez et al. (2014), who found, a higher proportion of extractable antioxidants in aqueous solution for the samples of Robusta silverskin, in comparison with Arabica. The differences found in the total antioxidant capacity values in ACS and RCS extracts may be related to those detected by analyzing their components, melanoidins and CGA since, as it has been suggested, these compounds contribute to the antioxidant properties of coffee silverskin.

Fig. 1 shows the effect of CS extracts, caffeine, and chlorogenic acid on the formation of fluorescent AGEs in glycation model systems composed by BSA and glucose treated at 37 °C for 21 days. Caffeine did not affect the formation of AGEs under our particular conditions. CGA showed antiglycative capacity being its IC_{50} value of 0.4 mg/mL. Kim et al. (2011) already described the antiglycative capacity of CGA in the formation of fluorescent AGEs and crosslinking of collagen. Arabica and Robusta CS extracts showed similar and dose-dependent rates of AGE inhibition with an IC_{50} of 0.6 mg/mL for both extracts. The only significant differences ($p < 0.05$) were found at the concentration of 0.71 mg/mL where ACS showed a significantly higher antiglycative activity than RCS.

Fig. 2 represents the antiglycative capacity of the Arabica and Robusta CS extracts, caffeine, and chlorogenic acid in glycoxidation model systems (BSA–MGO) at 37 °C for 14 days. MGO was reported as a potent agent for AGE generation and modified proteins irreversibly by targeting the side chains of arginine at a much faster rate than reducing sugars (Oya et al., 1999). Caffeine (1.4 mg/mL) exerted a weak antiglycative capacity corresponding to 10% of the inhibition of the formation of fluorescent AGEs. The antiglycative capacity of CGA was notably higher in the BSA–MGO assay (IC_{50} of 0.1 mg/mL) as compared with the BSA–glucose assay. However, the antiglycative capacity of Arabica and Robusta CS extracts was lower (IC_{50} of 1.3 mg/mL) as compared with the BSA–glucose assay. Again, significant differences between both CS extracts were not observed, and they behaved in a dose-dependent manner.

AGE formation can be suppressed by inhibitors at the post-Amadori stage which can scavenge carbonyl precursors. As a consequence, the direct MGO-trapping capacity of Arabica and Robusta CS extracts was determined. Fig. 3 shows the MGO trapping ability of the CS extracts at 168 h in a range of concentrations from 0.001 to 0.5 mg/mL. Pyridoxamine (0.1 mg/mL) was used as control since it reacts with methylglyoxal to form stable adducts (Nagaraj et al., 2002). IC_{50} value for PM was 0.006 mg/mL. Caffeine, chlorogenic acid, and the Arabica and Robusta CS extracts trapped MGO in a dose-dependent manner. The lowest effectiveness was shown by the caffeine whereas only 20% of the initial MGO was decreased in the presence of 0.5 mg/mL caffeine. MGO was effectively quenched by CGA with an IC_{50} of 0.14 mg/mL. Arabica and Robusta CS extracts showed significant differences in their ability to trap MGO with an IC_{50} of 0.055 and 0.65 mg/mL for

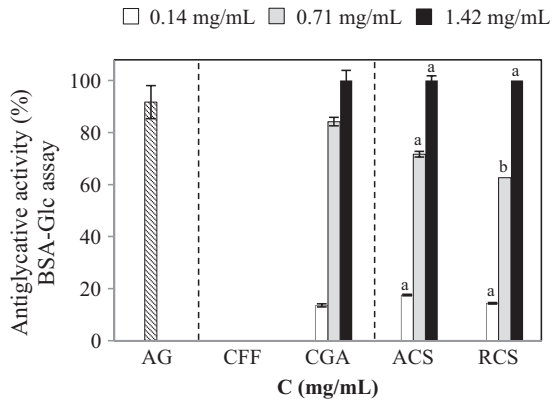

Fig. 1. Antiglycative capacity of Arabica and Robusta coffee silverskin extracts (ACS, RCS), caffeine (CFF) and chlorogenic acid (CGA) in the BSA–glucose assay. Concentrations assayed were 0.14, 0.71 and 1.42 mg/mL. Results are expressed as mean ± SD for $n = 4$. Aminoguanidine (AG) (0.6 mg/mL) showed an antiglycative activity of 91.2%. Different letters mean significant differences among the ACS and RCS extracts for a same concentration.

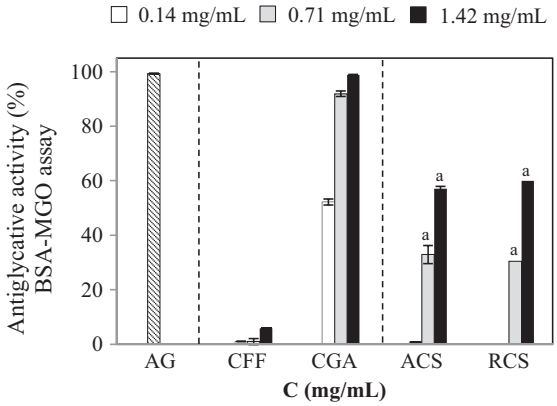

Fig. 2. Antiglycative capacity of Arabica and Robusta coffee silverskin extracts (ACS, RCS), caffeine (CFF), and chlorogenic acid (CGA) in the BSA–MGO assay. Concentrations assayed were 0.14, 0.71 and 1.42 mg/mL. Results are expressed as mean ± SD for $n = 4$. Aminoguanidine (AG) (0.6 mg/mL) showed an antiglycative activity of 99.2%.

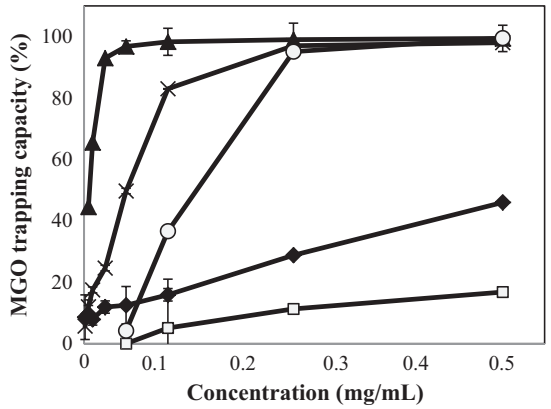

Fig. 3. Methylglyoxal trapping capacity of Arabica and Robusta coffee silverskin extracts (ACS, RCS), pyridoxamine (PM), chlorogenic acid (CGA), and caffeine (CFF) at 168 h. Results are expressed as mean ± SD for $n = 4$. PM (0.1 mg/mL) showed a MGO trapping capacity of 99.6%. ACS; RCS; PM; caffeine; CGA.

Robusta and Arabica CS extracts, respectively. Robusta CS extract was nearly 10-fold more active against MGO than Arabica CS extract.

To get more insight into the MGO trapping capacity of the extracts, kinetic evaluation for MGO-trapping capacity for 168 h at a concentration of 0.1 mg/mL was performed and compared with that of the PM (Fig. 4). PM and Robusta CS extract already trapped 50% of the initial MGO in the system at 27 and 66 h, respectively. In contrast, the Arabica CS extract only reached to trap around 14% of the MGO at 168 h, which showed a significant lower MGO-trapping capacity of this extract. The MGO-trapping capacity of the Arabica and Robusta CS extracts increased continuously during the time of incubation at 37 °C, following the same trend as to that of PM.

In the present study the antiglycative capacity measured as the inhibition of the formation of fluorescent AGEs, and mitigation of carbonyl radical stress (in terms of MGO-trapping capacity) of Arabica and Robusta CS aqueous extracts have been demonstrated. These results are in agreement with Verzelloni, Pellacani, et al. (2011) and Verzelloni, Tagliazucchi, et al. (2011) who reported that coffee contains molecules with *in vitro* antiglycative capacity. Our investigation demonstrates that antiglycative properties are also maintained in coffee by-products which may be related to phenolic compounds naturally present in coffee beans (Napolitano et al., 2007) and melanoidins formed through Maillard reaction during roasting (Delgado-Andrade & Morales, 2005). According to Borrelli et al. (2004) antioxidant compounds present in coffee can form covalent links with carbohydrates resulting in a fiber–antioxidant complex. Specifically the phenolic compounds from CS, mainly chlorogenic acid, may react with polysaccharide components forming melanoidins, which exerts the antioxidant activity (Borrelli, Visconti, Mennella, Anese, & Fogliano, 2002). According to Verzelloni, Pellacani, et al. (2011) and Verzelloni, Tagliazucchi, et al. (2011) the high molecular weight compound fraction of coffee has high antioxidant, chelating and antiglycative activities. Chlorogenic acid and fiber contents were significantly higher in Robusta CS extract as compared with Arabica CS extract.

Antiglycative effects of phenolic compounds have been previously reported. These compounds can exert their inhibition through their antioxidant properties, scavenging of free radicals, and quenching of carbonyl radical species (Delgado-Andrade & Morales, 2005; Kim & Kim, 2003; Sang et al., 2007; Wu & Yen, 2005). Wu, Hsieh, Wang, and Chen (2009) evaluated the ability of phenolic acids to inhibit glycation, concluding that these compounds were effective in the prevention of glucose-mediated protein modification, which are considered potent inhibitors of both AGE formation and the subsequent crosslinking of proteins. Among the phenolic acids studied by these authors, chlorogenic acid was considered to be one of the major antiglycative compounds, being in line with our results. In a similar way, Gugliucci, Bastos, Schulze, and Souza (2009) reported for yerba maté water extract containing chlorogenic acid and caffeic acid a high percentage of AGE fluorescence inhibition.

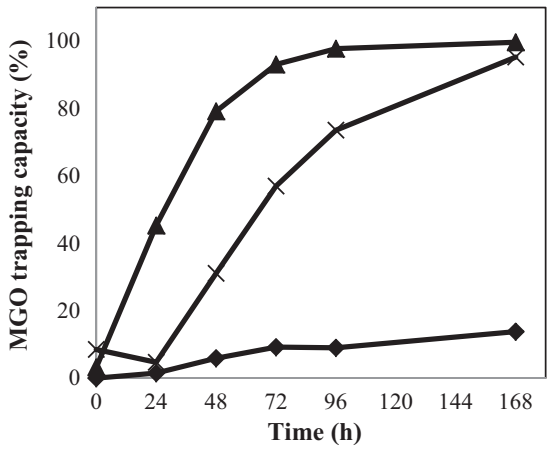

Fig. 4. Time-course MGO trapping capacity of the Arabica and Robusta coffee silverskin extracts (ACS, RCS). Results are expressed as mean ± SD for $n = 4$. Pyridoxamine (PM) (0.1 mg/mL) showed a MGO trapping capacity of 99.6%. PM; ACS; RCS.

As mentioned above, the present investigation was carried out using aqueous extracts in order to perform a cost-effective and environmentally friendly procedure, as well as being more interesting from safety and toxicological points of view for their industrial application. Several authors have evaluated the influence of the extraction process on the antioxidant capacity, concluding that water is highly efficient at extracting antioxidants. In this sense, Budryn et al. (2009) reported that both chlorogenic acids and melanoidins were found in higher amounts in aqueous extracts than in ethanolic extracts from green and roasted coffee, showing that these antioxidants were more soluble in water. In a similar way, Yen, Wang, Chang, and Duh (2005) indicated that the highest antioxidant yields in extracts were obtained with water from roasted coffee residues. Furthermore, Bravo, Monente, Juániz, De Peña, and Cid (2013) stated that water is necessary to extract more phenolic and nonphenolic antioxidants from spent coffee. Consequently, it can be supposed that the aqueous CS extracts contain the majority of the antioxidant compounds present in the CS including chlorogenic acid and, hence, it might justify the high antiglycative activity of the extracts.

Regarding caffeine, it has been shown that this compound has a low *in vitro* antioxidant activity (Somoza et al., 2003; Yen et al., 2005). In addition, caffeine has displayed a low inhibitory effect on AGE formation in *in vitro* assay systems (Nakagawa, Yokozawa, Tarasawa, Shu, & Juneja, 2002). These findings are in accordance with those found in the present study, since caffeine practically did not exhibit any antiglycative effect in the assays of BSA–glucose and BSA–MGO and a low MGO trapping capacity in comparison with that found for CS extracts. However, reactive oxygen species scavenging has been recently proposed for caffeine (Leon-Carmona & Golano, 2011).

Concerning the MGO-trapping capacity, Robusta CS extract trapped MGO more rapidly and efficiently as compared with Arabica CS extract. It might be indicated that the different compositions of the extracts influenced their MGO-trapping ability but not significantly the antiglycative capacity in the protein assays. Although FRAP and DPPH assays did not show large differences among Arabica CS and Robusta CS extracts, ABTS and ORAC exhibited a higher antioxidant capacity in the Robusta variety. In addition, despite no differences were observed in total phenolic content of the two studied samples, CGA content and specifically 3-CGA, 4-CGA and 5-CGA contents were also higher in the Robusta specie (8.2, 9.1 and 26.3 mg/g *versus* 1.5, 1.8 and 6.4 mg/g in Arabica CS), which could explain the major antiglycative capacity of this extract. On the other hand, it should be taken into account that the presence of several AGE inhibitors in an extract may have synergistic effects (Chompoo, Upadhyay, Kishimoto, Makise, & Tawata, 2011). During coffee roasting, a part of CGA is incorporated into the melanoidins (Moreira, Nunes, Domingues, & Coimbra, 2012) and the new structures may contribute to the overall antiglycative capacity of the CS extracts. Specifically, according to Rice-Evans, Miller, and Paganga (1996), phenolic compounds may have synergistic or antagonistic effects among them or with other constituents of an extract. Therefore, it can be supposed that chlorogenic acid together with other phenolic compounds, and CGA–melanoidins rich might contribute overall to the antiglycative activity of the extracts.

In spite of the antiglycative properties of the aqueous extract of coffee silverskin demonstrated in the *in vitro* assays in the present study, it cannot be assured that this effect also occur in *in vivo* experiments. According to Verzelloni, Pellacani, et al. (2011) and Verzelloni, Tagliazucchi, et al. (2011), after ingestion by humans polyphenolic compounds can be metabolized generating different metabolites in the

colon, which can or not exert inhibitory activity against AGE formation. These observations may be taken into account for future research.

4. Conclusions

The antiglycative capacity of coffee silverskin (CS) extracts, obtained from Arabica and Robusta varieties, was evaluated using different *in vitro* models. Both Arabica and Robusta CS extracts exhibited an anti-AGE capacity in BSA–glucose and BSA–MGO assays at concentrations ranging from 0.1 to 1.5 mg/mL. No significant differences among the Arabica and Robusta CS extracts were observed. On the other hand, the direct MGO-trapping assay showed that the CS extracts trapped MGO in a dose-dependent manner, but the Robusta CS extract showed a higher trapping capacity ($IC_{50} = 0.55$ mg/mL) in comparison with Arabica CS extract. Chlorogenic acid content in samples did not explain the antiglycative properties of the CS extracts, although the MGO trapping capacity was greatly related to the CGA content. The anti-AGE capacity of the samples might be related to the antioxidant capacity of the CS, explained by the phenolic compound content, especially chlorogenic acid, but also by the presence of high molecular weight polymers (melanoidins) formed through Maillard reaction during the roasting of coffee. The synergistic contribution of other constituents of the CS extracts to the antiglycative and carbonyl trapping properties *in vitro* should not be discarded. Further studies should be performed for evaluating the bioactivity of the extracts *in vivo*.

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Carbonyl trapping and antiglycative activities of olive oil mill wastewater

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The use of natural compounds as antiglycative agents to reduce the load of advanced glycation end products from diet is very promising. Olive mill wastewater is a by-product of the olive oil extraction processes with a high content of hydroxytyrosol, hydroxytyrosol derivatives and molecules containing *o*-dihydroxyl functions such as verbascoside. Two powders were obtained after the ultrafiltration and nanofiltration of olive mill wastewater, and successive spray drying with maltodextrin and acacia fiber. The samples were characterized by phenolic composition and antioxidant capacity. Antiglycative capacity was evaluated by *in vitro* BSA-glucose and BSA-methylglyoxal assays, formation of Amadori products and direct trapping of reactive dicarbonyls (methylglyoxal and glyoxal). Both ultrafiltered and nanofiltered olive mill wastewater powders had an activity comparable to quercetin and hydroxytyrosol against the inhibition of protein glycation (IC_{50} = 0.3 mg mL^{-1}). The antiglycative activity of the powder was further investigated after separation by reverse phase solid extraction. Fractions extracted with the methanol content higher than 40% and rich in hydroxytyrosol and verbascoside exerted the highest reactivity against dicarbonyls. Data confirmed that the direct trapping of dicarbonyl compounds is the main route explaining the antiglycative action rather than of the already known antioxidant capacity. Results support further investigations to evaluate the technological feasibility to use olive mill wastewater powders as antiglycative ingredients in foods or in pharmacological preparations in future.

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1. Introduction

The health benefits of a Mediterranean diet have been firmly established, including the lowering of degenerative pathologies,[1] cardiovascular diseases[2] and cancer.[3,4] In this respect, olive oil's effects, as an important component of the Mediterranean diet, are related not only to the peculiar fatty acid profile but also to the presence of a specific category of phenolic compounds named secoiridoids. The main phenolic compound in an olive plant (*Olea europaea* L.) is the oleuropein, which is hydrolyzed into hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) and tyrosol during the extraction of olive oil.[5] Several epidemiological studies suggest that HT is an efficient scavenger of peroxyl radicals[6] and prevents damage due to oxidative stress. In the *in vivo* and *in vitro* studies, HT has shown various biological activities with potential beneficial health effects. HT has been associated with anti-inflammatory, hypocholesterolemic, neuroprotective[7] and cardio protective activity as well as hypoglycemic effect which could prevent the glucose auto-oxidation reaction, a process related to the formation of AGEs.[8]

Advanced glycation endproducts (AGEs) are the outcome of a non-enzymatic glycation reaction between the carbonyl group of a reducing sugar and the free amino group of a protein. Several AGEs have been identified to date and many of them are fluorescent and yield protein crosslinking that alters the protein structure and their biochemical properties.[9] AGEs are a significant contributor to classical features of ageing and common chronic pathologies such as diabetes and its complications and Alzheimer's disease.[10] Many researchers have linked the oxidative reaction to the accelerating rate of AGE formation, since radicals and α-dicarbonyls, highly reactive intermediates such as methylglyoxal (MGO), glyoxal (GO) or 3-deoxyglucosone, are generated. Previous studies have suggested that antioxidants protect against glycation since they reduce the formation of reactive carbonyl species which is a consequence of oxidative stress generated by the formation of reactive oxygen species (ROS).[9] AGEs can also be introduced in the systems of living bodies by exogenous sources like diet. Basically, AGEs, also named Maillard reaction products, are formed during the thermal processing of food. The AGE

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content in processed foods has increased in the past 50 years and dietary AGEs contributed to the total human body AGE pool.[11]

Due to AGE involvement in the development of several health disorders, the search for AGE formation inhibitors in biological systems and in foods has been the target of many investigations. The use of synthetic compounds was discontinued because of the possible side effects and the use of aminoguanidine was also proposed. However the strategy of using natural compounds as antiglycative alternatives is very appealing. Some bioactive phytochemicals naturally occurring in plants and possessing redox properties have been related to the inhibition of the glycation process.[12] Studies of HT *in vivo* and *in vitro* have displayed it to possess cardio protective, anticancer and antidiabetic effects related to its ability to inhibit oxidative stress. Considering that the relationship between HT, ROS and tissue injury has been involved[13] with the pathogenesis of insulin resistance, the antiglycative activity of HT is plausible and noteworthy to be investigated. Recently, Troise *et al.* (2014)[14] concluded that phenol compounds from olive mill wastewater (OMW) are effective inhibitors of the extent of Maillard reaction products during the thermal processing of UHT milk.

In this framework, a food ingredient made from spray-dried OMW was selected as a promising candidate for the antiglycative action. OMW is a by-product of the olive oil extraction processes with a high content of HT, HT derivatives and molecules containing *o*-dihydroxyl functions such as verbascoside.[15] The aim of the present study was to assess the inhibitory capacity of different OMW powders on the formation of AGEs by using different *in vitro* models of MGO and glucose as inducers of the glycation reaction. Furthermore, the ability of OMW ingredients for trapping dicarbonyl compounds was also assessed. Antioxidant activity was examined in order to study their possible relationship with AGE-inhibitory activity.

2. Materials and methods

2.1. Materials

D (+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (MGO, 40% aqueous solution), glyoxal (GO, 40% aqueous solution), aminoguanidine (AG), quercetin (QE), verbascoside (VB), 5-methylquinoxaline (5-MQ), nitrotetrazolium blue chloride (NBT), *o*-phenyldiamine (OPD), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 2-2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) and phenolic acid standards were provided by Sigma (St Louis, MO, USA). Folin–Ciocalteu reagent and iron(III) chloride were purchased from Panreac (Madrid, Spain). Hydroxytyrosol standard (HT, purity > 99%) was acquired from Seprox Biotech (Madrid, Spain). Pyridoxamine (PM) was acquired from Fluka Chemical (Madrid, Spain). Sodium dihydrogen phosphate monohydrate, sodium carbonate anhydrous, formic acid, glacial acetic acid and high-performance liquid chromatography (HPLC)-grade methanol were purchased from Merck (Darmstadt, Germany). C18 cartridges Sep-Pak Plus were supplied by Waters (Ireland). The Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

2.2. Equipment

Synergy™ HT-multimode microplate reader with an automatic reagent dispense and temperature control from Biotek Instruments (VT, USA). HPLC Shimadzu (Kyoto, Japan) equipped with an LC-20AD pump, an SIL-20AHT autosampler, a CTO-10ASVP oven, and a DAD (SPD-M20A).

2.3. Preparation of olive mill wastewater powder

Olive oil polyphenol powders were obtained from Azienda Agricola Fangiano (Calabria, Italy) starting from the water fraction resulting from virgin-olive oil production (Carolea variety) as follows. Olive water was separated from the oil and olive paste by centrifugation; it was treated with pectinases for 2 hours at 37° C then fractionated by a filtration plant made up of three membranes having different cut offs. Olive water was forced to pass through the microfiltration (cut off 25 kDa), ultrafiltration (cut off 8 kDa), and nanofiltration (cut off 0.3 kDa) membranes. At each stage a retentate containing the compounds not passing through the membrane pores was collected while the permeate moved on to the next filtration step. For this investigation, the ultrafiltration and nanofiltration retentates were concentrated by inverse osmosis (cut off 0.1 kDa) up to 20% dry weight and spray dried adding maltodextrin and acacia fiber in a ratio 2 : 1 of the water mill dry weight (66.6% olive mill water and 33.3% maltodextrin and acacia fiber 1 : 1). Finally, a fine pale yellow ultrafiltered (UOMW) and nanofiltered (NOMW) powder with moisture content below 4% was obtained and used in this study.

2.4. Analysis of phenolic compounds in OMW powder

The analysis of the phenolic component was carried out as described by Kokkinidou & Peterson[16] with some modifications. Briefly, the OMW powder was dissolved in distilled water, in order to prepare a solution at a concentration of 20 mg mL^{-1}. To 1 mL of this solution, 10 μL of a 5 mg L^{-1} solution of butyl-4-hydroxybenzoate as internal standard were added. The phenolic fraction was extracted through the use of SPE cartridges Strata C18-E, and dried under a nitrogen stream. Thereafter the precipitate was recovered in 500 μL of a solution of water–methanol/95 : 5 (v/v) ready for HPLC analysis. The instrument used for chromatographic analysis was an LC-20AD HPLC with a UV-Vis detector SPD20A, set at 279 nm, and an SCL-20A controller (Shimadzu, Japan). The mobile phases were 0.1% formic acid in H_2O (A) and methanol (B). The flow was 0.8 mL min^{-1}. A Prodigy ODS3 column was used (250 × 4.60 mm, 5 micron, 100 A, Phenomenex, USA). The sample (20 μL) was separated with the next gradient as follows; 0 min (5% B); 4 min (5% B); 40 min (98% B); 43 min (98% B);46 min (5% B); 49 min (5% B). Hydroxytyrosol, tyrosol and verbascoside were quantified by external calibration with the

standards. Peaks were identified by retention time, DAD spectra and spiking the sample with pure standards.

2.5. Determination of total phenolic content

Total phenolic content (TPC) was estimated by the Folin–Ciocalteu method as described by Contini *et al.*[17] Absorbance readings were recorded using a microplate spectrophotometer and quantified using gallic acid as a standard. Results were expressed as mg gallic acid equivalent (GAE) per g sample and all measurements were carried out in triplicate. The limit of quantification was set at 0.5 mg GAE per g sample.

2.6. Determination of antioxidant capacity according to ABTS assay

The antioxidant activity was estimated in terms of the radical scavenging capacity of the extracts as described by Mesías *et al.*[18] Aqueous solutions of Trolox at different concentrations were used for calibration. The limit of quantification was set at 1.1 μmol TEAC g^{-1} sample. All measurements were performed in triplicate.

2.7. Determination of antioxidant capacity according to FRAP assay

The formation of the Fe^{2+}-TPTZ complex from the Fe^{3+}-TPTZ complex was determined as described by Morales *et al.*[19] Results are expressed as μmol Trolox equivalent antioxidant capacity (TEAC) per g sample.

2.8. Fractionation of the olive mill wastewater powder ingredient

The ultrafiltrate olive mill wastewater (UOMW) powder was further fractionated by reversed phase solid phase extraction (C18 SepPak cartridge) with sequential extraction in methanol. A solution of the UOMW powder (1 mg mL^{-1}) was loaded onto a pre-activated cartridge. The first fraction was eluted and collected and termed as fraction UOMW-A. Then 1 mL of formic acid (0.1%) with 5% of methanol was passed through the cartridge and the resulting fraction (UOMW-B) was collected. UOMW-C is the fraction that was eluted with 1 mL of formic acid with 40% of methanol. Finally, the UOMW-D fraction was eluted with 1 mL of methanol passed through the cartridge. Chromatographic separations of the whole extract and fractions were performed on a C18 Kinetex column (75 mm × 4.6 mm, 2.6 μm, Phenomenex, USA). The temperature was maintained at 40 °C and the flow rate was 0.6 mL min^{-1}. The mobile phases were 0.5% acetic acid in water (solvent A) and methanol (solvent B) and the total running time was 40 min. The gradient changed as follows: 5%B was held for 5 min, then increased to 100%B in 25 min, held for 1 min, and decreased to 5%B in 9 min.

2.9. Assessment of *in vitro* glycation of BSA by methylglyoxal and glucose

The BSA-MGO and BSA-GLC assays were carried out as described by Mesías *et al.*[18] Aminoguanidine, hydroxytyrosol and quercetin were used as standards at a concentration of 0.1 mg mL^{-1}, AG (0.57 mg mL^{-1}) being the positive control of the assay. The systems containing the samples/control/blank were incubated at 37 °C for 14 days (BSA-MGO assay) or 21 days (BSA-GLC assay). The reference was prepared without incubation and stored at −80 °C until analysis. In parallel, samples were dissolved in phosphate buffer (0.1 mol L^{-1}, pH 7.4) and incubated at 37 °C for 14 days (BSA-MGO assay) or 21 days (BSA-GLC assay) in order to measure their intrinsic fluorescence. The fluorescence intensity of all systems (sample/control/blank) was measured using a microplate spectrophotometer. AGE formation was characterized by measuring fluorescence with the excitation and emission maxima at 340 and 420 nm, respectively, for the BSA-MGO assay and at 360 and 420 nm, respectively, for the BSA-GLC assay. The intrinsic fluorescence of the samples incubated under the same conditions was subtracted from the overall fluorescence in each BSA-MGO or BSA-GLC system.

The percentage inhibition of AGE formation was calculated according to the following equation:

$$\text{inhibition } (\%) = \{1 - [(\text{fluorescence of solution with inhibitor} - \text{intrinsic fluorescence of sample})/\text{fluorescence of solution without inhibitor}]\} \times 100$$

IC_{50} (the concentration required to inhibit glycation by 50%) were calculated from the dose–response curve using the Microsoft-Excel computer software.

2.10. Fructosamine adduct assay

Fructosamine formation was tested following the procedure described by Baker *et al.*[20] with slight modifications. Fructosamine has the ability to reduce NBT to tetrazinolyl radical (NBT^+) in an alkaline solution forming a coloured compound called monoformazan (MF^+). Therefore, 50 μL of samples for the BSA-GLC assay were mixed with 450 μL of NBT and the mixture was incubated in the dark at 37 °C for 60 min. Then, in a microplate was added 100 μL of the mixture and 100 μL of the sodium carbonate buffer (0.1 mol L^{-1}, pH 10.35), in quadruplicate, except in the blank where samples were replaced by the sodium carbonate buffer. The presence of fructosamine was characterized by the absorbance of MF^+ at 350 nm recorded using a microplate spectrophotometer.

The percentage of inhibition of fructosamine formation was calculated using the following equation:

$$\text{inhibition } (\%) = 100 - [(\text{fluorescence of solution with inhibitor} - \text{intrinsic fluorescence of sample} - \text{fluorescence of blank}) \times 100/(\text{fluorescence control positive} - \text{intrinsic fluorescence of control positive} - \text{fluorescence of blank})]$$

2.11. Direct MGO and GO trapping capacity

Direct MGO or GO trapping capacity of the samples was determined as described by Mesías *et al.*[18] with modifications. The

mixture of MGO (0.04 mg mL^{-1}) or GO (0.03 mg mL^{-1}) with different concentrations of the extracts was incubated at 37 °C for 168 h. Pyridoxamine (PM, 0.1 mg mL^{-1}) were used as the positive control. OPD was added in order to derivatize the unreacted MGO or GO into 2-methylquinoxaline (2-MQ) or 1-quinoxaline (1-Q), respectively. 5-quinoxaline (5-MQ) was used as an internal standard. Chromatograms were recorded at 315 nm with the retention times of 2-MQ, 1-Q and 5-MQ being 4.1, 3.4 and 5.6 min respectively. The amount of unreacted MGO and GO was calculated from the ratio of 2-MQ or 1-Q and 5-MQ to the control at time zero after preparation.

The MGO trapping capacity was calculated from the following equation:

MGO decrease (%) = [(amount of MGO in control − amount of MGO in sample with tested standard or PM solution)/amount of MGO in control] × 100%. The IC_{50} value (mg mL^{-1}) was obtained from the dose–response curves of each experiment

Similarly, the GO trapping capacity was calculated.

2.12. Statistical analysis

Data were analysed by using the software Statgraphics Centurion XV Statistical program (Herndon, VA). Homogeneity of variances was assessed *via* Levene's test. Analysis of variance (ANOVA) followed Bonferroni's or Tamhane's test when the variances were homogeneous or non-homogeneous, respectively. Differences were considered to be significant at $P < 0.05$. Data from at least three independent assays were used to obtain a mean value and its standard deviation.

3. Results

A polyphenol-rich powder was obtained from olive mill wastewater through ultrafiltration/nanofiltration and spray dried, and it was investigated as a promising functional ingredient to decrease non-enzymatic protein glycation. Two types of powders were obtained, one after the ultrafiltration (UOMW) of the olive mill wastewater extract and the second from the retentate after nanofiltration (NOMW). Both extracts were spray dried adding maltodextrins and acacia fiber, and the powders were characterized by the total polyphenol content and antioxidant capacity (FRAP and ABTS) as summarised in Table 1. The content of polyphenolic compounds and the antioxidant capacity, regardless of the method used, was significantly ($P < 0.05$) higher in the UOMW powder as compared with the NOMW powder. It is known that certain bioactive compounds present in plants possess antioxidant capacity as well as the ability to inhibit protein glycation as flavonoids.[21] During the spray drying of extracts, the yield of the nanofiltration powder was lower than that of ultrafiltration, particularly when the concentration of the added dietary fibre was reduced to 33.3%. The average content in hydroxytyrosol, tyrosol and verbascoside was 33.0, 2.1 and 2.8 μg g^{-1} for the UOMW powder and 13.8, 0.2, and 1.3 μg g^{-1} for the NOMW powder, respectively. The higher concentration of polyphenols in the UOMW powder was due to the presence of bound polyphenols linked to the polysaccharide moiety (pectins and arabinans) that remain in the retentate of the extract.[22]

Table 1 Antioxidant activity and total phenol content of nanofiltered olive mill wastewater (NOMW), ultrafiltered olive mill wastewater (UOMW) powders and its fractions obtained by reverse-phase extraction. Results are expressed as mean ± SD for $n = 4$. Different letters in the same column denote significant differences $P < 0.05$. LoQ < 0.5 GAE (mg g^{-1}) or <1.1 TEAC (μmol g^{-1})

	TFC GAE (mg g^{-1})	FRAP TEAC (μmol g^{-1})	ABTS TEAC (μmol g^{-1})
Sample			
NOMW	49.9 ± 5.5^{b}	439.4 ± 15.5^{b}	333.7 ± 34.9^{b}
UOMW	61.8 ± 3.4^{a}	780.4 ± 23.0^{a}	605.6 ± 28.8^{a}
Fractions			
UOMW-A	<LoQ	<LoQ	2.0 ± 0.4^{c}
UOMW-B	<LoQ	5.0 ± 0.1^{c}	5.0 ± 0.3^{c}
UOMW-C	24.6 ± 0.2^{c}	199.2 ± 2.6^{d}	249.3 ± 29.6^{d}
UOMW-D	30.0 ± 0.2^{c}	241.7 ± 17.1^{d}	379.8 ± 34.0^{b}

The antiglycative properties of OMW powders were evaluated by the BSA-MGO and BSA-GLC assays. Fig. 1a showed the formation of fluorescent AGEs in the BSA-MGO assay (37 °C/14 days). The UOMW and NOMW powders behave in a dose-dependent manner and both reduced the formation of AGEs significantly, with an IC_{50} of 0.886 and 1.029 mg mL^{-1}, respectively for the ultrafiltrated and nanofiltrated powders (Table 2). But the ultrafiltrated powder was significantly more effective for antiglycative activity mediated by MGO. AG and QE were used as positive controls due to their well-known effectiveness against the glycation process. The suppression of dicarbonyl formation and direct reaction with dicarbonyls are the main mechanisms of action of AG and QE.[23,24] The antiglycative activities of AG, QE, HT, and VB solutions (0.14 mg mL^{-1}) in the BSA-MGO assay were 68, 86, 70%, and 84%, respectively. IC_{50} in the BSA-MGO assay were 0.090, 0.053, 0.086, and 0.053 mg mL^{-1} for AG, QE, HT, and VB, respectively (Table 2). The control for maltodextrin and acacia fiber (MD + AF) showed a residual 3.5% antiglycative activity for the BSA-MGO assay.

Fig. 1b showed the formation of fluorescent AGEs for the BSA-GLC assay (37 °C for 21 days). Ultrafiltration and nanofiltration powders presented a dose-dependent inhibitory activity with no significant differences between them (IC_{50} of 0.457 and 0.429 mg mL^{-1}, respectively) as described in Table 2. IC_{50} for the BSA-GLC assay were 0.100, 0.101, 0.400, and 0.243 mg mL^{-1} for AG, QE, HT, and VB, respectively (Table 2). HT and VB are also effective in the BSA-GLC system where the autoxidation of glucose is a side reaction to form reactive dicarbonyl compounds that promote the formation of AGEs. The control for maltodextrin and acacia fiber did not show any antiglycative activity for the BSA-GLC assay.

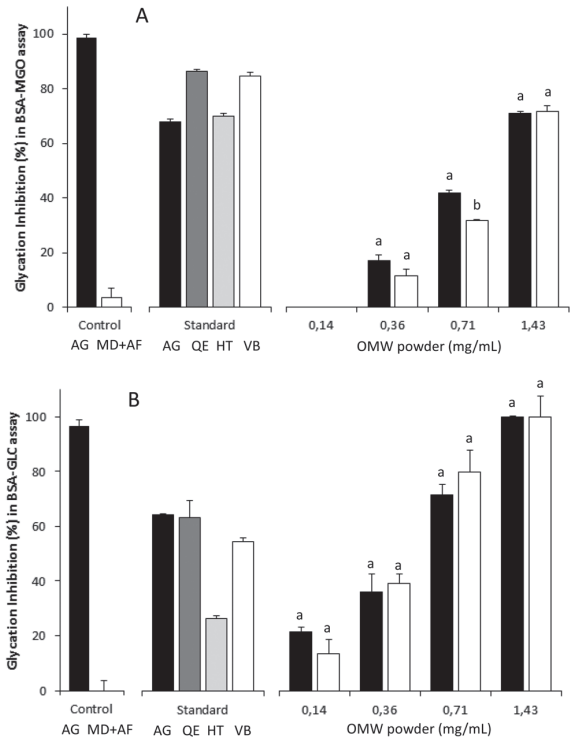

Fig. 1 Antiglycative activity of ultrafiltered (solid bar) and nanofiltered (empty bar) olive mill wastewater OMW extracts in the range from 0.14 to 1.43 mg mL^{-1} on the formation of fluorescent AGEs in (A) BSA-MGO and (B) BSA-GLC assays. Aminoguanidine (AG), quercetin (QE), hydroxytyrosol (HT), and verbascoside (VB) at 0.14 mg mL^{-1} were used as reference and showed an antiglycative activity of 67.7%, 86.3%, 69.8%, and 84.4% respectively in BSA-MGO and 64.3%, 63.1%, 26.5% and 54.4% respectively in BSA-GLC. AG was used as control of the assay. Maltodextrin and acacia fiber (MD + AF, 1.43 mg mL^{-1}) were used as control of the powder. Results are expressed as mean ± SD for n = 4. Different letters mean significant differences (P < 0.05) between samples for a same concentration.

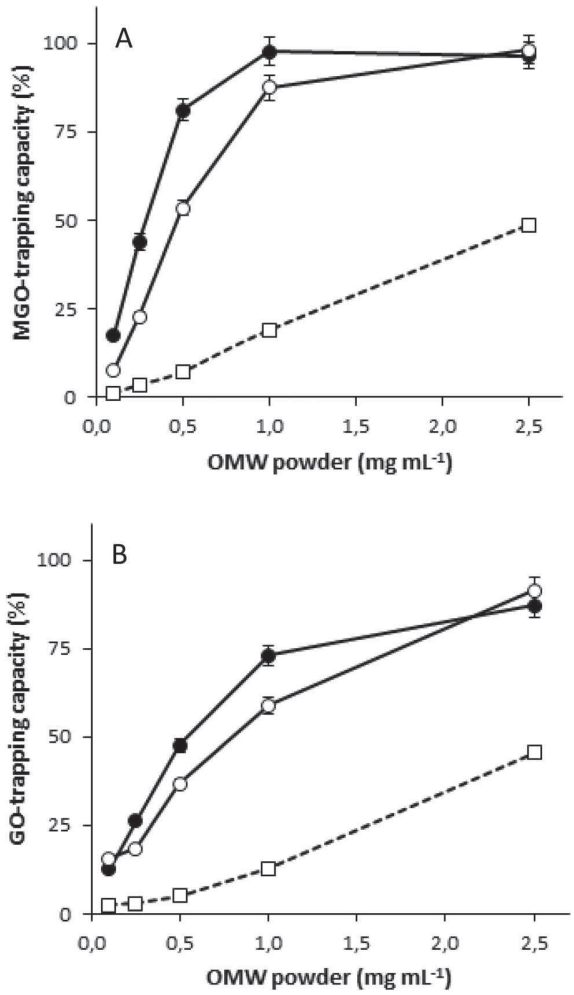

Fig. 2 MGO-trapping capacity (A) and GO-trapping capacity (B) of ultrafiltered olive mill wastewater (UOMW,●) and nanofiltered olive mill wastewater (NOMW, ○) powders (0.1–2.5 mg mL^{-1}) after incubation (168 h). Results are expressed as mean ± SD for n = 4. PM (0.1 mg mL^{-1}) was used as a control positive and presented MGO and GO trapping capacity of 99.6% and 14.5% respectively. Control for maltodextrin + acacia fiber depicted as dotted line.

Table 2 Concentration (mg mL^{-1}) of aminoguanidine, pyridoxamine, quercetin, hydroxytyrosol, tyrosol, verbascoside, and ultrafiltered (UOMW) and nanofiltered (NOMW) olive mill wastewater powders exerting the 50% inhibition of BSA glycation. Different letters in the same column denote significant differences P < 0.05 in the same column. IC_{50} higher than 1 mg mL^{-1} (n.a.)

	IC_{50}-antiglycative activity	
	BSA-GLC assay	BSA-MGO assay
Aminoguanidine	0.100^{a}	0.090^{a}
Pyridoxamine	0.283^{b}	0.046^{b}
Quercetin	0.101^{a}	0.053^{b}
Hydroxytyrosol	0.400^{c}	0.086^{a}
Tyrosol	n.a.	n.a.
Verbascoside	0.243^{b}	0.053^{b}
UOMW powder	0.457^{c}	0.886^{c}
NOMW powder	0.429^{c}	1.029^{d}

On the other hand, methylglyoxal (MGO) and glyoxal (GO) are the key promoters of the glycation process where the formation of AGEs and the irreversible modification of proteins is boosted. For this reason, the direct MGO and GO trapping capacities of the UOMW and NOMW extracts were investigated. Fig. 2a and b depicted the MGO and GO trapping abilities of the ultrafiltration and nanofiltration powders in the range from 0.1 to 2.5 mg mL^{-1}. Both extracts showed similar dose-dependent behavior and reached the highest MGO trapping abilities at 1 mg mL^{-1}. It is noteworthy that the control sample (maltodextrin + acacia fiber) showed a minor dicarbonyl trapping capacity, representing nearly 20% of the overall

Table 3 Percentage of inhibition of the formation of fructosamine by ultrafiltered olive mill wastewater (UOMW) and nanofiltrated olive mill wastewater (NOMW) powders in the range from 0.36–3.57 mg mL^{-1}. Results are expressed as mean ± SD for $n = 4$. Different letters in the same column denote significant differences $P < 0.05$

mg mL^{-1}	UOMW	NOMW
0.36	10.02 ± 5.45^{a}	10.54 ± 2.65^{a}
0.71	25.48 ± 1.90^{b}	33.52 ± 1.61^{b}
1.43	44.67 ± 2.92^{c}	43.67 ± 0.94^{c}
3.57	57.31 ± 1.90^{d}	56.75 ± 6.25^{d}

response. Results show that the efficacy of the OMW powder for trapping GO was lower as compared with the MGO trapping ability. IC_{50} for the GO-trapping assay was calculated to be 0.562 mg mL^{-1} and 0.811 mg mL^{-1} for the UOMW and NOMW powders, respectively. In contrast, both powders were more effective for trapping MGO with IC_{50} values of 0.294 and 0.473 mg mL^{-1}, respectively. The ultrafiltered powder was significantly ($P < 0.05$) more reactive against MGO and GO than the nanofilterd powder for a concentration higher than 0.1 mg mL^{-1}.

In the early phase of the glycation reaction, the reversible Schiff base leads to a rearrangement where a more stable Amadori product is formed. In the advanced phase of glycation, irreversible AGEs are formed. Therefore, the inhibition of fructosamine adducts could reduce AGE formation in foods and hence potentially ameliorate the diabetic complications *in vivo* by a restriction of the dietary AGE intake and a reduction of the circulating AGEs. The Amadori products were quantified principally on the basis of the reduction of NBT by Amadori products to monoformazan dye, which bears strong absorbance at 530 nm. Our results pointed out that the antiglycative activity and dicarbonyl trapping ability of the ultrafiltered and nanofiltered powders are relevant to the potential mitigation of diet-derived and endogenously formed AGEs. As shown in Table 3, both extracts performed their inhibitory effects at an early stage of glycation since a significant reduction in the formation of fructosamine was observed at all concentrations (0.36–3.57 mg mL^{-1}). The IC_{50} for the fructosamine assay was calculated to be 2.34 and 2.48 mg mL^{-1} for the ultrafiltered and nanofiltered powders, respectively.

Since the antiglycative activity of both ultrafiltered and nanofiltered powders was in the same order of magnitude, the ultrafiltered sample was selected for a more detailed investigation to elucidate the mechanisms of antiglycative action considering its higher yield during the production process and consequently the potential economic advantages in the scale up process.

Sequential fractionation of the UOMW powder by reverse phase extraction was applied for a further investigation of the antiglycative capacity. Fig. 3 described the chromatographic profile of the whole extract and the relative fractions A, B, C and D, eluted in a gradient with methanol. In the chromatograms were identified the peaks of HT, tyrosol and verbascoside. A hump corresponding to the high molecular weight polysaccharides present in the olive pulp was clearly detectable. Since the UOMW powder is particularly rich in HT (42% of the total polyphenols content), further investigations focused on the HT content. The highest concentration of HT (representing approx. 80% of the initial HT content) was collected in the fraction C which was eluted with 40% MetOH. Fraction D (corresponding to fraction eluted with 100% MetOH) contains the lowest amount of HT corresponding to approximately 20% of the initial content. However verbascoside is eluted at fraction D together with a major proportion of the polysaccharides rich in polyphenols.

The antiglycative activity of each fraction obtained by the solid phase extraction was estimated by the BSA-MGO (Fig. 4a) and BSA-GLC (Fig. 4b) assays. In both BSA-MGO and BSA-GLC assays, fractions A and B did not exert a protective effect on the formation of fluorescent AGEs, while C and D fractions showed an important antiglycative capacity. Fractions C and D were able to inhibit from 30 to 49% of the formation of fluorescent AGEs in the BSA-MGO assay, and 31.3 to 55.2% in the BSA-GLC assay. The antiglycative activity of the fraction eluted with methanol was significantly higher than the fraction eluted with 40% methanol. The partial contribution of the antiglycative capacity of the UOMW-C and UOMW-D fractions could explain the activity of the whole UOMW extract that involved 76.3 and 88.4% of inhibition in BSA-MGO, and BSA-GLC, respectively.

Fig. 5 shows the kinetic of the MGO trapping ability of the ultrafiltered powder and fractions over time. The UOMW-C and UOMW-D fractions were highly efficient in trapping MGO as compared with the UOMW-A and UOMW-B fractions. In fact, the UOMW-C and UOMW-D fractions reached a trapping capacity of 80.2 and 71.4% during incubation with MGO up to 168 h respectively. Both C and D fractions follow the same trend and a significant MGO-trapping capacity is recorded in the first 24 h of incubation. The MGO trapping capacity of the whole powder (1 mg mL^{-1}) peaked at 97.6% after 168 h of incubation, but the system was almost saturated (percentage of inhibition higher than 85%) at 72 h of reaction. In contrast, fractions A and B just accounted for 11 and 14.5% of the MGO trapping ability after 168 h, respectively. The reaction time was selected to be 48 h to compare the relative reactivity of the fraction with the whole UOMW powder. At 48 h, the UOMW powder reached an MGO-trapping capacity of 78.5% and the sum of fractions accounted to 99.3%.

Several assays have established a relationship between the antioxidant effects of certain plant extracts with the inhibition of protein modifications in the glycation process.[25] Jemai *et al.*[26] suggested that the antidiabetic effect of olive leaves might be due to the antioxidant activity of HT and oleuropein. In this regard, the total antioxidant capacity was evaluated in the samples by FRAP and ABTS. The total phenolic content of the powder and its fraction was assessed since phenolic compounds have been proposed as major

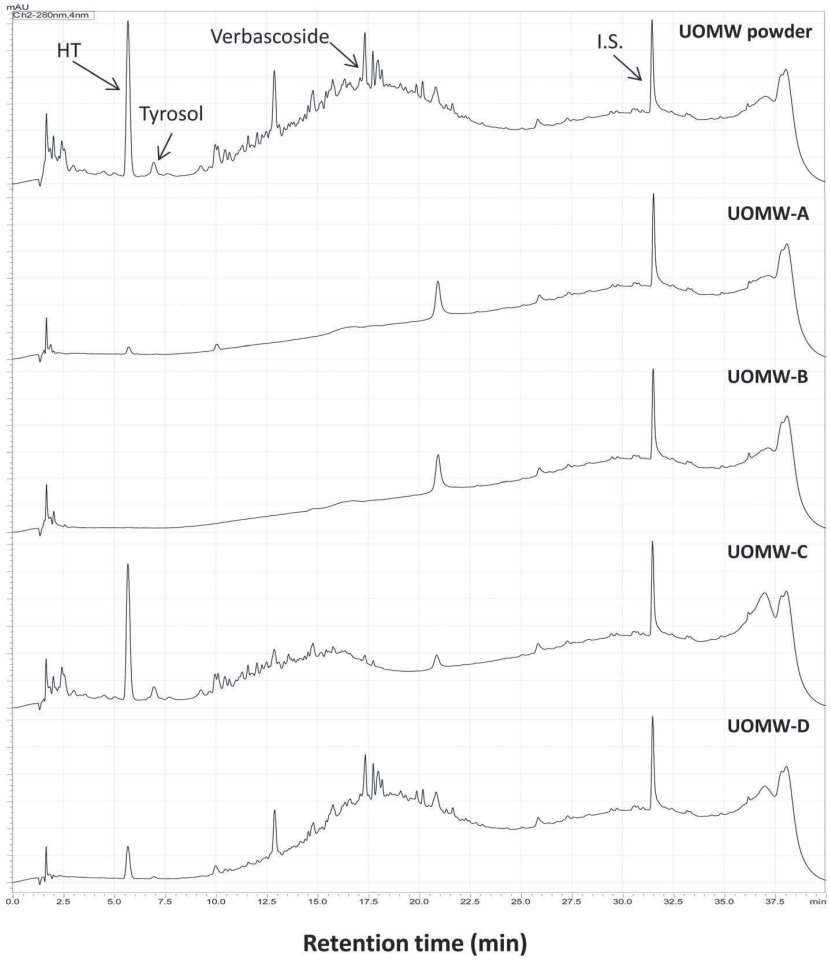

Fig. 3 Chromatographic profile of the ultrafiltered olive mill wastewater (UOMW) powder and (UOMW-A, OUMW-B, UOMW-C and UOMW-D) fractions. See Materials and methods for details. Butyl-4-hydroxybenzoate (IS).

contributors to antiglycative activity.[27] Table 1 summarized the results of TPC, FRAP and ABTS for the UOMW powders and its respective fractions A, B, C, and D. The reducing ability of the UOMW powder (780.4 μmol TEAC g^{-1}) was higher than the sum of the reducing activity of its UOMW-C and UOMW-D fractions (199.2 and 241.7 μmol TEAC g^{-1} respectively). ABTS results showed that UOMW powder had a TEAC value of 605.6 μmol g^{-1} and the free radical scavenging activity of UOMW was due to its UOMW-C and UOMW-D fractions. The total phenolic content of UOMW powder was 61.8 mg GAE g^{-1} and the UOMW-C and UOMW-D fractions accounted practically the total phenol content of the whole powder. Data suggested that the antiglycative action was related to the phenolic moiety of the ingredients, separating the polysaccharides that were added in the formulation steps, the biological activity is linearly correlated with the antioxidant ability.

4. Discussion

Olive mill wastewater is a by-product of the olive oil extraction industry posing a serious environmental problem due to its high organic load. However, OMW is also an important source of polyphenols, such as hydroxytyrosol, tyrosol, verbascoside and oleuropein, with potential health benefits. Consequently, during recent years, new technologies for the recovery of phenol compounds from OMW have been studied.[28]

It is known that under hyperglycaemic conditions, the glycation process leads to the formation and accumulation of AGEs, compounds able to modify the function and structure of proteins in tissues and organs inducing some metabolic diseases. Ramkissoon *et al.*[21] reported that many constituents in plants possess a high TPC and antioxidant potential as well as a high ability to inhibit protein glycation. The high incidence of chronic diseases such as diabetes, Alzheimer's or cardiovas-

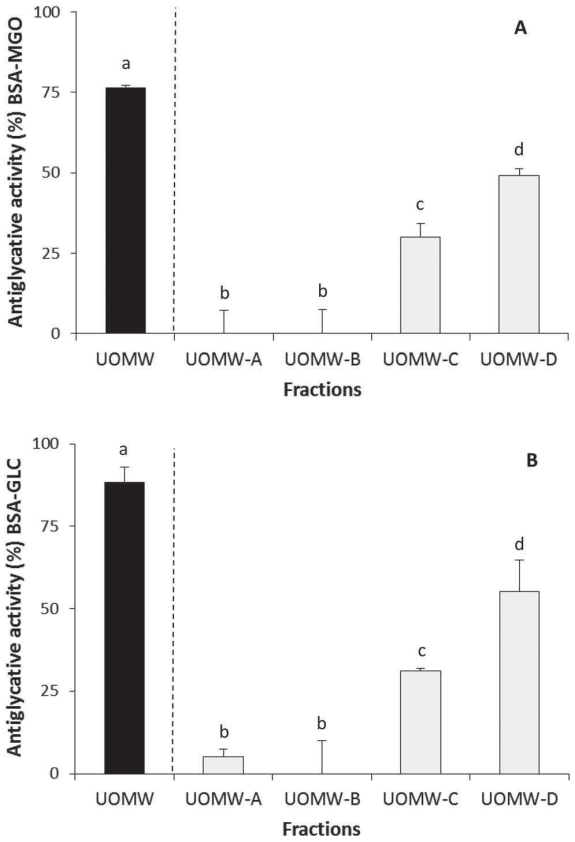

Fig. 4 Antiglycative activity of ultrafiltered olive mill wastewater (UOMW) powder and fractions in the BSA-MGO (A) and in BSA-GLC (B) assay. UOMW was used as a reference presenting an antiglycative activity of 76.3% and 88.4% for BSA-MGO and BSA-GLC, respectively. Control for maltodextrin and acacia fiber has no activity as described in Fig. 1. Results are expressed as mean ± SD for $n = 4$. Different letters denote significant differences $P < 0.05$.

cular diseases and the key role of the glycation process in causing these diseases has been intensively studied on the literature and several reports with a high relevance could be found thanks the beneficial effect of the polyphenols.[9–29] In an organism the total pool of circulating AGEs is the sum of those with dietary origin, including AGEs absorbed as such products as well as those formed *in vivo* from the absorbed dicarbonyl compounds, and those endogenously formed.[30] There is a direct correlation between the circulating AGE level, of which two-thirds remain in the body for 72 hours of those consumed.[11] Consequently, dietary AGE restriction is an efficient tool to ameliorate the pathological consequences of the glycation process *in vivo* until effective and safe drugs become available.[11] Our investigation points out that the by-products from olive oil must be considered as efficient inhibitors of the formation of AGEs, and in extension very likely will act as effective inhibitors of the glycation process. OMW extracts spray dried with maltodextrin and acacia fiber interfered with the formation of the Amadori product and also

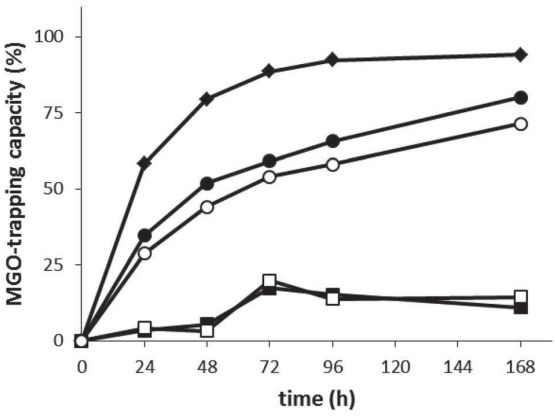

Fig. 5 Methylglyoxal trapping activity of ultrafiltrate olive mill wastewater powder and fractions over time. Ultrafiltered olive mill wastewater (UOMW) powder (◆) and UOMW-A (■), UOMW-B (□), UOMW-C (●), and UOMW-D (○) fraction.

reduced the formation of fluorescent AGE by trapping reactive dicarbonyls, such as MGO and GO. After partial fractionation of the OMW powder, hydroxytyrosol and verbascoside were revealed as the most active compounds exerting antiglycative and dicarbonyl trapping activity. Despite the promising data obtained *in vitro*, *in vivo* studies are needed as a further step and so are metabolic studies to clarify its mechanism of action.

5. Conclusion

Results suggest that the OMW powder would also act at the early stage of the glycation process being an effective inhibitor of fructosamine formation. The inhibition of dicarbonyl compound formation by OMW powder was in the same magnitude as that of pyridoxamine, hydroxytyrosol, and verbascoside. On the other hand, it is plausible that the antiglycative capacity and antioxidant properties of the extracts are due to their phenolic content, especially hydroxytyrosol and verbascoside, but also due to the presence of other high molecular compounds. High molecular weight compounds present in olives as cellulose, arabinans and pectins are present as well in the OMW powder and a significant part of the phenolic compounds is bound to the polysaccharide moiety[31] as shown by the fact that they are present in the ultrafiltration fraction. Olive mill wastewater powder could be considered as a potent antiglycative ingredient which was found to inhibit AGE formation at different stages of the pathway. In addition, the direct trapping of dicarbonyl compounds has been confirmed to be a novel mechanism of the action apart from the already known antioxidant capacity. Nevertheless, further research is ongoing in food models to confirm the mitigation of AGE formation and the mechanism of action before concluding firmly that the OMW powder is a diet AGE inhibitor.

Acknowledgements

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CAPÍTULO 2: *Estudio del mecanismo de acción antiglicante del hidroxitirosol en modelos in vitro. Obtención de un extracto de hoja de olivo como fuente de hidroxitirosol y evaluación de su capacidad antiglicante*

Introducción: Investigaciones *in vitro* e *in vivo* han confirmado que el HT, compuesto fenólico más característico de los productos del olivo, posee propiedades saludables frente al desarrollo de diversas patologías. El mecanismo de acción a través del cual el HT ejerce sus efectos beneficiosos para la salud se atribuye principalmente a su capacidad antioxidante, que es ejercida mediante el atrapamiento de RL y del incremento de la actividad de los sistemas de defensa endógena frente a estrés oxidativo. Sin embargo, determinadas patologías sobre las que puede actuar el HT como la diabetes y sus complicaciones tienen una génesis multifactorial, donde otros mecanismos de acción, como la capacidad antiglicante, no han sido investigados.

Objetivos: Análisis del potencial efecto antiglicante del hidroxitirosol y elucidación de un posible mecanismo de acción. Evaluación de la capacidad antiglicante de un extracto de hoja de olivo como fuente natural de hidroxitirosol.

Metodología: El estudio de la capacidad antiglicante del HT y sus derivados, hidroxitirosol acetato (HTA) y ácido 3,4-dihidroxifenilacético (DOPAC) se llevó a cabo mediante sistemas *in vitro* en condiciones fisiológicas simuladas (solución salina tampón 100 mM, pH 7.4, 37 ^{o}C). Como una primera aproximación, se utilizaron métodos indirectos para determinar la inhibición de AGEs inespecíficos formados bien a partir del producto de Amadori (sistema BSA-GLC) o bien a partir de la reacción directa con compuestos α-dicarbonilos (sistema BSA-MGO). Ya que los compuestos con actividad antiglicante pueden ejercer su acción en diversas etapas de la reacción de glicación, también se evaluó la actividad sobre las etapas tempranas mediante el bloqueo de grupos amino accesibles (método fluorimétrico OPA) y la determinación de la inhibición de la formación de productos de Amadori (método de determinación de fructosamina). El estudio del atrapamiento de C3- y C6-dicarbonilos ofrece información sobre el bloqueo de los principales inductores de la reacción de glicación. Se determinó la formación de CML y CEL como AGEs no fluorescentes más representativos en sistemas biológicos y alimentos formados a partir de la reacción de la lisina con el GO o MGO respectivamente. También se analizó ArgP, AGE fluorescente de creciente interés en sistemas biológicos y formado a partir de la reacción de la arginina con dos moléculas de MGO.

Con el objetivo de concretar un posible mecanismo a través del cual el HT podría ejercer su potencial efecto antiglicante, se realizó una cinética de incubación para monitorizar la estabilidad del HT en presencia y ausencia de α-dicarbonilo y la variación de su capacidad antioxidante y de atrapamiento de MGO. Adicionalmente su capacidad de atrapar α-dicarbonilos fue comparada con la de la lisina, arginina e histidina como precursores del proceso de glicación y con otros compuestos fenólicos de estructura química similar. Finalmente la confirmación de la formación de nuevas estructuras correspondientes a posibles aductos provenientes de la actividad de atrapamiento de

MGO se evidenció mediante el análisis de cromatografía de líquidos acoplada a espectrometría de masas de alta resolución (QTof).

Por otro lado, se obtuvo un extracto de hoja de olivo (OLE) como fuente natural de HT a través de maceración etanólica acidificada y posterior extracción con disolventes orgánicos. OLE fue caracterizado en función de su composición fenólica y se realizó un estudio antiglicante extrapolable al realizado previamente con el estándar de HT.

Resultados: El HT ejerció un efecto inhibitorio significativo sobre la formación de AGEs fluorescentes totales en los sistema *in vitro* BSA-MGO y BSA-GLC (IC_{50} de 0.48 y 2.30 µmol/mL respectivamente). Asimismo la acción ejercida por su derivado, el HTA, demostró ser significativamente inferior a la ejercida por el HT, mientras que el DOPAC mostró un efecto superior. Además, el HT fue capaz de proteger los restos ε-amino libres de la lisina reactiva de la proteína hasta en un 45% e inhibir la formación de fructosamina en su totalidad. Por otro lado, el HT ejerció una marcada capacidad de atrapamiento de MGO, 3-DG y 3-DGal, mostrando una preferencia por el atrapamiento de C3- frente a los C6-dicarbonilos aunque nuevamente el DOPAC mostró una actividad superior. Estos resultados constataron que el HT podría estar actuando en las diferentes etapas del proceso de glicación. Un estudio más específico sobre la inhibición de la formación de los AGEs más característicos demostró que el HT inhibió significativa la formación de CML, ArgP y CEL hasta en un 62, 71 y 21 % respectivamente, siendo la ArgP el AGE formado mayoritariamente. De manera complementaria se estudió el mecanismo por el cual el HT podría estar ejerciendo dicha actividad, verificando que su capacidad de atrapamiento de α-dicarbonilos fue superior a la de aminoácidos precursores de la glicación como la lisina, arginina e histidina. Se estableció que compuestos con estructuras similares al HT que mantuvieron el grupo hidroxilo en posición 2 en el anillo benceno compartían la capacidad de atrapamiento de MGO. El detrimento en el contenido de HT en un sistema incubado con y sin MGO en condiciones fisiológicas simuladas, la disminución de la actividad antioxidante y el aumento paralelo de la capacidad de atrapamiento de compuestos α-dicarbonilos dio lugar al establecimiento de la hipótesis de que el HT se estaba degradando hacia otros compuestos, entre ellos el DOPAC que presumiblemente ejercía la acción antiglicante. La actividad antiglicante del DOPAC fue confirmada en los modelos de glicación y mediante un estudio cinético de la formación de nuevas estructuras en un sistema incubado de HT-MGO. En los modelos se pudo constatar la formación de cuatro aductos DOPAC-MGO mediante una reacción de adición nucleofílica del MGO sobre el producto de oxidación del HT.

A continuación, se obtuvo un extracto de hoja de olivo (OLE) concentrado en compuestos fenólicos y concretamente en OLEU, HT y sus derivados para comparar la actividad del HT estándar con un

extracto natural fuente del mismo. El extracto de hoja de olivo mostró una inhibición eficaz de la formación de AGEs fluorescentes totales así como de la formación de fructosamina, aunque la inhibición de esta última fue en detrimento a medida que aumentó el tiempo de incubación. Estos resultados junto con una capacidad de atrapamiento similar para MGO, 3-DG y 3-DGal sugieren que la actividad antiglicante posiblemente estuviera ejerciéndose en fases intermedias-finales de la reacción. OLE inhibió notablemente la formación de AGEs específicos como la CML (61%) y en menor medida, CEL y ArgP (9.5% y 28.4% respectivamente). La presencia de los aductos DOPAC-MGO fueron también monitorizados al incubar tanto OLE como su compuesto fenólico mayoritario, oleuropeína, con MGO posiblemente debido a la degradación de la OLEU en HT y este último en DOPAC.

Conclusiones: El HT mostró una potente capacidad antiglicante en los sistemas de glicación estudiados, siendo además de amplio espectro ya que actuó en diferentes etapas de la glicación. En concreto, el HT manifestó una actividad relevante en la protección de los restos amino de la lisina, en la inhibición de la formación del producto de Amadori y en la formación de AGEs fluorescentes totales y AGEs específicos como CML, ArgP y CEL. Paralelamente, se ha podido evidenciar que también puede actuar bloqueando a los compuestos α-dicarbonilos de 2, 3 y 6 carbonos, aunque preferiblemente actúa atrapando los C3-dicarbonilos. Su mecanismo de acción fue confirmado mediante la monitorización de nuevos aductos formados por adición nucleofílica entre el DOPAC, compuesto de oxidación de HT y el MGO. Por último, se caracterizó un extracto obtenido a partir de hoja de olivo como fuente de HT, confirmando los efectos anteriormente descritos para el estándar. Los posibles sinergismos entre los compuestos fenólicos, la degradación de OLEU en HT, la propia presencia de HT y otros compuestos fenólicos con posibles efectos antiglicante hacen del OLE una buena alternativa como posible ingrediente o producto farmacológico anti-AGE.

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ORIGINAL PAPER

In vitro investigation on the antiglycative and carbonyl trapping activities of hydroxytyrosol

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Abstract Advanced glycation end products (AGEs) are involved in the aging and the development of common chronic diseases. Hydroxytyrosol (HT) and its acetate derivative (HTA) exert a significant inhibitory activity on the formation of fluorescent AGEs in bovine serum albumin glycation model systems induced by methylglyoxal (IC_{50} value of 0.48 and 0.58 μmol/mL, respectively) and glucose (IC_{50} 2.30 and 2.92 μmol/mL, respectively). Furthermore, HT and HTA showed a relevant carbonyl scavenging capacity toward methylglyoxal and glyoxal, which are the most potent promoters of the glycation in vivo, at molar reaction rates from 0.2 to 10 (carbonyl:phenol). However, carbonyl trapping capacity was significantly more effective against methylglyoxal (IC_{50} 0.19 μmol/mL) than glyoxal (IC_{50} 0.26 μmol/mL). At equimolar concentrations, the ester linkage did not significantly affect the antiglycative activity and carbonyl trapping capacity of the orthodiphenolic ring structure. Results were confirmed with the specific inhibition of the formation of the principal AGEs. Formation of carboxymethyl-lysine, argpyrimidine and carboxyethyl-lysine was significantly reduced by 61.9, 71.4 and 20.9 %, respectively. HT and its esters could be considered for upscaling studies as promising natural strategy against adverse consequences of protein glycation.

Keywords Hydroxytyrosol · Antiglycative activity · Advanced glycation end products · Carboxymethyl-lysine · Carboxyethyl-lysine · Argpyrimidine

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Abbreviations

AGEs	Advanced glycation end products
GLC	D(+)-Glucose
BSA	Bovine serum albumin
MGO	Methylglyoxal
GO	Glyoxal
AG	Aminoguanidine
5-MQ	5-Methylquinoxaline
NBT	Nitrotetrazolium blue chloride
OPD	*o*-Phenyldiamine
OPA	*o*-Phthaldialdehyde
HFBA	Heptafluorobutyric acid
HT	Hydroxytyrosol
HTA	Hydroxytyrosol acetate
PM	Pyridoxamine
CML	ε-*N*-carboxymethyl-L-lysine
CEL	ε-*N*-carboxyethyl-L-lysine
ArgP	Argpyrimidine
2-MQ	2-Methylquinoxaline
Q	Methylquinoxaline
PBS	Saline phosphate buffer
HPLC	High-performance liquid chromatography

Introduction

Glycation occurs physiologically in the course of aging with the formation of advanced glycation end products (AGEs) when a free amino group of protein interacts with the carbonyl group of glucose [1]. In addition, dicarbonyl compounds, such as methylglyoxal (MGO) and glyoxal (GO), are also formed from the sugar degradation being key intermediates in AGEs formation [2]. Human plasma levels for GO and MGO have been estimated to be in the 0.3–1.5 μmol/L range, and their concentrations are

increased in diabetes and chronic renal failure [3, 4]. AGEs are a group of complex and heterogeneous products with different fluorescent and non-fluorescent structures, such as *Nε*-carboxymethyl-lysine (CML), *Nε*-carboxyethyl-lysine (CEL), pentosidine, argpyrimidine (ArgP), glyoxal lysine dimers, or methylglyoxal lysine dimmers among others [1, 5, 6]. AGEs that accumulate in tissue altering irreversibly the functionality and protein structure by binding to certain amino acids such as lysine and arginine are structurally modified [2]. Therefore, AGEs might damage the tissues and organs underlying inflammation through specific receptor, and its accumulation is accelerated in diabetes, neurodegenerative and autoimmune diseases. AGEs have been associated with the process of normal aging and the development of common chronic diseases such as Alzheimer's disease, atherosclerosis or diabetes and its numerous long-term complications [1, 7].

The high incidence of these chronic diseases and the importance to maintain a healthy aging have caused that research focuses on the search for AGE inhibitors [8]. Initially, aminoguanidine (AG) was studied as synthetic AGE inhibitors, but due to toxic side effects it was discharged. Later, compounds from a natural source such as pyridoxamine, quercetin, curcumin, creatine or carnitine were considered as the most appropriate alternative [6, 9, 10]. Nowadays, certain bioactive phytochemicals are particularly relevant as natural AGE inhibitors since phenolic compounds have redox activity which could contribute to the inhibition of glycation process [11]. This hypothesis is supported by clinical studies where the development of type II diabetes might be ameliorated by natural antioxidants [12]. However, the specific biological consequences of the dietary AGEs are still under debate [13].

Hydroxytyrosol (HT, 2-(3,4-dihydroxyphenyl) ethanol) is an orthodiphenolic compound present in our diet, and in pharmacological preparations, as a secoiridoide derivative in free form or as an acetate ester. HT is essential component of oleuropein, present in olive oil and mill waste in appreciable quantities [14]. Dietary intake of olive oil polyphenols has been estimated to be around 9 mg (within 25–50 olive oil/day), where at least 1 mg of them is derived from free HT and 8 mg related to their elenolic esters and oleuropein [15]. Chemical structure of HT and its derivative, hydroxytyrosol acetate (HTA) (3,4-dihydroxyphenylethyl acetate), are shown in Fig. 1. Studies of HT in vivo and in vitro have shown various biological activities with potential beneficial health effects. For instance, it has been associated with anti-inflammatory, hypocholesterolemic, neuroprotective, antibacterial, cardioprotective, anticancer and insulin-stimulatory activities [14, 16]. Previous studies have linked former effects with the antioxidant activity of orthodiphenolic compounds. In vitro and in vivo studies proved that HT is a potent scavenger of free radicals and could exert an indirect protection by modulating the endogenous antioxidant defense system [15]. Although most of the studies refer to HT, it was found that HTA presents rather similar antioxidant activity than HT [17]. Restraint of oxidative stress by HT and prevention of glucose autooxidation also allowed performing a protection against certain metabolic diseases such as diabetes and its complications [17–19]. However, no alternative mechanisms for the antiglycative activity of HT have been elucidated apart of its antioxidant and antiradical properties. Recently, a number of in vitro studies have pointed out the direct relationship between the major constituents in olive leaf [20], or olive oil wastewater [21] and the inhibition of fluorescent AGEs and the mitigation of the carbonyl stress by trapping

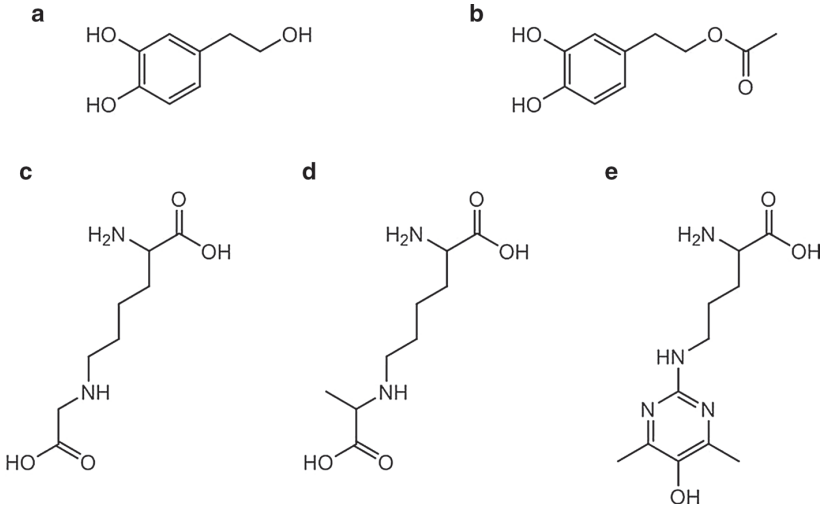


Fig. 1 Structure of hydroxytyrosol (**a**), hydroxytyrosol acetate (**b**), *ε-N*-carboxymethyl-L-lysine (**c**), *ε-N*-carboxyethyl-L-lysine (**d**) and argpyrimidine (**e**)

reactive dicarbonyls [22]. However, those studies just focussed on the inhibition of the formation of fluorescent-derived AGEs, but there is no information of the specific inhibition on the formation of AGEs structures as formed in vivo.

This study aimed to investigate the antiglycative activity of HT and its acetate derivative directly on the formation of fluorescent AGEs by applying different in vitro models, but also through the inhibition of major AGEs, such as CML, CEL and ArgP. Additionally, ability of HT and HTA against a direct trapping of MGO and GO, and the inhibition of the formation of fructosamine adducts were also evaluated.

Materials and methods

Chemical and materials

D(+)-Glucose (GLC), bovine serum albumin (BSA), MGO (40 % aqueous solution), GO (40 % aqueous solution), aminoguanidine (AG), 5-methylquinoxaline (5-MQ), nitrotetrazolium blue chloride (NBT), *o*-phenyldiamine (OPD), *N*α-acetyl-L-lysine, *o*-phthaldialdehyde (OPA), sodium borohydride, perfluoropentanoic acid (purity >97 %), heptafluorobutyric acid (HFBA) and standards of phenolic acids were provided by Sigma (St. Louis, MO, USA). HT and HTA (purity >99 %) were acquired from Seprox Biotech (Madrid, Spain). Pyridoxamine (PM) was acquired from Fluka Chemical (Madrid, Spain). Sodium dihydrogen phosphate monohydrate, sodium carbonate anhydrous, glacial acetic acid and high-performance liquid chromatography (HPLC)-grade methanol were purchased from Merck (Darmstadt, Germany). *N*ε-carboxymethyl-L-lysine (CML, ≥97 %), *N*ε-carboxyethyl-L-lysine (CEL, ≥97 %), CML-d_2, CEL-d_4 and argpyrimidine (ArgP) were obtained from PolyPeptide Laboratories (Strasbourg, France). The Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals were of analytical grade and supplied by Panreac Química (Barcelona, Spain).

In vitro glycation assay with bovine serum albumin induced by methylglyoxal and glucose

Two glycation models on BSA, one induced by MGO (BSA–MGO assay), and the other induced by glucose (BSA–GLC assay), were carried out as described by Mesias et al. [23]. The BSA solution (0.525 μmol/mL) was prepared in phosphate buffer (0.01 mol/L, pH 7.4), containing 0.1 g/mL sodium azide and penicillin G (0.05 g/mL) to ensure aseptic conditions and EDTA (0.026 g/mL) to prevent metal-catalyzed oxidation. The MGO stock solution (5.551 μmol/mL) and glucose stock solution (975.381 μmol/mL) were prepared in phosphate buffer (0.1 mol/L, pH 7.4). In the BSA–MGO assay, 200 μL of BSA solution was incubated with 400 μL of MGO solution (5.551 μmol/mL) at 37 °C for 14 days in the presence of 100 μL of the target compound. In the BSA–GLC assay, 200 μL of BSA solution was incubated with 400 μL of GLC solution at 37 °C for 21 days in the presence of 100 μL of the target compound. HT, HTA and phenolic acids were dissolved in methanol/water (60/40, v/v), and concentrations from 0.058 to 5.8 μmol/mL were prepared. In both assays, a blank without target compounds and a positive control with AG (7.694 μmol/mL) were incubated at the same conditions. A portion of each system at the initial time was kept at −80 °C until measurement. In parallel, standards (from 0.058 to 5.8 μmol/mL) were dissolved in phosphate buffer (0.1 mol/L, pH 7.4) and incubated at 37 °C for 14 days (BSA–MGO assay) or 21 days (BSA–GLC) in order to measure their intrinsic fluorescence.

The fluorescence intensity is as an index of the formation of fluorescent AGEs. A Synergy™ MX multimode microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) was used. The formation of AGEs was characterized by fluorescence with excitation and emission at 340 and 420 nm for the BSA–MGO assay and 360 and 420 nm for the BSA–GLC. The intrinsic fluorescence of the standards incubated under the same conditions was subtracted to the global fluorescence in each BSA–MGO or BSA–GLC system. The percentage inhibition of AGEs formation was calculated according to the following equation:

$$\text{Inhibition}\,(\%) = \{1 - [(\text{fluorescence of solution with inhibitor} - \text{intrinsic fluorescence ofsample})/\text{fluorescence of solution without inhibitor}]\} \times 100.$$

The concentration required to inhibit the glycation by 50 % (IC_{50}) was calculated from the dose–response curve using the Microsoft Excel computer software.

Fructosamine adduct assay

Procedure was carried out as described by Baker et al. [24] with slight modifications. The extent of glycation on BSA was determined by measuring the reducing activity of NBT for Amadori compound in alkaline solution. Under these conditions, fructosamine has the ability to reduce NBT to tetrazinolyl radical (NBT^+) forming a colored compound called monoformazan. The samples from the BSA–GLC assay were used for the determination. Thereby, 50 μL of sample was mixed with 450 μL of NBT and the mixture was incubated in darkness at 37 °C for 60 min. Then, in each well of the microplate were added 100 μL of mixture and 100 μL of sodium carbonate buffer (0.1 mol/L, pH 10.35) except wells of blank that only contented 200 μL of

sodium carbonate buffer (0.1 mol/L, pH 10.35). The presence of fructosamine was characterized by the absorbance of monoformazan at 530 nm using a BioTek microplate spectrophotometer. The inhibition of the fructosamine formation, expressed as percentage, was calculated taking into consideration the absorbance in the presence/absence of the inhibitor.

$$\text{Inhibition (\%)} = 100 - \big[(\text{absorbance of the solution with inhibitor} - \text{intrinsic absorbance of sample} - \text{absorbance of blank}) \times 100 / (\text{absorbance control positive} - \text{intrinsic absorbance of control positive} - \text{absorbance of blank})\big].$$

Determination of reactive lysine in BSA using OPA

The reactive lysine residues in BSA were determined as described by Goodno et al. [25]. BSA solution (200 µL, 35 mg/mL in phosphate buffer 0.01 mol/L, pH 7.4) was incubated at 37 °C for 1, 2 and 3 days in the absence or the presence of HT (100 µL, 4.112 µmol/mL in 60:40 methanol/water). The samples (20 µL) were mixed with 200 µL of protein buffer (50 % buffer borate (0.1 M, pH 10), 10 % SDS (20 %, w/w) and 40 % water). *Nα*-acetyl-lysine in formic acid 0.1 %, at various concentrations was used for calibration (1000–50 µM). The presence of reactive lysine was measured by fluorescence of at 360/460 nm (excitation/emission wavelength) and expressed as percentage of amino acid blockage.

Evaluation of direct MGO and GO trapping capacity

Direct MGO and GO trapping capacity was determined as described by Mesias et al. [23] with modifications. The MGO solution (5.551 µmol/mL) or GO solution (5.686 µmol/mL) was dissolved in phosphate buffer (0.1 mol/L, pH 7.4), 5-MQ (internal standard, 1 mg/mL) in 500 mL/L methanol and OPD (10.8 mg/mL) in methanol. PM solution (0.58 µmol/mL phosphate buffer, pH 7.4) was used as positive control, and the target compounds under evaluation were dissolved in methanol/water (60/40). The reaction media were formed by 750 µL of phosphate buffer (0.1 mol/L, pH 7.4), 50 µL of 5-MQ, 100 µL of the target compounds or PM solution or phosphate buffer (blank) and 100 µL of MGO or GO solution. The mixture was incubated at 37 °C up to 168 h. After that, 200 µL of OPD solution was added, rapidly vortexed for 5 s and was kept in darkness for 30 min where the derivatization reaction of unreacted dicarbonyl to corresponding quinoxaline derivative was complete. The quantification of Q, 2-MQ and 5-MQ was carried out on Shimadzu HPLC system equipped with an LC-20AD pump, an SIL-10ADvp autosampler, a CTO-10ASVP oven and an SPD-M20A diode array detector. Chromatographic separation was performed on a Mediterranean-Sea-ODS2 column (150 mm × 3 mm, 5 µm; Teknokroma, Barcelona, Spain). The injection volume was 10 µL, and the flow rate was 0.5 mL/min. The total run time was 7 min in isocratic conditions (mobile phase of 5 mL/L acetic acid/methanol, 50:50 v/v). The chromatograms were recorded at 315 nm being the retention times of Q, 2-MQ and 5-MQ, 3.4, 4.1 and 5.6 min, respectively. The amount of unreacted MGO was calculated from the ratio of 2-MQ and 5-MQ as compared with control at the initial time. The amount of unreacted GO was calculated from the ratio of Q and 5-MQ as compared with control at the initial time.

The percentage of inhibition of MGO or GO was calculated with the next formula.

$$\text{MGO or GO decrease (\%)} = [(\text{amount of MGO or GO in control} - \text{amount of MGO or GO in sample with tested standard or PM solution})/\text{amount of MGO or GO in control}] \times 100.$$

The concentration required to inhibit the glycation by 50 % (IC_{50}) was obtained from the dose–response curves using Microsoft Excel computer software.

Determination of CML and CEL by LC-MS/MS

The formation of CML and CEL was determined as described by Niquet-Léridon and Tessier [26] with minor modifications. Sample (1 mL, containing 10 mg of protein) was incubated with 1.5 mL of sodium borate (0.2 M, pH 9.2) and 1 mL of sodium borohydride (1 M in 0.1 M NaOH) for 4 h at room temperature. Then, samples were hydrolyzed with 5 mL of HCl (final concentration of 6 M) for 20 h at 110 °C, filtered and 500 µL was dried in a Speed-Vac concentrator (ThermoFisher Scientific, Courtaboeuf, France). Finally, residue was reconstituted in 1 mL of NFPA 5 mM (containing 0.1 mL of CML-d_2 or CEL-d_4 at 1 µg/mL). LC-MS/MS was performed with an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole Agilent

(G6410B) via electrospray ionization operating in positive mode. Sample (10 µL) was injected on Hypercarb column (100 mm × 2.1 µm, 5 µm, ThermoFisher Scientific). The flow rate was 0.5 mL/min, and the isocratic elution was applied using a mobile phase of 5 mM NFPA / acetonitrile (95:5 v/v). The product ion at *m/z* 84 was used for quantification of CML (*m/z* 205), CML-d_2 (*m/z* 207), CEL (*m/z* 219) and CEL-d_4 (*m/z* 223). The ratio of response factor of CML or CEL to that their respective labeled internal standards was used for calculation of CML or CEL in samples. Data were processed using Masshunter Data Acquisition and MassHunter Qualitative Analysis (Agilent Technologies). Results were expressed as µmol CML or CEL /g protein.

Determination of argpyrimidine by LC-fluorescence

Sample preparation was the same as described above for lysine-derived AGEs until obtaining the freeze-dried protein hydrolysate as described by Wilker et al. [27]. The analysis of ArgP (10 µL sample injection) was carried out on a Prominence HPLC system with an RF-20A XS fluorescence detector (Shimadzu Corporation, Kyoto, Japan). Sample was eluted onto a Mediterranean-Sea-ODS2 column (250 mm × 4 mm, 5 µm; Teknokroma, Barcelona, Spain) at a flow rate of 0.8 mL/min under gradient elution. The solvent system gradient was of HFBA (1 mL/L) as solvent A and ACN (500 mL/L) containing HFBA (1 mL/L) as solvent B. The following multistep linear gradient was applied: 0 min, 20 % B; 25 min, 100 % B; 26 min, 100 % B; and 27–37 min, 20 % B. ArgP was detected at 335 and 385 nm for excitation and emission wavelength, respectively, and eluted at 15.1 min. Calibration was carried out in the range 0.001–0.01 mg/mL with pure standard. Results were expressed as µmol ArgP/g protein.

Statistical analysis

Data were analyzed using the Statgraphics Centurion XV Statistical program (Herndon, VA, USA). Homogeneity of variances was evaluated by the test of Levene. Analysis of variance was performed using ANOVA procedures followed by a Bonferroni test or Tamhane test when the variances were homogeneous or non-homogeneous, respectively. Differences were considered significant at $p < 0.05$. Data from three independent assays were expressed as the mean value ± SD.

Results and discussion

The antiglycative activity of HT and HTA was evaluated by measuring the inhibition of the formation of fluorescent

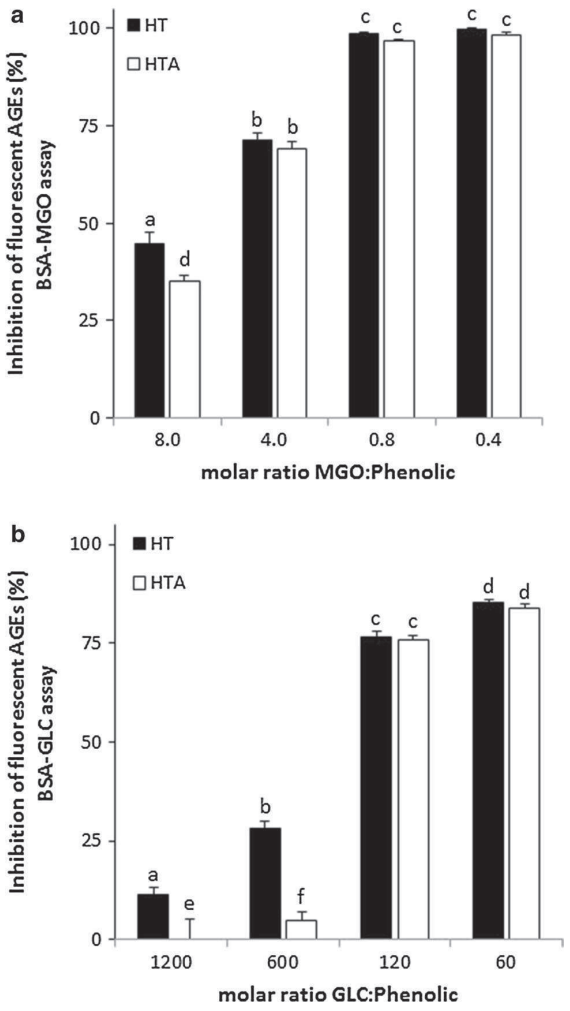


Fig. 2 Antiglycative activity of hydroxytyrosol (*solid bar*) and hydroxytyrosol acetate (*empty bar*) on the formation of fluorescent AGEs in the BSA–MGO assay (**a**) and in the BSA–GLC assay (**b**). Results are expressed as mean (percentage of inhibition respect to control) ± standard deviation for $n = 4$. *Different letters* denote significant differences ($p < 0.05$)

AGEs in a model of glycation with BSA under physiological conditions. Accumulation of AGE-specific fluorescence is a general measure of overall protein glycation damage, and it is a widely used tool for glycation research in in vitro and in vivo studies [6]. Since HT and HTA were dissolved in a methanolic solution (60 % v/v), the lack of potential interferences in the glycation models was evaluated. Different proportions of methanol:water up to 100 % after 14 or 21 days of incubation at 37 °C for BSA–MGO and BSA–GLC systems, respectively, were tested. In addition, the intrinsic fluorescence of HT and HTA was residual, accounting for <5 % of the total fluorescence in the glycation models.

Table 1 Determination of the inhibition of the formation of Amadori products by the fructosamine assay by hydroxytyrosol (HT) and hydroxytyrosol acetate (HTA) in the range from 0.405 to 8.282 µmol/L

Molar ratio Glucose:phenolic	Concentration µmol/mL	HT Inhibition (%)	HTA Inhibition (%)
1200	0.405	2.9 ± 2.3a	7.2 ± 2.0a
600	0.811	66.5 ± 7.4b	7.3 ± 2.1a
120	4.112	100 ± 8.0c	100 ± 2.5c
60	8.282	100 ± 6.7c	100 ± 7.7c

Results are expressed as mean ± SD for $n = 4$

Different letters in same column denote significant differences $p < 0.05$

The antiglycative capacity of HT and its acetate derivative in a range from 0.405 to 8.282 µmol/mL were assayed in the BSA–GLC (21 days/37 °C) and BSA–MGO (14 days/37 °C) systems. Glycation reaction in the BSA–GLC system proceeds slower than the BSA–MGO system since autoxidation of glucose to form dicarbonyl compounds is minimized in the presence of EDTA. Another limiting step in the BSA–GLC system is the conversion of glucose to its open-chain form to condensate with the amino residues [2]. Figure 2 shows the percentage of inhibition of the formation of fluorescent AGEs related to the molar ratio between the glycative stressor and the orthodiphenolic compound. Both HT and HTA exerted a dose-dependent antiglycative activity in BSA–MGO and BSA–GLC systems. Maximum inhibition (98.6 %) was reached at 4.112 µmol/mL in the BSA–MGO system (Fig. 2a), corresponding to a molar ratio (MGO:phenolic compound) of 0.8. At lower molar ratio, the activity was kept without significant differences ($p < 0.05$) among samples. The potency of the antiglycative capacity of HTA was similar to that HT at equimolar concentration of MGO, although response of HTA was significantly lower than HT at 0.405 µmol/mL (8 molar ratio MGO:phenolic compound). AG was used as positive control since it inhibits AGEs formation by reacting with carbonyl groups of reducing sugars, Amadori products and dicarbonyl intermediates [6]. AG reached 99.6 % of inhibition at the same molar ratio MGO:AG. IC_{50} values of 0.48 and 0.58 µmol/mL were calculated for HT and HTA in the BSA–MGO system, respectively. In the BSA–GLC system (Fig. 2b), although a similar trend was observed, the antiglycative activity of HT was significantly higher than HTA at higher molar ratio with GLC. Additionally, IC_{50} value of 2.30 and 2.92 µmol/mL was calculated for HT and HTA, respectively, for their antiglycative activity in the BSA–GLC system.

The extent of the sugar-induced modifications was further evaluated in the BSA–GLC system by the formation of the Amadori products using the fructosamine assay. In this assay, glucosone is the primary carbohydrate oxidation product formed from the Amadori compounds. In this sense, fructosamine assay is an efficient tool to identify at which level of the glycation cascade is acting our target compounds. Table 1 shows the inhibition of the formation of the Amadori products in the presence of different concentrations of HT and HTA (0.405–8.282 µmol/mL). Both HT and HTA are able to prevent the formation of the Amadori product but in an efficiently manner. Results are correlated with the antiglycative effect of HT and HTA in the BSA–GLC assay. HT was significantly more effective

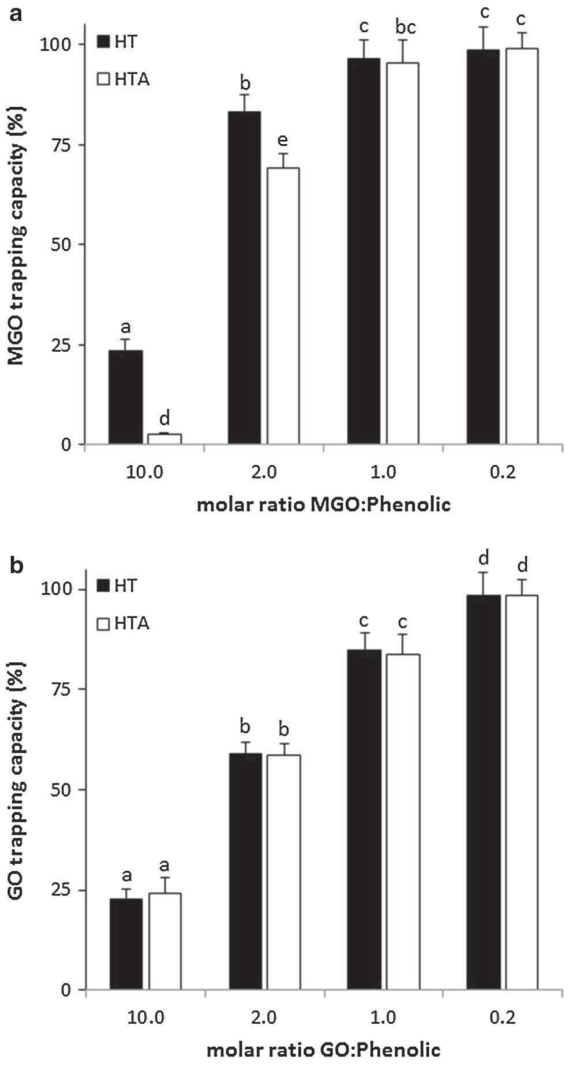


Fig. 3 Methylglyoxal (**a**) and glyoxal (**b**) trapping capacity of hydroxytyrosol (*solid bar*) and hydroxytyrosol acetate (*empty bar*) at different molar ratios. Results are expressed as mean ± standard deviation for $n = 4$. *Different letters* denote significant differences ($p < 0.05$)

Table 2 Methylglyoxal trapping capacity of hydroxytyrosol, hydroxytyrosol acetate, gallic acid, caffeic acid, protocatechuic acid, chlorogenic acid (CGA), sinapinic acid, ferulic acid, gentisic acid and *p*-coumaric acid

Standard	IC_{50} (μmol/mL)
Hydroxytyrosol	0.195
Hydroxytyrosol acetate	0.204
Pyridoxamine	0.059
Gallic acid	0.176
Caffeic acid	0.222
Protocatechuic acid	0.389
Chlorogenic acid	0.367
Sinapinic acid	0.892
Ferulic acid	1.648
Gentisic acid	3.179
p-Coumaric acid	2.985

Pyridoxamine is used as reference

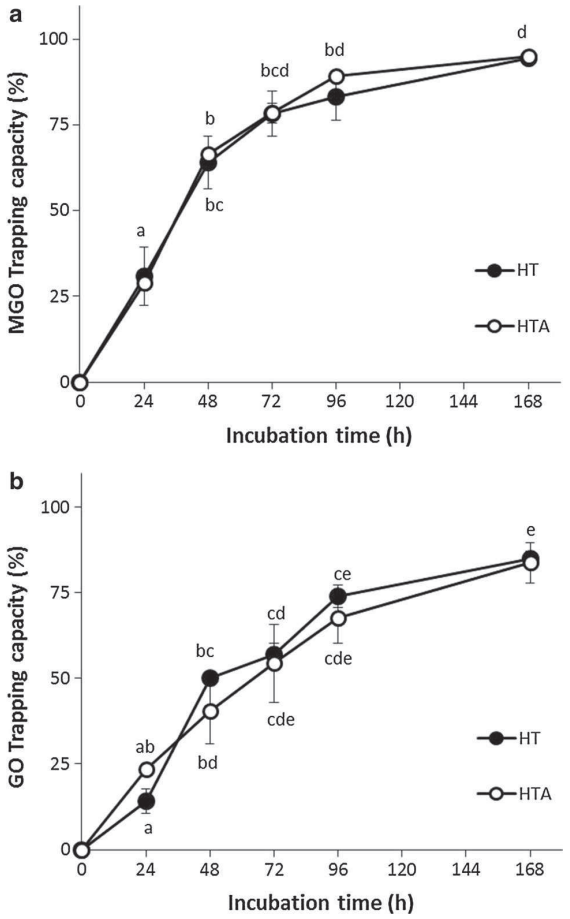


Fig. 4 Methylglyoxal (**a**) and glyoxal (**b**) trapping capacity of hydroxytyrosol (*solid circle*) and hydroxytyrosol acetate (*open circle*) incubated (37 °C for 168 h) at the equimolar conditions (0.581 μmol/mL). *Different letters* denote significant differences ($p < 0.05$)

than HTA in the prevention of the formation of Amadori products at a molar ratio (GLC:phenolic compound) of 600, whereas there were no differences at lower molar ratio since a complete inhibition was already reached. These results on fructosamine could explain the higher activity of HT than HTA exerted in the inhibition of the fluorescent AGEs in the BSA–GLC assay.

The protection of reactive lysine residues in the protein by HT was investigated. HT was able to protect the modification of the 24.3, 39.7 and 45.1 % of amino group in just 1, 2 and 3 days of incubation, respectively. Recently, Navarro and Morales [22] shown that the reactivity of orthodiphenolic compounds against MGO and GO was effective enough to compete with free lysine, arginine and histidine and subsequently to prevent the formation of carbonyl-derived AGEs. However, the mechanism of reaction was not concluded. Our results pointed out that ortodiphenolic compounds not only could mitigate the extension of the glycation process by reducing the formation of the Amadori products but also by protecting the lysine residues to initiate the glycation reaction.

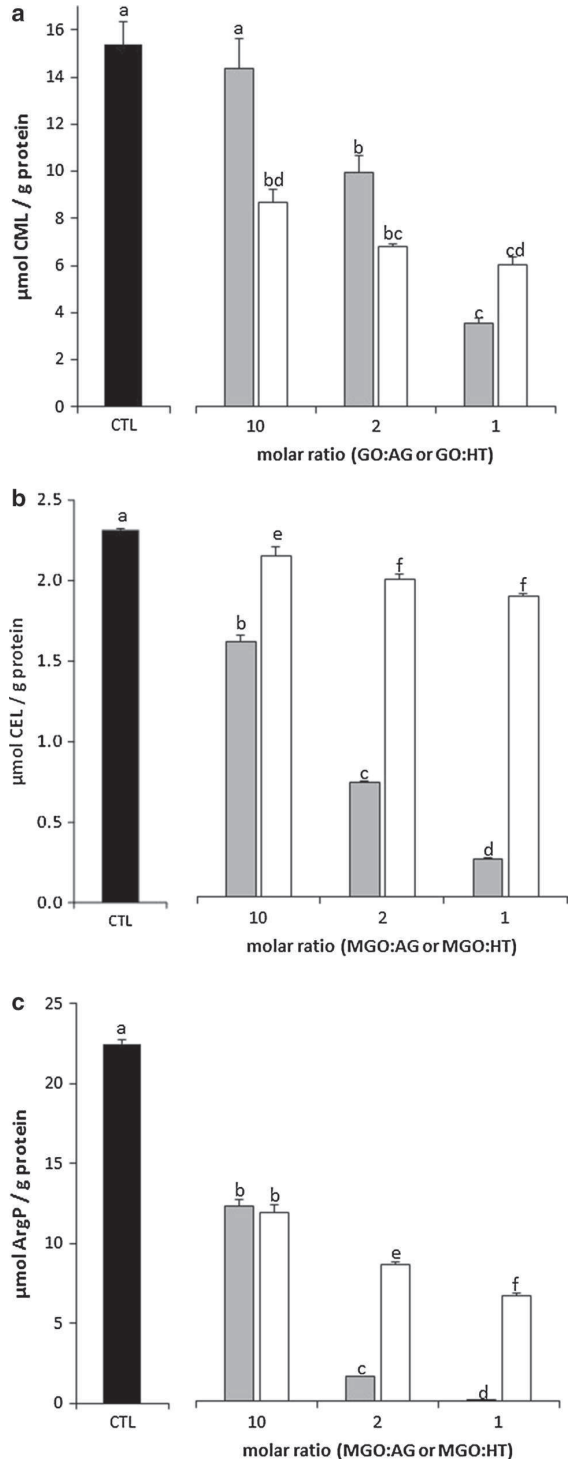
In the BSA–MGO system, our results showed the protective effect of HT on the formation of the fluorescent AGEs at similar molar ratio with MGO. The evaluation of the specific scavenging capacity of inhibitors toward dicarbonyl compounds gives a complementary view of the potential antiglycative activity of target compounds, and it can be used as an effective strategy to prevent protein modifications and AGEs formation [8, 28, 29]. The role of dicarbonyl compounds was further investigated in MGO and GO trapping model systems (Fig. 3) at different molar ratios (dicarbonyl:phenolic compound). Both HT and HTA displayed similar dicarbonyl trapping activity against GO regardless the molar ratio (Fig. 3b). However, significant differences between HT and HTA were found at molar ratio higher than 1 for trapping MGO (Fig. 3a). Dicarbonyls and particularly MGO and GO are key promoter in the glycation process [30], and they are important precursors in the formation of AGEs [1, 11]. Dicarbonyls are formed from oxidative reaction to sugars, Schiff bases, Amadori products and lipid oxidation. Then, dicarbonyls irreversibly and progressively modify proteins overtime and yield AGEs which are contributing to the complications associated with aging. These modifications involve lysine residues, but arginine is predominantly modified with the formation of fluorescent compounds [5]. Our results on direct dicarbonyl trapping are in line with the results obtained for the inhibition of fluorescent AGEs in the BSA–MGO assay shown in Fig. 2a.

Additionally, the trapping capacity, expressed as IC_{50}, of different structure-related phenolic compounds (gallic acid, caffeic acid, protocatechuic acid, chlorogenic acid, sinapinic acid, ferulic acid, gentisic acid and *p*-coumaric acid) is given in Table 2. Results confirm the promising dicarbonyl trapping ability of HT, and HTA as compared with other phenolic acids. It has been described that the hydroxyl groups in the phenyl ring have electron-donating properties and enhance the reactivity of MGO by just nucleophilic addition. Navarro and Morales [22] identified two major adducts during the incubation of HT with MGO at physiological conditions. In order to gain more insight into the dicarbonyl trapping abilities of HT and HTA, the kinetic of the trapping over time was investigated. Figure 4 shows the time course of the disappearance of dicarbonyl compounds (expressed as MGO or GO trapping capacity) in the reaction mixtures (100 mM PBS, 37 °C) at the same dicarbonyl:phenolic compound molar ratio. PM was used as a positive control of the reaction, and HT and HTA behave as PM did (data not shown). The kinetic of HT and HTA was similar, time dependent and progressively is reaching a maximum activity at 168 h of incubation (94.6 and 95.1 %, respectively). Again, it was observed that HT and HTA were quantitatively less effective for trapping GO as compared with MGO at any time of incubation.

The antiglycative properties of HT were further investigated on the specific inhibition of the formation of the most representative AGEs (Fig. 1). CML is the most abundant non-fluorescent AGEs and together with CEL has been used as markers of the extension of the glycation in foods and physiological systems [1, 2, 30]. In our BSA–GLC glycation system that contains EDTA, CML is basically formed by the condensation of glucose with the ε-amino group of lysine, where the Amadori rearrangement product fructosyllysine is unstable intermediate and undergoes oxidation to form CML. Then a BSA–GO model was carried out to enhance specifically the formation of CML. In contrast, the system BSA–MGO was used for the determination of CEL and ArgP. ArgP is the major fluorescent AGEs result of the reaction between two molecules of MGO and arginine [5], and CEL is a lysine-MGO-derived AGE [3]. In this investigation was not included pentosidine since it was not defined a model system with ribose. In the investigation with CML, CEL and ArgP, the incubation time of the glycation models was settled at 7 days for BSA–MGO and BSA–GO systems since the maximum concentration of these AGEs was already reached at that time. BSA–GO system was prepared in similar way to BSA–MGO.

The CML formation in the BSA–GO system (7 days of incubation at 37 °C) is shown in Fig. 5a. AG was used as a positive control at the same molar ratio than HT. The formation of CML was significantly reduced in the presence of HT at all the concentration assayed, being significantly stronger than AG at the highest molar ratio with GO. CEL is formed in low amounts in the BSA–MGO system, and AG is able to inhibit its formation very effectively in a dose–response manner. In regard to the formation of CEL (Fig. 5b), it was clearly observed a dose-dependent effective mitigation by AG, even at the highest MGO:AG molar ratio. However, HT was not efficient enough to impair the formation of CEL where only a 20.9 % of inhibition is reached at equimolar concentration. As described by Navarro and Morales [22], arginine is the preference site of reaction for MGO, followed by histidine, and lysine is less prone to react with MGO that it could explain the low rate of formation of CEL. As expected, the arginine-MGO-derived AGE ArgP was largely formed in the BSA–MGO (Fig. 5c) and ArgP formation is largely dependent on the presence of AG and HT. For both compounds, the formation of ArgP was significantly reduced to 47.4 and 49.2 % for AG and HT, respectively, as compared with the control at the highest MGO molar ratio. It is noteworthy to mention that the formation of ArgP at equimolar concentration was completely impaired by AG and significantly reduced at 71.4 % by HT. Two molecules of MGO are necessary for the formation of ArgP, and the presence of free MGO is the limiting factor in the reaction.

The protein glycation process can be divided into the early stage in which the Amadori products are formed, and the advanced stage in which Amadori products undergo a number of rearrangements to form AGEs. Our results on the fructosamine assay and blockage of reactive amino residues reveal that HT and HTA have also an inhibitory effect on the formation of the Amadori product at the early stage of the glycation. But, HT and HTA are also effective inhibitors of the advanced stage since the formation of fluorescent AGEs, CML and ArgP were effectively suppressed. Results are promising to upscale the in vitro experiments to in vivo systems since HT is abundant in olive oil and efficiently absorbed [15, 16]. In this regard, Miro-Casas et al. [18] investigated the bioavailability of HT in animals and humans administrated with virgin olive oil have demonstrated that HT absorption, plasmatic levels and urine excretion have a direct response to the intake. The maximum concentration of HT in plasma is reached at 32 min with a half-life of 2.43 h. Nearly 98 % of the HT appears in plasma as conjugated HT and its metabolites suggesting extensive intestinal/hepatic metabolism of the dietary HT [15]. Serra et al. [31] in an intervention experiment feeding rats with olive extract shown that HT was absorbed and rapidly distributed (56.8 nmol/L plasma after 1 h) practically to all organs through the blood stream more effectively than the luteolin derivatives (0.68 nmol/L plasma after 1 h). Other study in rats supports that after an intravenously injection of HT, it was quickly absorbed and distributed, being preferential kidney uptake. The



◀**Fig. 5** Effect of hydroxytyrosol (*empty bar*) on the inhibition of the formation of carboxymethyl-lysine (**a**), carboxyethyl-lysine (**b**) and argpyrimidine (**c**) at different molar ratios of methylglyoxal and glyoxal. Aminoguanidine was used as reference (*gray bar*). BLK corresponds to control (*solid bar*). Results are expressed as mean ± standard deviation for $n = 3$. *Different letters* denote significant differences ($p < 0.05$) with control

distribution of its different metabolites depends on the enzymatic conversion, being sulfo-conjugated forms, the metabolites from a sequential oxidation (3,4-dihydroxy-phenylacetaldehyde, 3,4-dihydroxy-phenylacetic acid, 4-hydroxy-3-methoxy-phenylacetic acid) and the methylated derivative form (4-hydroxy-3-methoxy-phenylethanol) the majority in kidney, brain, heart, liver and brain, respectively [32].

In conclusion, HT and its acetate derivate, HTA, were evaluated with the aim of investigating their antiglycative capacity using different in vitro models. These orthodiphenolic compounds exhibited a potent inhibitory activity on the formation of CML, and ArgP formation and very likely the carbonyl trapping capacity plays a major role. Results are in line with Navarro and Morales [22] who described the mechanism of action for trapping carbonyl species by HT due to electrophilic aromatic substitution of the orthodiphenyl ring. Additionally, the reactivity of amino residues and fructosamine formation was efficiently inhibited so it could be assumed that HT and HTA are also acting at the early stages of glycation process. This study has shown by first time that HT and its acetate ester act at multiple steps of the glycation process and the reactive part of the molecular structure is the orthodiphenyl ring without major relevance to the ester linkage. These in vitro results are promising and pointed out that it is realistic to define further steps in vivo before to propose HT as a natural AGEs inhibitor.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Human and animal rights This investigation does not contain studies with human or other animal subjects.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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Mechanism of reactive carbonyl species trapping by hydroxytyrosol under simulated physiological conditions

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3,4-Dihydroxyphenylacetic acid (PubChem CID: 547)
3,4-Dihydroxyphenylacetaldehyde (PubChem CID: 119219)
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ABSTRACT

This investigation reveals that hydroxytyrosol (HT) could compete with lysine, arginine and histidine to bind methylglyoxal (MGO) and reducing the formation of advanced glycation end products. Kinetic of the degradation of HT in presence/absence of MGO under simulated physiological conditions is monitored by HPLC coupled to a QTOF spectrometer. HT should previously be oxidized to DOPAC (3,4-dihydroxyphenylacetic acid) which reacts with MGO by electrophilic aromatic substitution of the ortho-diphenyl ring. DOPAC was detected as the major degradation product of HT under simulated physiological conditions. Ortho-hydroxyl groups are necessary to promote the nucleophilic addition of MGO by HT and related compounds. The formation of four adducts were described by mass spectrometry, but monoDOPAC-monoMGO adduct ($C_{11}H_{12}O_6$) was predominant. Results suggest that HT and its degradation product DOPAC could have a relevant role in preventing the formation of advanced glycation end products and therefore potentially mitigate the diabetic complications.

1. Introduction

The development of some chronic diseases, such as Alzheimer's disease and diabetes Mellitus and its long-term complications (atherosclerosis, cataracts, and nephropathies), have been associated with advanced glycation end-products (AGEs). AGEs are a chemically heterogeneous group of compounds which are result of the non-enzymatic glycation process (Poulsen et al., 2013; Singh, Barden, Mori, & Beilin, 2001). Glycation proceeds when a free amino group of proteins, lipids and nucleic acids interacts with carbonyl group of reducing sugars or α-dicarbonyl compounds such as methylglyoxal (MGO), glyoxal (GO) or deoxyglucosone. This reaction is also taking place during thermal processing of foods and named Maillard reaction. Generally, AGEs can be divided into two types on the basis of chemical structure, one type is fluorescent properties and crosslinking structures AGEs, and the other type is non-fluorescent and non-crosslinking AGEs (Wu, Huang, Lin, & Yen, 2011).

Glycation is a major source of ROS (reactive oxygen species) and RCS (reactive carbonyl species) that are generated by both oxidative and non-oxidative pathways. RCS such as 3-deoxyglucosone, glyoxal and methylglyoxal (MGO) are critical intermediaries formed during glycation of proteins in vivo or from diet (Baynes & Thorpe, 1999; Cai et al., 2002). Both clinical and pre-clinical studies shows that MGO is associated with hyperglycemia in type II diabetes and diabetes-related complications, and also referred as carbonyl stress (Rahbar & Figarola, 2003). MGO can bind to amino groups, thereby modifying biological molecules to form covalently

Abbreviations: HT, hydroxytyrosol; PBS, phosphate buffer saline; MGO, methylglyoxal; GO, glyoxal; DOPAC, 3,4-dihydroxyphenylacetic acid; BSA, bovine serum albumin; AGEs, advanced glycation end products.

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cross-linked aggregates. In healthy humans, plasma levels of MGO are lover than 1 μM, however these levels are elevated 2–4-fold in patients with diabetes (Wang, Meng, Gordon, Khandwala, & Wu, 2007). Thus, scavenging RCS has been used as an effective strategy to prevent protein modification and AGE formation.

Today, clinical results indicate that the development of diabetes and accumulation of AGEs may be reduced by the intake of natural antioxidants through the diet (Baynes, Monnier, Ames, & Thorpe, 2004; Montonen, Knekt, Järvinen, & Reunanen, 2004). The radical scavenging property of phenolics compounds has been previously associated with inhibiting the Maillard reaction in both biological and food systems, although the reaction mechanisms are poorly defined and commonly linked with oxidative-dependent Maillard pathways (Nagasawa et al., 2003). However, the direct reactivity of phenolic compounds with key Maillard carbonyl precursors is an alternative mechanism (carbonyl trapping) and may partly explain the inhibitory effect of phenolics compounds on the Maillard reaction (Totlani & Peterson, 2006). Several investigations have documented the trapping effect of MGO and GO by phenolics compounds, such as flavonoids, chalcones, stilbenes, isoflavones and phenolic acids. In general, flavones exhibit stronger inhibitory effect on the formation of AGEs when compared with flavonols, flavones, and isoflavones (Peng, Ma, Chen, & Wang, 2011).

Hydroxytyrosol (3,4-dihydroxyphenylethanol, HT) is the major phenolic compound in olive oil and has been exhaustively studied for its beneficial health properties. The free radical scavenging activity and increase the endogenous defence systems of HT and its metabolites could be involved in the prevention of different pathologies caused by oxidative stress such as inflammation, cancer, diabetes, cardiovascular or neurodegenerative diseases (Hu, He, Jiang, & Xu, 2014). Several researches focused on antidiabetic and hypolipidemic activities of HT through the inhibition of disaccharidase and lipase actions to retard the absorption of carbohydrates, free fatty acids and monoglycerides (Hamden et al., 2010). It has also been described that HT can affect the formation of AGEs and their interaction with respective receptors in cells, and modulates the complications associated to type II diabetes (Kawaguchi, Matsumoto, & Kumazawa, 2011), and preventing autoxidation of glucose (Hamden, Allouche, Damak, & Elfeki, 2009).

Several studies have highlighted that HT possesses antimicrobial, anti-inflammatory, anticarcinogenic activity, cardioprotective activity, and anti-diabetic activities by preventing oxidative stress situations (Hu et al., 2014; Pan, Lai, Tsai, Wu, & Ho, 2012). The antioxidant activity of this ortho-diphenol has been suggested to be the main mechanism of action. However, HT has not previously been considered as an antiglycative natural dietary compound. This investigation aims to examine the carbonyl trapping capacity of HT and its mechanism of action at simulated physiological conditions. The formation of adducts from HT or its degradation products with MGO is investigated.

2. Material and methods

2.1. Chemicals

D(+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (40% aqueous solution), glyoxal (40% aqueous solution), L-lysine, L-arginine, L-histidine, aminoguanidine (AG), 5-methylquinoxaline (5-MQ), *o*-phenyldiamine (OPD), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,4,6-tripyridyl-s-triazine (TPTZ), 2-2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), and phenolic acids standards were provided by Sigma (St. Louis, MO, USA). Folin–Ciocalteu reagent, iron (III) chloride was purchased from Panreac (Madrid, Spain). Hydroxytyrosol (purity > 99%) was acquired from Seprox Biotech (Madrid, Spain). Pyridoxamine (PM) was acquired from Fluka Chemical (Madrid, Spain). Sodium dihydrogen phosphate monohydrate, sodium carbonate anhydrous, glacial acetic acid and HPLC-grade methanol were purchased from Merck (Darmstadt, Germany). Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

2.2. Assessment of MGO and GO trapping capacity

Direct MGO or GO trapping capacity was determined as described by Mesias, Navarro, Gökmen, and Morales (2013). MGO (0.04 mg/mL) or GO (0.03 mg/mL) was incubated with amino acids (arginine, histidine and lysine), or phenolics compounds (hydroxytyrosol, acetate hydroxytyrosol, tyrosol, 2-phenylethanol, phenylacetic acid, 3,4-dihydroxyphenylacetic, pyrocatechol, caffeic acid and gallic acid), or pyridoxamine (PM, 0.1 mg/mL, as positive control) in a pH 7.4 100 mM saline phosphate buffer solution at 37 °C for 168 h. For kinetic studies, sampling was extended to 24, 48, 72, 96, and 168 h where samples ($n = 2$) were withdraw at that time. Incubations were performed at a molar rate of 1:1 and 5:1 (sample: dicarbonyl). For studying the effect of pH on trapping of MGO by HT, different PBS solutions (100 mM, 50 mM, 10 mM, saline, and pH), sodium acetate buffer (100 mM at pH 4.5 and 7.5), water and alkaline water (pH 8.5) were applied. After the incubation, samples were added with OPD in order to derivatise of unreacted MGO or GO into 2-methylquinoxaline (2-MQ) or 1-quinoxaline (1-Q), respectively. 5-quinoxaline (5-MQ) was used as internal standard. Remaining MGO or GO as their quinoxaline derivative were determined by HPLC-DAD. Chromatograms were recorded at 315 nm being the retention times of 2-MQ, 1-Q and 5-MQ, 4.1, 3.4 and 5.6 min, respectively. The amount of unreacted MGO and GO was calculated from the ratio of 2-MQ or 1-Q and 5-MQ to the control at time zero after preparation.

The percent inhibition of MGO was calculated from the following equation

$$\text{MGO decrease } (\%) = [(\text{amount of MGO in control} - \text{amount of MGO in sample with tested standard or PM solution})/\text{amount of MGO in control}] \times 100\%.$$

IC_{50} value (mg/mL) was obtained from the dose–response curves of each experiment. For kinetic studies, the time (h) required to trap MGO by 50% was calculated using Microsoft-Excel computer software. In the same manner, the percent inhibition of GO was calculated.

2.3. Assessment of in vitro glycation of BSA by methylglyoxal and glucose

The BSA-MGO and BSA-GLC assays were carried out as described by Mesias et al. (2013). Aminoguanidine (0.57 mg/mL) was used as positive control of the inhibition for the formation of fluorescent AGEs. Briefly, the mixtures (blank, control, or sample) were incubated at 37 °C for 14 days (BSA-MGO assay) or 21 days

(BSA-GLC assay) in PBS buffer (100 mM, pH 7.4). An aliquot was just taken before incubation and stored in at −80 °C as control to establish the fluorescent increase. In parallel, samples (0.07–1.43 mg/mL) were also dissolved in PBS buffer (100 mmol/L, pH 7.4) and incubated at 37 °C for 14 days or 21 days in order to establish their intrinsic fluorescence, if any. Fluorescence ($n = 4$) was measured in a microplate reader (Synergy™ HT-multimode Biotek, VT, USA) with an automatic reagent dispense and temperature control. AGEs formation was characterized by measuring fluorescence with excitation and emission maxima at 340 and 420 nm for the BSA-MGO assay and 360 and 420 nm for the BSA-GLC. The intrinsic fluorescence of the samples incubated without protein and MGO or glucose, was subtracted to the overall fluorescence in each BSA-MGO or BSA-GLC system. The percentage inhibition of AGEs formation was calculated according to following equation:

$$\text{Inhibition}\ (\%) = \{1 - [(\text{fluorescence of solution with inhibitor} - \text{intrinsic fluorescence of sample}) / \text{fluorescence of solution without inhibitor}]\} \times 100.$$

The concentration required to inhibit glycation by 50% (IC_{50}) was calculated from the dose–response curve using Microsoft-Excel computer software.

2.4. Determination of total phenolic content

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method as described by Singleton, Orthofer, and Lamuela-Raventos (1999). Absorbance was recorded using a microplate spectrophotometer and quantified using gallic acid as a calibration curve. Results were expressed as mg GAE equivalent/g sample. The limit of quantification was set at 4.5 mg GAE/g sample. Measurements were performed in triplicate.

2.5. Determination of antioxidant capacity according to ABTS assay

Antioxidant activity was estimated in terms of radicals scavenging capacity of the extracts as described by Re et al. (1999). Trolox calibration curve was used for quantification. Results were expressed as μmol TEAC/g sample. The limit of quantification was set at 1.1 μmol TEAC/g sample. Measurements were performed in triplicate.

2.6. Determination of antioxidant capacity according to FRAP assay

The formation of Fe^{2+}-TPTZ complex from Fe^{3+}-TPTZ complex was as described by Benzie and Strain (1996). Trolox calibration curve was used for quantification. Results were expressed as μmol Trolox equivalent antioxidant capacity (TEAC)/g sample. Measurements were carried out in triplicate.

2.7. Determination of browning

Microplate reader was used to measure the browning at 420 nm. HT (0.5 mg/mL) was incubated at 37 °C in PBS (100 Mm, pH 7.4) up to 168 h. Absorbance was measured after appropriate dilution in water to obtain an absorbance less than 1.5 arbitrary units. Results were expressed as absorbance units at 420 nm/g sample. Measurements were performed in triplicate.

2.8. Determination of hydroxytyrosol

The degradation of HT and formation of related compounds in the different models was monitored by HPLC-DAD as described by Rubio-Senent, Rodríguez-Gutíerrez, Lama-Muñoz, and Fernández-Bolaños (2012) with modifications. A HPLC Shimadzu (Kyoto, Japan) equipped with a LC-20AD pump, a SIL-20AHT autosampler, a CTO-10ASVP oven, and a DAD (SPD-M20A) was used. Chromatographic separation was carried out onto a Kinetex C18 column (75 mm × 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA). The flow rate was 0.6 mL/min and the injection volume was 10 μL. A binary solvent system gradient of acetic acid (5 mL/L) (A) and methanol (B) was used; 0 min, 0% B; 8 min, 5% B; 9 min, 5%; 21 min, 40% B; 25 min, 80% B; 26 min, 80% B; 27 min, 0%. B; 40 min, 0%. The total run time was 40 min and chromatograms were recorded from 190 to 330 nm, but reference wavelength was set at 280 nm. HT and DOPAC were eluted at 10.28 min and 12.08, respectively. Peaks were identified by time retention with standards and DAD scan. Calibration was carried out in the range (0.05–0.5 mg/mL).

2.9. HPLC–ESI-QTOF-MS

Analysis of the mixtures of HT or DOPAC with MGO after incubation at 37 °C were also conducted with an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a quadrupole time-of-flight mass spectrometer (Agilent Technologies) via an electrospray ionization jetstream probe. The HPLC system consisted of a quaternary pump (G1311A), autosampler (G1367B), column heater holder (G1316A), a variable wavelength UV/vis detector (G1315B). Samples (10 μL) were separated on a reverse phase C-18 Kinetex column (4.6 mm × 75 mm, 2.6 μm packing column; Phenomenex) maintained at temperature of 25 °C using a binary solvent system of acetic acid (5 mL/L) (A) and methanol (B) at 0.6 mL/min. The following gradient was applied: 0 min, 5% B; 1 min, 5% B; 20 min, 60% B; 21 min, 60% B; 22 min, 5% B; 30 min, 5%. B. The negative-ion polarity mode was set for ESI ion source with the voltage at 3 kV, cone voltage of 40V, 250 °C of desolvation temperature. The drying gas was at 300 °C with 10 L/min of flow with nebulizing gas pressure of 35 bar. The scan range was $-m/z$ 100–1000 Da. The scan time was 1.0 s, and the interscan delay was 0.010 s. The MS/MS acquisition was performed using collision energy from 20 to 40 V from QTOF. The structural information of HT, tyrosol, DOPAC, DOPAL (3,4-dihydroxyphanylacetaldehyde), catechol, and the major MGO adducts was obtained by tandem mass spectrometry (MS/MS). Data were processed using Masshunter Data Acquisition B.04.00 and Masshunter Qualitative Analysis B.04.00 (Agilent Technologies). Structural identification was further stablished by using NIST Standard Reference Databases (data v.NIST05).

2.10. Statistical analysis

Data were analysed by using the software Statgraphics Centurion XV (Herndon, VA, USA). Homogeneity of variances was assessed via Levene's test. Analysis of variance (ANOVA) was followed by Bonferroni's or Tamhane's test when the variances were homogeneous and non-homogeneous, respectively. Differences were considered to be significant at $p < 0.05$. Data from at least three independent assays were used to obtain a mean value and its standard deviation.

3. Results and discussion

3.1. Reactivity of hydroxytyrosol towards dicarbonyl compound as compared with amino acids

RCS, such as glyoxal and methylglyoxal, are critical intermediates in the glycation process and can bind to amino groups to form AGEs. Arginine, lysine, cysteine and histidine are primary target of

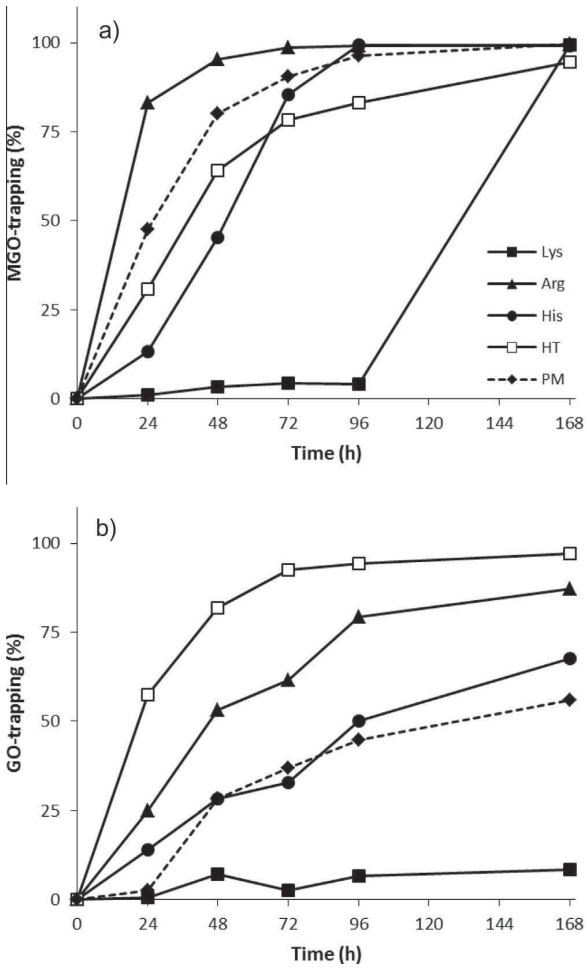


Fig. 1. Methylglyoxal-trapping capacity (a) and glyoxal-trapping capacity (b) of arginine, histidine, lysine, and hydroxytyrosol. Pyridoxamine (PM) was used as reference (dotted line). Reaction condition (target compound: dicarbonyl compound) was 1:1 mol for MGO-trapping and 5:1 mol for GO-trapping assay.

RCS, but presence of competitors could modulate the extent of the reaction and subsequently to ameliorate its unwanted nutritional and biological consequences (Poulsen et al., 2013; Singh et al., 2001). MGO trapping capacity of HT was compared with the most susceptible amino acids to form carbonyl–derived AGEs. Cysteine was not included in the study due to its instability. The time profile for MGO trapping capacity of lysine, arginine, histidine and HT in a molar ratio 1:1 under simulated physiological conditions (100 mM PBS, pH 7.4, 37 °C) is described in Fig. 1a. Concentration of reactants was 0.555 μmol/L. PM was used as reference of a classical carbonyl trapping compound (Lo, Westwood, McLellan, Selwood, & Thornalley, 1994). Arginine showed higher MGO trapping activity (IC_{50} 14.5 h), as compared with histidine (IC_{50} 50.5 h) and lysine (IC_{50} 130 h). It has been described that RCS reacts with the guanidino group of arginine to form hydroimidazolones, or the epsilon-amino group of lysine to form N-carboxyethyl-lysine (Poulsen et al., 2013). HT exhibited a relevant MGO trapping activity with an IC_{50} of 37.2 h. All samples were able to trap completely MGO within 168 h, but arginine reached a maximum trapping activity just after 72 h of incubation. Results showed that HT exerted reactivity against MGO to compete with lysine, arginine or histidine to prevent the formation of carbonyl-derived AGEs.

Fig. 1b displayed the time profile for GO-trapping capacity of lysine, arginine, histidine and HT in a molar ratio 5:1. At 1:1 molar ratio, the trapping of GO was very low and it did not reach the 50% of reactivity. In this case, HT had higher GO trapping activity (IC_{50} 21 h), as compared with arginine (IC_{50} 46 h), histidine (IC_{50} 99.5 h), while lysine activity was residual (<10% GO-trapping capacity). Differences observed in the reactivity of both low molecular weight carbonyl compounds are based in their stability in water aqueous solutions that influence the rate of the reaction (Nemet, Vikić-Topić, & Varga-Defterdarović, 2004). GO is more unstable than MGO in buffer solutions. The hydrated monomer of GO tends to polymerize more rapidly than MGO and consequently to slow down its reactivity (Wang & Ho, 2012). For next studies, investigation on dicarbonyl trapping mechanism of HT was focused on MGO.

3.2. Stability of hydroxytyrosol in presence/absence of methylglyoxal

It is important to highlight that the disappearance of HT in the MGO trapping system (100 mM PBS, pH 7.4) was linear over time of incubation, and almost decomposed (>98.2%) within 168 h of incubation. But chromatograms showing the HT disappearance in presence of MGO also becomes more complex with the appearance of two new peaks (data not shown) as compared without presence of MGO. Then, additional trapping models were designed with different buffers and pH to get more insight in the reaction mechanism and HT stability at 37 °C in presence of salts. There is a clear relationship between the HT degradation and the extent of the MGO trapping capacity of the solution (Supplemental Table 1). There was no trapping of MGO and degradation of HT was residual (1.14%) during incubation in milli-Q water. Thus, it is necessary that degradation of HT initiates in some extent to achieve detectable levels of trapping of MGO in the system. Regardless the buffer concentration and type of salt, the pH was a critical factor as reported by Zafra-Gómez, Luzón-Toro, Capel-Cuevas, and Morales (2011). These authors evidenced that HT is rather stable in milli-Q water, but degradation increased with the mineral content. In aqueous solutions, MGO is polymerized and hydrated, but at pH 7 the 56% of the MGO is in monohydrate form yet. MGO shows an enol-oxo tautomerization and the equilibrium is shifted towards the enol form by the increase of pH. Then, MGO trapping by HT is significantly reduced at slight acid conditions or in the absence of minerals but increased at mild alkaline conditions. Thus, the pH 7.4 can increase the nucleophilicity of the unsubstituted carbons in the dihydroxy phenyl ring of HT and facilitate the addition of MGO to form adducts (Shao et al., 2014).

However, it was noticed that HT also decreased in the control (without MGO) during incubation at simulated physiological conditions (100 mM PBS, pH 7.4, 37 °C). Then, a HR-QTOF analyzer was used as a powerful tool for the identification of HT and their derivatives as deprotonated molecules $[M-H]^-$. The extracted ion chromatograms (tolerance 0.01 Da) from HT incubated at 37 °C for 196 h was evaluated. In addition to HT (tr 7.627 min), the formation of DOPAC (9.638 min), DOPAL (8.302 min) were clearly identified (Supplemental Fig. 1). It was also observed residual levels of catechol (8.550 min). The calculated elemental composition for the identification of HT, DOPAL, DOPAC and catechol in the system was satisfactory (Supplemental Table 2). HT decomposed progressively during incubation time while DOPAL and DOPAC were formed. DOPAL was early detected after just one hour of incubation of HT and levels remains until 168 h of incubation. However, DOPAC increased linearly until 48 h, after that DOPAC slowly decreased in time (data not shown). Our results are in line with Pereira-Caro et al. (2012) who stated that DOPAC was already formed after gastrointestinal digestion of HT in the blank system (without the presence of enzymes) after incubation at 37 °C (pH

Table 1
Antioxidant activity, browning and MGO-trapping capacity during incubation of hydroxytyrosol (0.5 mg/mL) at 37 °C in 100 mM PBS pH 7.4 up to 168 h. Results are expressed as mean ± SD for $n = 4$. Different letters in the same column denote significant differences $P < 0.05$.

Time (h)	FRAP TEAC (μmol/g)	ABTS TEAC (μmol/g)	TFC GAE (mg/mL)	Browning increment ((%))	MGO-trapping (%)
0	6863 ± 162a	10187 ± 355a	6960 ± 142a	–	4.70 ± 6.65a
8	6537 ± 133b	9924 ± 215a	6710 ± 127a	6.3 ± 0.04a	50.85 ± 7.99b
24	4908 ± 73c	8300 ± 146b	5614 ± 151b	11.4 ± 0.03b	79.25 ± 1.77c
30	4563 ± 80d	7946 ± 151b	5191 ± 136c	13.8 ± 0.08b	86.2 ± 1.27c
48	2925 ± 67e	6340 ± 140c	3538 ± 67d	17.1 ± 0.11c	97.45 ± 0.78c
168	1375 ± 58f	4705 ± 122d	2141 ± 73e	19.1 ± 0.03d	98.25 ± 0.49c

7.5). Then, it could be plausible that any of the degradation products of HT in the PBS system at pH 7.4 could also be responsible of the trapping of MGO, instead of the HT itself.

The time-course of the degradation of HT was also characterized by the antioxidant activity (FRAP, ABTS, TFC), and browning (absorbance at 420 nm). Table 1 shows the antioxidant capacity was reduced as the incubation time was increased. In ABTS and TFC assays, 24 h of incubation were required to observed a significant differences while in FRAP assay significant differences were observed after 8 h of incubation. Antioxidant capacity of HT, and structural related substances, is attributed to the electron donating ability of hydroxyl groups in the ortho position and subsequent formation of stable intramolecular hydrogen bonds with the phenoxylic radical (Visioli, Bellomo, & Galli, 1998). As expected, browning increases with the incubation time and reaching a 19.1% for 168 h. It is known that *ortho*-diphenols, like HT, darken progressively due to oxidative reactions in presence of cations (García, Romero, Brenes, & Garrido, 1996), and *o*-quinone derivatives are formed. Additionally, Roche, Dufour, Mora, and Dangles (2005) described that HT shows a propensity for oligomerization with formation of dimers and trimmers under incubation at 37 °C in phosphate buffer in an oxidative environment promoted by hydrophilic diazo radicals. These results pointed out the potential oxidation or polymerization of HT during incubation under simulated physiological conditions and the formation of novel compounds with lower antioxidant capacity but higher MGO trapping capacity. However our lc-ms data evidences the rapid oxidative degradation of HT but the formation of HT polymers were not detected. This evidence is in line with Pereira-Caro et al. (2012) who do not found dimmers after incubation of HT and its esters under physiological condition without external prooxidative environment.

3.3. Methylglyoxal-trapping capacity of hydroxytyrosol-related compounds

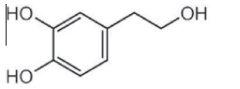
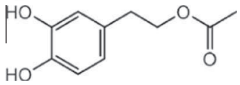
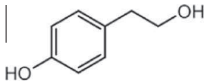
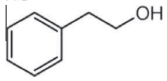
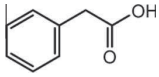
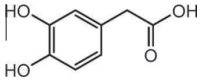
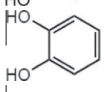
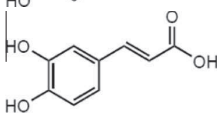
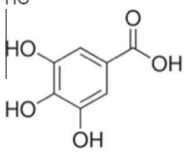
The MGO-trapping capacity of different HT structure-related compounds was evaluated with the aim to establish a relationship between their MGO-trapping capacity and chemical structure. As described in Table 2, MGO-trapping capacity was higher in compounds with a hydroxyl group in position 2, such as HT, HTA, DOPAC, catechol, caffeic acid and gallic acid. However compounds without a hydroxyl group in position 2, as tyrosol, 2-phenylethanol and phenylacetic acid, did not show ability to trap MGO. These results are in line with reported by Wu and Yen (2005), who concluded that the number of hydroxyl groups increases the inhibitory activity against AGEs formation. DOPAC was the main degradation product of HT during incubation at physiological conditions. It seems that the activity observed for HT is due to formation of degradation products like DOPAC which also exerted a relevant MGO trapping ability.

The potential antiglycative activity of DOPAC was further assessed by in vitro BSA-MGO and BSA-GLC assays. The reaction of MGO with bovine serum albumin (BSA) at 37 °C (pH 7.4) involved the reversible and irreversible formation of MGO-BSA adducts. The formation of MGO-modified proteins involves formation of fluorescent AGEs (Lo et al., 1994). Irreversible modification of BSA occurred mainly on arginine residues to form imidazolone, and argpyrimidine, but also with lysine to form methyl-glyoxal-lysine dimer (MOLD) and carboxyethyl-lysine. Fig. 2 depicted the inhibition of fluorescent AGEs formation using excitation and emission maxima of 340/420 nm for BSA-MGO and 360/420 nm for BSA-GLC at 37 °C for 14 and 21 days, respectively. DOPAC showed significant activity on the mitigation of the formation of fluorescent AGEs in a dose-dependent manner in both systems. However, the effectivity of DOPAC in the BSA-GLC assay was lower than BSA-MGO. In the BSA-GLC system, MGO is formed by fragmentation of 3-deoxyglucosone, which is formed by deprotonation, and dehydration of glucose or from the Schiff base. PBS buffer catalysed the deprotonation of glucose and subsequent autooxidation side reaction implicated in the formation of dicarbonyls. In addition, depending on temperature and type of salts, MGO could change from a less reactive non-carbonyl form to more reactive carbonyl and dicarbonyl forms (García et al., 1996). These results support the antiglycative activity of DOPAC is likely mediated by its carbonyl trapping capacity.

3.4. Structural identification of main compounds formed in the reaction of methylglyoxal and hydroxytyrosol under simulated physiological conditions

The main hypothesis arisen after obtaining former results was that HT was degraded into DOPAC and this compound was the direct responsible for the MGO trapping capacity and subsequently, the antiglycative action of HT. In a new model of reaction more concentrated, HT was incubated up to 168 h with MGO (5.55 mM) in a 1:2 molar ratio (HT:MGO) to enhance the potential formation of carbonyl adducts. The exact mass was obtained by high resolution Q-ToF and structural information of these products was obtained using ms/ms fragmentation under selective ion monitoring mode. Fragmentation was carried out at mild conditions (voltage on the ESI interface at 3 kV) to always maintain the trace of the parent ion. Fig. 3 depicted the total ion chromatogram of the system of HT incubated with MGO at 37 °C for 168 h in 100 mM PBS (pH 7.4), and the extracted ions of the new peaks obtained. Just after 24 h of incubation, HT decreased by nearly 30% and four new peaks were identified as compared with a control without MGO. The new peaks had molecular ions of 239.0561 $[M-H]^-$ (adduct-1), 225.0405 $[M-H]^-$ (adduct-3), 223.0612 $[M-H]^-$ (adduct-2), and 209.0455 $[M-H]^-$ (adduct-4). The molecular ions matched (>95% of confidence by software) with the next formulas; $C_{11}H_{12}O_6$ (adduct-1), $C_{10}H_{10}O_6$ (adduct-3), $C_{11}H_{12}O_5$ (adduct-2), and $C_{10}H_{10}O_5$ (adduct-4). Adduct-1 reached the highest signal, adduct-3 was secondary in intensity and adduct-2 and adduct-4 had a minor signal at any incubation time. The conjugation of one MGO group (72.0211 exact mass) to HT (154.0630 exact mass) did not clearly support the molecular ions obtained. However, direct conjugation of MGO with DOPAC (168.04226 exact mass)

Table 2
MGO-trapping capacity (0.1 mg/mL) of different compound with structures related to hydroxytyrosol.

Compound	MGO-trapping (%)	Structures
Hydroxytyrosol	97.2 ± 1.06	
Hydroxytyrosol acetate	97.1 ± 2.47	
Tyrosol	6.8 ± 2.47	
2-Phenylethanol	0.8 ± 1.13	
Phenylacetic acid	4.5 ± 2.47	
3,4-Dihydroxyphenylacetic acid	99.4 ± 0.28	
Pyrocatechol	99.6 ± 0.21	
Caffeic acid	90.6 ± 1.48	
Gallic acid	99.3 ± 0.71	

explained the formation of the major adduct (Supplemental Table 1). These results were further confirmed after incubation of the system DOPAC:MGO at 1:2 molar ratio, since the same four adducts and relatives intensities were detected, being adduct-1 the predominant ion. It is plausible that MGO trapping ability of HT is due to DOPAC reactivity.

Further investigation was focused on adduct-1. The ms/ms spectra of the major adduct of the HT and MGO incubation system was evaluated (Supplemental Fig. 2). The adduct-1 ($-m/z$ 239.0561) with fragments ion with $-m/z$ 211.0620 $[M-28-H]^-$, 193.0506 $[M-46-H]^-$, and 166.0281 $[M-73-H]^-$ was tentatively identified as monoDOPAC-monoMGO adduct. Major fragment matched with the decarbonylation (–CO) of the adduct-1 as DOPAC-MGO, and fragment at $-m/z$ 193.0501 could be obtained by subsequent dehydration. MGO could be attached to DOPAC at C1 position by electrophilic substitution, following a similar reaction scheme as proposed for trapping reactions of (−)-epigallocatechins, chalcones or stilbenes (Lo et al., 1994; Totlani & Peterson, 2005; Wu et al., 2013). Then, adduct-1 was assigned the molecular formula $C_{11}H_{12}O_6$ based on their relationship between experimental and theoretical exact mass, and the ms/ms fragmentation pattern. The plausible presence of tautomers should be described by applying one- or two-dimensional NMR analysis.

3.5. Hypothesis for a mechanism of reaction

The hypothesis for the mechanism of trapping of MGO by HT mediated by DOPAC at physiological conditions is depicted in Fig. 4. It was assumed that HT oxidized to DOPAL and later to DOPAC and it underwent electrophilic aromatic substitution with MGO. The unsubstituted carbon 1 on the DOPAC ring should be the major active site for trapping MGO, and likely to other RCS. The hydroxyl groups in the phenyl ring have electron-donating property and enhance the reactivity with MGO since this reaction is a nucleophilic addiction. Similar reaction mechanism has been previously suggested by Totlani and Peterson (2005, 2006) in epicatechin carbonyl-trapping reactions and also Wang, Yagiz, Buran, Nunes, and Gu (2011) for phytochemicals in berries and grapes. Shao et al. (2014) investigated the scavenging of reactive dicarbonyls species of flavonoids as an effective way to prevent diabetic complications. They concluded that the A ring is the active site of flavonoids in contributing the MGO-trapping efficacy, and the C-5 hydroxyl group on the A-ring enhances the trapping efficacy, but the number of hydroxyl groups on the B ring does not significantly influence the trapping efficacy.

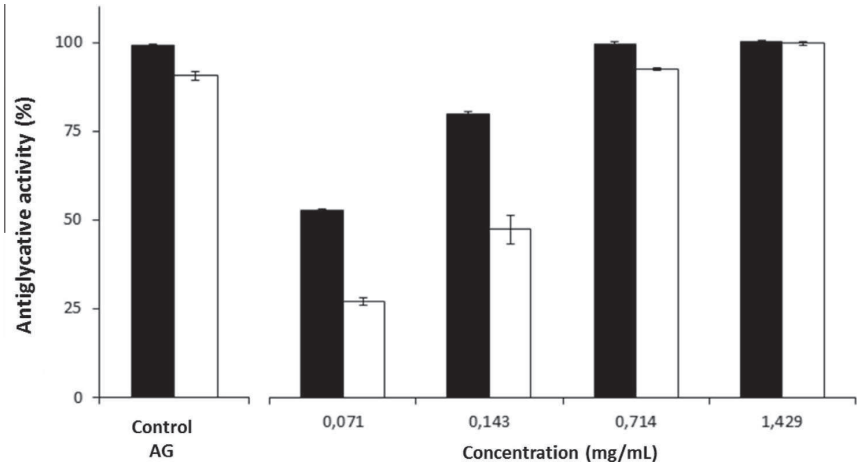


Fig. 2. Antiglycative activity of 3,4-dihydroxyphenylacetic acid (DOPAC, from 0.071 to 1.429 mg/mL) on the formation of fluorescent AGEs in both BSA-MGO (solid bar) and BSA-GLC (empty bar) assays. Aminoguanidine (AG) was used as positive control with an antiglycative activity of 99.30% in BSA-MGO and 90.57% in BSA-GLC.

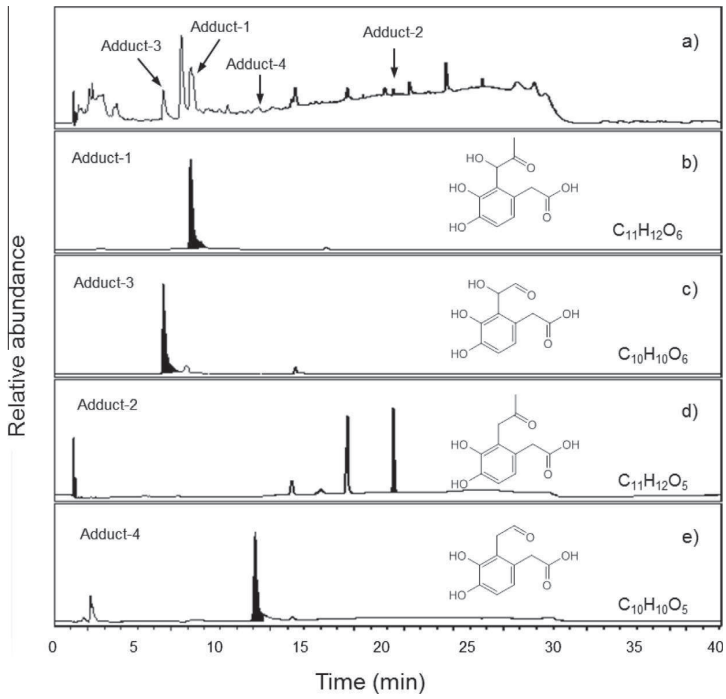


Fig. 3. Total ion chromatogram of hydroxytyrosol incubated with methylglyoxal in molar ratio 5:1 at 37 °C for 168 h in 100 mM PBS pH 7.4 (a), and extracted ions chromatograms $[M-H]^-$ of adduct-1 ($-m/z$ 239.0561) (b), adduct-3 ($-m/z$ 225.0405), (c), adduct-2 ($-m/z$ 223.0612) (d), and adduct-4 ($-m/z$ 209.0455) (e).

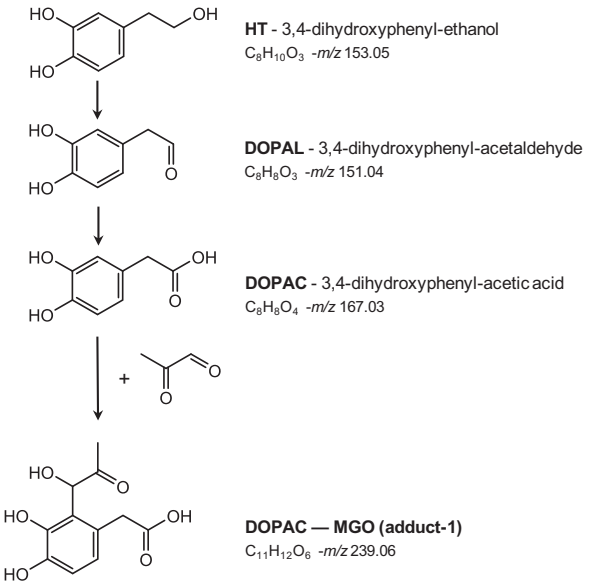


Fig. 4. Proposed mechanism of reaction for trapping of MGO by hydroxytyrosol under simulated physiological conditions.

Several authors stated that interventions against MR in foods and glycation in vivo may take place at different stages of the reaction with the final objective to impair the AGE formation (Peng et al., 2011; Shao et al., 2014; Wu et al., 2011). Most of the inhibitors to date have several sites of action, and dietary antioxidants have firstly been identified as AGE inhibitors due to antioxidant capacity, radical scavenging properties and transition metal chelation (Peyroux & Sternberg, 2006). Although glucose was originally thought to be the sole precursor of AGEs, current research indicates that RCS generated from carbohydrates, lipids, and amino acids degradation are even more reactive and potent precursors of AGEs (Rahbar & Figarola, 2003). Thus, very recently the dicarbonyl trapping capacity has been considerate as a valuable mechanism of action. An extensive investigation has been done with the prevention of diabetes complications by flavonoids (luteolin, rutin, (−)-epigallocatechin-3-gallate, and quercetin) and the mechanism of reaction is still unclear, although there is a growing evidence of the importance of trapping of RCS (Wu & Yen, 2005). To our knowledge HT has not previously been investigated as a potential antiglycative agent due to the complexity to understand the mechanism of reaction. However, a previous investigation pointed out in this line. Cervantes-Laurean et al. (2006) evidenced that rutin, metabolites, specifically its aglycone quercetin and its phenol derivatives (including DOPAC) was able to modulate the formation of non-fluorescent AGE (i.e. CML) and fluorescent AGE (i.e. pentosidine) biomarkers.

4. Conclusions

This investigation reveals that HT (3,4-dihydroxyphenylethanol) could compete with lysine, arginine and histidine to dicarbonyl compounds and avoid the formation of fluorescent protein-bound amino acid derivatives. It is plausible that the mechanism of action under simulated physiological conditions to exert the antiglycative activity is mediated by the trapping of RCS, such as MGO. However, HT should previously be oxidized to DOPAC which reacts with MGO by electrophilic aromatic substitution. The formation of a monoDOPAC-monoMGO adduct is pro-

posed but the plausible presence of a mixture of two tautomers should be further confirmed by ^{1}H and ^{13}C NMR experiments.

Conflict of interest

Authors declare not conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.11.117.

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ANNEX A: SUPPLEMENTARY DATA

SUPPLEMENTARY TABLES

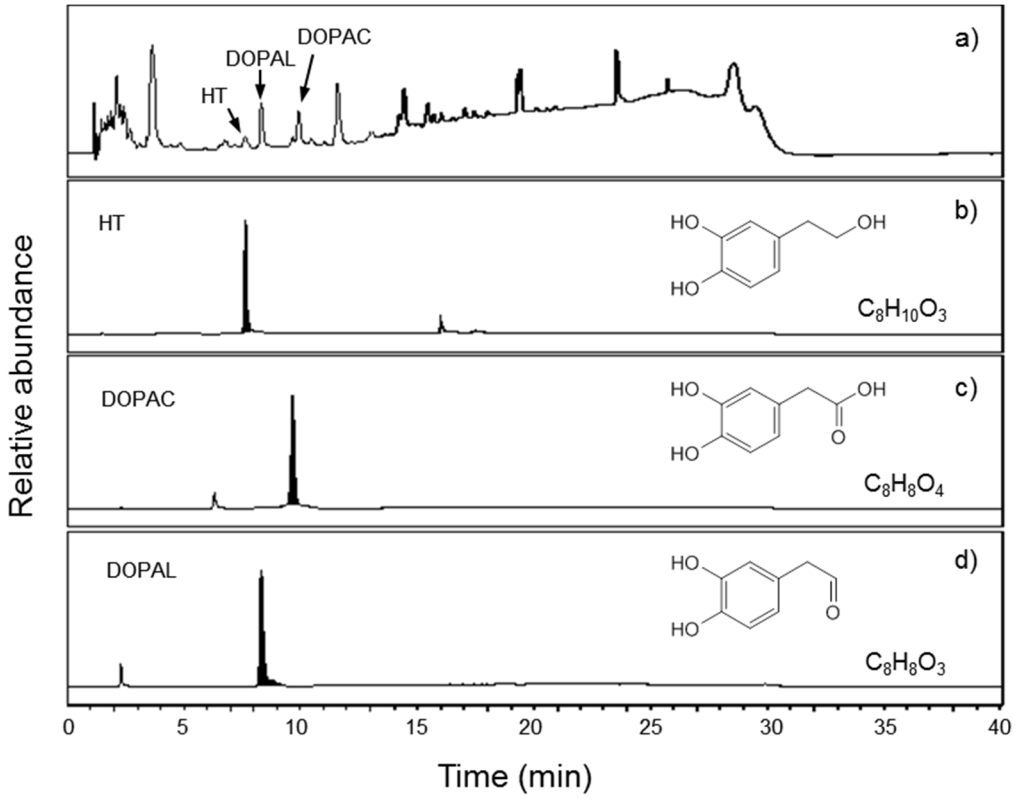
Supplementary table 1. Degradation of hydroxytyrosol (HT, initial 0.05 mg/mL; 0.324 µmol/mL) and methylglyoxal (MGO, initial 0.04 mg/mL; 0.555 µmol/mL) trapping after incubation at 37°C for 168 h in water and different buffers (phosphate, phosphate saline, and acetate). Different letters in the same column denote significant differences $P < 0.05$.

	HT degradation (%)	MGO trapping (%)
Water	1.90 ± 2.69a	7.07 ± 9.15a
Water + NaOH (8.5)	5.36 ± 1.49a	6.59 ± 8.65a
Sodium acetate (100 mM; 4.5)	3.41 ± 1.69a	6.90 ± 9.76a
Sodium acetate (100 mM; 7.4)	63.8 ± 2.35b	86.4 ± 4.25b
Sodium Phosphate (100 mM; 7.4)	98.2 ± 0.23c	99.0 ± 0.48c
Phosphate saline (10 mM; 7.4)	90.3 ± 0.63d	95.1 ± 0.77d
Phosphate saline (50 mM; 7.4)	99.0 ± 0.59c	98.5 ± 0.53c
Phosphate saline (100 mM; 7.4)	98.3 ± 0.11c	98.8 ± 0.67c
Phosphate saline (100 mM; 5.5)	25.5 ± 6.30e	53.7 ± 5.68e

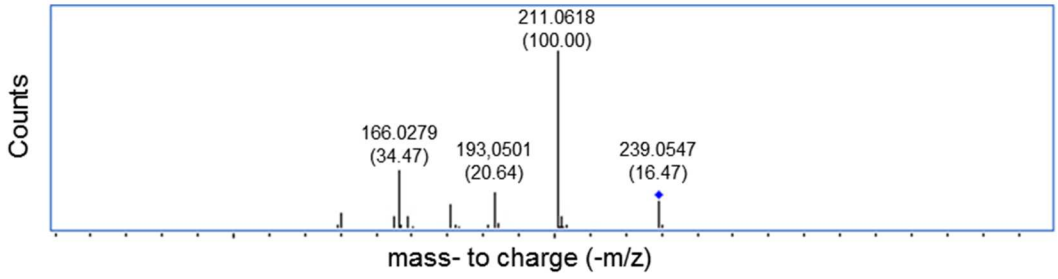
Supplementary table 2. Calculated elemental composition of HT degradation products and tentative HT-MGO adducts. * error in calculated elemental composition.

Structure	Rt (min)	*-m/z* $[M-H]^-$	M	Elemental composition	Error* (ppm)	Score (%)
HT	7.63	153.0557	154.0635	$C_8H_{10}O_3$	-3.06	98.60
DOPAC	9.64	167.0350	168.0433	$C_8H_8O_4$	6.39	94.02
DOPAL	8.30	151.0401	152.0472	$C_8H_8O_3$	-0.8	87.59
CATECOL	8.55	109.0295	110.0377	$C_6H_6O_2$	-8.2	94.48
Adduct-1	8.15	239.0561	240.0626	$C_{11}H_{12}O_6$	3.19	96.85
Adduct-3	6.52	225.0405	226.0472	$C_{10}H_{10}O_6$	2.56	95.88

Supplementary Fig. 1. Total ion chromatogram of hydroxytyrosol solution incubated at 37°C for 168h in 100 mM PBS pH 7.4 (**a**), and extracted ions chromatograms $[M-H]^-$ of hydroxytyrosol (-*m/z* 153.0575 (**b**), DOPAC (-*m/z* 167.0367 (**c**), and DOPAL (-*m/z* 151.0401, (**d**). DOPAC (3,4-dihydroxyphenylacetic acid), DOPAL (3,4-dihydroxyphenylacetaldehyde).



Supplementary Fig.2. LC/MS/MS spectra of adduct-1 from incubation of MGO and HT in 100 mM PBS buffer 7.4 at 37°C for 168h.



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Investigations on the Reaction of C3 and C6 α-Dicarbonyl Compounds with Hydroxytyrosol and Related Compounds under Competitive Conditions

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ABSTRACT: α-Dicarbonyl compounds are intermediates in reactions that lead to the formation of potentially harmful advanced glycation end-products. Carbonyl-trapping capacities of antiglycative substances have been traditionally limited to C2 and C3 α-dicarbonyl structures. Glyoxal (GO)-, methylglyoxal (MGO)-, 3-deoxyglucosone (3-DG)-, 3-deoxygalactosone (3-DGal)-, 3,4-dideoxyglucoson-3-ene-, and glucosone-trapping capacities of hydroxytyrosol (HT), hydroxytyrosol acetate (HTA), and 3,4-dihydroxyphenylacetic acid (DOPAC) in simple (phenolic/dicarbonyl) and competitive model systems (phenolic/dicarbonyl$_1$/dicarbonyl$_2$) were investigated. HT and HTA were more effective for MGO than 3-DG and 3-DGal. Furthermore, DOPAC exerted higher trapping capacity than HT and HTA for C3 and C6 α-dicarbonyl compounds. In the competitive systems, HT-related substances did not show preference for trapping 3-DG or 3-DGal and behaved as in the simple systems. In the presence of MGO, however, HT-related substances were more effective for trapping MGO than C6 structures. The results demonstrate the C6 α-dicarbonyl-trapping capacities of HT, HTA, and DOPAC, with DOPAC exerting the highest activity.

KEYWORDS: *hydroxytyrosol, α-dicarbonyl compounds, carbonyl-trapping capacity, 3-deoxyglucosone, 3-deoxygalactosone, methylglyoxal*

INTRODUCTION

α-Dicarbonyl compounds are key Maillard intermediates mainly formed during the oxidation, dehydration, and fragmentation reactions of saccharides in food products, but also they are produced in vivo.[1,2] α-Dicarbonyl compounds have been detected in cookies, beer, wine, Manuka honey, coffee, and drinks sweetened with the addition of high-fructose corn syrup,[3,4] and they could be an exogenous source to the systemic circulation which is however still under debate.[5]

Several pathways for the formation of C2, C3, and C6 α-dicarbonyl compounds have been described such as the Maillard reaction, autoxidation of glucose, and peroxidation of lipids as well as the polyol pathway in vivo. Among those, retro-aldol reaction and α- and β-dicarbonyl cleavage are the major pathways leading to the fragmentation of α-dicarbonyl compounds.[6,7] The fate of α-dicarbonyl compounds in the glycation process is pivotal because they are nearly up to 20,000-fold more reactive than glucose.[8] They can react with amino acid residues of proteins, especially with the sulfhydryl group of cysteine, the guanidino group of arginine, and the ε-amino group of lysine, to form advanced glycation end-products (AGEs) directly without involving a precursor.[9] The formation and accumulation of AGEs in vivo have drawn major attention because of their possible association with the aging process as well as the development of chronic diseases, such as Alzheimer's disease, atherosclerosis, chronic inflammatory diseases, cardiovascular diseases, or diabetes mellitus and its numerous long-term complications.[10−13] Human plasma levels for 3-deoxyglucosone (3-DG), methylglyoxal (MGO), and glyoxal (GO) have been estimated to reach 0.16−1.24 μmol/L, increasing to values of 4.09 μmol/L in diabetes and uremic patients and thus contributing to systemic carbonyl stress.[14]

Pharmaceutical interventions against glycation were initially carried out with substances such as aminoguanidine; however, their relevant side effects in clinical trials have led to the search for natural products with the same ability.[12,15] Natural plant extracts are the priority source for searching compounds that mitigate the formation of AGEs,[15] although recently the antiglycative activity of other naturally occurring substances such as creatine has been highlighted.[16] Because certain stages of the glycation process are driven by redox reactions, polyphenols have been largely investigated by applying their antioxidant capacity to mitigate the radical-mediated reactions. Moreover, the ability of certain polyphenols to trap reactive carbonyl species has recently been pointed out.[13,15,17] However, investigations on the carbonyl trapping activity have only been carried out with MGO and GO, whereas their activity against C6 α-dicarbonyl compounds has not been determined, although these compounds are mostly present in higher concentrations than C2 and C3 α-dicarbonyl structures.[4] Additionally, 3-DG is the most abundant C6 α-dicarbonyl in vivo, ranging from 35 to 56 pmol/mL in plasma of healthy subjects.[1,11]

Hydroxytyrosol (2-(3,4-dihydroxyphenyl)ethanol, HT) is a major phenol in olive oil, leaves, or mill wastewater, and it is

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present as esterified phenol with elenolic acid to form oleuropein aglycone and in free form.[18,19] Epidemiologic studies support the protective effect of HT against diseases with high prevalence such as cancer, diabetes, and neurodegenerative and cardiovascular diseases. Mostly, in vivo and in vitro studies associate its beneficial health effects with its antioxidant activity, for example, by scavenging of free radicals or the possible modulation of the endogenous antioxidant defense system.[20,21] Recently, Navarro and Morales[22] showed that HT and its acetate (hydroxytyrosol acetate; HTA) mitigate the formation of AGEs, such as carboxymethyllysine, carboxyethyllysine, and argpyrimidine. In addition, an antiglycative mechanism of action has been proposed mediated by trapping of C3 α-dicarbonyl compounds as confirmed by the detection of MGO adducts with HT-related substances.[23]

So far, studies on the mitigation of AGE formation through the trapping of reactive α-dicarbonyl compounds by phenolic compounds and particularly HT are limited to MGO and GO, whereas their reactivity toward C6 α-dicarbonyl compounds is unknown. Distler et al.[24] have shown that the glycation activity and specificity of various α-dicarbonyl compounds differ to a great degree, and consequently different biological consequences are expected. Therefore, different model systems of different complexity were designed in the present study with HT, HTA, and 3,4-dihydroxyphenylacetic acid (DOPAC) to investigate the direct trapping capacity of C6 α-dicarbonyls such as 3-DG, 3-deoxygalactosone (3-DGal), 3,4-dideoxyglucoson-3-ene (3,4-DGE), and glucosone (GLUCOS).

MATERIALS AND METHODS

Materials. GO (40% aqueous solution), MGO (40% aqueous solution), 5-methylquinoxaline (5-MQ, used as internal standard), *o*-phenylenediamine (OPD), pyranose oxidase from coriolus, catalase from bovine liver, ammonium formate, *p*-toluidine, DOPAC, and quercetin (QE) were provided by Sigma (Steinheim, Germany). HT and HTA (purity > 99%) were acquired from Seprox Biotech (Madrid, Spain). 3-DG (≥95%) was obtained from Chemos (Regenstauf, Germany). D-Glucose, D-galactose, benzoylhydrazine (Alfa Aesar, Germany), and diethyl ether were purchased from ApplyChem (Darmstadt, Germany). The water used for all experiments was obtained using a Synergi-185 labwater system (Millipore, Schwalbach, Germany). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade and supplied by Sigma (Taufkirchen, Germany), Fluka (Steinheim, Germany), or Acros (Geel, Belgium).

Synthesis of 3-Deoxy-D-*erythro*-hexos-2-ulose (3-Deoxyglucosone, 3-DG) and 3-Deoxy-D-*threo*-hexos-2-ulose (3-Deoxygalactosone, 3-DGal) Standards. The synthesis of 3-DG was carried out according to the method described by Madson and Feather[25] with minor modifications. Briefly, 20 g of D-glucose, 11 g of *p*-toluidine, and 450 mL of ethanol containing 22 mL of acetic acid was refluxed and mixed with 33 g of benzoylhydrazine for 7 h. Afterward, the mixture was cooled overnight, leading to a precipitate of crude 3-DG bis(benzoylhydrazone). The obtained powder was washed with ethanol and diethyl ether and was recrystallized from 100% ethanol and desiccated overnight. Five grams of 3-DG bis(benzoylhydrazone) was suspended in 400 mL of 37.5% aqueous ethanol, 22 mL of acetic acid, and 8 mL of benzaldehyde, and the mixture was stirred under reflux. The precipitate was filtered and the ethanol evaporated. Then, the filtrate was mixed with 30 g of Serdolit MB-2, concentrated, washed with diethyl ether, and mixed with Serdolit MB-2 again. Finally, the filtrate was evaporated. Synthesis of 3-deoxy-D-*threo*-hexos-2-ulose (3-deoxygalactosone, 3-DGal) was performed as described above for 3-DG, but using initially D-galactose instead of D-glucose.[26] For quantification of 3-DG and 3-DGal, commercially available 3-DG was used as standard as both α-dicarbonyls have the same chromophore, and therefore the coefficient of absorption is expected to be the same. Thus, purities of 92.5 and 95% for 3-DGal and 3-DG respectively were determined.

Synthesis of 3,4-Dideoxyglucoson-3-ene Standard. The synthesis of 3,4-DGE was carried out as described by Mittelmaier et al.[27] Briefly, 80 g of D-glucose was dissolved in 200 mL of ammonium formate buffer (0.1 M, pH 4.5) at 120 °C for 4 h. After cooling, the solution was divided in four aliquots and purified with an IsoluteENV+ solid phase extraction (SPE) cartridge (500 mg, 6 mL reservoir; Biotage AB, Uppsala, Sweden). Each cartridge was eluted with 5 mL of water, 60 mL of sample, 15 mL of water, and 5 mL of ethanol (10%). The ethanol fraction was concentrated and then transferred to another IsoluteENV+ cartridge by applying 5 mL of water, 2 mL of sample, 15 mL of water, and 2 mL of ethanol (10%, five times). The second ethanol fraction containing the highest concentration of 3,4-DGE was used for the experiments.

The concentration of the synthesized 3,4-DGE solution was determined by the UHPLC method described below using a calibration curve with 3,4-DGE quinoxaline standard. The synthesis of the 3,4-DGE quinoxaline standard was described by Mittelmaier et al.[27] The purity of this standard was determined by quantitative NMR.[27] Thus, a concentration of 1.60 mM was calculated for the here-synthesized 3,4-DGE solution.

Synthesis of D-*lyxo*-Hexos-2-ulose (Glucosone) Standard. The synthesis of GLUCOS was carried out as described by Mittelmaier et al.[28] Briefly, 5 g of D-glucose was mixed with 3 mg of pyranose oxidase (9.4 u/mg) and 3 mg of catalase (1927 u/mg), dissolved in 20 mL of water, and incubated with slight agitation (25 °C for 48 h, aerating the mixture every hour for 3 min). Finally, the enzymes were removed by ultrafiltration, and the solution was lyophilized. A purity of 76.03% was calculated in the same way as described for 3-DGal.

UHPLC Analysis of α-Dicarbonyls. 3-DG, 3-DGal, 3,4-DGE, and GLUCOS were quantitated after conversion of the compounds into quinoxaline derivatives. The process of derivatization and UHPLC analysis was carried out as described by Mittelmaier et al.[27] In short, 80 μL of the standards (3-DG (400 μM), 3-DGal (400 μM), glucosone (100 μM), or 3,4-DGE (50 μM)) was derivatized with 20 μL of derivatizing solution composed of internal standard (diacetylquinoxaline, 50 μg/mL) and OPD (4% in 1 M HEPES buffer, pH 7.0) (50:50 v/v). A UHPLC Ultimate 3000 RS system with degasser, binary pump, autosampler, column oven, and DAD (Dionex, Germering, Germany) was used for quantitation of the corresponding quinoxalines. The analytes were eluted using an ACQUITY UHPLC BEH phenyl column (100 × 2.1 mm, 1.7 μm; Waters, Eschborn, Germany) at 55 °C. The flow rate was 0.4 mL/min and the injection volume 10 μL. The mobile phase consisted of ammonium formate buffer (5 mM, pH 2.8; A) and methanol (solvent B), and the gradient program was as follows: −3 min, 10% B; 0 min, 10% B; 8.5 min, 25% B; 10 min, 50% B; 10.1 min, 80% B; 12 min, 80% B. The total run time was 12 min, and chromatograms were recorded at 316 nm for all α-dicarbonyls except 3,4-DGE, which was analyzed at 335 nm. For data acquisition and processing, Chromeleon 6.80 SR10 software was used.

Evaluation of α-Dicarbonyl Stability. Solutions of 3-DG (0.64 mM), 3-DGal (0.64 mM), GO (0.64 mM), MGO (0.64 mM), GLUCOS (0.64 mM), and 3,4-DGE (0.64 and 1.60 mM) were prepared in saline phosphate buffer (0.1 mol/L, pH 7.4, PBS). 5-MQ (internal standard at 0.5 mg/mL) was prepared in methanol/water (50:50, v/v) and OPD (10.8 mg/mL) in methanol. A mixture of 100 μL of an α-dicarbonyl solution with 850 μL of PBS and 50 μL of 5-MQ was incubated at 37 °C from 1 to 168 h. OPD solution (200 μL) was added and rapidly vortexed, and the mixture was kept in darkness for 2 h for complete derivatization of the unreacted α-dicarbonyl compounds to the corresponding quinoxaline derivative. Quantification was conducted using the UHPLC method described above.

Evaluation of the Direct Dicarbonyl-Trapping Capacity. Direct α-dicarbonyl-trapping capacity was tested using a method described by Mesías et al.[29] with modifications. Target phenol compounds (HT, HTA, and DOPAC) were prepared at different molar ratios in methanol/water (60:40, v/v) and QE (as positive control) in ethanol/water (50:50, v/v). The solutions above-described

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J. Agric. Food Chem. 2016, 64, 6327−6332

were employed to prepare a mixture of 100 μL of α-dicarbonyl solution, 750 μL of PBS, 50 μL of 5-MQ, and 100 μL of either PBS (negative control), solution of the target phenolic compound (1:1, 2:1, 5:1, 10:1 of phenol/dicarbonyl compound molar ratio). The mixture was incubated at 37 °C for 6, 24, and 72 h and rapidly derivatized. The quantitation of α-dicarbonyls was carried out as described for the evaluation of α-dicarbonyl stability. The amount of unreacted dicarbonyl was calculated from the ratio of the quinoxaline of the corresponding dicarbonyl and 5-MQ as compared with control before reaction. The percentage of inhibition of the α-dicarbonyl compound by the target compounds was calculated with following formula: dicarbonyl trapping (%) = [(amount of α-dicarbonyl compound in control (without target compound) − amount of α-dicarbonyl compounds in sample with target compounds or QE/amount of α-dicarbonyl compounds in control (without target compound)] × 100.

Statistical Analysis. Data were analyzed using the SPSS Statistics v.21 (IBM Corp., Armonk, NY, USA) statistical program. Homogeneity of variances was evaluated by the test of Levene. Analysis of variance was performed using a one-way ANOVA procedure followed by a Bonferroni test. Differences were considered significant at $p < 0.05$. Data from three independent assays were expressed as the mean value ± SD.

RESULTS AND DISCUSSION

Stability of Pure α-Dicarbonyls. α-Dicarbonyl compounds are derivatized by OPD to form stable quinoxaline derivatives, which are suitable for UHPLC-DAD analysis at 316 nm (GO, MGO, 3-DG, 3-DGal, GLUCOS) and at 335 nm for unsaturated structures (3,4-DGE). However, numerous investigations indicated that α-dicarbonyl compounds are easily degraded to yield short-chained structures due to their high reactivity,[6,30] and MGO and GO also tend to polymerize.[31] On the basis of these considerations, the stability of GO, MGO, 3-DG, 3-DGal, 3,4-DGE, and GLUCOS during incubation up to 168 h in 100 mM PBS, pH 7.4, at 37 °C was evaluated first (Figure 1). The stability decreased in the order 3-DG > GO > MGO > 3-DGal ≫ 3,4-DGE ≈ GLUCOS. 3-DG, GO, MGO, and 3-DGal content decreased by 13.9, 17.4, 24.0, and 36.3%, respectively, after 72 h, and by 22.3, 32.7, 40.3, and 64.8%, respectively, after 168 h. In contrast, 3,4-DGE and GLUCOS were very unstable under these conditions, reaching $t_{1/2}$ (the time required to decrease by 50%) at 3.75 and 5.26 h, respectively. In contrast, $t_{1/2}$ for 3-DGal was 108 and >168 h for 3-DG, GO, and MGO. The high degradation rate for 3,4-DGE was expected because its structure combines the dicarbonyl reactivity with the α,β-unsaturated carbonyl, yielding, for example, 3-DGal and 3-DG after the addition of water.[1] GLUCOS is further degraded to C5 α-dicarbonyl structures such as 3-deoxypentosone, 1-deoxypentosone, and pentosone (not measured).[7] 3-DG is revealed as the most stable α-dicarbonyl under simulated physiological conditions and together with MGO and GO will represent the priority targets in the search for carbonyl trapping agents.

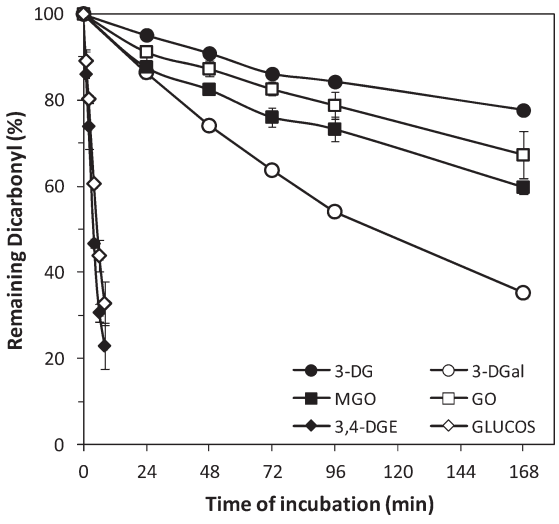

Figure 1. Degradation of glyoxal (GO), methylglyoxal (MGO), 3-deoxyglucosone (3-DG), 3-deoxygalactosone (3-DGal), glucosone (GLUCOS), and 3,4-dideoxyglucosone-3-ene (3,4-DGE) up to 168 h of incubation at 37 °C in saline phosphate buffer (100 mM, pH 7.4). Data are means ± SD ($n = 3$).

Direct Trapping of α-Dicarbonyl Compounds in a Simple System. In a previous study, Navarro and Morales[23] evaluated the MGO- and GO-trapping abilities of HT and HT-related substances demonstrating a high activity of HTA and DOPAC. However, their trapping capacity toward C6 α-dicarbonyl compounds is not yet known. Therefore, their trapping capacity against different α-dicarbonyl compounds was evaluated at 1:1, 2:1, 5:1, and 10:1 molar ratios (phenol/dicarbonyl).

Table 1 summarizes the trapping capacities of HT, HTA, and DOPAC toward 3-DG, 3-DGal, and MGO, when applied in a molar ratio of 1:1 or 2:1 (phenol/dicarbonyl). Results from molar ratios of 5:1 and 10:1 are not depicted, because α-dicarbonyl compounds were rapidly trapped at a high rate (nearly to 90%) in all combinations. The reaction was carried out for 72 h in 100 mM PBS at 37 °C. Under these conditions, GLUCOS and 3,4-DGE are rapidly degraded so that the trapping capacity toward these two compounds was evaluated after 6 h. A control was carried out at each time point by incubating the dicarbonyl compound without phenol. In addition, QE was used as reference because it is a naturally occurring phenol, with a chemical structure related to the target compounds under investigation, and its dicarbonyl-trapping

Table 1. Methylglyoxal (MGO)-, 3-Deoxyglucosone (3-DG)-, and 3-Deoxygalactosone (3-DGal)-Trapping Capacities of Hydroxytyrosol (HT), Hydroxytyrosol Acetate (HTA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), and Quercetin (QE) at 1:1 and 2:1 (Phenol/Dicarbonyl Compound) Molar Ratios[a]

	phenol/3DG 3-DG trapping (%)	phenol/3DGal 3-DGal trapping (%)	phenol/MGO MGO trapping (%)
	Ratio 1:1		
HT	26.1 ± 2.7aA	21.3 ± 2.45aA	35.9 ± 3.30aB
HTA	27.1 ± 0.6aAB	23.5 ± 2.50aA	35.0 ± 2.68aB
DOPAC	39.0 ± 0.6bA	54.5 ± 0.65bB	50.8 ± 1.92bB
QE	59.0 ± 0.7cA	50.4 ± 5.69bA	75.4 ± 0.79cB
	Ratio 2:1		
HT	33.2 ± 0.4dA	36.7 ± 4.4aA	49.4 ± 0.7bB
HTA	39.4 ± 0.4bA	50.7 ± 0.7bB	50.7 ± 2.1bB
DOPAC	68.4 ± 0.5eA	86.2 ± 0.2dB	73.4 ± 1.7cC
QE	72.8 ± 1.0eA	76.6 ± 6.1dA	88.2 ± 0.1dB

[a]The mixtures were incubated at 37 °C for 72 h. Results are expressed as the mean ± standard deviation for $n = 3$. Different lower case letters in the same column and capital letters in the same row denote significant differences ($p < 0.05$) at the same molar ratio.

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capacity has been well-described.[15] Similar to previous studies, significant differences between the MGO-trapping activities of HT and HTA were not observed, whereas the activity of DOPAC was significantly higher at both molar ratios. 3-DG- and MGO-trapping capacities of QE were higher than the activity of the HT-related substances, but 3-DGal-trapping capacity of DOPAC was similar to QE at both tested molar ratios. The 1:1 and 2:1 molar ratios showed a similar trend behavior for target phenol compound except the 3-DGal-trapping capacity of HT, which was similar at both molar ratios. Moreover, the 3-DGal-trapping capacity of HTA at the 2:1 molar ratio was similar to its MGO-trapping capacity.

When its activities toward different dicarbonyl compounds were compared, QE trapped MGO more efficiently than 3-DG and 3-DGal. Similar results are observed for HT and HTA, but DOPAC was more effective against 3-DGal. In contrast to the HT derivatives, QE is a flavonoid in which the A ring has been identified as the active site in the MGO-trapping capacity, which is enhanced by the presence of a hydroxyl group at C-5.[17] The formation of adducts with two molecules of MGO has been suggested for catechin[32] and quercetin.[17] However, only the formation of MGO monomer adducts has been described for HT-related substances.[23] In that study, the formation of monomer adducts of MGO and DOPAC was identified when HT was reacted with MGO. The higher dicarbonyl-trapping capacity of DOPAC compared to those of HT and HTA could be explained by the fact that HT should be previously degraded to DOPAC to exert its activity. IC_{50} values of 0.66, 1.04, and 0.85 mol/mL for MGO-, 3-DGal-, and 3-DG-trapping capacities, respectively, were obtained for QE, and 1.01, 0.92, and 1.36 mol/mL were obtained in the case of DOPAC. These results suggest the formation of dimers of MGO−QE is plausible, but only monomer adducts for DOPAC and C3 and C6 α-dicarbonyl compounds are expected.

Because of the instability of 3,4-DGE and GLUCOS, their trapping capacities were investigated after 6 h at 1:1, 2:1, 5:1, and 10:1 molar ratios, but trapping was detected only at a high excess of the phenols (molar ratio of 5:1 in the case of GLUCOS and 10:1 of 3,4-DGE) (Table 2). These results show that 3,4-DGE- and GLUCOS-trapping capacities of HT-related substances and QE were very low likely due to the short half-life of these α-dicarbonyl compounds or because the studied phenols need a longer incubation time to exert their activity. The IC_{50} values could not be calculated at these molar ratios because inhibition did not reach 50%, but QE was less effective than HT-related substances in trapping GLUCOS but more effective in trapping 3,4-DGE at the applied incubation conditions. Because it is known that 3-DGal and 3-DG are formed from 3,4-DGE, their trapping was further investigated in the system with 3,4-DGE (Table 3). Under these conditions, total degradation of 3,4-DGE and mainly 3-DGal formation was observed (0.105 mol of 3-DGal/mol of 3,4-DGE), which was trapped at >50% in all cases by the test compounds. However, the 3-DG formation from this system was lower, and the activity against this carbonyl could not be accurately measured. Therefore, it could be expected that 3,4-DGE-trapping by HT-related substances is not carried out directly, but is efficient after its conversion to 3-DGal.

Table 2. Glucosone (GLUCOS)- and 3,4-Dideoxyglucoson-3-ene (3,4-DGE)-Trapping Capacities of Hydroxytyrosol (HT), Hydroxytyrosol Acetate (HTA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), and Quercetin (QE) at 5:1 and 10:1 (Phenol/Dicarbonyl Compound) Molar Ratios[a]

	phenol/GLUCOS		phenol/3,4-DGE
ratio 5:1	GLUCOS trapping (%)	ratio 10:1	3,4-DGE trapping (%)
HT	20.6 ± 1.5ac	HT	6.7 ± 4.4b
HTA	20.6 ± 1.3ac	HTA	17.4 ± 0.7a
DOPAC	24.5 ± 1.0ac	DOPAC	
QE	7.1 ± 0.1b	QE	27.8 ± 1.9c

[a]The mixtures were incubated at 37 °C for 6 h. Results are expressed as the mean ± standard deviation for $n = 3$. Different letters denote significant differences ($p < 0.05$).

Table 3. Trapping of 3-Deoxygalactosone (3-DGal) Formed from the Degradation of 3,4-Dideoxyglucoson-3-ene (3,4-DGE) by Hydroxytyrosol (HT), Hydroxytyrosol Acetate (HTA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), and Quercetin (QE) at Molar Ratios of 1:1, 5:1, and 10:1 (Phenol/3,4-DGE)[a]

	3-DGal trapping (%)	
ratio 1:1	ratio 5:1	ratio 10:1
HT	57.7 ± 1.8ac	75.7 ± 11.9bc
HTA	63.0 ± 0.3ac	75.9 ± 10.1bc
DOPAC	80.2 ± 0.1bc	95.5 ± 0.2b

[a]The mixtures were incubated at 37 °C for 24 h. Different letters denote significant differences ($p < 0.05$).

These results confirm that dicarbonyl-trapping capacities of HT-related substances behave in a dose-dependent manner. DOPAC was more effective than HT and HTA for trapping C6 α-dicarbonyl compounds, which is in line with previous investigation with C3 α-dicarbonyl compounds.[23] HTA, HT, and its degradation product DOPAC may play a role in detoxifying C6 and C3 dicarbonyl compounds. Thus, HT and related substances could have a relevant role in preventing the formation of AGEs and therefore potentially mitigate the AGE-associated biological complications.

Direct Trapping of α-Dicarbonyl Compounds in a Competitive System (Cocktail Effect). A competitive system containing two α-dicarbonyl compounds was designed to get more insight into the preferences of the HT-related substances for dicarbonyl trapping. This cocktail effect was evaluated on 3-DG, 3-DGal, and MGO at two different molar ratios (2:1:1 and 4:1:1; phenol/dicarbonyl$_1$/dicarbonyl$_2$), and the results are summarized in Table 4. Although double concentration of each reactant was in the system as compared with the simple models, molar ratios of 1:1 and 2:1 (phenol/dicarbonyl) were maintained. The competitive system containing 3-DG and 3-DGal exerted the same response as the simple systems. Again, DOPAC and QE were more reactive against these dicarbonyl compounds than HT and HTA. 3-DG- and 3-DGal-trapping by the different compounds was similar, not showing preference for either dicarbonyl present in the combined system. However, the preference for MGO-trapping is evident in the cocktail systems (phenol/MGO:3-DG or 3-DGal) except in the case of HTA, which showed a similar trapping activity of 3-DG, and MGO and DOPAC, which exhibited a slight preference for 3-DGal over MGO. In general, MGO was preferably trapped by HT-related substances as compared to C6 dicarbonyl compounds in the competitive system. The competitive system is more realistic because the

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Table 4. Methylglyoxal (MGO)-, 3-Deoxyglucosone (3-DG)-, and 3-Deoxygalactosone (3-DGal)-Trapping Capacities of Hydroxytyrosol (HT), Hydroxytyrosol Acetate (HTA), 3,4-Dihydroxyphenylacetic Acid (DOPAC), and Quercetin (QE) at Molar Ratios of 2:1:1 and 4:1:1 (Phenol/Dicarbonyl Compound$_1$/Dicarbonyl Compound$_2$)[a]

	phenol/3DGal/3DG		phenol/MGO/3DG		phenol/MGO/3DGal	
	3-DGal trapping (%)	3-DG trapping (%)	MGO trapping (%)	3-DG trapping (%)	MGO trapping (%)	3-DGal trapping (%)
			Ratio 2:1:1			
HT	40.0 ± 2.5aA	43.1 ± 1.3aA	42.4 ± 3.6aA	29.9 ± 5.5aB	40.6 ± 0.9aAB	28.4 ± 0.5aB
HTA	50.9 ± 3.8bA	48.9 ± 1.2bA	46.5 ± 2.6aA	46.0 ± 0.1bAB	45.4 ± 1.4bAB	37.2 ± 0.7bB
DOPAC	80.4 ± 2.0cA	80.4 ± 1.9cA	64.7 ± 5.8bBC	50.5 ± 3.5bcB	69.9 ± 0.1aAC	77.0 ± 0.3cA
QE	83.6 ± 2.2cA	80.4 ± 2.0cA	87.8 ± 2.9cB	77.0 ± 4.0dA	83.5 ± 0.6dAB	75.6 ± 1.1ceA
			Ratio 4:1:1			
HT	71.4 ± 3.5dA	68.1 ± 2.0dAB	66.9 ± 1.2bAB	51.5 ± 2.6bcC	65.2 ± 0.1eAB	58.6 ± 2.8dBC
HTA	80.7 ± 0.4cdA	80.6 ± 0.4cA	70.8 ± 1.3bB	62.8 ± 0.2cC	68.4 ± 1.2ceB	70.7 ± 0.1eB
DOPAC	>90eA	>90eA	>90cA	86.9 ± 0.3deB	>90fA	>90fA
QE	88.4 ± 0.9cA	89.3 ± 0.1fA	>90cB	>90eB	86.5 ± 0.3dA	85.2 ± 0.1gA

[a]The mixtures were incubated at 37 °C for 72 h. Results are expressed as the mean ± standard deviation for $n = 3$. Different capital letters in the same row and different lower case letters in the same column denote significant differences ($p < 0.05$).

phenol compounds will exert their activity in food and biological systems with a variety of dicarbonyl compounds present. However, further investigations considering kinetic modeling are necessary for major understanding of the chemistry behind these competitive systems.

The contents of HT and other bioactive compounds such as luteolin, verbascoside, or oleuropein in several products from the olive farming have been pointed out as a promising source of potential inhibitors of AGEs.[33,34] However, before any pharmacological application, the fate of HT in vivo should be further addressed. A considerable number of studies have been carried out to elucidate the bioavailability and metabolism of HT. In humans, the HT absorption was >55−66%, although this depends on the matrix administration.[35] Among its metabolites, DOPAC has been described as a HT-related substance with high antiglycative activity in vitro.[18,23] Absorption studies of olive oil phenols in humans suggested that oleuropein is metabolized into HT and other components, increasing the HT circulating levels.[35]

This study shows for the first time that HT, HTA, and DOPAC are able to scavenge C6 α-dicarbonyl compounds to a similar extent as MGO and GO under simulated physiological conditions. In addition, it is noticeable that there is a preference for trapping short-chain dicarbonyl compounds in the cocktail systems constituted by a binary combination of C3 and C6 dicarbonyl compounds. Previously, Navarro and Morales[23] proposed that the mechanism by which HT could act as an antiglycative compound was its MGO-trapping activity mediated by electrophilic aromatic substitution to the orthodiphenyl ring. It is expected that this mechanism proceeds in a similar way with C6 dicarbonyl compounds, which must, however, be further confirmed. The present results support the relevance of HT and its derivatives as antiglycating substances beyond their C3 α-dicarbonyl trapping capacities and may contribute to the detoxification of α-dicarbonyl compounds. Further investigations are necessary to confirm the formation of 3-DG and 3-DGal adducts with HT, HTA, or DOPAC.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

HT, hydroxytyrosol; HTA, hydroxytyrosol acetate; QE, quercetin; DOPAC, 3,4-dihydroxyphenylacetic acid; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; 3-DGal, 3-deoxygalactosone; 3,4-DGE, 3,4-dideoxy-glucoson-3-ene; GLUCOS, glucosone

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Evaluation of olive leaf extract as a natural source of antiglycative compounds

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ABSTRACT

Thermally processed foods contain dietary advanced glycation endproducts (AGEs), which may have adverse physiopathological effects. Therefore, mitigating the formation of AGEs may be an effective strategy to avoid such effects. In this work, an Olive Leaf Extract (OLE) obtained under acidic conditions was characterized by LC–MS/MS and subsequently fractionated by solid-phase extraction to assess its antiglycative capacity as inhibitor of the formation of Amadori products at an early stage and of AGEs at a late stage, as well as of side reactions by trapping dicarbonyl compounds. The OLE and its fractions substantially inhibited the formation of fluorescent AGEs in reaction models of bovine serum albumin. However, the inhibition rate of fructosamine formation decreased over time; this suggests that the OLE acts by trapping different reactive dicarbonyl compounds (glyoxal, methylglyoxal, 3-deoxyglucosone and 3-deoxygalactosone), preferentially at a late glycation stage. The extract efficiently inhibited carboxymethyl-lysine but was not so active against argpyrimidine or carboxyethyl-lysine. This natural extract is therefore an efficient antiglycative agent at different stages of the glycation process which acts mainly by trapping reactive dicarbonyl compounds.

Keywords: Olive leaves; antiglycative activity; advanced glycation end-products; dicarbonyl compounds; Glycation

Abbreviations: Olive leaf extract (OLE); fractions 1 and 2 of olive leaf extract (OLE-F1 and OLE-F2); hydroxytyrosol (HT); 3,4-dihydroxyphenylacetic acid (DOPAC); methylglyoxal (MGO); aminoguanidine (AG); glyoxal (GO); 3-deoxyglucosone (3-DG); 3-deoxygalactosone (3-DGal).

1. INTRODUCTION

Advanced Glycation End-products (AGEs) are a group of heterogeneous compounds with a wide variety of chemical structures that can be roughly classified by their fluorescent properties and cross-linking abilities (Schmitt, Schmitt, Münch, & Gasic-Milencovic, 2005). Although glycation is the main pathway to AGE formation, other side routes such as glucose autoxidation, lipid peroxidation or polyol reactions produce dicarbonyl compounds which are highly efficient intermediates of reactions with amino residues and the direct formation of AGEs (Peyroux & Stenberg, 2006). Thus, glyoxal, methylglyoxal and 3-deoxyglucosone can lead to the formation of

carboxymethyl-lysine (CML), carboxyethyl-lysine (CEL), argpyrimidine (ArgP) or pyrraline (Thorpe & Baynes, 2003).

The biological significance of AGEs arises from their accumulation *in vivo*; in fact, AGEs can bind to lysine, histidine and arginine residues in proteins to modify their structure and functionality (Poulsen et al., 2013). Some health-related conditions such as Alzheimer's disease (Münch, Thome, Foley, Schinzel, & Riederer, 1997), cardiovascular and renal diseases (Schmitt, Schmitt, Münch, & Gasic-Milencovic, 2005), and diabetes mellitus and its pathological complications (Vlassara & Palace, 2002; Vlassara & Uribarri, 2014), have been associated with AGEs to a varying extent. The high prevalence of these diseases has aroused interest in mitigating AGE formation, particularly in medical and food science research (Luevano-Contreras C, & Chapman-Novakofski K., 2010).

Some natural constituents of foods (specifically, phytochemicals) exert a protective effect against the formation and accumulation of AGEs (Khangholi, Majid, Berwary, Ahmad, & Aziz, 2016; Peng, Ma, Chen, & Wang, 2011). In general, their antiglycative activity involves trapping reactive dicarbonyl species, thereby preventing glycoxidative reactions and blocking the glycation process at an early or late stage (Wu & Chen, 2005; Kontogianni *et al.*, 2013; Chandler *et al.*, 2010).

Olea europaea L. leaves are an olive farming by-product containing high proportions of phenolic compounds with antihypertensive, anticarcinogenic, anti-inflammatory, hypoglycemic, antimicrobial and hypocholesterolemic properties (Kawaguchi, Matsumoto, & Kumazawa, 2011; Hayes, Allen, Brunton, O'Grady, & Kerry, 2011). Thus, olive leaf extracts (OLEs) have so far been used in a number of medicines, cosmetics, pharmaceuticals or even commercially valuable nutraceuticals (Kawaguchi, Matsumoto, & Kumazawa, 2011). Olive leaves are especially rich in oleuropein but additionally contain minor amounts of other compounds including ligstroside, oleuroside, verbascoside (Quirantes-Piné *et al*., 2013), flavonoids (e.g., rutin, luteolin-7-glucoside, apigenin-7-glucoside and their aglycones) and simple phenols (e.g., hydroxytyrosol, tyrosol, ferulic acid, gallic acid) (Lee *et al.*, 2009; Goday-Caballero, Acedo-Valenzuela, & Galeano-Díaz, 2012). Like hydroxytyrosol (HT), oleuropein, which is an ester of elenolic acid and HT, is a potent antioxidant, hypoglycemic, hypolipidemic and antiatherogenic agent (Ahmadvand, Noori, Dehnoo, Bagheri, & Cheraghi, 2014; Hu, He, Jiang, & Xu, 2014).

The aim of this study was to assess the antiglycative capacity of a chemically characterized OLE. For this purpose, various *in vitro* glycation models were used to examine the formation of fructosamine, total fluorescent AGEs and specific AGEs (CML, CEL and ArgP). In parallel, direct dicarbonyl trapping by the OLE was investigated as a potential antiglycative mechanism and also to identify the formation of specific adducts.

2. MATERIAL AND METHODS

2.1. Chemicals

Olive leaves (*Olea Europaea*, Picual variety, Córdoba, Spain), D (+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (MGO) and glyoxal (GO) (40% aqueous solution), aminoguanidine (AG), 5-methylquinoxaline (5-MQ), Nitrotetrazolium Blue chloride (NBT), *o*-phenylenediamine (OPD), sodium borohydride, perfluoropentanoic acid (purity > 97%), heptafluorobutyric acid (HFBA) and standards of phenolic compounds were supplied by Sigma (St Louis, MO, USA). 3-Deoxyglucosone (3-DG, purity > 95%) and 3-deoxygalactosone (3-DGal, purity > 95%) were obtained from Professor Monika Pischetsrieder (University of Erlangen-Nürnberg, Denmark) and HT (purity > 99%) was purchased from Seprox Biotech (Madrid, Spain). Nε-carboxymethyl-L-lysine (CML, ≥ 97%), Nε-carboxyethyl-L-lysine (CEL, ≥ 97%), CML-d2, CEL-d4 and argpyrimidine (ArgP, ≥ 97%) were obtained from PolyPeptide Laboratories (Strasbourg, France). All other chemicals were analytical-grade and supplied by Panreac Química (Barcelona, Spain) or Merck (Darmstadt, Germany).

2.2. Equipment

The hardware used included a Synergy™ HT-multimode microplate reader equipped with an

automatic reagent dispenser and temperature control from Biotek Instruments (Winooski, VT, USA); and an HPLC instrument from Shimadzu (Kyoto, Japan) fitted with a quaternary pump (LC-20AD), an autosampler (SIL-20AHT), an oven (CTO-10ASVP), a diode-array detector (SPD-M20A) and a fluorescence detector (RF-20AXS). LC–MS/MS was performed with a 1200 HPLC system from Agilent Technologies (Palo Alto, CA, USA) coupled to a G6410B triple quadrupole mass spectrometer, also from Agilent Technologies, using electrospray ionization and operating in the negative mode. The LC–MS/MS combination was also coupled to a quadrupole time-of-flight mass spectrometer (MSD-6530A, Agilent Technologies) via an electrospray ionization jetstream probe.

2.3. Preparation of the Olive Leaf Extract (OLE)

An OLE containing a high proportion of phenolic compounds was obtained by using the procedure of Lee *et al*. (2009) with slight modifications. Thus, an amount of 250 g of fresh olive leaves was washed, dried at 40 ^{o}C for 2 days and ground, and 10 g of the resulting powder was mixed with 100 mL of ethanol:water:HCl (80:19.75:0.25; pH 2.5) and shaken at 37 ^{o}C in the dark for a week. Then, the pH was readjusted to 2.5 with 2 N HCl and the mixture kept in the dark for another 3 h before filtering and drying of the supernatant in a vacuum evaporator (Strike 300, Steroglass, Perugia, Italy). The dried fraction was extracted with hexane first (25 mL, 3 times) and ethyl acetate then (50 mL, 5 times). The ethylacetate fraction (250 mL) was again dried under vacuum and dissolved in a methanol/water solution (60:40 % v/v) to obtain a final OLE concentration of 1 mg/mL.

2.4. Fractionation of the extract

The OLE was fractionated by solid-phase extraction (C18 SepPak cartridge, 1 mL, Waters Corporation, Milford, MA, USA). To this end, an OLE stock solution (1 mg/mL in ethanol:water, 60:40, v/v) was directly loaded onto a pre-activated cartridge. The first eluate was collected and labelled fraction OLE-F1. Then, 1 mL of methanol was passed through the cartridge and the resulting fraction collected and labelled OLE-F2. The procedure was repeated 10 times, and fractions were pooled and evaporated on a Speed Vac (Savant SPD131, Thermo Scientific, Milford, MA, USA) and reconstituted in methanol:water (60:40, v/v).

2.5. Determination of phenolic compounds by HPLC-DAD–ESI-QTOF-MS/MS

Phenolic compounds were characterized according to Peralbo-Molina, Priego-Capote, & Luque de Castro (2012). The sample was injected (10 µL) onto a Mediterranean-Sea-C18 column (250 × 0.4 mm, 5 µm, Tecknokroma, Barcelona, Spain) operating at 0.8 mL/min. The mobile phase consisted of formic acid in water (0.1%, phase A) and acetonitrile (phase B). The running time was 55 min and the following gradient elution programme applied: 0 min, 4% B; 4 min, 4% B; 40 min, 100% B; 43 min, 100% B; 45 min, 4% B; 55 min, 4% B. Chromatograms were recorded at 280 nm and coupled to a mass spectrometer. The ESI source was operated in the negative ionization mode and the voltage set at 3.5 kV. The fragmentor, skimmer and octopole voltages were 175, 65 and 750 V respectively. Structural information for the compounds was obtained from the fragments of the target compounds identified by tandem mass spectrometry (MS/MS). Data were processed by using Masshunter Data Acquisition B.04.00 and Masshunter Qualitative Analysis B.04.00 (Agilent Technologies). Structural identification was further established by using NIST Standard Reference Databases (data v.NIST05).

2.6. Fructosamine assay

Fructosamine was measured as described by Navarro, Fiore, Fogliano, & Morales (2015). OLE at a concentration 0.07 or 0.14 mg/mL, and its fractions (obtained from the whole extract at 0.07 mg/mL), were incubated in a BSA–GLC glycation model for 1–21 days. The results were expressed as percent inhibition relative to the control.

2.7. In vitro *glycation assay with bovine serum albumin induced by methylglyoxal, glyoxal and glucose*

The glycation models of BSA with MGO (BSA–MGO assay), GO (BSA–GO assay) and GLC (BSA–GLC assay) were used according to Mesias, Navarro, Gökmen, & Morales (2013). Two different concentrations of OLE (0.07 and 0.14

mg/mL), and its fractions, obtained from the whole extract at 0.07 mg/mL, were incubated for 1–14 days (BSA–MGO or GO) or 1–21 days (BSA–GLC). Aminoguanidine (0.57 mg/mL) was used as positive control to reach more than 90% inhibition. The results are expressed as percent inhibition relative to the control.

2.8. Determination of CML and CEL by LC–ESI-MS-QQQ

CML and CEL were determined as described by Navarro & Morales (2016), using the LC–MS system in combination with a triple quadrupole and a porous graphitic carbon column (Hypercarb, 100 mm × 2.1 µm, 5 µm, ThermoFisher Scientific). The flow rate was 0.5 mL/min and isocratic elution applied by using a mobile phase of 5 mM NFPA/acetonitrile (95:5 v/v). The product ion at *m/z* 84 was used to quantify CML (*m/z* 205), CML-d2 (*m/z* 207) and CEL (*m/z* 219), and that at *m/z* 88 was used for CEL-d4 (*m/z* 223). Quantitation was based on the response factor of CML or CEL relative to their respective labelled internal standards. The calibration range was 0.005–0.1 µg/mL for CEL and 0.05–1 µg/mL for CML. Data were processed by using Masshunter Data Acquisition and Masshunter Qualitative Analysis (Agilent Technologies). The results were expressed as µmol CML or CEL/g protein.

2.9. Determination of argpyrimidine by LC-fluorescence

ArgP formation was examined according to Navarro and Morales (2016). The compound was analysed by HPLC with fluorescence detection, using an injected volume of 10 µL and a flow rate of 0.8 mL/min. Solvent A was HFBA (1 mL/L) and solvent B ACN (500 mL/L) containing HFBA (1 mL/L); the two were used under the following gradient elution conditions: 0 min, 20% B; 25 min, 100% B; 26 min, 100% B; 27-37 min, 20% B. ArgP was detected at an excitation wavelength of 335 nm and an emission wavelength of 385 nm, and eluted at 15.1 min. The calibration curve was constructed from pure standards spanning the concentration range 0.02–5 µg/mL. The results were expressed as µmol ArgP/g protein.

2.10. Evaluation of direct dicarbonyl trapping capacity

The direct dicarbonyl trapping capacity of OLE and its fractions was determined by using the procedure of Mesias, Navarro, Gökmen, & Morales (2013) with some modifications. Briefly, 750 µL of phosphate buffer (0.1 mol/L, pH 7.4), 50 µL of 5-MQ (0.5 mg/mL, internal standard), 100 µL of OLE and its fractions (7–70 µg/mL in methanol:water, 60:40) or PM solution in methanol:water (60:40) as positive control or methanol:water (60:40) as negative control plus 100 µL of MGO, GO, 3-DG or 3-DGal (0.64 mM) were used. Quinoxaline derivatives were analysed on a Shimadzu HPLC-DAD instrument. The mixtures were injected (10 µL) on an analytical phenyl–hexyl column (100 mm × 4.6 mm, 2.6 µm; Kinetex, Phenomenex, Torrance, CA, USA) at 40 ^{o}C and chromatograms recorded at 315 nm. The retention times for the quinoxalines of 3-DG, 3-Dgal, GO and MGO and 5-MQ were 18.3, 18.9, 22.0, 23.2 and 24.4 min, respectively. The amount of unreacted dicarbonyl was calculated from the ratio of dicarbonyl quinoxaline and 5-MQ to the control. The percent inhibition of dicarbonyl was calculated as follows:

Dicarbonyl decrease (%) = [(amount of dicarbonyl in control – amount of dicarbonyl in sample with extract or HT solution)/amount of dicarbonyl in control] × 100.

2.11. LC–ESI-QTOF-MS

Mixtures of OLE, HT or oleuropein incubated at 37 ^{o}C with or without MGO in PBS (50 mM, pH 7.4) for 24–96 h were analysed according to Navarro & Morales (2015). Samples (10 µL) were separated on a reverse phase C-18 Kinetex column (75 × 4.6 mm, 2.6 µm; Phenomenex) at 25 ^{o}C. The flow rate was set at 0.6 mL/min and a binary solvent delivery system consisting of 0.5 % (v/v) acetic acid in water as solvent A and methanol as solvent B was used. The gradient elution programme was as follows: 0 min, 5% B; 1 min, 5% B; 20 min, 60% B; 21 min, 60% B; 22 min, 5% B; 30 min, 5%. B. The ESI ion source was operated in the negative-ion polarity mode, using a voltage of 3 kV, a cone voltage of 40 V and a desolvation temperature of 250 ^{o}C. MS/MS spectra were acquired by using a collision energy of 20–40 V from the QTOF.

Structural information for phenolic compounds in the OLE was obtained, oleuropein degradation after different times of incubation was assessed and major MGO adducts were identified by using tandem mass spectrometry (MS/MS). Data were processed with Masshunter Data Acquisition and Qualitative Analysis.

2.12. Statistical analysis

Measurements were made at least in triplicate. Statistical analyses were performed by using the software IBM SPSS Statistics v. 21.0. Data were expressed as mean value ± SD. Analysis of variance (ANOVA) and the Bonferroni test were used to identify differences between means. Differences were considered to be significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Identification of phenolic compounds in the extract

Figure 1a depicts the chromatogram at 280 nm for the OLE components eluted at 12–24 min by using an increasing ACN gradient from 25 to 57%. A total of thirty compounds were identified in the acid-treated OLE. Characterization involved using high-resolution Q-TOF to determine the exact mass of each compound as confirmed by the MS/MS spectra, the NIST standard reference database and previously reported data (Quirantes-Piné *et al.*, 2013; Kawaguchi, Matsumoto, & Kumazawa, 2011; Peralbo-Molina, Priego-Capote, & Luque de Castro, 2012). HT, oleuropein, rutin, verbascoside and tyrosol were further confirmed by comparison with commercial standards. **Table 1** summarizes the characteristics of the compounds identified (formula, retention time, experimental and theoretical *m*/*z*, score, error and representative fragment ions).

HT (peak 4) and its derivatives hydroxytyrosol hexose isomer a (peak 1) and b (peak 2), and hydroxytyrosol rhamnoside (peak 12), were all unambiguously identified. In peaks 1, 2, and 4, one of the fragments for the HT derivatives corresponded to HT in free form (–*m*/*z* 153.0555) and the other to HT with the loss of a CH_2O group (–*m*/*z* 123.0454). The content in free form of HT was higher than that of its glycosylated forms, probably as a result of the acid treatment applied during the maceration step. Consistent with previous results (Quirantes-Piné *et al.*, 2013), oleuropein (peak 24) was the main secoiridoid. This compound was identified at –*m*–*z* 539.1770, and so were its isomers (peaks 25, and 26) and derivatives (peaks 10, 13, 14, 17, 19, 30). Elenolic acid was also identified in the extract, but only in glycosylated form (peaks 9 and 11), with a retention time of 15.46 min for isomer a and 16.21 min for isomer b, and a respective –*m*–*z* value of 403.1261 and –*m*/*z* 403.1256. Other typical tyrosol-derived secoiridoids included oleoside (peaks 3 and 5) and its glycosylated form (peak 7). Verbascoside (peak 16) and 3,4-DHPEA-EDA (peak 28) were found in relatively low proportions. Flavonoids were represented mainly by luteolin derivatives (peaks 18 and 22), apigenin derivatives (peaks 20 and 21) and, to a lesser extent, luteolin in free form (peak 29) and rutin (peak 15).

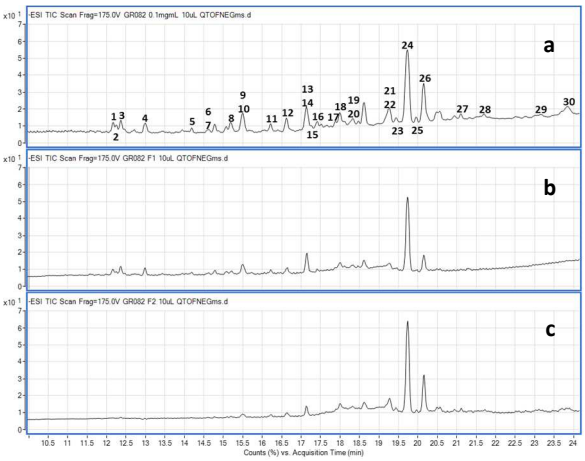

Figure 1: Chromatogram for the olive leaf extract (OLE) (a) and its two fractions: OLE-F1 (b) and OLE-F2 (c). The UV chromatogram was obtained by LC-DAD–QTOF-MS/MS at 280 nm. Peaks (1–30) are identified in Table 1.

The distribution of the previous phenolic compounds in the acid-treated OLE fractions F1 and F2 differed markedly. **Figures 1b** and **1c** show the chromatographic profile for OLE-F1 and OLE-F2, respectively. **Table 1** shows the distribution of compounds in F1 and/or F2. OLE-F1 was rich in the more polar phenolic compounds and hence exhibited an outstanding phenolic front (peaks 1–8). HT and its derivatives were concentrated in OLE-F1, whereas oleuropein was almost identically distributed between OLE-F1 and OLE-F2. OLE-F2 contained slightly more oleuropein

Peak	Compound	Fraction	Formula	RT (min)	*m/z* (–) theoretical	*m/z* (–) experimental	Score	Diff (ppm)	Fragments
1	Hydroxytyrosol hexose isomer a	F1	$C_{14}H_{20}O_8$	12.16	315.1085	315.1106	91.86	–4.86	153.0555 123.0454
2	Hydroxytyrosol hexose isomer b	F1	$C_{14}H_{20}O_8$	12.26	315.1085	315.1099	93	–4.3	153.0555 123.0454
3	Oleoside isomer a	F1	$C_{16}H_{22}O_{11}$	12.36	389.1085	389.1097	98.30	–1.93	227.0554 183.0675 121.0673
4	Hydroxytyrosol	F1/F2	$C_8H_{10}O_3$	12.97	153.0557	153.0562	98.64	–2.99	123.0460
5	Oleoside isomer b	F1	$C_{16}H_{22}O_{11}$	14.17	389.1085	389.1106	90.55	–4.41	227.0554 183.0675 121.0673
6	Loganin	F1	$C_{17}H_{26}O_{10}$	14.58	389.1453	389.1461	97.87	–1.98	309.1739 165.0514 121.0629
7	Oleoside glucoside	F1	$C_{22}H_{32}O_{16}$	14.71	551.1618	551.1614	80.02	0.62	
8	Tyrosol	F1	$C_8H_8O_3$	15.21	137.0608 ($C_8H_{10}O_2$)	151.0403	99.18	–1.6	
9	Elenolic acid glucoside isomer a (Oleoside-11-methylester)	F1/F2	$C_{17}H_{24}O_{11}$	15.46	403.1246	403.1261	95.46	–3.14	245.1147 223.0525 179.0729
10	Oleuropein aglycon	F1/F2	$C_{16}H_{26}O_{10}$	15.51	377.1453	377.1462	95.92	–2.41	197.0824 153.0927

11	Elenolic acid glucoside isomer b (Oleoside 11-methyl ester)	F1/F2	$C_{17}H_{24}O_{11}$	16.21	403.1246	403.1256	94.07	–2.77	245.1147 223.0525 179.0729
12	Hydroxytyrosol rhamnoside	F1/F2	$C_{20}H_{34}O_{13}$	16.61	481.1927	481.1929	99.26	–0.5	271.5145 197.8319 143.0400
13	Demethyloleuropein (Oleuropein acid)	F1/F2	$C_{24}H_{30}O_{13}$	17.12	525.1618	525.1618	99.03	–0.79	
14	10-Hydroxyoleuropein	F1/F2	$C_{25}H_{32}O_{14}$	17.17	555.1719	555.1733	94.65	–2.81	291.0832 223.0623 151.0439
15	Rutin	F1	$C_{27}H_{30}O_{16}$	17.35	609.1461	609.1473	97.53	–1.15	413.1338 301.0361 149.0457
16	Verbascoside	F1	$C_{29}H_{36}O_{15}$	17.40	623.1981	623.1997	96.65	–2.03	461.1583 212.9809 161.0223
17	Oleuropein diglucoside isomer a	F1/F2	$C_{31}H_{42}O_{18}$	17.80	701.2298	701.2313	96.42	–2.07	
18	Luteolin 7-glucoside isomer a	F1/F2	$C_{21}H_{20}O_{11}$	17.98	447.0933	447.0950	93.24	–3.81	287.0563 254.9668 151.0042
19	Oleuropein diglucoside isomer b	F1/F2	$C_{31}H_{42}O_{18}$	18.25	701.2298	701.2289	94.09	1.26	
20	Apigenin rutinoside	F1/F2	$C_{27}H_{30}O_{14}$	18.32	577.1563	577.1576	73.95	–2.21	
21	Apigenin 7-glucoside	F1/F2	$C_{21}H_{20}O_{10}$	19.24	431.0979	431.0979	98.71	1.01	269.0433

22	Luteolin 7-glucoside isomer b	F1/F2	$C_{21}H_{20}O_{11}$	19.24	447.0933	447.0926	98.76	1.57	285.0389 254.9668 168.9877
23	Chrysoeriol-7-*O*-glucoside	F1/F2	$C_{22}H_{22}O_{11}$	19.50	461.1089	461.1102	96.3	–2.62	
24	Oleuropein	F1/F2	$C_{25}H_{32}O_{13}$	19.69	539.1770	539.1789	93.09	–3.19	377.1279 307.0858 275.0944
25	Oleuropein isomer a	F1/F2	$C_{25}H_{32}O_{13}$	19.96	539.1770	539.1783	89.18	–2.3	377.1279 307.0858 275.0944
26	Oleuropein isomer b	F1/F2	$C_{25}H_{32}O_{13}$	20.14	539.1770	539.1778	98.76	–1.38	377.1279 307.0858 275.0944
27	Ligstroside	F2	$C_{25}H_{32}O_{12}$	21.10	523.1821	523.1836	94.71	–2.86	
28	4-Hexenoic acid 3,4-DHPEA-EDA	F2	$C_{17}H_{20}O_6$	21.70	319.1187	319.1199	91.67	–3.81	139.0729
29	Luteolin	F2	$C_{15}H_{10}O_6$	23.17	285.0405	285.0407	99.22	–0.57	151.0039 133.0298
30	Oleuropein aglycone? (3,4-DHPEA-EA)	F2	$C_{19}H_{22}O_8$	23.80	377.1242	377.1256	93.12	–4.16	307.0815 275.0582 139.0378

Table 1: Compounds identified in the olive leaf extract (OLE) and its fractions (OLE-F1 and OLE-F2) by LC-DAD–QTOF-MS/MS.

isomer b (peak 26), oleuropein isomer a (peak 25) and oleuropein (peak 24) than did OLE-F1. A residual peak for luteolin-7-glucoside isomer a (peak 18) was identified in both fractions, but apigenin-7-glucoside (peak 21) and luteolin 8-glucoside isomer b (peak 22) were only found in OLE-F2. In contrast, ligstroside, 3,4-DHPEA-EDA, luteolin and 3.4-DHPEA-EA were only detected in OLE-F2. Based on the chromatograms for OLE-F1 and OLE-F2, most phenolics were extracted by 60/40 methanol:water but elution of the less polar compounds in OLE-F2 required using pure methanol.

Based on previous results for the *in vitro* antiglycative properties of pure HT and its esters (Navarro & Morales, 2016), our investigation focused on the phenolic constituents of the OLE relating to HT. The extract contained 120 mg/g HT and 815 mg/g oleuropein. OLE-F1 contained a 100.0 mg/g concentration of the former and one of 441.2/g mg of the latter, whereas OLE-F2 contained 16.6 mg/g HT and 431.4 mg/g oleuropein. HT recovered from the fractions accounted for 97.2% of the total amount (83.3% in OLE-F1 and 16.7% in OLE-F2). Oleuropein, which was completely recovered, was equally distributed between both fractions (ca. 50% in each).

3.2. Antiglycative capacity of the extract

3.2.1. Inhibition of fructosamine

Because fructosamine is a marker of the formation of Amadori products, its inhibition requires antiglycative activity at an early stage of the glycation process. Amadori products are formed by condensation of glucose with amino residues in BSA and conversion of the resulting Schiff base. Under mild reaction conditions, an Amadori product is stable and forms no AGEs. As can be seen from **Table 2**, the OLE inhibited almost complete (> 90%) formation of fructosamine within the first day of incubation; however, the degree of inhibition decreased with time. The effect was dose-dependent; thus, inhibition at 21 days amounted to 24.9% with 0.07 mg/mL and to 40.1% with 0.14 mg/mL in both OLE-F2 and OLE-F1. Based on the results, the OLE and its fractions have a limited ability to inhibit the formation of Amadori products at long incubation times.

Sample (mg/mL)	1 day	7 days	14 days	21 days
OLE (0.14)	> 90 ^{aA}	74.7 ± 4.5^{aB}	52.4 ± 2.7^{aC}	40.1 ± 3.2^{aD}
OLE (0.07)	> 90 ^{aA}	41.1 ± 3.9^{bB}	17.7 ± 3.7^{bC}	24.9 ± 4.6^{bC}
OLE-F1 (0.07)	13.4 ± 5.1^{bAB}	5.5 ± 1.6^{cA}	4.7 ± 2.3^{cA}	17.8 ± 5.9^{bB}
OLE-F2 (0.07)	35.6 ± 4.0^{cA}	9.8 ± 3.8^{cB}	6.3 ± 3.6^{cB}	7.2 ± 1.1^{cB}

Table 2: Inhibition of fructosamine formation at 1, 7, 14, and 21 days of incubation. The olive leaf extract (OLE) was assayed at 0.07 and 0.14 mg/mL, and both fraction 1 (OLE-F1) and 2 (OLE-F2) at 0.07 mg/mL. The results are expressed as percent inhibition (mean ± standard deviation for n = 4). Different lowercase letters in the same column and uppercase letters in the same row denote significant differences ($p < 0.05$).

3.2.2. Inhibition of fluorescent AGEs

Fluorescent AGEs formed by the glycation models containing GLC, GO and MGO as initiators and incubated for 1–14 days were examined. The time of incubation for the BSA–GLC model was extended to 21 days because it evolved more slowly than the BSA–GO and BSA–MGO models owing to the high reactivity of the dicarbonyl compounds and their different formation pathway. **Figure 2a** depicts the formation of fluorescent AGEs in the BSA–MGO assay after 1, 7 and 14 days of incubation. The OLE (0.07 mg/mL) reached 70.7% inhibition within the first day of incubation and 88.0% within 14 days. A similar trend was observed with a dose of 0.14 mg/mL. Likewise, OLE-F1 and OLE-F2 exhibited substantial activity during the first day of incubation (33.1% and 56.2%, respectively) and their activity increased slightly over the next 14 days (to 48.9% in OLE-F1 and 68.7% in OLE-F2).

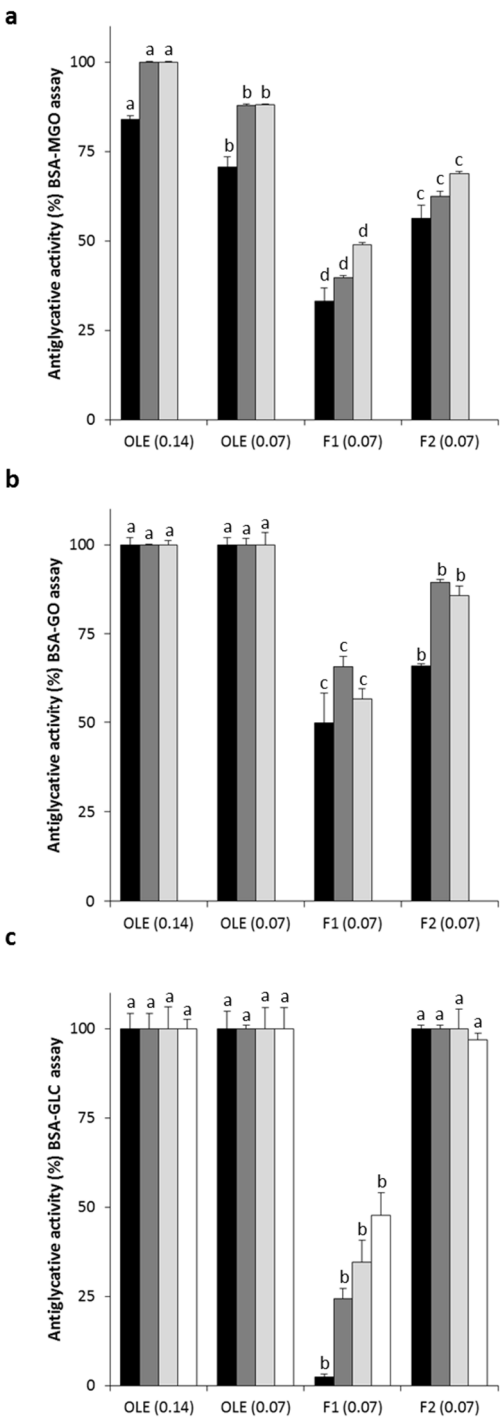

Figure 2: Antiglycative activity of the olive leaf extract (OLE) at 0.14 mg/mL and 0.07 mg/mL, and of its two fractions (OLE-F1 and OLE-F2) as obtained from 0.07 mg/mL OLE on the formation of fluorescent AGEs in the BSA–MGO (A), BSA–GO (B) and BSA-GLC assay (C). Aminoguanidine (AG) at 0.57 mg/mL was used as reference. Bars represent incubation times: 1 day (black), 7 days (dark grey), 14 days (pale grey) and 21 days (white). The results are expressed as mean ± SD (n = 4). Different letters denote significant differences at the same incubation time ($p < 0.05$).

Figure 2b illustrates the inhibition of fluorescent AGEs in the BSA–GO assay after 1, 7 and 14 days of incubation. Whichever the dose, the OLE reached 100% inhibition and retained its activity throughout the incubation period. The antiglycative activity of the OLE fractions followed the same trend as in the BSA–MGO system, with 49.9% in OLE-F1 and 66.1% in OLE-F2.

A similar trend was also observed in the BSA–GLC assay from the first day of incubation (**Figure 2c**). OLE-F1 was scarcely active on the first day (2.3%), but much more so after 21 days (47.8%). Interestingly, the BSA–GLC assay exhibited the greatest differences in inhibitory activity between fractions, the activity of OLE-F2 being similar to that of the whole extract.

3.2.3. Inhibition of CML, CEL, ArgP

CML, which forms from lysine and GO, is the most widely studied AGE and has been used as marker of glycation in foods and biological systems. CEL and ArgP are AGEs of the reaction between MGO and lysine or two molecules of MGO and arginine, respectively. This led us to use the BSA–GO assay to investigate the formation of CML, and the BSA–MGO assay to examine that of CEL and ArgP.

The OLE inhibited CML formation at all times studied (1, 7 and 14 days), the degree of inhibition ranging from 47.3 to 61.0% (**Figure 3a**) and both fractions (OLE-F1 and OLE-F2) exhibiting similar inhibitory activity (38.7–46.0%). However, the extract and its fractions had very limited inhibitory activity on the formation of CEL relative to CML. CEL formation in the BSA–MGO system was reduced by 14.1% and 16.1% by an OLE dose of 0.07 and 0.14 mg/mL, respectively, after 1 day of incubation and by 9.5% by a dose of 0.14 mg/mL after 14 days (**Figure 3b**). Only with the highest dose, however, did OLE significantly inhibit ArgP formation in the BSA–MGO system (by 28.3%, 26.7% and 28.4% after 1, 7 and 14 days of incubation, respectively) (**Figure 3c**). In any case, CEL formation was less marked than ArgP formation because MGO tends to prefer the guanidine group in arginine to the amino group in lysine (Ahmed, Dobler, Dean, & Thornalley, 2005).

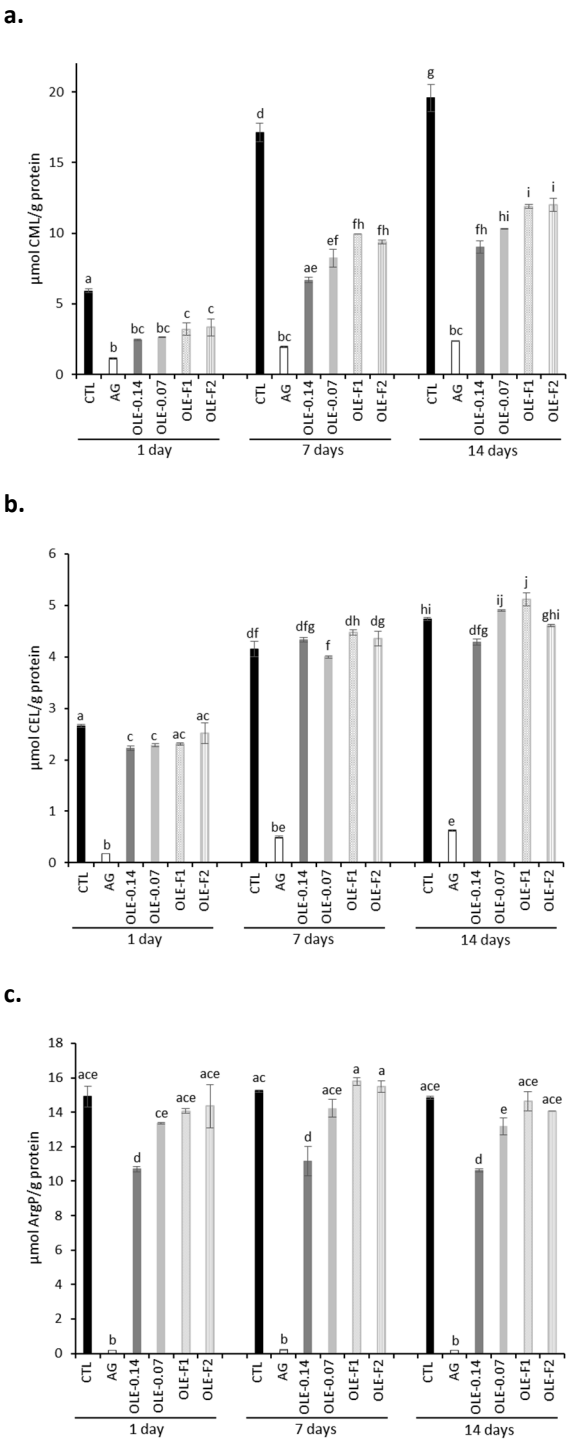

Figure 3: Effect of the olive leaf extract (OLE) at 0.14 and 0.07 mg/mL, and its fractions 1 (OLE-F1) and 2 (OLE-F2) at 0.07 mg/mL, on the inhibition of carboxymethyl-lysine (A), carboxyethyl-lysine (B) and argpyrimidine formation (C) at different times of incubation (1, 7 and 14 days). The white bars correspond to aminoguanidine (AG, the reference) and the black bars to the control. The results are expressed as mean ± standard deviation (n = 3). Different letters denote significant differences (p < 0.05) from the control.

3.2.4. Dicarbonyl trapping capacity

Since dicarbonyl compounds are key promoters of the glycation process (Peyroux, & Steinberg, 2006), direct dicarbonyl trapping capacity of OLE and its fractions were investigated as an antiglycative mechanism. OLE showed a dose-dependent dicarbonyl-trapping capacity in the range between 7-35 µg/mL for all the dicarbonyls tested and it was saturated (> 90%), or close to saturation, at 70 µg/mL for MGO, 3-DG and 3-DGal (**Table 3**). The activity of OLE against the different dicarbonyls was further compared by estimating the concentration able to reach the 50% of inhibition in the reaction mixture. 3-DG and 3-DGal were more effectively trapped by OLE (13.7 µg/mL) than MGO (18.8 µg/mL) and GO (30.0 µg/mL). The concentrations of oleuropein and HT —the major components of the OLE— inhibiting MGO by 50% were 85 and 28 µg/mL, respectively. These results suggest that HT is more effective in trapping MGO than is oleuropein, which is consistent with previous reports (Navarro & Morales, 2016; Navarro, Fiore, Fogliano, & Morales, 2015). The dicarbonyl trapping capacity of OLE and its fractions was found to be dose-dependent and similar; thus, the three were more effective against 3-DG and 3-DGal than they were against MGO and GO irrespective of dose.

3.2.5. Identification of new compounds in the reaction between OLE and MGO

According to Navarro & Morales (2015), the dicarbonyl trapping ability of HT is mediated by oxidation to DOPAC, which undergoes electrophilic aromatic substitution with MGO to form a monoDOPAC–monoMGO adduct. Under this premise, a mixture consisting of PBS (50 mM, pH 7.4), MGO (5.5 mM) and OLE (0.1 mg containing 0.1 mM HT) was incubated at 37 ºC for up to 96 h and analysed for the monoDOPAC-monoMGO structure. **Figure 4** shows the total ion chromatogram for the OLE–MGO system and the extracted ions for the new peaks as compared with incubation in the absence of MGO. The highest signal was a peak at $-m/z$ 225.0405 ($C_{10}H_{10}O_6$) that was accompanied by smaller signals at $-m/z$ 239.0561 ($C_{11}H_{12}O_6$), $-m/z$ 223.0612 ($C_{11}H_{12}O_5$) and $-m/z$ 209.0455 ($C_{10}H_{10}O_5$). These signals were all recorded within

		Dicarbonyl trapping capacity			
Sample	**µg/mL**	**MGO**	**GO**	**3-DG**	**3-DGal**
OLE	7	13.7 ± 7.2^{aA}	12.3 ± 0.1^{aA}	26.7 ± 3.5^{aA}	22.8 ± 6.2^{aA}
	17	46.5 ± 10.7^{bdAB}	29.8 ± 2.3^{bA}	60.6 ± 7.7^{bB}	63.4 ± 5.3^{bB}
	35	81.7 ± 5.1^{cA}	57.8 ± 1.3^{cB}	$>90^{cA}$	$>90^{cA}$
	70	89.5 ± 4.6^{cA}	67.0 ± 0.4^{cB}	$> 90^{cA}$	$>90^{cA}$
OLE-F1	17	26.4 ± 0.5^{adAB}	17.6 ± 1.5^{aA}	34.8 ± 3.1^{aB}	37.3 ± 7.2^{aB}
	35	59.5 ± 10.2^{bcA}	33.0 ± 2.7^{bB}	84.0 ± 0.2^{cC}	85.1 ± 0.5^{cC}
OLE-F2	17	24.6 ± 5.1^{adA}	13.6 ± 0.8^{aA}	29.7 ± 3.3^{aA}	26.0 ± 4.2^{aA}
	35	47.9 ± 7.7^{bdA}	17.2 ± 4.6^{aB}	$>90^{cC}$	88.3 ± 3.4^{cC}

Table 3: Methylglyoxal (MGO), glyoxal (GO), 3-deoxyglucosone (3-DG) and 3-deoxygalactosone (3-DGal) trapping capacity of the olive leaf extract (OLE) at concentrations of 7–70 µg/mL, and dicarbonyl trapping capacity of fractions 1 (OLE-F1) and 2 (OLE-F2) at 17–35 µg/mL. The system was incubated at 37 °C for 96 h. The results are expressed as mean ± standard deviation (n = 3). Different uppercase letters in the same row and lowercase letters in the same column denote significant differences ($p < 0.05$).

one hour of incubation of the OLE–MGO system and peaked after 24 h. This result is consistent with those of Navarro & Morales (2015), who identified four different adducts formed by conjugation of MGO with standard HT and DOPAC, whether directly (–m/z 239.0561, adduct 1) or upon the loss of CH_2 (–m/z 225.0405, adduct 3), O (–m/z 223.0612, adduct 2) or CH_2O (m/z 209.0455, adduct 4).

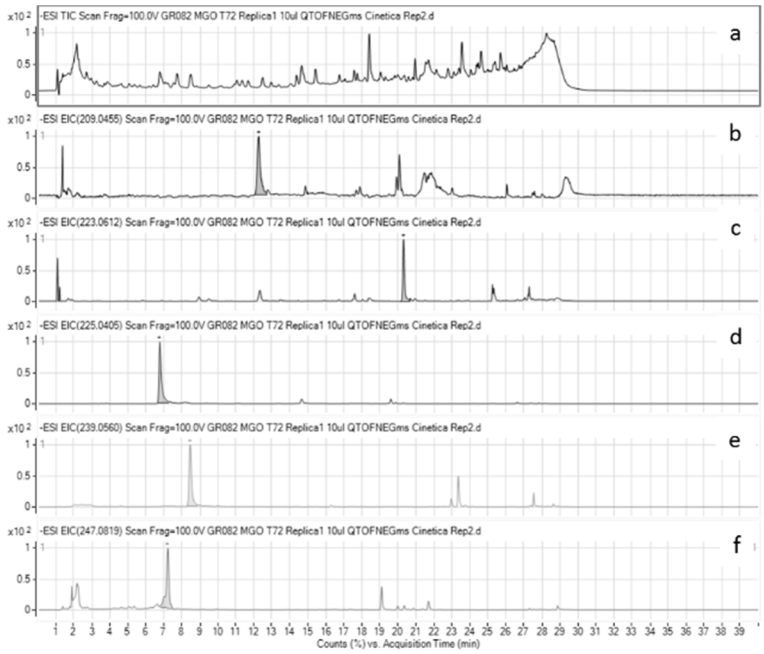

Figure 4. Total ion chromatogram for the olive leaf extract (OLE) as incubated with methylglyoxal (MGO) in 50 mM PBS at pH 7.4 for 72 h (a), and extracted ion chromatograms for DOPAC–MGO adduct-4 (–m/z 209.0455) (b), DOPAC–MGO adduct-2 (–m/z 223.0612) (c), DOPAC–MGO adduct-3 (–m/z 225.0405) (d), DOPAC–MGO adduct-1 (–m/z 239.0560) (e), an unknown OLE–MGO adduct (–m/z 247.0819) (f). DOPAC denotes 3,4-dihydroxyphenyl-acetic acid.

Oleuropein incubated under identical conditions in the presence and absence of MGO degraded rapidly under simulated physiological conditions and nearly 70% of the initial content was lost as the HT and DOPAC concentration was raised (results not shown); also, the mixture was found contain DOPAC. Interestingly, a new peak at $-m/z$ 247.0819 ($C_{10}H_{16}O_7$) was identified in the OLE–MGO and oleuropein–MGO systems but not in the absence of MGO. This peak may be due to another adduct formed by conjugation of MGO with an oleuropein degradation compound. Various possible fragments and structures were investigated but no plausible mechanism accounting for the new peak could be established.

4. CONCLUSION

An olive leaf extract (OLE) obtained under acidic conditions proved an effective inhibitor of glycation under physiological conditions by hindering the formation of fluorescent AGEs and specific structures of CML, and also, to a lesser extent, CEL and ArgP. The inhibitory mechanism probably involves direct trapping of dicarbonyl compounds. However, the fact that the inhibition rate of fructosamine formation decreased over time suggests that the extract acts preferentially by trapping reactive dicarbonyl compounds at a late glycation stage. In fact, the identification of adducts formed between DOPAC, degradation products of HT, and MGO, is consistent with a trapping mechanism. Our results provide for the first time evidence that an olive leaf extract possesses dose-dependent dicarbonyl trapping capacity against the most important C_2 (GO), C_3 (MGO) and, especially, C_6 dicarbonyl compounds (3-DG, 3D-Gal).

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Conflict of Interest

The authors declare no conflict of interest.

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CAPÍTULO 3: *Evaluacion del hidroxitirosol y del extracto de hoja de olivo como potencial ingrediente antiglicante en un modelo de galleta*

Introducción: En los últimos años, diversas investigaciones *in vitro* han evidenciado un efecto antiglicante de subproductos alimentarios ricos en distintas familias de fitoquímicos. Estos resultados podrían tener una aplicación directa en nutracéuticos con el objeto de mitigar la formación de AGEs endógenos, siendo objeto de estudio en el ámbito farmacológico. Sin embargo hay evidencias que demuestran que la dieta contribuye al incremento de las tasas de AGEs circulantes al aportar los AGEs procedentes de los alimentos. En este sentido, es escasa la información que se dispone sobre la capacidad de estos compuestos bioactivos para inhibir la formación de los AGEs de los alimentos en una matriz alimentaria sometida a un tratamiento térmico.

Objetivo: Introducir el HT y OLE como ingredientes alimentarios en una formulación convencional de galleta y determinar su capacidad para reducir la formación de AGEs del alimento durante el horneado.

Metodología: El horneado de galletas representa un modelo reproducible de alimento para investigar el desarrollo de la RM y fue seleccionado para llevar a cabo la investigación. Se elaboró una formulación estándar con harina de trigo (galleta control) sobre la que se incorporaron pequeñas proporciones (0.25 – 0.75 % p/p) del compuesto fenólico objeto de estudio (quercetina, ácido gálico, HT) u OLE. Posteriormente, las masas fueron horneadas a 180 ^{o}C durante 20 min alcanzándose un valor de humedad acorde al producto. Se realizó una caracterización genérica de las galletas mediante el valor de pH, actividad de agua, humedad, color y sólidos solubles totales. Específicamente, la capacidad de inhibición de la RM por parte del HT y OLE como ingrediente a diferentes concentraciones (0.15-12.31 mg/g materia seca) se determinó y se comparó con los resultados obtenidos en las galletas formuladas con quercetina (QE) y ácido gálico (GA), dos conocidos compuestos fenólicos con capacidad inhibitoria del proceso. En una primera aproximación, se evaluaron los AGEs fluorescentes no ligados a proteínas que se formaron durante la elaboración de las galletas. Posteriormente, se determinó el contenido en pentosidina como AGE fluorescente ligado a proteínas mediante HPLC-fluorescencia previa hidrólisis ácida, así como CML y CEL como AGEs no fluorescentes que se analizaron mediante HPLC-MS/MS. La presencia de compuestos α-dicarbonilos como importantes precursores de la RM fueron cuantificados como quinoxalinas derivadas mediante HPLC-DAD y previa derivatización con OPD. Por último, y para ampliar la información de la actividad inhibitoria del extracto y HT sobre otros compuestos intermediarios y característicos de la RM, se evaluó la formación de 5-hidroximetilfurfural (HMF) y furosina mediante HPLC-DAD.

Resultados: Tanto la introducción de compuestos fenólicos como del OLE en la formulación de las galletas no ocasionó una alteración significativa en los parámetros fisicoquímicos evaluados con respecto a las galletas control, salvo un descenso significativo en el pH de la galleta a la que se le había adicionado GA. Contrariamente, se produjo un cambio significativo en el color de las galletas formuladas con GA, HT y OLE (estas últimas a las mayores concentraciones ensayadas) posiblemente debido a la propia contribución del compuesto o extracto adicionado. El color de las galletas formuladas con QE se modificó significativamente hasta el punto de perder los parámetros cromáticos característicos de la galleta. Con respecto a las consecuencias de la adición de estos compuestos sobre la RM que se desarrolla durante el horneado, compuestos tempranos como la furosina, intermediarios como los α-dicarbonilos y HMF o finales como los AGEs fueron evaluados. La furosina en el modelo de galleta presentó unos valores por debajo de los límites de cuantificación posiblemente debido a la composición de la masa y al estado avanzado de la RM, mientras que la formación de HMF fue reducida hasta en un 47.4% al adicionar HT a la formulación. Los α-dicarbonilos presentes en las galletas no mostraron diferencias significativas en su contenido con respecto a la galleta control, donde el MGO fue el α-dicarbonilo mayoritario (11.89 μg/g galleta control). A este respecto y como única excepción, las galletas formuladas con HT redujeron en un 41.6% el contenido de 3-DG con respecto a la galleta control, estableciendo una correlación positiva entre el contenido de HMF y 3-DG. Por otro lado, la determinación de AGEs identificó al CEL como el AGE que mayoritariamente se formó en el modelo de galleta ensayado (60.91 μg/g galleta control) y que fue inhibido en un 49% y 42% al adicionar GA y OLE como ingredientes. Los AGEs fluorescentes libres fueron inhibidos significativamente por la QE y el HT, siguiendo la misma tendencia la pentosidina salvo en las galletas formuladas con GA y OLE, que pese a que no mostraron actividad inhibitoria frente a los AGEs fluorescentes libres, redujeron significativamente el contenido de pentosidina. Por último, la formación de CML no sufrió variación alguna al introducir los compuestos fenólicos o el extracto en la formulación de la galleta horneada a 180 ^{o}C/20 min.

Conclusión: La incorporación de ingredientes con efectos inhibitorios sobre la RM en la formulación de galletas podría modular la formación de AGEs en un modelo alimentario sometido a un tratamiento térmico controlado. En nuestro modelo de galleta, la introducción de HT en la formulación redujo significativamente el contenido de AGEs fluorescentes libres y ligados a proteínas como la pentosidina, mientras que el OLE tuvo una actividad más amplia al reducir significativamente la formación de pentosidina y CEL, el AGE formado mayoritariamente en el modelo. Por otra parte, se pudo constatar una correlación positiva entre la reducción de 3-DG y HMF tras la adición de HT a la masa. Por todo ello, el HT y el OLE podrían ser propuestos como

ingredientes alimentarios con capacidad de mitigar la formación de AGEs en productos de panadería, y consecuentemente capaces de reducir la ingesta de AGEs dietéticos. No obstante, estudios más amplios centrados en la aceptabilidad del producto final por los consumidores y la viabilidad tecnológica y comercial serían necesarios en una siguiente etapa.

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Effect of hydroxytyrosol and olive leaf extract on 1,2-dicarbonyl compounds, hydroxymethylfurfural and advanced glycation endproducts in a biscuit model

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ABSTRACT

The antiglycative activity of hydroxytyrosol (HT) and olive leaf extract (OLE) was investigated in wheat-flour biscuits. Quercetin (QE) and gallic acid (GA) were used as reference of antiglycative activity of phenolic compounds. HT, OLE, QE and GA were added in the range of 0.25–0.75% (w/w). Samples were compared against a control recipe baked at 180 °C/20 min. HT biscuit was able to inhibit efficiently the formation of hydroxymethylfurfural (HMF) and 3-deoxyglucosone (3-DG), as well as reduced the formation of overall free fluorescent AGEs and pentosidine. The inhibition of the 3-DG and HMF formation was directly and significantly correlated under controlled baking conditions. However, samples formulated with OLE exerted similar antiglycative capacity against pentosidine and Nε-carboxyethyl-lysine, although the amount of HT in the biscuit was 100-fold lower than the biscuit formulated with HT. Methylglyoxal, 3-DG, and glyoxal were the predominant 1,2-dicarbonyl compounds after baking but only 3-DG was significantly reduced by HT.

1. Introduction

The development of the Maillard Reaction (MR) plays a pivotal role as responsible of the organoleptic, textural, and nutritional properties during thermal processing of foods (O'Brian & Morrisey, 1989). Basically, MR is a complex cascade of consecutive and parallel reactions initiated by the condensation of an amino residue of protein and a carbonyl group of a sugar, leading the formation of a myriad of compounds named as Maillard reaction products (MRPs). However, 1,2-dicarbonyl compounds are not only intermediates but also potent promotors of the MR that through its interaction with lysine and arginine side chains of proteins lead to the formation of a set of structures named advanced glycation endproducts (AGEs) (Poulsen et al., 2013). 1,2-dicarbonyls may also form from other pathways, such as the autoxidation of glucose and peroxidation of lipids being also named as advanced

Abbreviations: MR, Maillard reaction; AGEs, advanced glycation endproducts; ALEs, advanced lipoxidation endproducts; HT, hydroxytyrosol; OLE, olive leaf extract; MGO, methylglyoxal; GO, glyoxal; 3-DG, 3-deoxyglucosone; HMF, hydroxymethylfurfural; CML, N^{ε}-carboxymethyl-lysine; CEL, N^{ε}-carboxyethyl-lysine; QE, quercetin; GA, gallic acid.

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lipoxidation endproducts (ALEs) (Vistoli et al., 2013). This is the case of N^{ε}-carboxymethyl-lysine (CML) and N^{ε}-carboxyethyl-lysine (CEL) that both are AGEs/ALEs. However, pentosidine is a crosslinking AGE formed from the oxidative reaction of lysine and arginine residues with pentoses, hexoses, ascorbate or 3-deoxyglucosone (3-DG) (Henle, Schwarzenbolz, & Klostermeyer, 1997). Since the chemical structure of dietary AGEs is shared by those produced in the organism, it is believed that the consumption of thermally treated food rich in AGEs could increase the total body AGEs load (Birlouez-Aragon et al., 2010; Poulsen et al., 2013). Studies have reported that a typical diet provides 25–75 mg of AGEs per day and approximately 10% of ingested AGEs are absorbed (Sebekova & Somoza, 2007; Yamagishi, Matsui, & Nakamura, 2008). Since a significant correlation has been found between ingested and circulating AGEs in humans, dietary AGEs are suggested to be implicated in the development of glycation and inflammation associated with the aging process, and complications associated to chronic pathologies such as diabetes, atherosclerosis, and neurodegenerative disease, among others (Van Nguyen, 2006). However, the biological consequences of the dietary AGEs are still under debate.

Nowadays the research on natural glycation inhibitors has taken a special interest. A number of phenolic compounds have been reported to exert an antiglycative action under simulated physiological conditions (Mesias et al., 2014; Peng, Ma, Chen, & Wang, 2011). However, there are few studies addressing the relationship between phenolic compounds and the inhibition of the formation of dietary AGEs in thermally processed foods (Srey et al., 2010; Zhang, Chen, & Wang, 2014). Bakery products, and particularly biscuits and bread, have been used as a reproducible food models. Biscuit composition (sugar, protein, shortening, leaving agents), baking conditions (temperature, time), in parallel with the pH, moisture, and water activity are the most important variables contributing to the extent of the MR (Charissou, Ait-Ameur, & Birlouez-Aragon, 2007; Gökmen, Açar, Serpen, & Morales, 2008). Generally, the antiglycative activity of the phenolic compounds it has been attributed to dicarbonyls-trapping capacity and antioxidant activity through the free radical scavenging and metal ion chelation (Peng et al., 2011; Srey et al., 2010).

The European Food Safety Authority (EFSA Panel on Dietetic Products, Allergies, & protection of LDL particles from oxidative damage, 2011) concluded a positive health opinion on the contribution of olive oil polyphenols against the oxidative stress. The olive leaf is a by-product with high content in phenolic compounds such as oleuroside, verbascoside, luteolin, rutin, hydroxytyrosol (HT), tyrosol or ferulic acid (Lee et al., 2009; Quirantes-Piné et al., 2013) being the oleuropein the majority secoiridoid. The HT can be present in free form but mostly in its glycosylated form and esterified with elenolic acid forming the oleuropein. Then, the olive leaf has recently received particular attention as ingredient bioactive in pharmaceuticals, cosmetics and nutraceuticals. Navarro and Morales (2015) described the antiglycative activity of HT is mainly mediated by the trapping of 1,2-dicarbonyl compounds in simulated physiological conditions which is relevant at a physiological scale. However, information on the effectiveness of the antiglycative capacity of HT in a food model against specific AGEs is still not conclusive.

Recently, Mateos et al. (2016) pointed out that HT is a promising functional ingredient in biscuits since it is highly bioavailable and lowers postprandial oxidized-LDL levels. Since HT maintains their bioactivity after baking, it could be plausible that its antiglycative capacity will remain as well. Then, the aim of the present study was to investigate the efficacy of HT and an olive leaf extract (OLE) with high content in HT to mitigate the fluorescent AGEs formation and specifically of AGEs as CML, CEL and pentosidine in different biscuit formulations. In parallel, dicarbonyl trapping capacity and inhibition of the formation of HMF were investigated to get more insight into the mechanism of action.

2. Material and methods

2.1. Chemical and materials

The ingredients of model biscuits were purchased from local supermarkets. Olive leaves (*Olea Europaea* Picual variety, Córdoba, Spain), MGO (40% aqueous solution), GO (40% aqueous solution), 5-methylquinoxaline (5-MQ), o-phenylenediamine (OPD), sodium borohydride, perfluoropentanoic acid (purity > 97%), heptafluorobutyric acid (HFBA), perfluoropentanoic acid (purity > 97%), Hydroxymethylfurfural (HMF), quinine sulphate, quercetin (QE) and gallic acid (GA) standards were provided by Sigma (St Louis, MO, USA). 3-deoxyglucosone (3-DG) was provided by Prof. Pischetsrieder (University of Erlangen-Nuremberg, Germany) and HT (purity > 99%) was acquired from Seprox Biotech (Madrid, Spain). Ethanol, hexane, ethyl acetate, hydrochloric acid were obtained from Panreac (Madrid, Spain). Potassium hexacyanoferrate, zinc acetate, formic acid, glacial acetic acid and high-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were from Merck (Darmstadt, Germany). N^{ε}-carboxymethyl-l-lysine (CML, $\geqslant$97%), N^{ε}-carboxyethyl-l-lysine (CEL, $\geqslant$97%), CML-d2, CEL-d4, and pentosidine were obtained from PolyPeptide Laboratories (Strasbourg, France). The Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals were of analytical grade and supplied by Panreac Quimica (Barcelona, Spain).

2.2. Equipment

Synergy™ HT-multimode microplate reader with an automatic reagent dispense and temperature control from Biotek Instruments (Winooski, VT, USA). HPLC Shimadzu (Kyoto, Japan) equipped with a quaternary pump (LC-20AD), an autosampler (SIL-20AHT), an oven (CTO-10ASVP), a diode-array detector (SPD-M20A) and a fluorescence detector (RF-20AXS). LC-MS/MS was performed with a 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole (G6410B, Agilent Technologies) via electrospray ionization operating in positive mode.

2.3. Preparation of the Olive Leaf Extract (OLE)

An OLE containing a high proportion of HT was obtained according to Lee et al. (2009) with some modifications. Fresh olive leaves were washed, dried (40 °C/2 days) and ground. The powder (10 g) was mixed with 100 mL of ethanol:water:HCl (80:19.75:0.25; pH 2.5), and kept under shaking in darkness (37 °C/7 days). Then, pH was readjusted to 2.5 with 2 N HCl and the mixture kept in the dark for another 3 h before filtering. The supernatant was dried in a vacuum evaporator (Strike 300, Steroglass, Perugia, Italy) and then was extracted with hexane (25 mL, 3 times) and ethyl acetate (50 mL, 5 times). The ethyl acetate fraction (250 mL) was again dried under vacuum and dissolved in a methanol/water solution (60:40% v/v) to obtain a final OLE concentration of 1 mg/mL (stock solution) with a HT content of 0.15 mg/mL extract.

2.4. Preparation of biscuits

Model biscuits were prepared as described by Gökmen, Açar et al. (2008) with some modifications. Recipes were formulated with 95 g of wheat flour, 35 g of sucrose, 25 g of deionized water, 24 g of sunflower oil, 1.2 g of sodium bicarbonate, 1 g of salt

(control dough) and individual phenolic compound (HT, QE, GA) or OLE at different concentrations up to 0.75% (w/w) of the total solid content. Models were identified as CTL (without addition of phenol compounds), and HT-1, HT-2, HT-3, QE, GA, OLE-1, OLE-2 containing 2.554 mg HT, 5.108 mg HT, 10.219 mg HT, 5.000 mg QE, 5.638 mg GA, 0.127 mg OLE, 0.537 mg OLE/g dough, respectively. The ingredients were thoroughly mixed and dough was rolled out to discs with the diameter of 5.5 cm and the thickness of 2 mm. Then, samples were baked at different temperatures (180 °C and 200 °C) for different times (20 and 10 min). A natural convection oven (Memmert UNE 400, Germany) was used and the biscuits were prepared by triplicate.

2.5. Measurement of pH

Ground biscuits (0.25 mg) were mixed with 25 mL of water and vortexed for 3 min. The mixture was held at room temperature for 1 h to separate solid and liquid phases. After carefully removing the supernatant layer, the pH was measured using a CG-837 pHmeter (Schott, Mainz, Germany).

2.6. Determination of moisture and water activity

The moisture of samples was determined gravimetrically. Ground biscuits (1 g) were dried to constant weight in an oven at 105 °C for 24 h. The water activity was measured at 25 °C by an AquaLAB CX-2 (Decagon Devices Inc., Pullman, WA).

2.7. Determination of soluble browning compounds

Soluble browning compounds were measured according to Rufián-Henares, Delgado-Andrade, and Morales (2006) using a microplate reader. Results were as absorbance units at 420 nm/g dry matter.

2.8. Determination of colour

The colour of biscuits were measured in a Konika Minolta reflectance spectrophotometer CM-3500d (Konica Minolta Sensing INC, Osaka, Japan) and the results expressed with the CIELab colour system. Three independent measurements of a^* (readness), b^* (yellowness), and L^* (lightness) parameters were performed at different areas of the biscuit surface. The E value was calculated according the equation $E = (L^{*2} + a^{*2} + b^{*2})^{1/2}$. The equipment was calibrated with a standard calibration white plate CR-A43 (L^* 93.80; a^* 0.3156; b^* 0.3319).

2.9. Determination of free fluorescent AGEs

The free fluorescent AGEs were determined as described by Delgado-Andrade, Rufián-Henares, and Morales (2006) at an excitation/emission wavelength of 340/420 nm using a microplate reader. Samples (0.3 g) were extracted three times with deionized water, clarified with 300 μL of Carrez solutions (potassium ferrocyanide, 15% w/v; zinc acetate, 30% w/v), centrifuged (4500 g, 10 min, 4 °C), and filtered (0.45 μm). A quinine sulphate solution (0.639–6.386 μmol/L) was used for calibration and the results were expressed in dry matter as quinine sulphate equivalent (μg/g biscuit). The percentage inhibition of AGE formation was calculated using the following equation: inhibition (%) = 100 − (SQE biscuit with phenol × 100/SQE control biscuit).

2.10. Determination of pentosidine by LC-fluorescence

Pentosidine was determined following the HPLC method described by Wilker, Chellan, Arnold, and Nagaraj (2001) with slight modifications. Ground biscuit (0.5 g) was incubated with 1.5 mL of sodium borate (0.2 M, pH 9.2) and 1 mL of sodium borohydride (1 M in 0.1 M NaOH) for 4 h at room temperature. Then, samples were hydrolyzed with 5 mL of HCl (final concentration of 6 M/20 h/110 °C) and 500 μL was dried in a Speed-Vac concentrator (ThermoFisher Scientific, Courtaboeuf, France). The residue was reconstituted in 1 mL of formic acid (1 mL/L) and the pentosidine of samples was measurement by HPLC coupled to a fluorescence detector. Samples (10 μL) were eluted onto a Mediterranean-Sea-ODS2 column (250 mm × 4 mm, 5 μm; Teknokroma, Barcelona, Spain) at a flow rate of 0.8 mL/min under gradient elution. The mobile phase consisted of HFBA (1 mL/L) (solvent A) and acetonitrile (500 mL/L) containing HFBA (1 mL/L) as solvent B and the applied gradient program was as follows: 0 min, 20% B; 25 min, 100% B; 26 min, 100% B; and 27–37 min, 20% B. Pentosidine was detected at excitation and emission wavelength of 335 and 385 nm respectively, and eluted at 15.9 min. Calibration was carried out in the range 0.0005–0.05 μg/mL with a pure standard. Results were expressed as μg pentosidine/g dry matter.

2.11. Determination of CML and CEL by LC-MS/MS

CML and CEL were determined as described by Navarro and Morales (2016). LC-MS/MS was coupled to a triple quadrupole and the samples (10 μL) were injected on Hypercarb column (100 mm × 2.1 μm, 5 μm, ThermoFisher Scientific). The flow rate was 0.5 mL/min and the isocratic conditions (mobile phase of 5 mM NFPA/acetonitrile, 95:5 v/v) were applied. The product ion at m/z 84 was used for quantification of CML (m/z 205), CML-d2 (m/z 207) and CEL (m/z 219) while m/z 88 was used for CEL-d4 (m/z 223). The ratio of response factor of CML or CEL to that their respective labelled internal standards was used for quantitation. Data were processed using MassHunter Data and MassHunter Qualitative Analysis (Agilent Technologies). Results were expressed as μg CML or CEL/g dry matter.

2.12. Determination of glyoxal, methylglyoxal and 3-deoxyglycosone by LC-UV

The 1,2-dicarbonyl compounds were determined according to Arribas-Lorenzo and Morales (2010) with some modifications. 5-MQ was used as internal standard (0.5 mg/mL) and treated with 200 μL of OPD (10.8 mg/mL). The quantification of the corresponding quinoxalines of the dicarbonyls was conducted using a Shimadzu HPLC and the chromatographic separation was carried out on a Kinetex-Phenyl-Hexyl column (100 mm × 2.6 mm, 4.6 μm; Phenomenex, Torrance, CA, USA). The flow rate was 0.8 mL/min and the injection volume was 10 μL. The total run time was 37 min and the mobile phase consisted of acetic acid (5 mL/L) (solvent A) and methanol (solvent C) with the following gradient program: 0 min, 0% C; 5 min, 10% C; 11 min, 10% C; 13 min, 15% C; 15 min, 15% C; 17 min, 50% C; 20 min, 50% C; 23 min, 80% C; 26 min, 80% C; 27 min, 0% C. The chromatograms were recorded at 315 nm leading to retention times of 18.29, 22.04, 23.19 min for the quinoxalines of 3-DG, GO, MGO respectively and 24.37 min for 5-MQ. 1,2-dicarbonyl standard solution were derivatized with OPD, held in darkness for 2 h and were used as calibration in the range 0.52–0.05 μg/mL. The percentage decrease in dicarbonyls was calculated using the following equation: dicarbonyls decrease (%) = [(amount of dicarbonyl in biscuit without tested phenol or OLE − amount of dicarbonyl in biscuit with tested phenol or OLE)/amount of dicarbonyl in biscuit without tested phenol or OLE] × 100%.

2.13. Determination of 5-hydroxymethylfurfural by LC-DAD

Determination of HMF was performed according to Rufián-Henares et al. (2006). Calibration curve was established in the range 0.2–5 mg/L with HMF standard. Results were expressed as μg/g dry matter and the limit of quantification was set at 0.6 μg/g.

2.14. Determination of furosine by ion-pairing-LC-DAD

Furosine determination was performed according to the method described by Delgado-Andrade, Rufián-Henares, and Morales (2007). Calibration curve was established in the range 1–10 mg/L with furosine standard. Results were expressed as mg/kg dry matter and the limit of quantification was set at 20 mg/kg.

2.15. Statistical analysis

Statistical analyses were performed using the IBM SPSS Statistics program (version 21.0). Data were expressed as the mean value ± SD. All data are expressed in dry matter otherwise indicated. Analysis of variance (ANOVA) and the Bonferroni test were applied to determine differences between means. Measurements were carried out at least by duplicate, for chromatographic analysis, and at least by quadruplicate for fluorescence analysis, and the differences were considered to be significant at $p < 0.05$.

3. Results and discussion

Seven dough recipes for biscuit were formulated by adding different amount of HT in the range 0.25–0.75% (w/w), QE and GA (0.25% w/w) and OLE (0.01 and 0.05% w/w) to a standard recipe (wheat flour, sugar, salt, shortening, sodium bicarbonate, and water) that were kept constant. The level of fortification in HT was established after considering that the range of losses of phenolic compounds are 20–47% (Reis, Rai, & Abu-Ghannam, 2014), and specifically a 20% loss of HT during conventional baking of biscuits (Mateos et al., 2016). Additionally, QE and GA were used as reference phenol compounds since it has been described that exert antiglycative activity (Zhang et al., 2014).

Firstly the models were baked at 200 °C between 10 and 15 min but the reproducibility, particularly 1,2-dicarbonyl compounds content and moisture, was not satisfactory in the samples fortified with QE and GA likely due to browning proceeds very rapid (data not shown). The reference conditions for baking were settled at 180 °C for 20 min which all samples reached similar moisture content. The water activity, moisture, pH, soluble browning, and colour of biscuits were measured (Table 1). The water activity was in a range of 0.08–0.12 and the moisture was in the range of 1.06–1.53 g/100 g, but no significant differences were obtained when compared to CTL. The pH value ranged from 6.9 to 7.7, and the formulation with GA showed a significant lower pH value with respect to CTL. The addition of phenol compounds impacts on the colour of the biscuits (*Supplementary material 1*). HT-2, HT-3, OLE-2, GA, and QE were significantly darker than the CTL biscuit. Additionally, the levels of soluble browning compounds were also significantly increased for the same samples. QE samples achieved the highest levels of soluble browning compounds and colour change as described in Table 1. These results were expected and they are in concordance with those reported by Kohyama et al. (2009) who suggested that compounds with catechol or pyrogallol structures stimulated the browning by baking of the dough. Similar changes in the colour were obtained for Peng et al. (2010) by adding to a bread and biscuit formulations different amount of green tea, grape seed extract and quercetin.

GO, MGO and 3-DG are highly reactive 1,2-dicarbonyl compounds capable to boost the MR towards the formation of dietary AGEs after reaction with amino residues or even other intermediary compounds as HMF by dehydration (Poulsen et al., 2013). While 3-DG is formed from hexoses, MGO and GO are breakdown products formed by via retro-aldol reactions. Then, MGO, GO may form through the MR, caramelization and lipid oxidation during baking. Since wheat flour has a low content in free amino acids and a relatively high content of reducing sugar in the biscuit formulation, caramelization has been considered as the main route in the formation of 1,2-dicarbonyl compounds during baking (Kocadağlı et al., 2015). The formation of 1,2-dicarbonyl compounds were studied in the different biscuit models and summarized in Table 2. The CTL biscuit showed a GO and MGO formation of 3.29 ± 0.36 μg/g, and 11.89 ± 3.37 μg/g, respectively. Levels are similar to other described in the literature for biscuits and ranging from 4.8–26.0 and 3.7–81.4 mg/kg, for GO and MGO, respectively (Arribas-Lorenzo & Morales, 2010). It was not observed significant differences with CTL in GO, and MGO regarding the fortification with phenol compounds. A number of studies have been carried out to elucidate the 1,2-dicarbonyl trapping capacity of phenol compounds obtaining contradictory results (Li, Zheng, Sang, & Lv, 2014; Peng et al., 2011; Zhang et al., 2014). However, most of the studies have been developed in simple model systems where the 1,2-dicarbonyl and protein interacted directly with potential inhibitors at moderate temperature conditions. In the case of bakery products, other compounds and other pathways related to intermediates compounds of MR play a key role in their complex matrix where the reactions are accelerated due to the application of high temperatures (Kocadağlı et al., 2015). Zhang et al. (2014) reported in a biscuit model baked at 200 °C that several polyphenols, including quercetin, were effective in inhibiting the formation of GO but they did not have effect on MGO levels. However our data show that MGO and GO were not inhibited by presence of HT, QE, GA and OLE in the recipe as compared with CTL.

3-DG ranged from 5.4 to 7.8 μg/g in CTL sample (Table 2) which it is in line for similar bakery products reported in literature (Degen, Hellwig, & Henle, 2012). A significant reduction was

Table 1
Identification of control biscuit doughs (CTL) and formulated with hydroxytyrosol (HT-1, HT-2, HT-3), quercetin (QE), gallic acid (GA), and olive leaf extract (OLE). Levels of the water activity, moisture, pH, soluble browning compounds, and colour of biscuits after baking at 180 °C/20 min. Different letters in the same column mean significant differences ($p < 0.05$).

Sample	Phenol added mg/g d.m	Phenol added μmol/g d.m.	Water activity	Moisture (g/100 g)	pH	Soluble browning A420/g	Colour E value
CTL	–	–	0.10 ± 0005^{ab}	1.37 ± 0.21^{a}	7.5 ± 0.2^{a}	1.28 ± 0.10^{a}	59.3 ± 1.3^{a}
HT-1	3.08	20	0.08 ± 0.001^{a}	1.53 ± 0.01^{a}	7.3 ± 0.1^{ab}	1.18 ± 0.04^{a}	57.8 ± 0.1^{ab}
HT-2	6.15	40	0.09 ± 0.001^{ab}	1.06 ± 0.04^{a}	7.5 ± 0.1^{a}	1.69 ± 0.01^{b}	55.7 ± 0.2^{bc}
HT-3	12.31	80	0.12 ± 0.001^{b}	1.43 ± 0.08^{a}	7.4 ± 0.1^{ab}	2.11 ± 0.01^{c}	56.1 ± 0.3^{bc}
QE	6.02	20	0.10 ± 0.001^{ab}	1.28 ± 0.01^{a}	7.6 ± 0.1^{a}	4.75 ± 0.04^{d}	49.2 ± 0.8^{d}
GA	6.79	40	0.10 ± 0.002^{ab}	1.52 ± 0.02^{a}	6.9 ± 0.1^{b}	1.78 ± 0.10^{b}	52.3 ± 0.6^{e}
OLE-1	0.15	0.15*	0.08 ± 0.001^{a}	1.34 ± 0.01^{a}	7.7 ± 0.1^{a}	1.31 ± 0.06^{a}	57.8 ± 0.3^{ab}
OLE-2	0.65	0.63*	0.10 ± 0.002^{ab}	1.31 ± 0.02^{a}	7.3 ± 0.1^{ab}	2.11 ± 0.03^{c}	54.1 ± 0.5^{ce}

* Amount is referred to HT content in OLE.

Table 2
Formation of methylglyoxal (MGO), glyoxal (GO), and 3-deoxyglucosone (3-DG) in biscuit samples formulated with hydroxytyrosol (HT-1, HT-2, HT-3), quercetin (QE), gallic acid (GA), olive leaf extract (OLE-1, OLE-2) and in control biscuit (CTL). Sample description as in Table 1. Different letters in the same column mean significant differences ($p < 0.05$). Data expressed in dry matter (d.m.).

Sample	MGO (μg/g d.m.)	GO (μg/g d.m.)	3-DG (μg/g d.m.)
CTL	11.89 ± 3.37^{ab}	3.29 ± 0.36^{a}	6.20 ± 0.77^{a}
HT-1	11.41 ± 1.73^{ab}	3.17 ± 0.06^{a}	4.89 ± 0.81^{ab}
HT-2	12.52 ± 0.29^{ab}	3.43 ± 0.07^{a}	3.85 ± 0.79^{b}
HT-3	12.91 ± 0.29^{ab}	3.20 ± 0.13^{a}	3.62 ± 0.37^{b}
QE	9.70 ± 0.44^{a}	3.18 ± 0.01^{a}	5.79 ± 0.39^{ab}
GA	15.77 ± 0.76^{b}	3.46 ± 0.08^{a}	7.97 ± 0.37^{c}
OLE-1	9.75 ± 0.74^{a}	3.35 ± 0.08^{a}	6.77 ± 0.22^{a}
OLE-2	11.22 ± 2.28^{b}	3.59 ± 0.01^{a}	5.29 ± 0.21^{ab}

observed for the 3-DG content of biscuit formulated with HT as compared with CTL. When the dough was formulated with 6.15 mg HT/g (HT-2 sample) and 12.31 mg HT/g (HT-3 sample) the formation of 3-DG was significantly reduced by 37.9% and 41.6%, respectively. As opposite, a significant increase of 3-DG was observed in the sample GA and reaching a value of 7.97 ± 0.37 μg/g. Similar results were described for chlorogenic acid and analogues such as caffeic acid or *p*-coumaric acid which increased the 3-DG and MGO formation but also the HMF formation when they were heated with fructose at 90 °C for 48 h (Zhang et al., 2016). Formation of 1,2-dicarbonyl compounds in a sugar Maillard system is dependent on the pH value. Since biscuit formulated with GA showed the lowest pH, it could be plausible that the degradation of the Amadori Product was enhanced through the 1,2-enaminol route and more 3-DG was formed in this sample. Other investigation in biscuits formulated with flours of white wheat, hull-less barley and yellow, dark-red, blue, or dark-blue coloured corns showed higher amounts of 3-DG with increasing levels of polyphenols but not for MGO or GO (Kocadağlı et al., 2015). 1,2-dicarbonyl compounds impact positively on flavour and colour of biscuits. Then, any modification in the biscuit recipe with a potential consequence on desirable sensorial attributes has to be carefully evaluated in a consumer acceptance test beyond their effects on health.

HMF is formed after 1,2 enolization, dehydration and cyclization reactions from sugar caramelization and Amadori product degradation at the advanced stage of the MR. In our samples, sucrose hydrolyses to yield glucose and fructose, which interconvert into each other through isomerization reactions. Formation of HMF in the different samples is depicted in Fig. 1. HMF content was in line with those reported for commercial biscuit in Spain and France, being 14.4 μg/g (Delgado-Andrade, Rufián-Henares, & Morales, 2009) and 24.1 μg/g (Ait-Ameur, Trystram, & Birlouez-Aragon, 2006), respectively. The control biscuit and biscuit formulated with phenol compounds showed a positive correlation between the HMF content and 3-DG content ($r = 0.950$, $p = 0.004$; *Supplementary material 2*). However, the recipes with OLE did not match this correlation since HMF was already present in the OLE sample due to the maceration under acid conditions and the rupture of carbonyl moiety of the glycosylated phenol compounds. The initial HMF content was 3.13 and 13.58 μg/g dough for OLE-1 and OLE-2, respectively, corresponding to 25 and 108 nmol/g d.m. If the initial amount of HMF in the dough is subtracted from the OLE samples, the formation of HMF and 3-DG after baking followed the similar correlation than CTL and samples fortified with HT, QE, and GA. During baking, 3-DG is also form from caramelization and MR. 3-DG as a stable intermediate can accumulate, due to delayed generation of HMF (Degen et al., 2012).

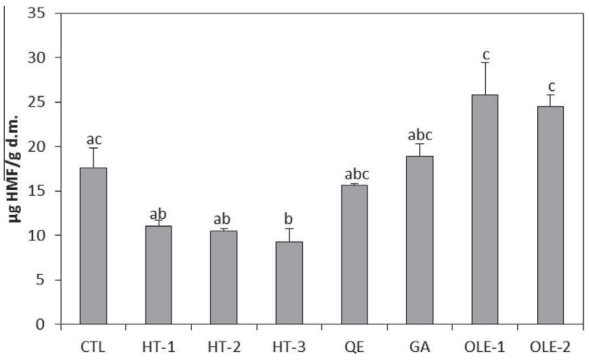

Fig. 1. Formation of hydroxymethylfurfural (HMF) in biscuit control (CTL) and formulated with hydroxytyrosol (HT), quercetin (QE), gallic acid (GA), or olive leaf extract (OLE). Sample description as in Table 1. Different letters mean significant differences ($p < 0.05$). Data expressed in dry matter (d.m.). Error bar denoted standard deviation.

Furosine is well-known marker of the transient Amadori product formed at the early stage of the MR. The formation of furosine was quantitatively very low and close to the limit of quantitation (20 mg/100 g). These results are in concordance with that reported by Gökmen, Serpen, Açar, and Morales (2008) where the levels of furosine were low in biscuits formulated with sucrose and sodium bicarbonate as leavening agent compared with glucose and ammonium bicarbonate. The usefulness of furosine for understanding the antiglycative activity of phenol compounds in biscuit is very limited due to the baking conditions applied and the moisture content that will accelerate the evolution of the MR to the advanced stage.

The Amadori product undergoes dehydration and fission and yields colourless reductones and fluorescent compounds in the advanced stage before the formation of colour. Compounds contributing to fluorescence have been identified as AGEs and they could be in free form and bound to the protein backbone (Delgado-Andrade et al., 2006). The ratio of the total and free Maillard-derived fluorescent compounds was dependent on the baking conditions but remains constant, nearly to 10.4-fold, at moderate baking conditions for wheat-flour but increased significantly in rice-flour samples (Calvarro, Gökmen, & Morales, 2009). Then, the formation of free Maillard derived fluorescent compounds was analysed in the different biscuit formulations. QE sample was the most effective against the formation of fluorescent AGEs (64.9% of inhibition) followed by HT that exhibited an inhibitory activity of 28.8% at the same concentration although its activity was not dose dependent. The biscuits added with GA and OLE showed an inhibitory activity below 10%, and there were not statistically different to the CTL sample (Fig. 2a). These results are in concordance with that reported by Zhang et al. (2014) who showed that QE was able to inhibit above 80% of fluorescent AGEs formation in a biscuit model. Similarly, Culetu, Fernandez-Gomez, Ullate, Del Castillo, and Andlauer (2016) identified that polyphenols enriched fractions from decaffeinated tea dust added in the formulation of bread allowed to reduce the formation of fluorescent AGEs.

To get more insight into the formation of specific AGEs in the biscuit samples, pentosidine, CEL, and CML were evaluated. Pentosidine is a protein-crosslinking fluorescent AGE that it is formed in reactions of pentoses with lysine and arginine, but also formed from glucose, fructose, or Amadori compounds, through an oxidative route (Henle et al., 1997). Level of pentosidine in the CTL sample was 0.90 ± 0.07 μg/g, corresponding to 17.5 ± 1.27 μg/g protein, which is in line with the levels reported up to 35 μg/g protein for bakery products (Henle et al., 1997). Fig. 2b depicts the formation of pentosidine in the different biscuit samples. A similar trend to that free fluorescent AGE was observed for samples formulated

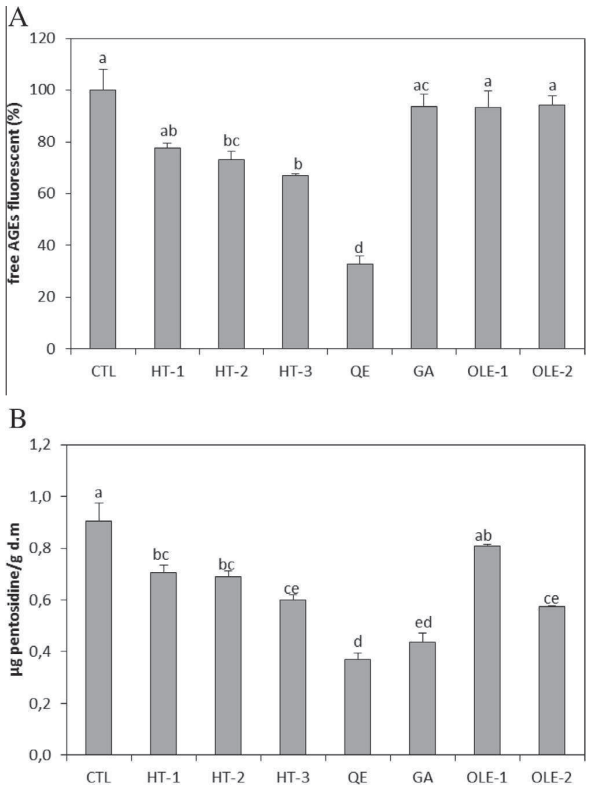

Fig. 2. Inhibitory capacity of hydroxytyrosol (HT), quercetin (QE), gallic acid (GA), or olive leaf extract (OLE) on the formation of free fluorescent AGEs (percentage with respect to CTL) (A), and formation of pentosidine (µg/g) in biscuit samples (B). Sample description as in Table 1. Different letters mean significant differences ($p < 0.05$). Data expressed in dry matter (d.m.). Error bar denoted standard deviation.

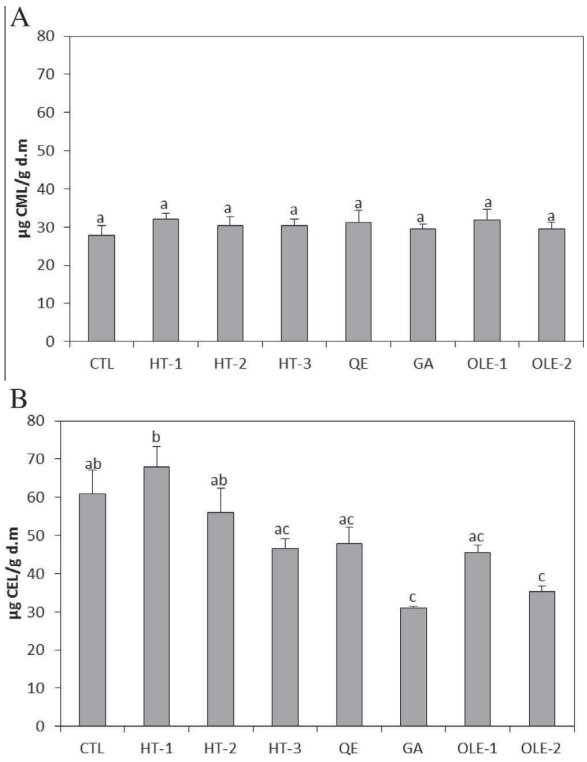

Fig. 3. Formation of Nε-carboxymethyl-lysine (CML) (A) and Nε-carboxyethyl-lysine (CEL) (B) in biscuits formulated with hydroxytyrosol (HT), quercetin (QE), gallic acid (GA), or olive leaf extract (OLE) and biscuit control (CTL). Sample description as in Table 1. Different letters mean significant differences ($p < 0.05$). Data expressed in dry matter (d.m.). Error bar denoted standard deviation.

with HT and QE. HT was able to inhibit 22.8% up to 34.3% in a concentration range of 3.08–12.31 mg HT/g while QE at 6.02 mg/g inhibited a 59.5% of the formation of pentosidine as compared with CTL. However, the sample formulated with GA was very efficient against the formation of pentosidine as opposite that result from free fluorescent AGEs, since pentosidine formation was inhibited by 52.2% versus 9.5% of inhibition of free fluorescent AGEs. Similarly, OLE-2 sample displayed a 37.3% of pentosidine inhibition but showed no activity against the free fluorescent AGEs. GA and OLE showed a clear preference against fluorescent AGEs linked to protein backbone.

Non-fluorescent AGEs such as CML and CEL were identified and quantified in biscuit samples. CML is formed by the reaction between GO and the epsilon-amino group of lysine. CML formation in CTL sample was 27.74 ± 2.55 µg/g, corresponding to 531.3 ± 48.9 µg/g protein. Fig. 3a depicts the formation of CML in the different biscuit samples. It was not found significant differences on the formation of CML among the biscuit samples with different phenol compounds. In this sense, our results are not in line with Peng et al. (2010) who reported that grape seed extract fortification inhibit CML formation generation in bread. That investigation was carried out in a bread model where the contribution of the lipid oxidation was residual as opposite to our biscuit model system. It is known that GO, CML formation precursor, has different routes of formation such as the oxidation of the fructosyl-lysine, the oxidation of glucose or lipid peroxidation. Then the contribution of the lipid oxidation to CML formation in our biscuit model cannot be excluded. Therefore, this fact could explain the results since there is an alternative source of reactants to the CML formation that cannot efficiently be delayed by the phenol compounds added in the formulation.

CEL is formed by the reaction between MGO and lysine. The amount of CEL in the different biscuit formulations was higher than CML and pentosidine probably due to MGO was the dicarbonyl more abundant in the biscuit models. In control biscuit, the CEL content was 60.91 ± 6.20 µg/g which is 2.2-fold higher than CML (27.74 ± 2.55 µg/g) and 67-fold higher than pentosidine (0.90 ± 0.07 µg/g). The information on the CEL amounts in food is very limited, but the results are in line with He, Zeng, Zheng, He, and Chen (2014) that reported levels from 12.16 to 50.70 µg/g for biscuits, although they depends on the biscuits ingredients and processing temperatures. The dough fortified with HT showed an inhibitory activity until 23.30% at the highest concentration and 21.50% when the dough was fortified with QE although without differences with respect to control biscuit. The biscuit fortified with GA and OLE (0.65 mg OLE/g) exhibited the highest activity entailing a decrease of CEL formation by 49.19% and 42.16% respectively (Fig. 3b).

In accordance to literature, the biscuit formulation, moisture and the binomial time-temperature applied are parameters to consider in the generation of AGEs/ALEs in bakery products. Our dough was formulated with sunflower oil, sucrose and sodium bicarbonate. Srey et al. (2010) established that the type of fat used in bakery products was more important than the presence of sucrose as precursor of CML formation, probably due to the generation of GO from lipid oxidation. It is worth noting that the fundamental

characteristic of seed oil is its high content of polyunsaturated fatty acids and specifically linoleic acid representing the 60% of total fatty acids. It is well-known that the polyunsaturated fatty acids are more vulnerable to the oxidation than monounsaturated or saturated fatty acids and this fact could lead to an increase of ALEs formation through GO generation. Nevertheless, although the levels of CEL formed were higher than levels of CML, the importance of other pathways for CEL formation are unknown and it should be explored. Our results pointed out that effectiveness of the antiglycative activity of HT, QE, GA, and OLE against the formation of CML could be reduced by the use of unsaturated oils as shortenings in the biscuit formulation.

On the other hand, it was reported that the losses of phenolic compounds by volatilization or thermal decomposition are the main causes of reduction of phenolic compounds content after being exposed to elevated temperatures (Soong & Barlow, 2004). This fact can be interpreted as a decline of their antioxidant activity against peroxidation lipid or sugar oxidation and therefore as a lack of inhibitory activity on the 1,2-dicarbonyl formation. Moreover, the effective inhibition of the formation of pentosidine and fluorescent AGEs by HT, QE, GA, and OLE could give an idea that the inhibition of oxidation of RM intermediates as Amadori Product towards the AGEs formation could be the main way of action. This conclusion agrees with the results of Zhang et al., 2014 that different polyphenols, including quercetin, were able to inhibit efficiently fluorescent AGEs. Results on OLE concentrated in HT (0.15 mg HT/mL OLE) proved to be more effective in the inhibition of AGEs/ALEs formation that the HT standard since the HT content in OLE was below 100 times and its inhibitory activity was more potent against pentosidine and CEL formation. The presence of other phenolic compounds with anti-AGEs capacity and the possible synergism that can be established between HT and these compounds can explain this enhanced antiglycative behaviour.

4. Conclusion

This investigation confirms that biscuit ingredients could modulate the formation of specific dietary AGEs/ALEs under controlled baking conditions. Biscuit formulated with HT in the range of 0.25–0.75% (w/w) reduced significantly the formation of free fluorescent AGEs and pentosidine, a characteristic fluorescent AGE linked to protein backbone. However, the use of polyunsaturated oils in the recipe is an additional source for short chain 1,2-dicarbonyls, mainly GO, during baking that will limit the efficacy of phenol compounds against the formation of CML. In parallel, it was clearly identified a direct relationship between the decreased of 3-DG and HMF. However the biscuits formulated with OLE, that contained levels 100-fold less of HT that the biscuit formulated with standard HT, was able to exert similar antiglycative activity for the pentosidine and higher for the CEL, AGE more abundant in our biscuit model. The OLE activity suggested that other structures could contribute synergistically to the antiglycative activity of HT. Before any recommendation to use ingredients rich in phenol compounds as a strategy for mitigation of dietary AGEs/ALEs in bakery products, it is necessary to identify its role in the formation of potentially harmful compounds, such as HMF.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.09.039.

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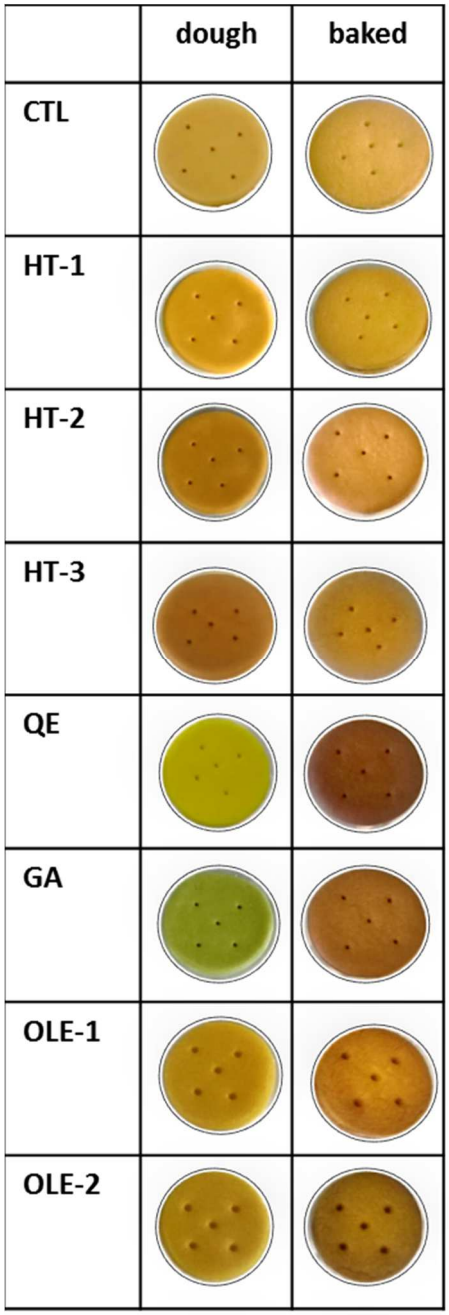
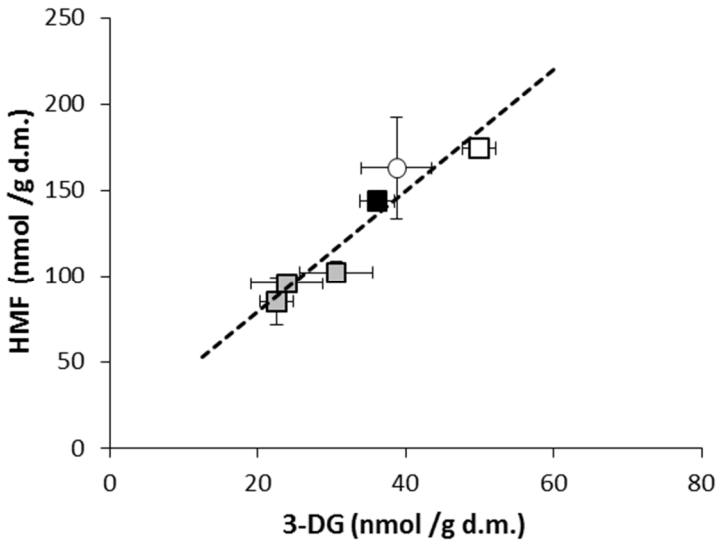
SUPPLEMENTARY MATERIAL

Supplementary material 1. Images of the doughs and the respective biscuits at 180ºC/20min. Control biscuit (CTL), and formulations with hydroxytyrosol (HT), quercetin (QE), gallic acid (GA), and olive leaf extract (OLE).



Supplementary material 2. Relationship between hydroxymethylfurfural (HMF, nmol/g d.m.) and 3-deoxyglucosone (3-DG, nmol/g d.m.) in biscuits control (empty circle) and formulated with hydroxytyrosol (grey square), quercetin (black square), and gallic acid (empty square). Data expressed in dry matter (d.m.). Error bar denoted standard deviation.



Capítulo 4: *Evaluación in vitro de la capacidad antiglicante de dos extractos de hoja de olivo con diferente composición fenólica. Estudio de la actividad antiglicante de un extracto de hoja de olivo concentrado en hidroxitirosol en un modelo celular de estrés carbonílico*

Introducción: El proceso de glicación en el organismo se desarrolla tanto a nivel celular como tisular y se encuentra acelerado en condiciones de hiperglucemia. La glicación se caracteriza por la formación de AGEs, que están asociados con el avance y complicaciones de determinadas enfermedades crónicas y, de manera genérica, con el envejecimiento. Es por ello que la investigación en busca de compuestos capaces de inhibir o mitigar la formación de AGEs haya experimentado un auge en los últimos años. En sus orígenes, los compuestos con actividad antiglicante han sido seleccionados en base a sus propiedades antioxidantes ya que algunas de las reacciones implicadas en la formación de AGEs son oxidativas. Sin embargo, la capacidad de atrapamiento de α-dicarbonilos al ser los promotores más potentes en la generación de AGEs, es otra vía antiglicante a considerar. Estudios previos de esta memoria de tesis apuntan a que las hojas de olivo pueden ser una fuente de compuestos antiglicantes. Sin embargo, la información sobre el efecto anti-AGEs *in vivo* o a nivel celular de los extractos de hoja de olivo es muy limitada o incluso inexistente.

Objetivos: Evaluación de la capacidad antiglicante *in vitro* y en un modelo celular de HepG2 de dos extractos de hoja de olivo (OLE) concentrados en OLEU o HT.

Metodología: Se propusieron dos extractos de hoja de olivo con diferente composición fenólica como fuente natural de compuestos fenólicos con capacidad antiglicante. La obtención de los extractos tuvo lugar tras una maceración etanólica seguida de una extracción por disolventes orgánicos (extracto OLE-A) y una acidificación adicional para la obtención del segundo extracto (extracto OLE-B). El objetivo de la última etapa de acidificación más intensa fue favorecer la degradación de las estructuras fenólicas más complejas como la OLEU en otras más sencillas como el HT. De esta manera, el extracto OLE-A se concentró en OLEU y el extracto OLE-B en HT. Se cuantificó el contenido en OLEU, HT, el contenido fenólico total y la capacidad antioxidante de los extractos OLE-A y OLE-B. Posteriormente se realizó una primera aproximación para evaluar la capacidad antiglicante *in vitro* mediante la inhibición de la formación de AGEs fluorescentes totales en tres modelos de glicación (BSA-GLC, BSA-MGO y BSA-GO) y se estimó la capacidad de atrapar α-dicarbonilos como posible mecanismo de acción. En todos los modelos de reacción se utilizaron como patrones de referencia HT y OLEU. Finalmente el extracto de hoja de olivo que presentó la mayor actividad anti-AGE (OLE-B) se seleccionó para el estudio de su efecto antiglicante en un modelo celular. Las HepG2 fueron seleccionadas como modelo fiable, bien caracterizado y ampliamente utilizado para estudios bioquímicos y nutricionales. Las células fueron incubadas durante 20 h con diferentes concentraciones de OLE-B (0.3-264 μg/mL) para estudiar el efecto sobre la viabilidad celular. Seguidamente el extracto fue fraccionado mediante extracción en fase sólida en función de la polaridad de sus constituyentes y se obtuvieron dos fracciones OLE-BF1 y

OLE-BF2. A continuación se evaluó el efecto de OLE-B (13 µg/mL), sus fracciones (OLE-BF1 y OLE-BF2) y un estándar de HT (5 µM) sobre la carbonilación de las proteínas celulares tras su preincubación con células HepG2 (20 h) y tras un estrés carbonílico inducido por MGO (2 mM, 6 h). La inhibición ejercida por OLE-B de la formación de CML, CEL y ArgP fue estimada a partir de las proteínas celulares sometidas a una hidrólisis ácida (HCl 6N, 110 ^{o}C, 20 h) y cuantificadas mediante HPLC-MS/MS (CML y CEL) o HPLC-fluorescencia (ArgP).

Resultados: Dos extractos de hoja de olivo con distinto perfil fenólico fueron obtenidos para investigar la influencia de dicho perfil en las propiedades antiglicantes del extracto. El estudio se centró en la OLEU y el HT ya que la OLEU es el principal compuesto fenólico presente en el extracto de hoja de olivo y el HT es su principal producto de degradación. El contenido en OLEU del extracto OLE-A fue de 93.9 mg/g OLE y de 1.82 mg/g OLE en el extracto OLE-B, mientras que este último presentó un elevado contenido de HT (54.5 mg/g OLE). OLE-B mostró un contenido fenólico total y una actividad antioxidante 3.4 y 1.5 veces superior al extracto OLE-A respectivamente, debido probablemente a la hidrólisis de los compuestos fenólicos más complejos en otros más sencillos. El efecto antiglicante de los extractos siguió una tendencia similar a su capacidad antioxidante, donde OLE-B ejerció nuevamente el mayor efecto (IC_{50} de 0.25-0.29 mg/mL) en los diferentes modelos de glicación *in vitro* ensayados. Como posible mecanismo de inhibición del proceso de glicación se estudió el atrapamiento de uno de sus promotores clave, el MGO. Como era de esperar, OLE-B mostró la mayor capacidad de atrapamiento (IC_{50} de 0.16 mg/mL) acorde a la actividad mostrada por el estándar HT que fue significativamente superior a la actividad de la OLEU (IC_{50} de 0.03 mg/mL frente a 0.08 mg/mL). En base a los resultados obtenidos, el efecto anti-AGE del OLE-B se evaluó en un modelo celular. Inicialmente se constató que un tratamiento de las HepG2 con OLE-B en un rango de concentración de 0.3-66 µg/mL no ocasionó ningún efecto sobre la viabilidad celular. Se seleccionó una concentración de 13 µg/mL, correspondiendo con un contenido de HT de 5 µM para la realización de los siguientes experimentos. Los resultados mostraron una reducción de un 21.1-29.6 % de la carbonilación de las proteínas celulares tras un pretratamiento de las células con OLE-B u OLE-BF1, fracción con mayor contenido en HT (41.4 mg/g OLE). Contrariamente, OLE-BF2 y el estándar de HT no redujeron significativamente la carbonilación de las proteínas celulares, por lo que posiblemente la acción en el extracto esté condicionada a los posibles sinergismos del HT con otros compuestos fenólicos de similar polaridad. La formación de CEL y ArgP, AGEs obtenidos a partir de MGO, fue de 278 y 213 µg/g de proteína respectivamente en las células sin pretratar, siendo reducida únicamente la formación de ArgP en un 26 % al pretratarse con OLE-B. En cuanto a la reducción de la formación de CML no se obtuvieron resultados concluyentes, puesto que su formación fue inferior al límite de cuantificación.

Conclusión: El extracto de hoja de olivo concentrado en HT (OLE-B) mostró una capacidad antioxidante y antiglicante en los modelos de glicación *in vitro* superior al extracto concentrado en OLEU (OLE-A). En el modelo celular HepG2, el extracto OLE-B no mostró citotoxicidad en un rango de concentración fisiológica y confirmó su actividad antiglicante en un modelo celular de estrés carbonílico, inhibiendo la carbonilación de proteínas celulares y específicamente la formación de ArgP. El estándar de HT y OLE-BF2 no presentaron inhibición de la carbonilación de las proteínas celulares, si bien OLE-BF1 ejerció una actividad similar a la del extracto original sin fraccionar, posiblemente debido a la presencia de otros compuestos con similar polaridad al HT que potencian su actividad. Esta investigación muestra por primera vez la efectividad de la actividad antiglicante de un extracto de hoja de olivo en un modelo celular de estrés carbonílico. Antes de recomendar su uso como nutracéutico, los resultados deben ser confirmados en modelos *in vivo*.

Olive leaf extract concentrated in hydroxytyrosol attenuates protein carbonylation and the formation of advanced glycation end products in human HepG2 cells

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ABSTRACT

The glycation takes place both at the cellular level and at the extracellular matrix and generates, consequently, advanced glycation end products (AGEs) associated with chronic diseases and the aging process. Two olive leaf extracts (OLE) concentrated in i) oleuropein (OLE-A; 93.9 mg oleuropein/g) and ii) concentrated in hydroxytyrosol (OLE-B; 54.5 mg hydroxytyrosol/g) were evaluated according to their antiglycative and antioxidant capacity *in vitro*. OLE-B exerted the highest anti-AGEs effect in different glycation models (IC_{50}: 0.25-0.29 mg/mL). OLE-B showed the highest antioxidant capacity and methylglyoxal-trapping capacity (IC_{50} 0.16 mg/mL). OLE-B showed a significant inhibitory effect against protein carbonylation (21%) and the generation of argpyrimidine (26%) in a hepatocyte cellular carbonyl stress model evoked by MGO. OLE-B was further fractionated by solid phase-extraction and the protection against protein carbonylation was only exerted by the fraction containing hydroxytyrosol. However, hydroxytyrosol standard, at the same concentration than in the extract, inhibited the protein carbonylation below 10% but not significantly. The results point to antiglycative activity of OLE in cells could be due to a synergic effect of hydroxytyrosol and other minor compounds with similar polarity. This investigation demonstrated by first time the antiglycative activity of an olive leaf extract in a cellular model in a situation of carbonyl stress. Forthcoming investigations are necessary to confirm these promising results in vivo before to propose OLE as a natural anti-AGEs agent.

Keywords: Olive leaf extract; hydroxytyrosol; oleuropein; antiglycative activity; advanced glycation end products; dicarbonyl compounds; human hepatoma HepG2

Abbreviations: Olive leaf extract (OLE); hydroxytyrosol (HT); oleuropein (Oleu); methylglyoxal (MGO); glyoxal (GO); carboxyethyl-lysine (CEL); carboxymethyl-lysine (CML); argpyrimidine (ArgP).

INTRODUCTION

Glycation involves a series of complex reactions that are initiated when a carbonyl group of a reducing sugar condenses with an amino group of protein, lipid or nucleic acid. The glycation in living bodies takes place both at the cellular level and at the extracellular matrix and culminates in the formation of advanced glycation end products (AGEs) (Thornalley, Battah, Ahmed, Karachalias, Agalou, Babaei-Jadidi, et al. (2003)).

AGEs have been associated with chronic diseases such as Alzheimer, cardiovascular diseases or diabetes mellitus and its complications, as well as with the aging process (Münch, Thome, Foley, Schinzel, and Riederer (1997); Nowotny, Jung, Hohn, Weber, and Grune (2015); Baynes (2001)).

Currently, the high incidence of these diseases has motivated numerous investigations conducted to search compounds capable of inhibiting the formation of AGEs generally, by the blockage of some key step of the glycation reaction (Chinchansure, Korwar, Kulkarni, and Joshi (2015)). Thus, the antiglycative properties most frequently reported have been the dicarbonyl trapping activity and antioxidant capacity through metal chelation or free radical scavenging (Peng, Ma, Chen, and Wang (2011)).

α-Dicarbonyls are the key precursors of AGEs and are generated *in vivo* during the glycation process, but also from other pathways such as lipid peroxidation, autoxidation of glucose or the glucose metabolism (Shangari and O'Brien (2004); Shao, Chen, Zhu, Sedighi, Ho, and Sang (2014)). Reaction of glyoxal (GO) and methylglyoxal (MGO) with lysine and arginine residues in proteins give rise to the formation of AGEs such as carboxymethyllysine (CML), carboxyethyllysine (CEL) and argpyrimidine (ArgP). Indeed, CML is formed from the condensation of lysine and GO or CEL and ArgP are formed from reaction between lysine or arginine with MGO, respectively. Therefore, the term "carbonyl stress" has been suggested to describe the unusual accumulation of reactive carbonyl species due to disturbance of their production or cellular metabolism. The accumulation of α-dicarbonyl leading to increased protein modification and formation of AGEs contributes to cell and tissue dysfunction in ageing (Miyata, Izuhara, Sakai, and Kurokawa (1999); (Rabbani & Thornalley, 2015)). The MGO has been considered as a common intermediate implicated in the AGE formation *in vivo* (Oya, Hattori, Mizuno, Miyata, Maeda, Osawa, et al. (1999)). In fact, it has been showed that MGO is 20000-fold more reactive than glucose in the glycation process (Thornalley (2005)). The concentrations of MGO in human plasma is 50-150 nmol/L increasing until 212 and 312 nmol/L in patients with diabetes and with diabetic nephropathy, respectively (Handl, Filova, Kubala, Lansky, Kolacna, Vorlicek, et al. (2007); (Rabbani & Thornalley, 2015)). Cells under normal physiological states are able to detoxify MGO to D-lactate by the glyoxalase system. However, during a situation of oxidative stress, intracellular levels of dicarbonyls can be increased, which could overwhelmed this enzymatic system leading to a cellular damage (Yang, Qiang, Delaney, Mehta, Bruce, and O'Brien (2011); Shangari and O'Brien (2004)).

It is well known the relationship of the Mediterranean diet with several health promoting effects, such as the lower incidence of cancer or cardiovascular diseases. The use of olive oil, the most characteristic component of this diet, plays a pivotal role in the beneficial effects which it is attributed to its lipids and phenolic profile (Bulotta, Celano, Lepore, Montalcini, Pujia, and Russo (2014);Cicerale, Lucas, and Keast (2010)). However, other by-products of olive obtained during the processing of olive oil, such as olive leaf or olive oil mill wastewater, are also characterized by their high content of phenolic compounds. Oleuropein (Oleu) and hydroxytyrosol (HT) are the most representative compounds of the phenolic fraction of olive leave and oil, respectively. Their biological and pharmacological effects have mainly been attributed to their antioxidant activity in several preclinical disease models (Bulotta, et al. (2014); Cicerale, et al. (2010); Visioli, Poli, & Gall, (2002)). Thus, several studies *in vivo* have evidenced a strong relationship between the antioxidant and hypoglycemic activities of the olive leaf extract (OLE), which have mainly been attributed to its high content of HT and Oleu (Jemai, El Feki, and Sayadi (2009); Wainstein, Ganz, Boaz, Bar Dayan, Dolev, Kerem, et al. (2012); Bulotta, et al. (2014)). However, it remains unknown whether constituents of the OLE could exert an antiglycative activity *in vivo*, and subsequently to reduce the reactivity of dicarbonyl compounds involved in the formation of AGEs.

Despite previous investigations have reported the effect of MGO on the protein carbonylation in hepatocytes (Shangari and O'Brien (2004); Dong, Banaich, and O'Brien (2010); Yang, Qiang, Delaney, Mehta, Bruce, and O'Brien (2011)), its implication on the formation of different AGEs has not been studied. Two olive leaf extracts concentrated in Oleu or HT were screened on their capacity to inhibit the formation of fluorescent AGEs and MGO-trapping in simulated

physiological conditions. Then, the most active extract was selected to evaluate in a HepG2 cellular carbonyl stress model evoked by MGO the potential inhibitory effect against protein carbonylation and generation of CML, CEL and ArgP.

MATERIALS AND METHODS

Materials and chemicals. Olive leaves (*Olea Europaea* variety Picual, Córdoba, Spain). D (+)-Glucose (GLC), bovine serum albumin (BSA), methylglyoxal (MGO, 40% aqueous solution), glyoxal (GO, 40% aqueous solution), aminoguanidine (AG), Oleuropein (Oleu), 5-methylquinoxaline (5-MQ), o-phenylenediamine (OPD), trolox (6-hydroxy-2,5,7,8-etramethylchroman-2-carboxylic acid), 2,4,6-tris(2- pyridyl)-s-triazine (TPTZ), 2-2´-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), heptafluorobutyric (HFBA), 2,4-dinitrophenylhydrazine, gentamicin, penicillin, streptomycin and crystal violet were provided by Sigma (St Louis, MO, USA). Folin–Ciocalteu reagent, iron (III) chloride and sodium dodecyl sulfate salt were purchased from Panreac (Madrid, Spain). Hydroxytyrosol standard (HT, purity > 99%) was acquired from Seprox Biotech (Madrid, Spain). Pyridoxamine (PM) was acquired from Fluka Chemical (Madrid, Spain). Glacial acetic acid and high performance liquid chromatography (HPLC)-grade methanol were purchased from Merck (Darmstadt, Germany). Carboxymethyl-L-lysine (CML, ≥ 97%), carboxyethyl-L-lysine (CEL, ≥ 97%), CML-d2, CEL-d4, and argpyrimidine (ArgP, ≥ 97%) were obtained from Laboratories (Strasbourg, France). Bradford reagent was from BioRad Laboratories S.A. (Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Lonza (Madrid, Spain), respectively. The Milli-Q water was obtained by an Elix3 water purification system coupled to a Milli-Q Advance 10 module (Millipore, Molsheim, France). All other chemicals and reagents were of analytical grade.

Equipment. Synergy™ HT-multimode microplate reader with an automatic reagent dispense from Biotek Instruments (Winooski, VT, USA). HPLC (Shimadzu, Kyoto, Japan) equipped with a quaternary pump (LC-20AD), an autosampler (SIL-20AHT), an oven (CTO-10ASVP), a diode-array detector (SPDM20A) and a fluorescence detector (RF-20AXS). LC-MS/MS was performed with a 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to a triple quadrupole (G6410B, Agilent Technologies) via electrospray ionization operating in positive mode.

Preparation of the Olive Leaf Extract (OLE). An OLE concentrated on phenolic compounds was obtained according to Lee, Lee, Lee, Lee, Son, Park, et al. (2009) with some modifications. Procedure is summarized in **Figure 1**. Fresh olive leaves (250 g) were gently washed in water and dried (40ºC for 2 days) in an air-forced oven. Then the leaves were ground and 10 g were mixed with 100 mL of ethanol:water (80:20 v/v) by duplicate and kept under shaking for a week in darkness at 37ºC. The supernatant was removed by paper filtration to get the olive leaf extract A (OLE-A). Additionally, the pH of half of the supernatant was readjusted to 2.5 with HCl and kept under shaking for another 3 hours under darkness at room temperature to obtain the olive leaf extract B (OLE-B). Both supernatants were dried in a vacuum evaporator (Strike 300, Steroglass, Perugia, Italy) and the dried fraction was extracted with hexane (25 mL, 3 times) and then with ethyl acetate (50 mL, 5 times). The ethyl acetate fraction (250 mL) of OLE-B was again kept in darkness and pH readjusted to 2.5 with HCl. Finally, ethyl acetate fractions were dried under vacuum and subsequently dissolved in methanol/water solution (60:40, v/v). The final concentration of OLE-A and OLE-B was 12 mg/mL.

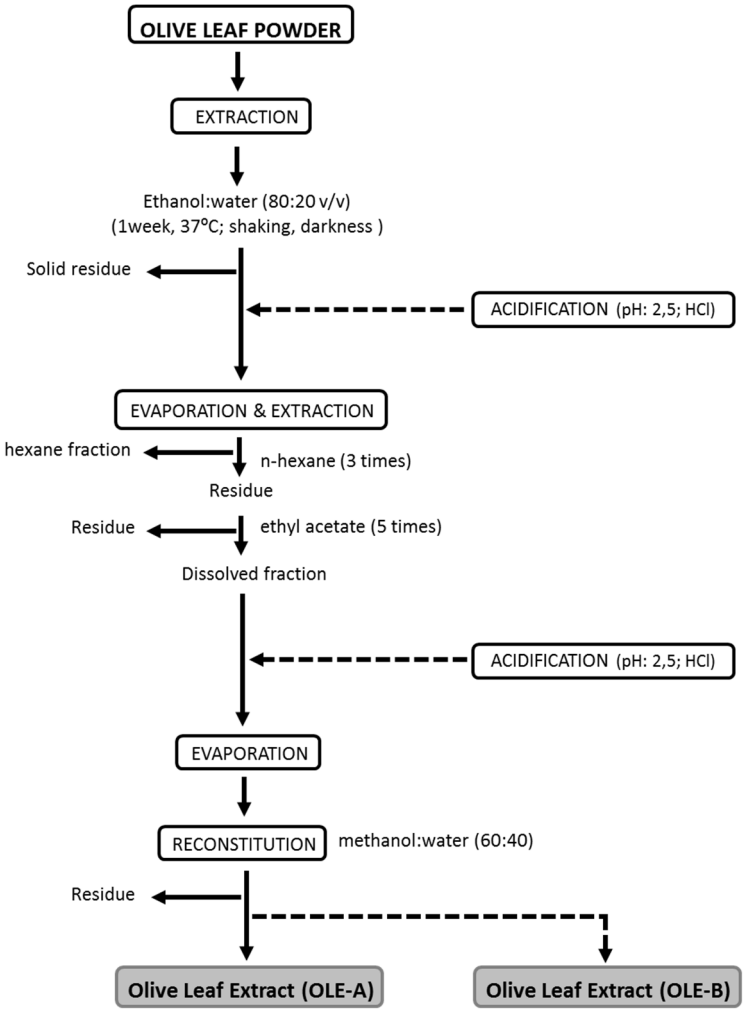

Figure 1: Schematic diagram of the obtention of olive leaf extracts concentrated in oleuropein (OLE-A) or hydroxytyrosol (OLE-B). Dotted line corresponds to additional steps on OLE-B.

Fractionation of the Olive Leaf Extract. OLE-B was further fractionated by reversed phase solid extraction (C18 SepPak cartridge, 1 mL, Waters Corporation, Milford, MA, USA). Briefly, 1 mL of OLE-B solution (12 mg/mL in metanol:water, 60:40, v/v) was directly loaded onto a pre-activated cartridge. The first eluate was collected and named as OLE-BF1. Then 1 mL of methanol was passed through the cartridge and a second fraction was collected (OLE-BF2). The procedure was repeated at least 10 times and the fractions were pooled, vacuum evaporated (Speed Vac, Savant SPD131, Thermo Scientific, Milford, MA, USA), and reconstituted in methanol:water (60:40, v/v).

Determination of phenolic compounds by HPLC-DAD. HT and Oleu in the OLE samples was determined by HPLC-DAD using a Shimadzu HPLC system. The samples were injected (10 µL) onto a Kinetex-C18 column (100 mm x 4.6 mm, 2.6 µm; Kinetex, Phenomenex, Torrance, CA, USA) operating at 0.6 mL/min. The mobile phases used were acetic acid in water (0.5%, phase A) and methanol (phase B). The running time was 30 min and the gradient method was as follows: 0 min, 5%B; 1min, 5%B; 20min, 60%B; 21min, 60%B;

22min, 5%B; 30min, 5%B. The chromatograms were recorded at 280 nm with a retention time of 5.4 min and 20.8 min for HT and Oleu, respectively. Calibration curves were obtained from pure standards.

Determination of total phenolic content. Total phenolic content (TPC) was measured by the Folin–Ciocalteu assay according to Singleton, Orthofer, and Lamuela-Raventós (1999). Results were expressed as mg gallic acid equivalent (GAE) per g sample and all measurements were performed in quadruplicate. The limit of quantification was set at 0.5 mg GAE per g sample.

Determination of antioxidant capacity according to ABTS assay. The antioxidant capacity of extracts by the ABTS assay was determined as described by Mesias, Navarro, Gokmen, and Morales (2013). Results were expressed as µmol Trolox equivalent antioxidant capacity (TEAC) per g sample. The limit of quantification was set at 1.1 µmol TEAC g−1 sample. All measurements were carried out in quadruplicate.

Determination of the antioxidant capacity according to FRAP assay. The ability to reduce Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex was determined according to the method described by Morales, Martin, Açar, Arribas-Lorenzo, and Gökmen (2008). Results were expressed as µmol Trolox equivalent antioxidant capacity (TEAC) per g sample. All measurements were carried out in quadruplicate.

In vitro glycation assay with bovine serum albumin induced by methylglyoxal, glyoxal and glucose. The glycation models of BSA with MGO (BSA-MGO assay), GO (BSA-GO assay) and GLC (BSA-GLC assay) were carried out as described by Mesias, et al. (2013) with slight modifications. The range of concentration of OLE-A and OLE-B in the mixture was 0.14-0.71 mg/mL and the concentrations of reactants were 0.23 mg/mL for MGO and GO, 100 mg/mL for GLC and 10 mg/mL for BSA. The formation of AGEs was characterized by fluorescence with excitation/emission at 340/420 nm for the BSA-MGO or BSA-GO and 360/420 nm for BSA-GLC. Results were expressed as percentage of inhibition of AGEs formation, and it was calculated according to following equation: Inhibition (%) = 1 - [(fluorescence of solution with inhibitor – intrinsic fluorescence of sample) / fluorescence of solution without inhibitor] x 100. The concentration required to inhibit the glycation by 50% (IC_{50}) was calculated from the dose-response curve using the Microsoft-Excel computer software.

Evaluation of direct MGO trapping capacity by HPLC-DAD. Direct MGO trapping capacity was determined as described by Mesias, et al. (2013) after MGO conversion into the respective quinoxaline derivative (2-MQ). The range of concentration of OLE-A and OLE-B in the mixture was 0.05-0.25 mg/mL for HT, and 0.01-0.1 mg/mL for Oleu. The incubation was carried out at 37ºC for 168 h in PBS (100 mM, pH 7.4). The amount of unreacted MGO was calculated from the ratio of 2-MQ and 5-MQ (internal standard) as compared with the control. The percentage of inhibition of MGO was calculated with the next formula: MGO decrease (%) = [(amount of MGO in control – amount of MGO in sample with OLE or phenol compounds standard)/amount of MGO in control] x 100. The concentration required to trap MGO by 50% (IC_{50}) was obtained from the dose-response curves using Microsoft-Excel computer software.

Cell culture and treatments. Human HepG2 cells were grown in DMEM containing 5.5 mM D-glucose and 2 mM glutamine and supplemented with 2.5% fetal bovine serum (FBS) and 50 mg/L antibiotics (gentamicin, penicillin and streptomycin). Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

To evaluate the protective effect of the OLE-B extract (13 µg/mL in methanol:water, 60:40 v/v), its fractions and HT (5 µM in methanol:water, 60:40 v/v) against MGO challenge (2 mM, 6 h), the mentioned substances were added to the cells for 20 h. To the control cells the methanol:water solution was added in the same proportion that was used to dissolve the tested sample. Then, the medium was discarded and fresh medium containing 2 mM MGO was added for additional 6 h. Later, cells were harvested and carbonyls were analysed.

Cell viability. The viability of HepG2 cells was determined using the crystal violet assay (Granado-Serrano, Martin, Izquierdo-Pulido, Goya, Bravo, and Ramos (2007). Cells were seeded in 96-well plates at low density (10^4 cells per well), grown for 20 h with different concentrations of olive leaf extract (0.3-264 µg/mL) and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water and 1% sodium dodecyl sulfate was added. The absorbance was measured at 570 nm using a SynergyTM HT-multimode microplate spectrophotometer. Results were expressed as the relative percentage of crystal violet stained control/untreated cells.

Determination of protein carbonylation. The content of protein carbonyl products generated by glycoxidation of hepatic cells was measured as previously described (Cordero-Herrera, Martin, Goya, and Ramos (2015)). Briefly, cells were lysed in PBS (pH 7.4) with an ultrasonic Processor (Vibra-Cell, Connecticut, USA). Then, the extracts were centrifuged (10000g, 15 min) and supernatants were collected. Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22000 nmol/L/cm. Protein concentration by using the Bradford reagent.

Determination of CML and CEL by LC-ESI-MS-QQQ. The formation of CML and CEL in cells was determined as described by Navarro and Morales (2016). Samples (10 µL) were separated on a porous graphitic carbon column (Hypercarb, 100 mm x 2.1 µm, 5 µm, ThermoFisher Scientific) at a flow rate of 0.5 mL/min. The isocratic elution was applied using a mobile phase of 5 mM NFPA / acetonitrile (95:5 v/v). The product ion at *m/z* 84 was used for quantification of CML (*m/z* 205), CML-d2 (*m/z* 207) and CEL (*m/z* 219) while m/z 88 was used for CEL-d4 (*m/z* 223). The ratio of response factor for CML or CEL to that of their respective labelled internal standards was used for quantitation. CML and CEL calibration was carried out in the range of 0.01-1 µg/mL and data were processed using MassHunter Data Acquisition and MassHunter Qualitative Analysis (Agilent Technologies). Results were expressed as µmol CML or CEL /g protein.

Determination of argpyrimidine by HPLC-fluorescence. The determination of ArgP formation in cells was carried out as described by Navarro and Morales (2016). Samples (10 µL) were eluted onto a Mediterranean-Sea-ODS2 column (250 mm × 4 mm, 5 µm; Teknokroma, Barcelona, Spain) at a flow rate of 0.8 mL/min. The mobile phase was performed with HFBA (1 mL/L) (solvent A) and ACN (500mL/L) containing HFBA (1 mL/L) (solvent B) under the following gradient elution: 0 min, 20% B; 25 min, 100% B; 26 min, 100% B; 27-37 min, 20% B. ArgP was detected at 335 nm and 385 nm for excitation and emission wavelength, respectively, and eluted at 15.1 min. Calibration was carried out in the range 0.01-0.5 µg/mL with pure standard. Results were expressed as µmol ArgP/g protein.

Statistical analysis. Statistical analyses were performed using the IBM SPSS Statistics program (version 21.0). Data were expressed as the mean value ± SD. Analysis of variance (ANOVA) and the Bonferroni test were applied to determine differences between means. Differences were considered to be significant at $P < 0.05$.

RESULTS

3.1. Quantification of main phenolic compounds in two olive leaf extracts and evaluation of their antioxidant activity.

Olea europaea L. leaf is considered a by-product of olive with a high content of phenolic compounds whose profile has been studied for their potential health benefits. Olive leaf is rich in secoiridoids, especially in oleuropein that constitute the main compound of interest. Other components may occur in appreciable amount such as hydroxytyrosol, oleoside, apigenin and luteolin derivatives as luteolin-7-*O*-glucoside or luteolin-4-*O*-glucoside (Peralbo-Molina, Priego-Capote, and Luque de Castro (2012); Quirantes-Pine, Lozano-Sanchez, Herrero, Ibanez, Segura-Carretero, and Fernandez-Gutierrez (2013)). On the basis of the above considerations two extracts of olive leaf with different content in Oleu and HT were obtained. The contents in Oleu, HT and an approximate composition of total phenolic (TPC), are summarized in **Table 1** for OLE-A and OLE-B.

	OLE-A	OLE-B
HT (mg/g OLE)	3.6 ± 0.2	54.5 ± 5.0
Oleu (mg/g OLE)	93.9 ± 12.8	1.8 ± 0.1
TPC (mg GAE/g)	93.8 ± 27.1[a]	320.8 ± 46.0[b]
ABTS (TEAC µmol/L)	1409.8 ± 46.4[a]	2061.1 ± 23.3[b]
FRAP (TEAC µmol/g)	2409.0 ± 55.1[a]	3603.6 ± 28.6[b]

Table 1: Hydroxytyrosol (HT), oleuropein (Oleu), total phenolic content (TPC), and antioxidant capacity of olive leaf extract-A (OLE-A) and olive leaf extract-B (OLE-B). Results are expressed as mean ± SD for n = 4. Different letters in the same row denote significant differences P < 0.05. LoQ < 0.5 GAE (mg/g) or <1.1 TEAC (µmol/g).

TPC in the OLE-B extract was 3.4-fold higher than OLE-A (320.8 ± 46.0 versus 93.8 ± 27.1 mg GAE/g). As expected, the OLE-A extract contained 93.9 mg of Oleu per gram of OLE while the OLE-B, which was additionally macerated with diluted HCl, included a high amount of HT (54.5 mg HT/g OLE) to the detriment of Oleu, which was reduced until 1.82 mg Oleu/g OLE.

Since there is an established relationship between the health beneficial effects and the antioxidant capacity of phenolic constituents (Poulsen, Hedegaard, Andersen, de Courten, Bugel, Nielsen, et al. (2013); Bulotta, Celano, Lepore, Montalcini, Pujia, and Russo (2014)), the antioxidant capacity of OLE was measured by ABTS and FRAP assays. The OLE-B showed an antioxidant capacity significantly higher than OLE-A for both *in vitro* assays.

3.2. Inhibition of the formation of fluorescent AGEs in vitro.

Previous studies have proved that several phenolic compounds present in olive leaves could exert a strong *in vitro* antiglycative capacity (Chinchansure, Korwar, Kulkarni, and Joshi (2015); Navarro and Morales (2016); Kontogianni, Charisiadis, Margianni, Lamari, Gerothanassis, and Tzakos (2013); Navarro, Fiore, Fogliano, and Morales (2015)). To gain further insight into the influence of the Oleu and HT concentration as the major phenolic compounds in the two extracts obtained (OLE-A, OLE-B) on their antiglycative capacity, a first screening was carried out through the formation of fluorescent AGEs in different models of glycation (BSA-GLU, BSA-MGO, BSA-GO) under simulated physiological conditions. The **Table 2** displays the concentration necessary of extract to inhibit the 50% of the formation of fluorescent AGEs (IC_{50}). OLE-B reached an IC_{50} from 0.248 to 0.294 mg/mL in all the glycation model systems, whereas OLE-A reached a concentration from 0.425 to 0.462 mg/mL for the BSA-MGO and BSA-GO respectively, and 0.716 mg/mL for BSA-GLC. Antiglycative capacity of OLE-B was significantly higher than OLE-A for the three models of glycation. Additionally, the antiglycative activities of OLE-A and OLE-B kept a similar trend when compared to their antioxidant activity; therefore, it is likely that the antiglycative capacities of OLE-A and OLE-B extracts could be related to their antioxidant capacity.

	OLE-A	OLE-B
BSA-MGO IC_{50} (mg/mL)	0.425 ± 0.008[a]	0.259 ± 0.002[b]
BSA-GO IC_{50} (mg/mL)	0.462 ± 0.039[a]	0.248 ± 0.011[b]
BSA-GLC IC_{50} (mg/mL)	0.716 ± 0.012[a]	0.294 ± 0.003[b]

Table 2: Antiglycative capacity of olive leaf extract-A (OLE-A) and olive leaf extract-B (OLE-B) against the formation of fluorescent AGEs in BSA-MGO, BSA-GO and BSA-GLC models. Results are expressed as concentration of sample that exerts the 50% inhibition of the glycation. Different letters in the same row denote significant differences P < 0.05. Aminoguanidine (0.57 mg/mL) was used as a positive control and presented an inhibition of 98.4%, 93.9% and 88.5% for BSA-MGO, BSA-GO and BSA-GLC models, respectively.

3.3. α-dicarbonyl-trapping capacity of olive leaf extract.

MGO is a key promoter of the glycation process and it could be formed from oxidation of glucose

and lipids, as well as intermediates of the glycation process to finally generate AGEs**. Figure 2** depicts the MGO-trapping capacity of the extracts.

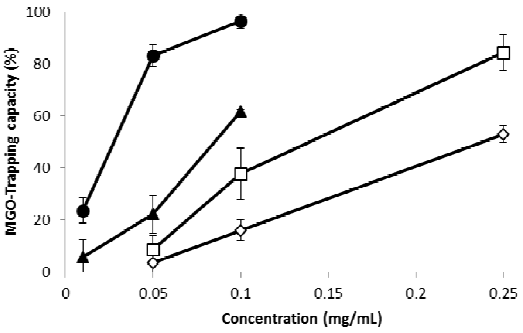

Figure 2: MGO-trapping capacity of hydroxytyrosol (HT) and oleuropein (Oleu) standards (0.01-0.1 mg/mL) and olive leaf extracts (OLE-A and OLE-B) (0.05-0.25 mg/mL) after incubation at 37°C for 168 h. Results are expressed as mean ± SD for n = 4. Pyridoxamine (0.1 mg/mL) was used as a positive control and presented MGO-trapping capacity of 99.5%. HT (solid circle), Oleu (solid triangle), OLE-A (open box), OLE-B (open diamond)

OLE-A and OLE-B extracts showed a dose-dependent response to MGO-trapping. The IC_{50} were 0.238 and 0.159 mg/mL for OLE-A and OLE-B, respectively. MGO-trapping capacity of OLE-B was significantly higher than OLE-A. Furthermore, the α-dicarbonyl-trapping capacity of the major phenolic compounds in OLE-A and OLE-B was also evaluated with standards. The Oleu and HT standard displayed an IC_{50} value of 0.085 mg/mL and 0.028 mg/mL respectively. The MGO-trapping activities of pure compounds were significantly more efficient than OLE-B and OLE-A at the same concentration although the Oleu and HT content in OLE-A and OLE-B extracts reached the IC_{50} value at 0.025 and 0.008 mg/mL, respectively. Therefore, the relative concentrations of Oleu and HT in the extracts were significantly lower than the IC_{50} value reached by their respective standards.

3.4. Treatment of HepG2 cells with olive leaf extract

3.4.1. Cell viability.

In view that OLE-B extract showed the highest antiglycative, carbonyl-trapping and antioxidant capacities in the *in vitro* models at simulated physiological conditions, OLE-B extract was selected in order to investigate the antiglycative activity in a hepatocyte cellular model. The human HepG2 cells have been used previously by other authors for the study of protein carbonylation and this cell line has been widely used for biochemical studies to be considered a well characterized model (Goya, Mateos, and Bravo (2007); Shangari and O'Brien (2004); (Yang, Qiang, Delaney, Mehta, Bruce, and O'Brien (2011)).

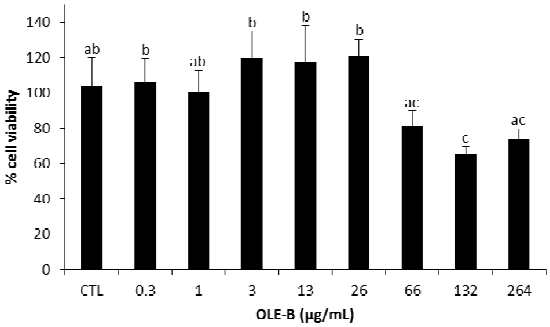

Figure 3: Effect of olive leaf extract-B (OLE-B) on HepG2 cell viability after a 24 h-treatment with different concentrations (0.3-264 mg/mL). Cell viability is expressed as relative percentage of control cells stained with crystal violet. Different letters denote statistically significant differences (P<0.05)

After a 24 h-treatment with different concentrations of OLE-B (0.3-264 µg/mL) cell viability was evaluated by the crystal violet assay. As shown in **Figure 3**, OLE-B did not provoke cell injury at the range of concentration from 0.3 to 66 µg/mL, which corresponds to 0.1-25 µM HT. However, a significant decrease in the number of viable cells was observed at higher concentrations of OLE-B. An intermediate concentration of 13 µg/mL was selected for further experiments. These results are in line with those of Goya, et al. (2007) reported for pure HT which did not produce cell injury in HepG2 below 50 µM.

3.4.2. Inhibition of protein carbonylation in HepG2 cells induced by methylglyoxal

To elucidate the potential inhibitory effect of HT on the protein carbonylation a hepatocyte cellular model of carbonyl stress induced with a MGO (2 mM) was assessed.

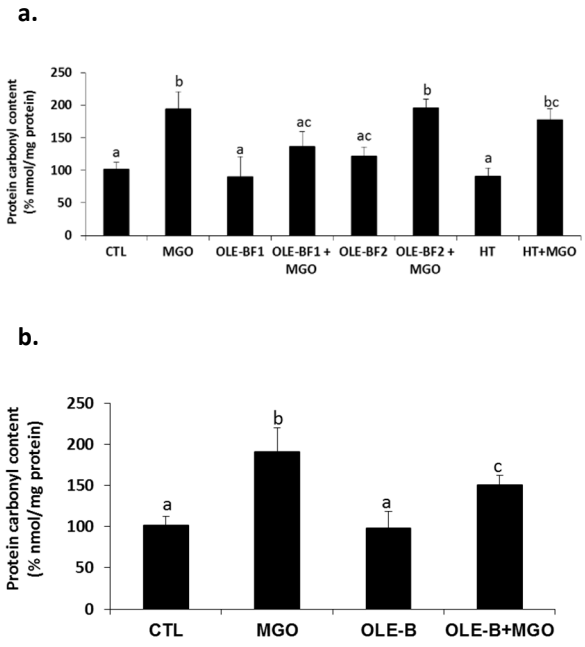

Figure 4: Protective effect of olive leaf extract-B at 13 µg/mL (OLE-B) [A]; and OLE-B fraction-1 (OLE-BF1), OLE-B fraction-2 (OLE-BF2) and hydroxytyrosol (HT, 5 µM) [B] on the hepatocyte protein carbonylation induced by methylglyoxal (MGO) in HepG2 cells. Results are expressed as protein carbonyl content per mg of protein. Different letters denote statistically significant differences (P<0.05)

As shown in **Figure 4a**, MGO evoked a protein carbonylation nearly 2-fold higher than in HepG2 control cells. The incubation of cells with 13 µg/mL of OLE-B showed no significant differences with respect to the controls, but the protein carbonylation was significantly decreased (21.15%) when cells were previously treated with OLE-B and later submitted to MGO challenge. OLE-B showed a protective effect on the protein carbonylation induced by MGO in HepG2 cells.

OLE-B was further fractionated under solid-phase extraction into fraction-1 (OLE-BF1) and fraction-2 (OLE-BF2). OLE-BF1 contained the higher fraction of HT (41.42 mg/g OLE) and other minor constituent with similar polarity. OLE-BF2 stands out for its content in compounds less polar and therefore low HT content (7.63 mg/g OLE). Then the potential inhibition of the carbonyl stress evoked by MGO in HepG2 cells treated with OLE-BF1 and OLE-BF2 was assessed (**Figure 4b**). As happened with the OLE-B, OLE-BF1 and OLE-BF2 alone did not affect to the carbonyl protein content when compared to untreated control cells. After incubating the cells with MGO, OLE-BF1 was able to reduce significantly the protein carbonylation similarly to the unfractionated extract. However, OLE-BF2 was not able to protect the cell against protein carbonylation. The result confirms that the fraction concentrated in HT, OLE-BF1, was responsible of the protection at the cellular level. In order to gain more insight, the effect of pure HT at the same concentration in which is present in the OLE-B was evaluated. The results showed an inhibition below 10% being not statistically significant.

3.4.3. Inhibition of CML, CEL and ArgP formation in HepG2 cells

To further elucidation of the antiglycative properties of OLE in HepG2 as cellular model the levels of specific AGEs was assessed. As shown, MGO provoked carbonyl stress in HepG2 cells; thus, the AGEs formed from MGO, such as CEL and ArgP were the most representative in this model. CEL, is a non-fluorescent AGE generated from lysine and MGO, whereas ArgP is a fluorescent AGE originated from the reaction between two molecules of MGO and arginine. On the other hand, CML formed by condensation of a lysine with GO, was also investigated to be considered as marker of glycation in many biological systems and food. The formation of the mentioned AGEs in cells under physiological conditions (untreated control cells) was lower than their respective values of LOQ (0.79 µg/mL, 0.24 µg/mL and 0.38 µg/mL for CEL, ArgP and CML, respectively). After the incubation of HepG2 cells with MGO, CEL values was 278 µg CEL/g protein, and the formation of ArgP was slightly lower (213 µg ArgP/g protein). OLE-B (13 µg/mL) was able to prevent the ArgP formation in 26% but did not inhibit CEL formation **(Figure 5).** As expected, the formation of CML was lower than its LOQ value (data not shown).

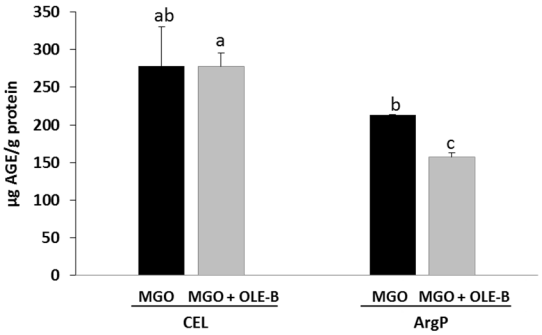

Figure 5: Antiglycative effect of olive leaf extract-B (OLE-B, 13 µg/mL) on the formation of carboxyethyllysine (CEL) and argpyrimidine (ArgP) induced by MGO in HepG2 cells**.** Results are expressed as mean ± standard deviation for n=3. Different letters denote significant differences ($P < 0.05$)

Discussion

A considerable number of studies have evidenced the relationship between phenolic compounds derived from the olive leaves and the health beneficial effects such as antihypertensive, anticarcinogenic, anti-inflammatory, hypoglycemic, antimicrobial and hypocholesterolemic properties (Kawaguchi, Matsumoto, and Kumazawa (2011); Hayes, Allen, Brunton, O'Grady, and Kerry (2011); de Bock, Thorstensen, Derraik, Henderson, Hofman, and Cutfield (2013)). Recently, it has been reported that several phenolic compounds naturally present in this by-product, such as luteolin, rutin, HT, verbascoside and Oleu, displays antiglycative activity (Kontogianni, Charisiadis, Margianni, Lamari, Gerothanassis, and Tzakos (2013), Pashikanti, de Alba, Boissonneault, and Cervantes-Laurean (2010)).

In the present study, two olive leave extract with different content in the major phenolic compounds were prepared. An extract was concentrated in Oleu (OLE-A) and the second extract was concentrated in HT (OLE-B). It is known that Oleu and HT exert hypoglycemic, hypolipidemic and antiatherogenic effects, which have been attributed to its antioxidant activity (Jemai, El Feki, and Sayadi (2009); (Visioli, Galli, Bornet, Mattei, Patelli, Galli, et al., 2000)). However, few studies have established a relationship between health beneficial properties and both antioxidant and antiglycative abilities through the reduction of the reactive dicarbonyl formed by the oxidative stress (Poulsen, et al. (2013)). In a previous study, Navarro and Morales (2016) reported that HT exerted an antiglycative activity in several *in vitro* models and specifically an inhibition of CML, CEL and ArgP formation through the dicarbonyls-trapping. In addition, it should be taken into account that Oleu is an ester of elenolic acid with HT so that the differences in Oleu and HT content between extracts could be due to the breakdown of Oleu into HT under mild acidic conditions used for the preparation of the OLE-B. On the basis of the above considerations, the extract concentrated in HT displayed the highest antioxidant, antiglycative and carbonyl-trapping capacity. These results are in line with those reported by Navarro and Morales (2015) in whose study the olive mill wastewater exerted an antiglycative capacity by direct α-dicarbonyls-trapping and was mostly attributed to the high content of hydroxytyrosol and secondly to the presence of verbascoside among others.

This investigation demonstrated by first time the antiglycative activity of an olive leave extract in a cellular model in a situation of carbonyl stress. The carbonylation of proteins, as well as the ArgP formation evoked by MGO were significantly reduced by preincubating the cells with OLE-B. In previous studies, where GO and MGO triggered hepatocyte protein carbonylation, several dicarbonyl scavengers such as aminoguanidine and penicillamine were assayed in order to prevent the protein carbonylation and cytotoxicity (Yang, et al. (2011)). However, studies with natural extracts carried out to elucidate a similar antiglycative effect in *in vitro* and *in vivo* assays have acquired special relevance to avoid the side effects of synthetic drugs such as aminoguanidine. Recent studies suggested that olive oil administration reduced the inflammatory response, peroxidation of lipids and carbonylated proteins in mice that have been chronically stressed and also stimulated the wound healing of pressure ulcers (Rosa Ados, Bandeira, Monte-Alto-Costa, and Romana-Souza (2014); Donato-Trancoso, Monte-Alto-Costa, and Romana-Souza (2016)). In the OLEA project, the olive leaf extract has been the target of several investigations where it has been reported that olive leaf extract

exerted cytoprotective and anti-inflammatory effects. In addition, this extract demonstrated a better protection against β-cell toxicity at a lower concentration than the doses tested for each isolated phenolic compound, such as Oleu or HT, pointing to the potential synergic effects of phenolic compounds (Ergin, Hariry, and Karasu (2013)).

In accordance to literature, olive mill waste polyphenols and especially HT significantly reduced hyperglycemia and oxidative stress produced by diabetes in rats (Hamden, Allouche, Damak, and Elfeki (2009)). In the same way, the administration of olive leaf extract rich in HT and Oleu (8 and 16 mg/kg body weight for 4 weeks) to alloxan-diabetic rats showed an antidiabetic activity attributed to its antioxidant effects, since it decreased the glucose and cholesterol levels and was able to restore the antioxidant defense system (Jemai, et al. (2009)). Perez-Herrera, Delgado-Lista, Torres-Sanchez, Rangel-Zuniga, Camargo, Moreno-Navarrete, et al. (2012) described in obese individuals that oils rich in phenols (0.45 mL/kg body weight) inhibited of NFκB in the postprandial state and, consequently, reduced the postprandial inflammation. Additionally, their beneficial effects were attributed mainly to the HT content, major compound in the hydrolysate olive leaf extract tested.

The HT was tested at realistic concentrations which are within the range recommended for *in vitro* studies (0.1-10 μM) (Kroon, Clifford, Crozier, Day, Donovan, Manach, et al. (2004)). At the concentration of 5 μM, a pure standard of HT did not inhibit the carbonylation of proteins induced by MGO. However, OLE-B at a concentration that corresponds to the dose of HT (13 μg/mL OLE-B contains 5 μM HT) exerted a slight decrease in the levels of protein carbonylation probably due to synergisms of HT with other minor phenol compounds present in the extract. In agreement to literature, other minor phenolic compounds present in the olive leaves such as luteolin, apigenin or gallic acid have been described as scavenging of reactive dicarbonyl species (Shao, et al. (2014)). In this line, it is reasonable to assume that HT is just one of the many bioactive substances present in the extract and that the synergic effects of phenolic compounds in foodstuffs should be taken into account. At this point it should be noted that the possible synergism established between the more polar phenolic compounds as HT present in OLE-BF1 could be exerting the inhibitory activity of HepG2 protein carbonylation not being the constituents of OLE-BF2 relevant for this activity. However, to elucidate whether the health beneficial properties of OLE-B were exerted by HT or the compounds derived from its metabolism require further studies.

In vivo and *in vitro* studies have demonstrated that the HT and related compounds are readily bioavailable. It has been shown that HT is absorbed in a dose-dependent manner after ingestion and its absorption depends on the administration matrix (Cicerale, et al. (2010)). In this line, studies in HepG2 and Caco-2 cells, as well as clinical trials in humans and animals, have demonstrated that HT is metabolized and absorbed (Mateos, Goya, and Bravo (2005), Tuck and Hayball (2002)). In addition, (Visioli, et al. (2000)) reported a HT excretion in humans of 30-60% of HT administered. Further, it is known that HT can be excreted unchanged or as metabolite. Indeed, the metabolites generated, such as its sulfate or glucuronide conjugate, homovanillic acid, homovanillic alcohol, 3,4-dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxyphenylacetaldehyde have been described. Homovanillic acid and homovanillic alcohol demonstrated to be strong radical scavengers while DOPAC displayed a boosted antiglycative capacity in physiological conditions *in vitro*.

In conclusion, the olive leaf extract concentrated in HT (OLE-B) demonstrated a boosted antiglycative and antioxidant capacity with respect to olive leaf extract concentrated in Oleu (OLE-A). Additionally, the antiglycative activity of OLE-B was evaluated in a HepG2 cell model no showing cytotoxicity within a realistic range of concentration and exhibiting a slight inhibition of protein carbonylation, and a selective inhibition of the formation of ArgP under a carbonyl stress condition. The fractionation of the OLE-B evidenced that OLE-BF1, fraction containing HT among other minor constituents, presented a

reduction of protein carbonylation similar to that of OLE-B. This inhibitory effect was not observed in OLE-BF2 or HT standard at the same concentration. It is plausible that the antiglycative activity of OLE in cells could be due to a synergic effect of HT and other minor phenolic compounds. However, further research is necessary to confirm the antiglycative activity *in vivo* and its mechanism of action before being proposed as a natural AGE inhibitor.

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Conflict of Interest

The authors declare no conflict of interest.

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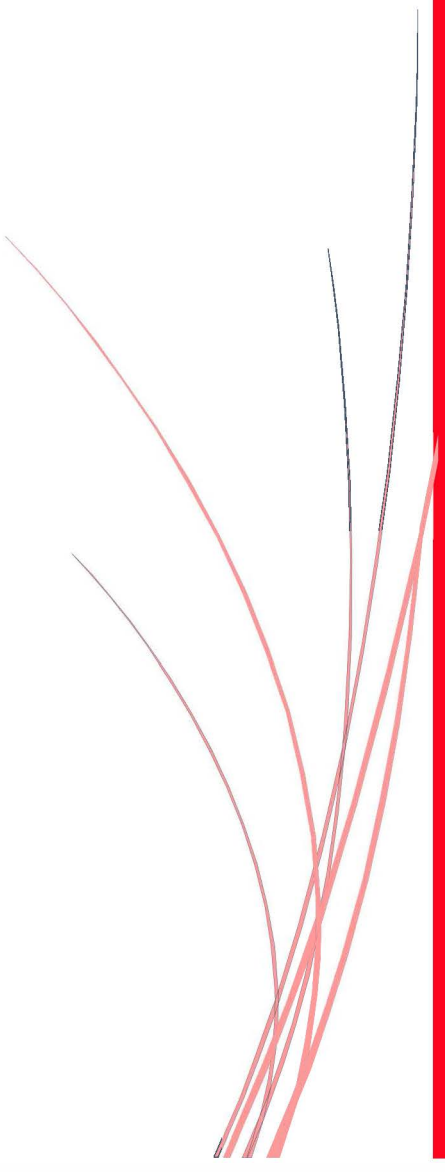
VI. DISCUSIÓN INTEGRADORA



1. Búsqueda de potenciales inhibidores naturales del proceso de glicación

La glicación constituye un complejo proceso de reacciones no enzimáticas que tienen lugar a nivel de células, órganos o tejidos. Es una de las reacciones más comunes de modificación de proteínas biológicas, afectando aproximadamente al 0.1-0.2% de los residuos de lisina y arginina (Garay-Sevilla y col., 2016). La glicación se considera un proceso lento, que culmina en la formación irreversible de los productos de la etapa avanzada de la glicación, los AGEs. Estos productos se han asociado con el desarrollo y las complicaciones de numerosas enfermedades crónicas que hoy día son consideradas enfermedades de alta prevalencia en las sociedades desarrolladas, como la diabetes mellitus, las enfermedades cardiovasculares y el Alzheimer. Como se ha mencionado en la revisión bibliográfica, los AGEs se acumulan durante el proceso de envejecimiento, acopio que se ve incrementado tanto en condiciones de hiperglucemia como por una deficiente clarificación renal. Las proteínas del cristalino o el colágeno son una muestra de las proteínas dianas que padecen las consecuencias del acúmulo de AGEs debido a su bajo recambio, al ser proteínas de vida media larga, pero también al contener un número elevado de residuos de lisina, hidroxilisina y arginina (Odjakova y col., 2012).

Debido a las evidentes repercusiones negativas para la salud del desarrollo de la glicación, las investigaciones se centraron en la búsqueda de productos naturales con efectos inhibitorios en la formación de AGEs. De esta manera se salvan los efectos secundarios que presentaban los primeros productos de síntesis propuestos por la industria farmacéutica. Estudios *in vitro* han atribuido efectos antiglicantes a diversos extractos naturales ricos en polifenoles, oligo y polisacáridos, carotenoides o ácidos grasos insaturados (Kokila y col., 2010; Sun y col., 2010; Chinchansure y col., 2015). Durante miles de años, las plantas han sido utilizadas en la medicina tradicional como remedio de múltiples enfermedades. En este sentido, numerosas especies del reino vegetal se han investigado por sus propiedades hipoglucemiantes, hipolipemiantes y antioxidantes, entre otras, todas ellas implicadas en el potencial efecto preventivo de la diabetes y sus complicaciones (Odjakova y col., 2012). Sin embargo, dado el carácter multifactorial de enfermedades crónicas como la diabetes, o incluso del proceso de envejecimiento, las investigaciones actuales buscan nuevos mecanismos de acción a través de los cuales los compuestos con efectos beneficiosos o preventivos puedan actuar. En consecuencia, la actividad antiglicante de determinadas sustancias obtenidas a partir de extractos naturales ha suscitado un gran interés dada la relación establecida entre la acumulación de AGEs y el agravamiento de patologías asociadas a la diabetes.

Los polifenoles son un grupo amplio y diverso de compuestos bioactivos que están mayoritariamente presentes en frutas, vegetales, cereales, semillas y bebidas como el café, vino y

té. Estos compuestos han sido estudiado en profundidad desde muchos ámbitos y se les han atribuido efectos beneficiosos frente al cáncer, enfermedades neurodegenerativas, cardiovasculares y diabetes (Tripoli y col., 2005; Nair y col., 2006; Viuda-Martos y col, 2010; Vilaplana-Pérez y col., 2014). Quizás la propiedad antioxidante de los polifenoles ha sido la actividad biológica más investigada en este grupo de compuestos, por lo que fue considerada la explicación más probable para justificar la capacidad antiglicante en los primeros trabajos en el área. Sin embargo, la capacidad antioxidante no explica por si sola la capacidad antiglicante de los compuestos fenólicos, ya que el entramado de reacciones que conllevan a la formación de AGEs es muy complejo. Los mecanismos de acción por los que actúan los compuestos antiglicantes pueden implicar a una o varias de las tres etapas de la glicación.

Durante la etapa inicial, la condensación de la glucosa con los restos amino de la proteína y la posterior reorganización hacia las correspondientes bases de Schiff y productos de Amadori, es un proceso oxidativo que implica la generación de radicales libres y la formación de compuestos α-dicarbonilos. Por tanto, las estrategias antiglicantes para actuar en la etapa inicial de la glicación estarán dirigidas a reducir la glucosa en sangre como principal reactante *in vivo*, y al bloqueo de radicales hidroxilo y superóxido que son catalizadores del proceso (Kocadaglı y col., 2016). Se han descrito una amplia variedad de fuentes naturales que inciden en la etapa inicial de la glicación. Jain y col. (2009) reseñaron que una suplementación de curcumina redujo significativamente los marcadores de inflamación vascular como consecuencia de una disminución de los niveles de glucosa en sangre, de la HbA_{1C} y del estrés oxidativo en un modelo de ratas diabéticas. Otros ejemplos destacados son el tomate, cuyo contenido en compuestos bioactivos antioxidantes inhiben la oxidación de la glucosa, (Kiho y col., 2004) o la canela, capaz de mimetizar la actividad de la insulina (Peng y col., 2008).

En la etapa intermedia de la glicación, la actuación preventiva estaría centrada en el atrapamiento de los α-dicarbonilos generados a partir de productos de Amadori, bases de Schiff u otras vías alternativas como la oxidación de la glucosa o la peroxidación lipídica. El atrapamiento de α-dicarbonilos hoy día se considera un mecanismo de antiglicación importante debido, en parte, a la gran relevancia que tienen estos compuestos en el avance de la glicación y como nexo de unión con otras vías para la formación de AGEs. Nuevamente, en este punto del proceso, compuestos que incidan tanto en el bloqueo de radicales libres como en la quelación de metales de transición involucrados en los procesos oxidativos deben ser considerados como potenciales agentes antiglicantes. Algunos polifenoles presentes en la manzana, té verde, uva tinta o soja presentan la capacidad de quelar metales o atrapar α-dicarbonilos como mecanismos de inhibición de la carbonilación de proteínas (Lo y col., 2006; Harsha y col., 2016; Rysman y col., 2016; Wang y col.,

2016). En nuestro cribaje inicial de extractos hidrosolubles de subproductos agroalimentarios (*Artículos 1, 3, 4*) como la cascarilla del grano de café, alpechín o cascarilla de la semilla de granada, se concluyó que la capacidad de atrapamiento de MGO podría ser considerado uno de los principales mecanismos de acción antiglicante junto con la capacidad antioxidante. El efecto antiglicante fue atribuido al contenido fenólico y especialmente al contenido mayoritario en ácidos clorogénicos, hidroxitirosol y ácido gálico, respectivamente. En línea con estas observaciones, Liu y col. (2014) reportaron que un extracto de granada y sus constituyentes fenólicos mayoritarios, la punicalagina, el ácido elágico y el ácido gálico, ejercieron un efecto anti-AGEs más potente que la aminoguanidina. Otros autores destacaron el potente efecto antiglicante de diferentes extractos obtenidos a partir del alpechín y del grano de café. La capacidad antiglicante fue mayoritariamente justificada a través de la capacidad de sus componentes fenólicos de atrapar compuestos α-dicarbonilos y ROS (Verzelloni y col., 2011; Troise y col., 2014).

Una vez avanza la glicación, los AGEs quedan ligados de manera irreversible a la estructura de la proteína. En este punto las investigaciones se centran por una parte en romper los entrecruzamientos con proteínas y por otra, en bloquear la interacción de los AGEs con RAGE. Como muestra de este planteamiento, recientemente se ha constatado que el licopeno, un carotenoide característico del tomate, mostró una protección parcial frente al desarrollo de nefropatía diabética al detener el eje AGE-RAGE y por tanto reducir el estrés oxidativo y la respuesta inflamatoria (Tabrez y col., 2015).

Sin embargo, no hay que olvidar que los inhibidores de la glicación podrían estar actuando simultáneamente en varias etapas de la reacción, por lo que diferentes mecanismos de acción para un mismo extracto o compuesto investigado en esta memoria son factibles. En el *artículo 4* el extracto acuoso obtenido a partir de alpechín destacó por su composición fenólica, considerándose al hidroxitirosol y al verbascósido como los principales responsables del efecto anti-AGEs. En la etapa inicial de la glicación, el extracto fue capaz de inhibir de manera dosis-dependiente la formación de productos de Amadori. La capacidad antioxidante de los extractos podría estar relacionada con la inhibición del proceso en este punto aunque posiblemente la interacción de sus compuestos fenólicos con los reactantes iniciadores de la glicación sea otro aspecto a considerar. El atrapamiento de α-dicarbonilos como MGO y GO tanto por parte del extracto, como por el hidroxitirosol y el verbascósido, interfirió en la evolución de la glicación hacia la etapa final, y consecuentemente hacia la formación de AGEs que fue reducida eficazmente.

Es frecuente encontrar en la literatura científica la propuesta de algún extracto agroalimentario como inhibidor de la glicación proteica en varias de sus etapas. En línea con nuestros resultados,

otros autores evaluaron la acción antiglicante de un extracto obtenido a partir de arándanos que ejerció su efecto inhibitorio a través de su contenido en procianidinas, antocianidinas y quercetina. Su efecto inhibitorio se hizo extensible a las tres etapas del proceso al ejercer una acción inhibitoria en la formación de productos de Amadori, un atrapamiento de α-dicarbonilos y una reducción de la generación de AGEs responsables de la activación de NF-kB y de la transcripción de factores proinflamatorios (Nair y col., 2006; Wang y col., 2011).

Sin embargo, son muchas las características que condicionan la actividad antiglicante de un extracto natural. En primer lugar, el agente extractivo utilizado y las condiciones de reacción empleadas para la obtención del extracto condicionarán el rendimiento y perfil de compuestos fenólicos (Lapornik y col. 2005). En el caso del estudio de la capacidad antiglicante de los extractos de semillas, cascarilla de café y alpechín que recoge la presente tesis (*Capítulo 1*) se utilizó como medio de extracción el agua con la idea de valorizar los subproductos de la manera más compatible con el medio ambiente, económica y segura para ser empleado en la industria alimentaria. El uso de metanol y etanol en la obtención de extractos naturales condiciona la obtención de extractos con mayor rendimiento en polifenoles que el uso de agua como método extractivo, aunque dependerá de la polaridad de los compuestos fenólicos presentes. El etanol y metanol al ser menos polares que el agua ejercen una mayor eficiencia en la degradación de semillas y paredes celulares lo que ocasiona que determinados polifenoles sean liberados de las células (Lapornik y col., 2005). Tehranifar y col. (2011) reportaron que el contenido fenólico y la consiguiente actividad antioxidante que presentó el extracto metanólico de piel de granada fue superior al presentado por el extracto acuoso y a otras partes de la granada, como la semillas u hojas. Por el contrario, diversos estudios muestran que tanto el extracto de café verde, tostado y sus subproductos obtenidos a partir de una extracción acuosa obtuvieron una mayor concentración de ácidos clorogénicos, compuestos responsables de su mayor rendimiento antioxidante (Budryn y col., 2009; Bravo y col., 2013).

Otro factor a considerar a la hora de cuantificar el efecto antiglicante de un extracto es su perfil fenólico. En este sentido, en el cribado inicial de extractos naturales presentados en el *Capítulo 1* se evaluó la capacidad antiglicante del extracto y de algunos de los compuestos fenólicos presentes. De los compuestos fenólicos estudiados, se observó que el ácido clorogénico de la cascarilla de café, el ácido gálico de la granada y el hidroxitirosol del alpechín, presentaron un IC_{50} de 0.4, inferior al límite del ensayo y 0.4 mg/mL, respectivamente, en el modelo BSA-GLC y de 0.10, 0.13 y 0.09 mg/mL, respectivamente, en el modelo BSA-MGO. Sin embargo, se debe considerar que los compuestos fenólicos presentes en los extractos podrían tener efectos sinérgicos o antagónicos entre ellos o con otros constituyentes que formen parte del extracto, justificando así la diversidad

de resultados obtenidos *in vitro* (Rice-Evans y col., 1996). Por otro lado, el contenido de compuestos fenólicos vendrá determinado por otros parámetros como la variedad, el grado de madurez y la parte de la planta que se esté valorando. En este sentido, nuestra investigación (*Artículo 3*) demostró que la cascarilla de café de la variedad robusta presentó una mayor capacidad de atrapamiento de MGO frente a la variedad arábica, posiblemente relacionado con su mayor contenido en clorogénicos. En la literatura se hallan numerosos ejemplos de cómo estos parámetros influyen en el contenido de compuestos bioactivos y, en consecuencia, de su actividad (Budryn y col., 2009; Tehranifar y col., 2011; Harsha y col., 2016).

La capacidad antioxidante es una de las propiedades más relevantes de los compuestos fenólicos. Es por ello que las primeras investigaciones en este ámbito relacionaron el contenido de compuestos fenólicos y su capacidad antioxidante con el efecto antiglicante que presenta un determinado extracto (Peng y col., 2008; Sun y col., 2010). Sin embargo este axioma no siempre se cumple como hemos demostrado en el estudio de los extractos hidrosolubles de diferentes semillas (Artículo 1). Otros autores, como Povichit y col. (2010) mostraron resultados similares en un estudio de 15 plantas medicinales donde encontraron una correlación entre el contenido fenólico y su capacidad antioxidante pero no con el efecto antiglicante.

No obstante, pese al gran número de investigaciones de las propiedades antioxidantes y antiglicantes de numerosos extractos naturales, pocos son los estudios realizados para valorar la posible toxicidad en ensayos tanto *in vivo* como *in vitro*. Existe cierta controversia en cuanto a los efectos tóxicos y de seguridad que pueden presentar determinados extractos naturales. En el caso de los extractos de granada, varios estudios han señalado efectos genotóxicos en extractos obtenidos a partir de una extracción hidroalcohólica e incluso se han descrito efectos apoptóticos en extracciones acuosas, si bien sólo a concentraciones elevadas (Settheetham e Ishida, 1995; Sánchez-Lamar y col., 2008). En contraposición a estos resultados, otros estudios con extractos hidrosolubles de granada, no revelaron toxicidad alguna (Amorin, 1995; Patel y col., 2008). En nuestro estudio sobre la posible toxicidad del extracto acuoso de un subproducto de granada, concretamente proveniente de la cascarilla de la semilla, no se observó citotoxicidad en las células HepG2 pero sí una acción protectora frente al efecto citotóxico y de estrés oxidativo ejercido por *t*-BOOH (*Artículo 2*). Hiraganahalli y col. (2012) obtuvieron resultados similares, donde la piel de granada ejerció una protección frente a *t*-BOOH y tetracloruro de carbono a nivel hepático. Estudios *in vivo* mostraron un restablecimiento de la estructura hepática y mejora de la actividad de enzimas hepáticas implicadas en la protección frente al estrés oxidativo (Murthy y col., 2002; Hiraganahalli y col., 2012).

2. Estudio del hidroxitirosol como nuevo compuesto antiglicante y evaluación de su mecanismo de acción. Propuesta de un extracto de hoja de olivo como fuente de hidroxitirosol con vistas a su valorización.

Como ya se ha comentado anteriormente, los compuestos fenólicos se han relacionado con efectos beneficiosos para la salud, aunque estos efectos dependerán de su absorción y posterior distribución en tejidos y células diana. Por otro lado, en la última década, el estudio de la farmacocinética y metabolismo de estos compuestos ha adquirido una importancia relevante y ha permitido acceder a nueva información reevaluando algunos de los efectos beneficiosos observados *in vitro*. Williams y col. (2004) propusieron que las propiedades antioxidantes así como los efectos moduladores en la señalización celular de los flavonoides debían ser valoradas, entendiendo el posible mecanismo de acción de estos compuestos a nivel *in vivo* y cómo sus metabolitos influían en dichos efectos beneficiosos. En este sentido, numerosos estudios confirmaron que los compuestos fenólicos sufren un metabolismo a nivel gastrointestinal, aunque existen diferencias en función del tipo de compuesto fenólico presente en la dieta. De forma general los polifenoles sufren una extensa metabolización a nivel del tracto gastrointestinal y, concretamente, en el lumen del intestino delgado los polifenoles pierden sus restos de azúcar y son sometidos a glucuronidaciones, metilaciones o sulfataciones (Verzelloni y col., 2011b). Estos metabolitos pueden pasar directamente al flujo sanguíneo, aunque pueden sufrir otra metabolización de fase II en el hígado y otros tejidos (Verzelloni y col., 2011b). Bajo estas premisas, los metabolitos del compuesto fenólico mayoritario en un determinado alimento no tienen por qué ser los que alcancen la mayor concentración en el organismo. En este sentido, cobran especial relevancia aquellos polifenoles que no son absorbidos a nivel del intestino delgado, sino que son capaces de alcanzar el intestino grueso, donde sufren una serie de modificaciones mediadas fundamentalmente por la flora colónica para posteriormente pasar al torrente sanguíneo y ser excretados finalmente en orina. Los principales catabolitos que se obtienen a este nivel suelen ser ácidos fenólicos de bajo peso molecular, que son encontrados en concentraciones considerablemente superiores a las de los metabolitos absorbidos en el intestino delgado. Por ello algunas investigaciones han centrado su atención en el estudio de estos metabolitos y sus implicaciones en los efectos atribuidos a su predecesor. Ejemplo de ello es el estudio de los metabolitos colónicos del ácido elágico como las urolitinas A y B o del ácido gálico como el pirogalol, que demostraron ejercer un efecto antiglicante en un modelo *in vitro* (Verzelloni y col., 2011b).

Centrando la atención en el HT, existe una gran controversia en cuanto a su biodisponibilidad. Es conocido que el HT es absorbido de manera dosis-dependiente aunque dependerá de la matriz en la que se administre, siendo su absorción más significativa si se administra como aceite de oliva

(Cicerale y col., 2010). La polaridad del HT hace factible que su absorción tenga lugar a nivel intestinal, aunque no está del todo claro el porcentaje que es absorbido y excretado. El aceite de oliva es utilizado como vehículo de HT en los estudios farmacocinéticos sin considerar en algunos casos que esta matriz contiene otros compuestos como la oleuropeína que son degradados a HT a su paso por el tracto gastrointestinal resultando en una sobreestimación de su absorción (Vissers y col., 2002). En estudios *in vitro* e *in vivo* con animales, el HT y sus metabolitos han mostrado ser potentes antioxidantes. Sin embargo, es importante establecer una relación entre la capacidad antioxidante que presenta el HT *in vivo*, su concentración en alimentos y la que se obtiene a nivel plasmático (Visioli y col., 2002 y 2011; Tripoli y col., 2005). Otra dificultad añadida es el método de obtención del HT en los fluidos o muestras biológicas, ya que no hay unanimidad y existen diferencias significativas entre los estudios que emplean ácido clorhídrico para la hidrólisis de los derivados de HT o un sistema enzimático como β-glucuronidasa (Cicerale y col., 2010). Lo que parece claro es que, aunque es absorbido, no lo hace mayoritariamente en forma libre sino en sus conjugados mediante procesos de glucuronidación, metilación y sulfonilación. Estas modificaciones tienen lugar en los enterocitos del epitelio del intestino y en el hígado (Vissers y col., 2002). Otros estudios han descrito la presencia de otros compuestos derivados del metabolismo del HT que han sido cuantificados en diferentes órganos. D´Angelo y col. (2001) administraron HT marcado vía intravenosa en ratas cuyo análisis farmacocinético indicó una rápida absorción en tejidos y órganos diana con una mayor preferencia por el riñón. Los principales metabolitos marcados que se obtuvieron fueron los derivados sulfo-conjugados, MOPET (4-hidroxi-3-metoxifeniletanol), DOPAC, DOPAL (3,4-dihidroxifenilacetaldehido) y HVA (ácido 4-hidroxi-3-metoxifenilacético, ácido homovanílico). Los metabolitos del HT se distribuyeron principalmente en plasma, cerebro, corazón, riñón, hígado y pulmón y fueron también cuantificados en orina.

En nuestros sistemas *in vitro* de incubación de MGO y HT en condiciones fisiológicas simuladas, observamos un atrapamiento del α-dicarbonilo (*Artículo 5*). Sin embargo, tanto en la incubación en presencia como en ausencia de MGO se evidenció la desaparición gradual de HT en el medio de reacción y la paulatina formación de compuestos de degradación entre los que destacó el DOPAC. La presencia de 3,4-dihidroxifenil-acetaldehído (DOPAL) a los tiempos de incubación más cortos como otro de los compuestos de degradación del HT en el sistema nos lleva a hipotetizar que el HT se puede estar oxidando a DOPAL y éste a su vez a DOPAC (*Artículo 6*). El pH y el contenido salino del sistema son factores críticos para la degradación del HT, hecho que fue demostrado en nuestro estudio en línea con las investigaciones de Pereira-Caro y col. (2012). Estos autores describieron una oxidación de HT en DOPAC después de una incubación a 37 ^{o}C y pH 7.5. El estudio de la capacidad antiglicante del HT *in vitro* nos llevó a suponer que el HT estaba ejerciendo su actividad

a través de su oxidación a DOPAC, compuesto que fue incrementándose en el sistema hasta las 48 h de incubación, sin embargo, no se descarta que otros compuestos de la degradación del HT jugaran un papel en la capacidad de atrapamiento presentada. Pashikanti y col. (2010) afirmaron que metabolitos colónicos de la rutina como el DOPAC y el 3,4-dihidroxitolueno ejercieron una inhibición de la formación de CML y ArgP *in vitro*. Otro estudio *in vitro* ha confirmado que el DOPAC, uno de los metabolitos colónicos de las antocianinas, presentó una actividad inhibitoria de formación de AGEs (Verzelloni y col., 2011b). Por otro lado, en nuestro estudio comprobamos que al incrementar la incubación y por tanto la degradación del HT, se obtenían dos efectos a destacar: la disminución de la capacidad antioxidante y el incremento de la capacidad de atrapamiento del MGO, hecho que vuelve a demostrar que no siempre los compuestos con mayor capacidad antioxidante muestran la mayor capacidad antiglicante. En este sentido, cabe destacar que la capacidad antioxidante del HT y sus derivados está relacionada con la habilidad de sus grupos hidroxilos en posición orto para donar electrones (Visioli y col., 1998b). En esta tesis se demostró además, que la capacidad de atrapamiento de α-dicarbonilos estaba relacionada con la presencia de un grupo hidroxilo en posición 2, en concordancia con los resultados de Wu y Chen (2013), quienes indicaron que el número de grupos hidroxilos era un factor importante en la actividad antiglicante de los flavonoides.

A nivel *in vivo*, se ha descrito la metabolización del HT a DOPAL y su posterior transformación en DOPAC a través de la acción del aldehído alcohol deshidrogenasa en hígado. Sin embargo no hay que olvidar que tanto el HT como el DOPAC son obtenidos endógenamente a través del sistema dopamina, representando una fuente endógena importante. En nuestra investigación (*Artículo 6*) planteamos la hipótesis que el MGO se incorpora al DOPAC mediante una reacción de adición nucleofílica. Este hecho se constató mediante un análisis con HPLC-QTOF de las nuevas estructuras emergentes en el sistema incubado de MGO-HT y que no aparecían en el mismo sistema sin MGO, por lo que pensamos que serían productos específicos de la reacción. El análisis estructural nos confirmó al menos cuatro aductos formados a partir de MGO y DOPAC. En la incubación MGO-OLE, aunque más complejo y con mayor actividad que el estándar HT, se confirmaron igualmente la presencia de los aductos anteriores, aunque con distintas intensidades a las presentadas en la incubación con el estándar HT, y la detección de un nuevo aducto sin una procedencia clara (*Artículo 8*).

En resumen, en la investigación que dio lugar al *Artículo 6* se determinó que la reacción del MGO con el DOPAC tuvo lugar por adición nucleofílica y los grupos hidroxilo del anillo aromático reforzaron esa reactividad posiblemente debido a la propiedad de donar electrones. Por otro lado se estableció que el C1 del anillo aromático podría ser el sitio activo donde se unirá el MGO, aunque

esta hipótesis deberá ser confirmada con un análisis de resonancia magnética nuclear ya que estéricamente la adición también podría tener lugar en el C4. En concordancia con estos resultados, otros autores han descrito mecanismos similares de atrapamiento de compuestos α-dicarbonilos a partir de otros compuestos fenólicos. Shao y col. (2014) analizaron la capacidad de los flavonoides para atrapar las RCS e impedir que estos contribuyan activamente en las complicaciones diabéticas. El estudio concluyó que el anillo A de los flavonoides, como la quercetina, luteolina o epicatequina, así como de otros subcomponentes de los flavonoides como el ácido gálico, pirogalol o pirocatecol fue el sitio activo para la unión del MGO favorecido por la presencia de un grupo hidroxilo en posición 5. Recientemente, Wang y col., (2016) detectaron *in vivo* la presencia de dos aductos formados por el MGO y la genisteína y hasta 6 aductos del MGO con sus metabolitos.

Los estudios presentados en esta Tesis se han centrado principalmente en la captación de α-dicarbonilos como principal mecanismo de inhibición del avance de la glicación. Por este motivo, y dada la relevancia de estos compuestos, estudiamos la capacidad de atrapamiento del HT y sus derivados, HTA y DOPAC frente a α-dicarbonilos C6 como 3-DG, 3-DGal, 3,4-DGE y GLUCOS (*Artículo 7*). Además, se diseñaron por primera vez y se propusieron sistemas competitivos en condiciones fisiológicas simuladas entre α-dicarbonilos C6 y MGO para conocer la preferencia de atrapamiento de los compuestos fenólicos. Los resultados mostraron un atrapamiento significativamente superior de MGO frente a los α-dicarbonilos C6 por parte de HT, HTA y DOPAC, siendo este último el más activo. De nuevo, estos resultados refuerzan la hipótesis que la actividad antiglicante del HT podría estar mediada por uno de sus productos de degradación a través del atrapamiento de compuestos α-dicarbonilos. En cuanto a los sistemas competitivos, se mantuvo la preferencia por el atrapamiento de MGO, aunque éste no se incrementó en detrimento del atrapamiento de 3-DG o 3-DGal. No obstante, pese a los resultados obtenidos, la formación de aductos del HT con otros dicarbonilos podría ser objeto de una investigación futura para obtener una mayor comprensión del mecanismo de acción. Por otro lado, es relevante comentar que el 3-DG presentó la mayor estabilidad en nuestros sistemas en condiciones fisiológicas simuladas, seguido muy de cerca por el GO, MGO y 3-DGal. Por el contrario, el 3,4-DGE y GLUCOS presentaron una estabilidad reducida de horas por lo que se podría explicar la ausencia de actividad de los compuestos fenólicos ensayados en el atrapamiento de estos dos últimos α-dicarbonilos. Previamente, Vistoli y col. (2013) observaron que el 3,4-DGE presentaba una alta tasa de inestabilidad debido a su estructura α,β insaturada combinaba con su alta reactividad como dicarbonilo. Por otro lado, GLUCOS se ha descrito como un α-dicarbonilo inestable que será degradado en α-dicarbonilos C5 como 3-deoxipentosona, 1-deoxipentosona y pentosona (Smuda y col., 2013). En este sentido, nuestros resultados constataron que tras una incubación de 24 h el 3,4-DGE se degradó en su totalidad y dio

lugar a la aparición de nuevos picos en el cromatograma, entre ellos al 3-DGal. HT, HTA y DOPAC incubados durante 24 h con el 3,4-DGE atraparon el 3-DGal que se formó a partir del 3,4-DGE.

Como ya se ha comentado, los solventes utilizados para la extracción de compuestos fenólicos determinan el rendimiento y el perfil fenólico obtenido. Adicionalmente, el extracto obtenido puede concentrarse en determinados compuestos fenólicos de interés en función de su solubilidad, como es el caso del uso de acetato de etilo y que en nuestra investigación se empleó para concentrar el OLE en HT (*Artículo 8*). Otro aspecto a considerar es el pH que se utiliza durante la extracción. En algunos casos, en la obtención del OLE se empleó ácido clorhídrico diluido (pH 2.5) tanto durante la maceración etanólica como posteriormente en el extracto líquido, concentrado aún más en HT. Se sabe que el empleo de condiciones ácidas mejora la extracción de los polifenoles con solventes orgánicos, y a su vez puede provocar la hidrólisis de los polifenoles glicados o acilados hacia estructuras agliconas. Por otro lado, el uso de condiciones acidificadas en la obtención de extractos puede tener como objetivo la obtención de estructuras fenólicas más sencillas como el HT a partir de otras más complejas como la oleuropeína (Tsao, 2010).

El HT es un compuesto fenólico con propiedades saludables ampliamente estudiadas y atribuidas en su mayoría a su capacidad antioxidante. Sin embargo, los estudios relativos a sus efectos beneficiosos no han valorado otros posibles mecanismos de acción mediante los cuales el HT pudiera estar ejerciendo dichos efectos. En consonancia con estas consideraciones y valorando el contenido en HT y compuestos fenólicos derivados del HT con similares efectos saludables, un gran número de investigaciones centraron su interés en el estudio de los productos y subproductos del olivo, fuente principal de estos compuestos.

La industria agroalimentaria genera ingentes cantidades de subproductos derivados del procesado de los alimentos cuya eliminación supone un problema económico y medioambiental. La tendencia actual y futura lleva a que estos subproductos sean valorizados y puedan ser convertidos en productos de alto valor añadido con aplicaciones diversas. Las hojas de olivo son considerados una fuente importante de compuestos fenólicos con un amplio espectro de propiedades saludables. El cultivo del olivo, su mantenimiento y la obtención de su producto estrella, el aceite de oliva, genera anualmente grandes cantidades de subproductos con limitado retorno económico. El mantenimiento anual de cada olivo genera alrededor de 25 kg de subproductos, entendiendo éstos como hojas y ramas y constituyendo el 10% del peso que llega a la almazara (Herrero y col., 2011). Los compuestos fenólicos presentes en las hojas de olivo son muy diversos y numerosos, destacando su contenido en secoiridoides como la oleuropeína y ligstrósido, verbascósido, flavonoides como la rutina, luteolina, apigenina y diosmetin , ácidos fenólicos, flavonoides y

especialmente fenoles sustituidos como el hidroxitirosol o el tirosol (Herrero y col., 2011). A lo largo de la literatura se han descrito multitud de efectos beneficiosos atribuidos a las hojas de olivo que se han utilizado tradicionalmente como ungüento natural para el tratamiento de ciertas enfermedades y actualmente se encuentra en auge su consumo como infusión. Sus efectos saludables radican en su rica composición fenólica y concretamente en la capacidad antioxidante de estos compuestos bioactivos. Su efecto antiglicante expuesto en esta memoria y concretamente el efecto ejercido por sus componentes fenólicos mayoritarios (HT y oleuropeína), así como los posibles sinergismos que puedan establecerse con otros componentes minoritarios pero con efectos antiglicantes probados como el verbascósido o luteolina (Shao y col., 2014) explican la actividad potenciada del extracto. Por todo ello, podría proponerse como nutracéutico, ingrediente funcional en la formulación de alimentos o incluso para otros usos farmacológicos.

3. Acción antiglicante de un extracto de hoja de olivo fuente de hidroxitirosol a nivel celular y en un modelo de alimento.

Como ya se ha comentado previamente, la RM puede desarrollarse tanto en el alimento como en el organismo y en ambos casos la consecuencia final será la formación de AGEs. Tanto los AGEs formados endógenamente en sistemas biológicos, como los AGEs de la dieta comparten la misma estructura química. Sin embargo y pese a los diversos estudios realizados hasta el momento, existe cierta controversia de cómo la ingesta de estos AGEs formados en el alimento pueden contribuir en el total de AGEs endógenos del organismo. Los detractores del establecimiento de esta asociación centran su oposición en la necesidad de realizar estudios a largo plazo, con métodos analíticos más robustos y mayor tamaño muestral de los ya existentes (Kellow y Coughlan, 2015). Sin embargo, en los últimos años, varias investigaciones apuntan hacia una correlación significativa entre los AGEs ingeridos a partir de la dieta y el nivel de AGEs circulantes en el organismo (Uribarri y col., 2005). En este sentido, el consumo de AGEs generados en los alimentos se ha relacionado con un incremento del estrés oxidativo y de la respuesta inflamatoria en individuos sanos y especialmente en individuos de edad avanzada (Uribarri y col., 2007). Otros estudios apuntan que la restricción dietética de AGEs podría estar relacionada con la disminución de la resistencia a la insulina y de las complicaciones diabéticas (Vlassara y col., 2002; Zheng y col., 2002). Koschinsky y col. (1997) afirmaron que la ingesta dietética de AGEs en individuos sanos podría producir una elevación de los niveles de AGEs en suero de manera directamente proporcional a la ingesta realizada. Se estimó que la excreción renal de los mismos normalmente es incompleta, representando aproximadamente el 30% de los AGEs absorbidos. Asimismo, concluyeron que el consumo de AGEs a través de la ingesta de alimentos por pacientes con nefropatía diabética provocó una reducción del 5% en la excreción renal de los mismos, pudiendo contribuir

negativamente a las complicaciones renal-vasculares vinculadas a la diabetes mellitus. Por otro lado, Seiquer y col. (2014) evidenciaron que el consumo de una dieta rica en PRM podría modular la composición de la microbiota intestinal tanto en humanos como en ratas, siendo en la actualidad una línea de investigación esperanzadora para avanzar en la comprensión del metabolismo de los AGEs.

Los productos de panadería y, particularmente, las galletas y pan se han utilizado en numerosos estudios como un modelo de alimento reproducible para el estudio de la RM. La formación de AGEs en la dieta viene condicionada por la composición del alimento y las principales variables del procesado, como la temperatura-tiempo de horneado, pH, actividad de agua o humedad (Martins y col., 2001). A este respecto son pocos los estudios existentes sobre el efecto de la adición de potenciales inhibidores de AGEs en alimentos con características favorables para el desarrollo de la RM. Sin embargo, es importante resaltar que pese a que el desarrollo de la RM desencadena la formación de compuestos indeseables, como el HMF, furfural o AGEs, su progreso es deseable en muchos alimentos, ya que la formación de otros compuestos le confiere las características típicas de sabor, olor y aroma. Por tanto, es conveniente buscar sustancias que sean capaces de inhibir la formación de compuestos perjudiciales pero sin ocasionar un detrimento de las características organolépticas del mismo. En este sentido, determinados productos de la RM presentan una dicotomía en cuanto a su posible reducción en el alimento. Es el caso de los α-dicarbonilos, compuestos altamente reactivos que se forman durante el progreso de la RM y que, pese a que son unos de los principales responsables de la formación de AGEs, también son los responsables de conferir al alimento aromas y sabores únicos.

De acuerdo a estas premisas y una vez comprobada la potencial capacidad antiglicante del extracto de hoja de olivo *in vitro*, nos planteamos investigar, por un lado, la repercusión de introducir el OLE y un estándar de HT, como ingredientes funcionales en la elaboración de un modelo de galleta y por otro lado, cómo podría influir en la inhibición de la glicación de proteínas intracelulares en un modelo de estrés oxidativo celular. Para ello, elaboramos un modelo de galleta a partir de una formulación estándar a la que se introdujo el OLE y el HT a distintas concentraciones (*Artículo 9*). Las galletas formuladas con OLE y HT se compararon con una formulación control y con otras de referencia formuladas con QE y GA al ser potentes agentes antiglicantes. La investigación confirmó que la formulación de la masa con HT moduló la formación de determinados AGEs, y concretamente los AGEs fluorescentes libres y pentosidina (AGE fluorescente ligado a proteínas más representativo). Sin embargo, no se observaron diferencias significativas en la formación de la CML. Por otro lado, el HMF y 3-DG mostraron una correlación positiva ($r = 0.950$; $p = 0.004$) y ambos fueron reducidos significativamente al introducir HT en la formulación de la galleta. La galleta

formulada con OLE inhibió significativamente la formación de pentosidina y también fue capaz de inhibir la formación de CEL, AGE mayoritario del modelo de galleta. Los resultados obtenidos con OLE en este modelo refuerzan la hipótesis de que otros compuestos fenólicos presentes en el extracto están ejerciendo una acción anti-AGEs, y/o potenciando la actividad del HT de manera sinérgica.

Trabajos recientes han reflejado que el enriquecimiento de productos alimentarios con ciertos polifenoles podrían ejercer alguna actividad inhibitoria de la formación de AGEs en el alimento. Zhang y col. (2014) evidenciaron que la formulación de galletas con compuestos fenólicos como la quercetina, epicatequina, naringenina o ácido rosmarínico en una proporción de 0.25% p/p inhibió significativamente la formación de AGEs fluorescentes totales. En el mismo sentido, Culetu y col. (2016) observaron una reducción de AGEs fluorescentes totales en la elaboración de un pan al que se le adicionó una fracción enriquecida en polifenoles de té en polvo. Sin embargo, no hay que olvidar que otros parámetros dependientes del desarrollo de la RM o del propio ingrediente anti-AGEs han de ser tenidos en cuenta como los posibles cambios de color, sabor, textura y aroma del alimento, así como la estabilidad del compuesto adicionado. En nuestras galletas, la mayor modificación de color se obtuvo con la adición de QE y aunque se observaron diferencias significativas con una concentración superior de HT y OLE, visualmente no fueron relevantes. No obstante, en un futuro se hace necesario ampliar la investigación para conocer el impacto sobre las características organolépticas en el alimento al inhibir la formación de los AGEs, así como la estabilidad de los compuestos anti-AGE durante el procesado.

En base a la dualidad dietética y biológica que presentan los AGEs, y una vez investigada la capacidad antiglicante del HT y del OLE en un modelo de alimento, se planteó estudiar la actividad del HT y OLE en mitigar la formación de AGEs intracelulares. Para este propósito, se utilizó un modelo celular bien caracterizado y empleado en multitud de estudios bioquímicos y nutricionales como son las células de hepatoma humano (HepG2). Las HepG2 fueron sometidas a un estrés carbonílico con MGO y pretratadas con HT y OLE para comprobar la posible repercusión en la carbonilación de las proteínas intracelulares y la posible formación de AGEs como CML, CEL y ArgP (*Artículo 10*). Estudios previos con cultivos de hepatocitos probaron que tras un estrés carbonílico con MGO y GO, el nivel de carbonilación de las proteínas celulares fue reducido significativamente al pretratar las células con agentes antiglicantes como la AG y penicilina (Yang y col., 2011). En nuestro modelo celular de estrés carbonílico partimos de las concentraciones del agente estresante empleadas por Yang y col. (2011). Sin embargo, la información disponible en la literatura científica sobre el análisis de AGEs específicos en líneas celulares es muy escasa, por lo que un trabajo previo fue validar las determinaciones de AGEs específicos en el modelo celular.

El uso de subproductos del olivo como el alpechín y hojas en ratas diabéticas redujo el estrés oxidativo y la hiperglucemia y restableció el sistema de defensa antioxidante (Hamden y col., 2009; Jemai y col., 2009) todo ello íntimamente relacionado con el proceso de glicación. Datos reveladores obtenidos durante la investigación *in vitro* recogida en el Capítulo 4 nos llevaron a hipotetizar que la actividad antiglicante podría ser ejercida principalmente por el HT como principal compuesto fenólico de OLE-B. Sin embargo en el estudio de la protección de HepG2 frente a un estrés carbonílico, OLE-B mostró una protección significativa frente a la carbonilación proteica y concretamente frente a la formación de ArgP, resultados que no se obtuvieron para un estándar de HT a la misma concentración. En contraposición con las observaciones obtenidas con el estándar de HT, Cao y col. (2014) mostraron que los niveles elevados de proteínas carboniladas en el hígado de ratas alimentadas con una dieta rica en grasas e inductora de obesidad, hiperglucemia, hiperlipemia y resistencia a la insulina eran reducidos de manera dosis-dependiente al introducir una suplementación de HT (10-50 mg/kg/día). Otro estudio de carbonilación de proteínas en un cultivo celular de epitelio pigmentario de la retina humano (ARPE-19) confirmó que un tratamiento de HT a una dosis de 100 µmol/L fue capaz de reducir en un 48% la carbonilación de proteínas inducida por 75 µmol/L de acroleína (Liu y col., 2007).

Para poder explicar nuestros resultados, nuevamente es importante resaltar que OLE contiene otros compuestos fenólicos con probadas capacidades antiglicantes y que podrían ejercer un efecto potenciador o sinérgico con el HT. En base a las conclusiones obtenidas con el OLE en la inhibición de AGEs procedentes de la dieta e intracelulares, OLE podría ser propuesto como un agente antiglicante de amplio espectro para su uso como ingrediente alimentario, nutracéutico o uso farmacológico. Sin embargo, es necesario llevar la investigación a un nivel superior y probar si dicho efecto es ejercido *in vivo* y si la reducción conseguida por su adición en el alimento puede tener alguna repercusión en el organismo.

VI. CONCLUSIONES

De los resultados obtenidos en la presente Tesis Doctoral se puede concluir que:

1. La actividad antiglicante que mostraron los extractos naturales obtenidos a partir de subproductos agroalimentarios de semillas (pimiento verde, albaricoque, melocotón, guinda, granada, avellana, almendra y sésamo), del tostado del café (cascarilla de café) y de la elaboración del aceite de oliva (alpechín), no se correlacionó con la capacidad antioxidante que aportaron mayoritariamente los compuestos fenólicos, indicando que éstos podrían estar actuando sobre vías no oxidativas de la glicación.

2. El hidroxitirosol mostró una capacidad antiglicante de amplio espectro ya que es efectivo en las diferentes etapas de la glicación, si bien su efecto más directo derivó en una mitigación de la formación de AGEs fluorescentes totales, y más concretamente en la formación de AGEs tan relevantes como la carboximetil-lisina, carboxietil-lisina y argipirimidina. A su vez, se propuso el atrapamiento de compuestos α-dicarbonilos por parte del hidroxitirosol y sus derivados como uno de los principales mecanismos de inhibición de la glicación, donde el atrapamiento de α-dicarbonilos C3 (metilglioxal) predominó frente a α-dicarbonilos C2 (glioxal) y C6 (3-deoxiglucosona, 3-deoxigalactosona, 3,4-dideoxiglucosona-3-ene y glucosona). Adicionalmente se constató que existe una relación estructura-actividad entre los compuestos fenólicos similares al hidroxitirosol y la capacidad de atrapamiento de metilglioxal, donde el grupo hidroxilo en posición 2 resultó clave para ejercer dicho efecto.

3. La hipótesis del atrapamiento de compuestos α-dicarbonilos como uno de los principales mecanismos antiglicantes del hidroxitirosol fue constatada tras la identificación de cuatro aductos procedentes de la reacción de adición nucleofílica directa del metilglioxal con el producto de oxidación del hidroxitirosol, el ácido 3,4-dihidroxifenil-acético. Este hallazgo propuso al ácido 3,4-dihidroxifenil-acético como el responsable final de la capacidad antiglicante descrita. Por extensión, se plantea que los compuestos α-dicarbonilos C6 sigan un mecanismo de reacción similar.

4. El posible uso del hidroxitirosol como ingrediente antiglicante constituye una propuesta de valorización de las hojas de olivo como fuente natural de dicho compuesto. El empleo de hidroxitirosol en la formulación de un modelo de galleta redujo significativamente los niveles de AGEs fluorescentes libres y ligados a proteína así como también los niveles de 5-hidroximetilfurfural y 3-deoxiglucosona. Sin embargo la formulación de la galleta con el extracto de hoja de olivo en un rango de concentración de hidroxitirosol 100 veces inferior inhibió significativamente la formación de pentosidina y de carboxietil-lisina, siendo este último el AGE más abundante en el modelo de galleta. La posible actividad antiglicante de otros

compuestos fenólicos cuantitativamente minoritarios, la degradación de la oleuropeína en hidroxitirosol y los posibles sinergismos que se establezcan entre ellos no son descartados en base a los resultados obtenidos.

5. El pretratamiento de células humanas HepG2 con el extracto de hoja de olivo concentrado en hidroxitirosol (13 µg/mL) ejerció una protección frente a un estrés carbonílico inducido por metilglioxal y redujo la formación de argipirimidina. Sin embargo, no se pudo establecer una relación con su contenido en hidroxitirosol, por lo que los resultados apuntan de nuevo hacia posibles sinergismos establecidos entre el hidroxitirosol y otros compuestos minoritarios en el extracto con polaridad similar.

Conclusión general:

Los resultados recogidos en esta memoria ponen de manifiesto que el uso de subproductos agroalimentarios ricos en compuestos fenólicos podrían considerarse una alternativa segura, económica y medioambientalmente sostenible para la obtención de ingredientes funcionales o nutracéuticos con actividad antiglicante. Concretamente, los subproductos derivados de la obtención del aceite de oliva constituyen una importante fuente de hidroxitirosol, compuesto fenólico que ejerce un potente efecto antiglicante en las distintas etapas de la glicación. El hidroxitirosol, previa oxidación a ácido 3,4-dihidroxifenilacético atrapó eficazmente compuestos α-dicarbonilos reduciendo así la carga de AGEs y, por consiguiente, suponiendo un nuevo mecanismo de acción más allá de su potente capacidad antioxidante frente a enfermedades tan prevalentes como la diabetes mellitus. El extracto de hoja de olivo redujo la formación de AGEs tanto en un modelo de alimento como a nivel celular, planteando la posibilidad de ser propuesto como ingrediente funcional, nutracéutico e incluso para uso farmacológico. Futuras investigaciones *in vivo* se hacen necesarias para esclarecer el mecanismo de acción en el organismo.

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ANEXO

Parte de los resultados expuestos en la presente Memoria de Tesis Doctoral han sido presentados en CONGRESOS:

POSTERS:

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PREMIOS:

- **Navarro** M, Morales FJ. Evaluación in vitro de la capacidad antiglicante de un extracto de hoja de olivo concentrado en hidroxitirosol. XX Jornadas Internacionales de Nutrición Práctica. Madrid (España, 2016).

OTRAS PUBLICACIONES:

- REVISTAS NO INCLUIDAS SCI

- **Navarro M**, Morales FJ (2015) Evaluación in vitro del efecto protector del hidroxitirosol frente a la formación de N^{ε}-(carboximetil)lisina. Nutrición Clínica y dietética Hospitalaria. 35 (supl.1):111-112
- **Navarro M**, Aguayo E, Morales FJ (2014) Evaluación *in vitro* del efecto protector de un extracto de corteza de sandía sobre el estrés carbonílico. Nutrición Clínica y dietética Hospitalaria. 34 (supl.1):110
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