María del Mar Castro López

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Estudio del comportamiento de antioxidantes naturales adicionados a poliolefinas en aplicaciones industriales

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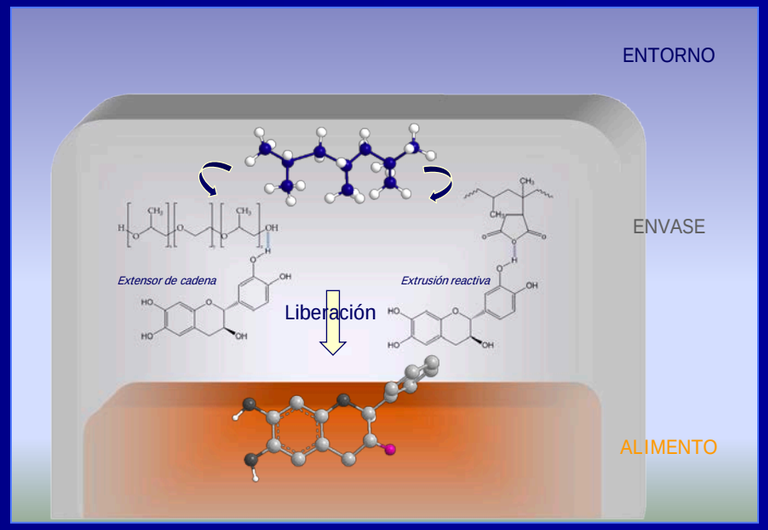
ESTUDIO DEL COMPORTAMIENTO DE ANTIOXIDANTES NATURALES ADICIONADOS A POLIOLEFINAS EN APLICACIONES INDUSTRIALES



TESIS DOCTORAL
Departamento de Física

María del Mar Castro López

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Departamento de Física

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ESTUDIO DEL COMPORTAMIENTO DE ANTIOXIDANTES NATURALES ADICIONADOS A POLIOLEFINAS EN APLICACIONES INDUSTRIALES

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Ferrol, Julio 2013

UNIVERSIDADE DA CORUÑA

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AUTORIZAN

A María del Mar Castro López a presentar la memoria titulada: "Estudio del comportamiento de antioxidantes naturales adicionados a poliolefinas en aplicaciones industriales", que ha sido realizada bajo su dirección en el Laboratorio de Química del Centro de Investigacións Tecnolóxicas para optar al grado de Doctora por la Universidad de A Coruña.

Y para que así conste a los efectos oportunos firman la presente en Ferrol, a 31 de mayo de 2013.

Fdo. **M.V. González R.** **J.M.L. Vilariño** **M.S. Dopico García**

Hace ya unos cuantos años, comenzaba una nueva etapa de mi vida científica con amplias ilusiones y esperanzas en un futuro lleno de aprendizaje, desarrollo científico y personal. Ahora, esa fase culmina con la presentación de esta tesis doctoral y con la escritura de estas últimas líneas, que aún siendo el principio de la memoria son la últimas en ser tecleadas.

El largo camino recorrido, a veces pedregoso, a veces frustrante, a veces estresante,…, aunque muchas otras (afortunadamente, la mayoría) ilusionante y satisfactorio, no habría llegado a este reconfortante fin sin la inestimable ayuda de todas aquellas personas que de un modo u otro me he encontrado en esta senda científica y todas aquellas otras que más personalmente me han tendido una mano cada vez que lo he necesitado. Por todo ello, en estos folios en blanco, empezados a ser cubiertos ahora por estas líneas, me gustaría dejar patente mi agradecimiento a todos aquellos que de un modo u otro han contribuido a la consecución de este fin.

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Al Departamento de Física de la Universidad de A Coruña y en especial al grupo de polímeros y, por extensión, a todos sus miembros, por permitir la realización de esta tesis doctoral.

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¡¡¡¡MUCHAS GRACIAS A TODOS!!!!!

En esta tesis doctoral se ha abordado el uso de antioxidantes naturales como agentes activos para su uso tanto en la protección de polímeros frente a su degradación oxidativa, como en envases activos con el objetivo de proteger tanto al polímero como al alimento envasado.

Se han desarrollado varios sistemas cromatográficos y electroforéticos para la identificación y cuantificación de catequinas y quercetina tanto en muestras naturales como en los simulantes alimentarios, y sistemas de purificación y preconcentración de catequinas mediante el uso de polímeros impresos molecularmente.

Derivado de su alto uso en el sector del envase y embalaje, se ha empleado polipropileno como material base para el desarrollo de los sistemas activos por extrusión, incorporando catequinas, quercetina, tocoferoles y extractos de té verde como antioxidantes naturales con el objetivo de retrasar el deterioro causado por los procesos oxidativos.

La escasa o nula capacidad de liberación de los antioxidantes desde la matriz de polipropileno limita su uso en envases activos, por lo que en esta tesis se propone el uso de dos sistemas como modificadores de la matriz polimérica: extensores de cadena/plastificantes y polipropileno modificado superficialmente mediante extrusión reactiva. Se han caracterizado las propiedades físico-químicas de interés de los materiales desarrollados, el efecto de la incorporación de varios antioxidantes naturales (catequinas, quercetina, tocoferoles y extractos de té verde) y las modificaciones en la capacidad de liberación de los antioxidantes provocadas por cada una de las modificaciones introducidas en diferentes simulantes alimentarios, así como la actividad antioxidante tras el proceso de liberación.

Nesta tese doutoral abordouse o emprego de antioxidantes naturais como axentes activos para o seu uso tanto na protección de polímeros fronte a súa degradación oxidativa, como en envases activos co obxectivo de protexer tanto ó polímero como ó alimento envasado.

Desenvolvéronse varios sistemas cromatográficos e electroforéticos para a identificación e cuantificación de catequinas e quercetina tanto en mostras naturais como nos simulantes alimentarios, e sistemas de purificación e preconcentración de catequinas mediante o uso de polímeros impresos molecularmente.

Derivado do seu alto uso no sector do envase e da embalaxe, empregouse o polipropileno como material base para o desenvolvemento dos sistemas activos por extrusión, incorporando catequinas, quercetina, tocoferois e extractos de té verde como antioxidantes naturais co obxectivo de retrasalos procesos oxidativos.

A escasa ou nula capacidade de liberación dos antioxidantes dende a matriz de polipropileno limita o seu uso en envases activos, polo que nesta tese proponse o emprego de dous sistemas como modificadores da matriz polimérica: extensores de cadea/plastificantes e polipropileno modificado superficialmente mediante extrusión reactiva. Caracterizáronse as propiedades físico-químicas de interese dos materiais desenvolvidos, o efecto da incorporación de varios antioxidantes naturais (catequinas, quercetina, tocoferoles e extractos de té verde) e as modificacións na capacidade de liberación dos antioxidantes provocadas por cada unha das modificacións introducidas en diferentes simulantes alimentarios, así como a actividade antioxidante tralo proceso de liberación.

The present thesis dissertation has focused on the use of natural antioxidants as active agents both to protect the polymer against its own oxidative degradation and as active ingredients into antioxidant active packaging to preserve the packaged food.

Several chromatographic and electrophoretic methods were developed and compared for separation and quantification of the studied antioxidants into natural samples and food simulants. Molecularly imprinted polymers were also developed and tested aimed to purify and concentrate catechins from those natural samples.

To delay food deterioration caused by oxidative processes, catechins, quercetin, tocopherols and extracts of green tea were selected as natural antioxidants and incorporated as active agents by extrusion into polypropylene matrix, highly used into packaging sector.

The use of polypropylene in active packaging is limited by its low or null capacity for the release of antioxidants. Therefore, this doctoral thesis concerns the development and assessment of two suitable strategies to modify the polymeric matrix and the mechanism by which antioxidants were released: use of chain extenders/plasticizers and polypropylene with modified surface trough reactive extrusion. The physicochemical properties of the materials, the effect of the incorporation of several antioxidants (catechins, quercetin, tocopherols and extracts of green tea), the modifications over the controlled release mechanisn within the polymer packaging to gradually deliver effective antioxidants into different food simulants, as well as the antioxidant activity after the release process were also evaluated.

CAPÍTULO I. INTRODUCCIÓN

CAPÍTULO III. RESULTADOS Y DISCUSIÓN

3.1. Determinación y cuantificación de antioxidantes naturales

- **Artículo 1.** Analytical determination of flavonoids aimed to analysis of natural samples and active packaging applications

3.2. Purificación de extractos naturales: polímeros de impresión molecular

- **Artículo 3.** Preparation, evaluation and characterization of quercetin-molecularly imprinted polymer for preconcentration and clean-up of catechins

3.3. Adición de antioxidantes naturales a muestras poliolefínicas

- **Artículo 4.** Natural extracts as potential source of antioxidants to stabilize polyolefins

3.4. Desarrollo de materiales capaces de realizar una cesión controlada de antioxidantes: envases activos y extrusión reactiva

- **Artículo 5.** Effect of PPG-PEG-PPG on the tocopherol-controlled release from films intended for food-packaging applications

- **Artículo 6/Patente.** Procedimiento de obtención de un material polimérico

- **Artículo 7.** Improving the capacity of polypropylene to be used in antioxidant active films: incorporation of plasticizer and natural antioxidants

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- **Artículo 8.** Interaction and release of catechin from anhydride maleic grafted polypropylene films

AA	acrilamida
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AIBN	2,2'-azo-bis-isobutironitrilo, 1,1,1-Tris(hydroxymethyl)-propantrimethacrylat tech
ATM	microscopía de fuerza atómica
ATR	attenuated reflection mode
BEH	Bridge ethylene hybrid
BET	adsorción/desorción de nitrógeno, Brunauer–Emmett–Teller method
BHA	tercbutilhidroxianisol, butylated hydroxytoluene
BHT	dibutilhidroxitolueno, butylated hydroxyanisole
BJH	Barret-Joyner-Halenda method
$\mathbf{B_t}$	parámetro de Boyd
C	catequina, (+)-catechin hydrate
$\mathbf{C_0}$	concentración inicial de soluto
Caff	caffeine
CAT	catechin
$\mathbf{C_e}$	concentración del soluto en condiciones de equilibrio en la disolución
CG	catequina galato, (-)-catechin gallate
CMC	critical micellar concentration
Cr	crosslinker
$\mathbf{C_t}$	concentración de soluto en fase líquida a tiempo t
CZE	electroforesis capilar de zona
D	diameter
D	diffusion coefficient
DC	degree of monomeric conversion
DPPH	2,2-Diphenyl-1-picrylhydrazyl

DSC	differential scanning calorimetry
DVB	divinilbenceno
EC	epicatequina, (-)-epicatechin
ECG	epicatequina galato, (-)-epicatechin gallate
EGC	epigalocatequina, (-)-epigallocatechin
EGCG	epigalocatequina galato, (-)-epigallocatechin gallate
EGDMA	etilenglicol dimetacrilato, ethylene glycol dimethacrylate
EPI	epicatechin
ESI	elestrospray ionization
EtOH	ethanol
EVOH	etilen vinil alcohol, ethylene vinyl alcohol
F	representación de la fracción de soluto adsorbido a cualquier tiempo t
FL	detector de fluorescencia, fluorescence detector.
FTIR	espectroscopia infrarroja con transformada de Fourier, Fourier transform infrared spectroscopy
GA	gallic acid
GC	galocatequina
GCG	galocatequina galato, (-)-gallocatechin gallate
GRAS	sustancias generalmente reconocidas como seguras, substances generally recognized as safe
h_1	primera velocidad de adsorción
h_2	segunda velocidad de adsorción
HPLC	cromatografía líquida de alta resolución
I	imprinting factor
I1010, Irganox 1010	pentaerythritoltetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate

I168, Irgafos 168	tris(2,4-di-tert-butylphenyl)phosphate
IP	efecto del grosor de la capa limítrofe
IR	espectro infrarrojo
Irganox 1076	octadecyl-3-(3,5-di-tertbutyl-4-hydroxyphenyl)-propionate
K	partition coefficient
$\mathbf{k_1}$	constante de velocidad de pseudo-primer orden
$\mathbf{k_2}$	constante de velocidad de pseudo-segundo orden
$\mathbf{K_{DR}}$	relacionado con la energía libre de adsorción por molécula de adsorbato cuando es transferida a la superficie del sólido desde el infinito en la disolución
$\mathbf{k_{ext}}$	parámetro de la velocidad de difusión para el modelo de difusión
$\mathbf{K_F}$	capacidad de adsorción del adsorbente
$\mathbf{k_i}$	constante de velocidad por el modelo de difusión intra-partícula
$\mathbf{K_{K1\text{-}K2}}$	media aparente de afinidades
$\mathbf{K_L}$	constante relacionada con la energía libre de adsorción
$\mathbf{K_m}$	constante de enlace
$\mathbf{K_p}$	distribution coefficient
l	film thickness
L	length
LDPE	low-density polyethylene
LOD	límite de detección, detection limit
LOQ	límite de cuantificación, quantification limit
Lp	film thickness
LTQ	linear ion trap
m(t)	mass of the migrant in the food at a particular time

m	intensidad de adsorción o heterogeneidad de superficie
M	monomer
MAA	ácido metacrílico, methacrylic acid
MAH	maleic anhydride
MDPE	polietileno de media densidad
MEKC	cromatografía electrocinética micelar
$M_{F,\infty}$	mass of migrant in the food at equilibrium
MFI	melt flow index
MIP	polímero de impresión molecular, molecularly imprinted polymers
MISPE	extracción en fase sólida sobre MIPs, SPE involving a molecular imprinted polymer
M_P	initial loading of antioxidants in the film
MS	mass spectrometry
M_s^f	mass of the migrant in the food at equilibrium
M_t	mass of the migrant in the food at a particular time t
N	number of data points
n	relacionado con el número de capas de enlace
NaOH	sodium hydroxide
nd	not detected
NIP	polímero no impreso molecularmente, non-imprinted polymers
N_{K1-K2}	número aparente de sitios
NMR	nuclear magnetic resonance
OIT	oxidation induction time
PDA	detector de red de diodos, photodiode array detector
PE	poliethylene

PEAD	polietileno de alta densidad
PEBD	polietileno de baja densidad
PEBDL	polietileno de baja densidad lineal
PEG	polyethylene glycol
PE-PEG	polyethylene-block-poly(ethylene glycol)
PET	polietilentereftalato, polyethylene terephthalate
PG	galato de propilo
PLA	ácido poliláctico
PLGA	ácido poliláctico co-glicólico
PP	polipropileno, polypropylene
PPG	polypropylene glycol
PPG-PEG-PPG	poly(propylene glycol)-block-poly(ethylene glycol)-block-poly(propylene glycol)
PPMAH	maleic anhydride modified polypropylenes
preI	initial imprinting value for the template during the synthesis step
PTFE	teflón, polytetrafluoroethylene
PVC	policloruro de vinilo
$\mathbf{q_{e,cal}}$	calculated adsorption uptake at the equilibrium
$\mathbf{q_{e,exp}}$	experimental adsorption uptake at the equilibrium
$\mathbf{q_e}$	cantidad del soluto adsorbido en condiciones de equilibrio
$\mathbf{q_m}$	máxima capacidad de adsorción del adsorbente, lo que representa el número total de sitios de enlace
QqQ	triple quadrupole mass detector
$\mathbf{q_{t,cal}}$	calculated adsorption uptake
$\mathbf{q_{t,exp}}$	experimental adsorption uptake
$\mathbf{q_t}$	cantidad de adsorbato adsorbida a cualquier tiempo

Quer	quercetin
R (tablas 6 y 3.6)	constante de los gases ideales
R (figura 3.3)	recovery
RL	factor de separación o parámetro de equilibrio
RMN	resonancia magnética nuclear
Rs	resolution
S	selectivity factor
$\mathbf{S_b}$	amount of substrate bound to the polymer
SB	standard deviation of the blank
SDS	sodium dodecyl sulphate
SEM	scanning electron microscope, microscopía electrónica de barrido
SIM	selective ion monitoring
SML	specific migration limit
SPE	solid phase extraction, extracción en fase sólida
T	temperatura absoluta
T	template
t	tiempo
$\mathbf{t_{1/2}}$	tiempo requerido para la adsorción de al menos la mitad del compuesto adsorbido en el equilibrio
TBHQ	ter butil-hidroquinona
$\mathbf{T_c}$	crystallization temperature
TGA	thermogravimetric analysis
Thy	Thymol
$\mathbf{T_m}$	melting temperature, temperatura de cristalización
TRIM	trimetilpropano timetacrilato, 2,2`-Azobis(2-methylpropionitrile)

UPLC **UHPLC**	cromatografía de líquidos de alta resolución; ultra-high performance liquid chromatography
UV-Vis	espectroscopia ultravioleta-visible
V_P	polymer volume
V_S	solution volume
X_f	concentration of substrate remaining in solution after adsorption to the polymer
yB	blank signal
α	velocidad inicial de adsorción
α-TOCO	alpha-tocopherol
β	relacionado con la extensión de cobertura de la superficie y con la energía de activación para quimisorción
ΔH_c	crystallization enthalpie
ΔH_m	melting enthalpie
Δq_t	normalized standard deviation
4-Vp	4-vinilpiridina, 4-vinylpiridine

CAPÍTULO I. INTRODUCCIÓN

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1.1. POLÍMEROS EN EL ENVASADO ALIMENTARIO

Aunque la preocupación general de los consumidores por su salud ha existido desde los primeros tiempos, interesándose por conocer qué alimentos podían suponer un riesgo; en las últimas décadas, la preocupación por la seguridad y toxicidad de los mismos se ha visto incrementada. El binomio consumo-seguridad alimentaria, ha cobrado una mayor importancia como resultado de la innovación en las condiciones de producción, transformación y distribución de los alimentos, así como de los cambios en los hábitos de consumo, llevando a un avance del conocimiento de los sistemas de envasado y de los peligros y riesgos alimentarios a los que el consumidor se encuentra expuesto. Esta creciente preocupación e interés han supuesto nuevas demandas para la industria alimentaria con la búsqueda de nuevos envases que atraigan al consumidor, que le den la máxima información sobre el alimento y que provoquen reducciones mínimas en las características propias de los alimentos, conservando sus propiedades y calidad sensorial intactas, pero proporcionando al mismo tiempo, alimentos lo más seguros posibles, tanto desde el punto de vista higiénico como de salud, y con una prolongada vida útil (Zink, 1997; van der Valk y col., 2005; Palzer, 2009).

Los alimentos son productos no inertes cuyos componentes son responsables de sus propiedades y características organolépticas y de su valor nutritivo. Los procesos de degradación progresivos de los alimentos durante las etapas de transporte y almacenamiento (reacciones de oxidación, degradación enzimática, cambios físicos por pérdida o ganancia de humedad, entre otras) se traducen en una pérdida de la calidad nutricional, sensorial y fisicoquímica de los alimentos. De ahí que de los objetivos básicos del envase alimentario sean la protección de los alimentos frente a las agresiones

externas (aire, agua, olor, microorganismos, luz, etc.), y su acción como barrera frente a la pérdida de características deseables del producto (aroma, sabor, estructura, etc.) sin proporcionarle al alimento otras características no deseables con la finalidad de alargar su vida útil, conservando sus principales características fisicoquímicas, sensoriales y nutricionales (Mateo, 2001).

Tradicionalmente, materiales como la cerámica, el vidrio, el acero, el aluminio o el cartón han sido ampliamente utilizados en el sector del embalaje alimentario. Sin embargo, en las últimas décadas, se ha producido un incremento substancial en el empleo de plásticos (materiales poliméricos). Tal y como se observa en las Figuras 1 y 2, el sector del envase y embalaje representa un 39% de la demanda europea de plásticos (PlasticsEurope, 2012) y el plástico representa un 37% del total de materiales empleados en este sector (Centro Español de Plásticos, 2012).

El uso generalizado de los plásticos puede atribuirse a su alta versatilidad de forma, facilidad de procesado y modelado, ligereza, facilidad de impresión, buena inercia química, resistencia mecánica adecuada y su relativo bajo coste (Rodríguez-Pin y col., 2003).

Dentro del sector del envase y embalaje, más de la mitad del total de la producción de plásticos se destina a alimentación. Actualmente, en la industria del envasado se emplean más de 30 tipos diferentes de plásticos. Los más comúnmente empleados son aquellos basados en poliolefinas, copolímeros de etileno, olefinas substituídas, poliésters, policarbonatos, poliamidas, acrilonitrilos, celulosas o ácido poliláctico (Siracusa, 2012). De todos ellos, las poliolefinas, entre las que se encuentran el polietileno de baja densidad (PEBD), de baja densidad lineal (PEBDL), de alta densidad (PEAD) y el polipropileno (PP) representan más del 47% del consumo total de plástico

en la zona oeste de Europa (PlasticsEurope, 2012), lo cual se atribuye a su versatilidad, bajo precio, fácil procesabilidad, excelentes propiedades barrera frente a la captación y pérdida de vapor de agua, inercia química, sellabilidad a temperaturas relativamente bajas, sencilla producción de films retráctiles y buena resistencia al impacto (Mateo, 2001).

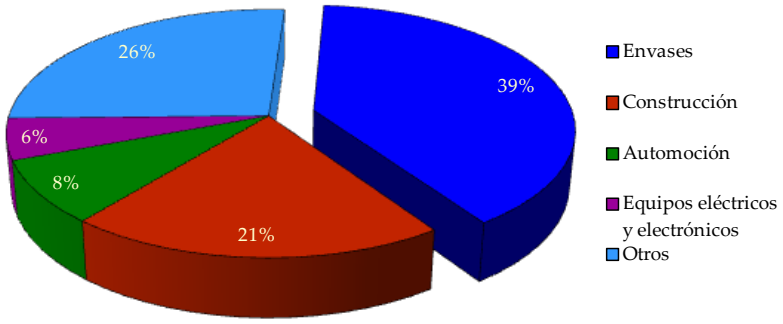


Figura 1. Demanda europea de plásticos por sectores (PlasticsEurope, 2012).

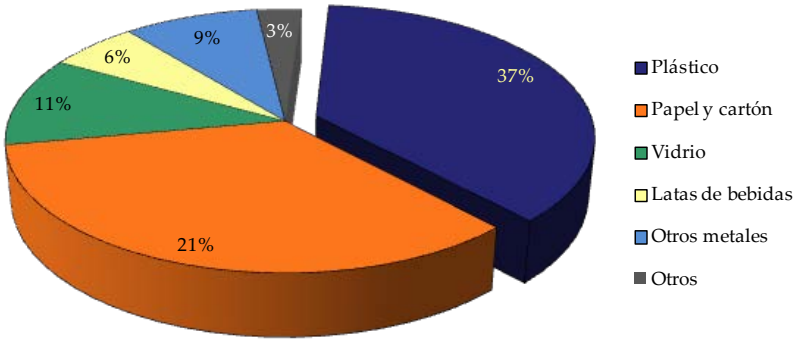


Figura 2. Materiales empleados en la fabricación de envases y embalajes - año 2012 (Centro Español de Plásticos, 2012).

Sin embargo, la existencia de ciertas interacciones envase-alimento, ha conducido a un cierto conflicto entre la innovación en el envase alimentario, nuevas aplicaciones y nuevos materiales de embalaje, y restricciones para garantizar una satisfactoria protección del consumidor (Vergnaud, 1998).

El conocimiento de los procesos de permeación (intercambio de sustancias a través del envase entre el ambiente exterior y el interior), sorción (retención en el material del envase de sustancias inicialmente presentes en el alimento) y migración (transferencia de sustancias del envase al alimento) resulta de elevada importancia en el caso de envases alimentarios, en los cuales dicha interacción entorno-envase-alimento y el consecuente intercambio de moléculas de bajo peso molecular puede ser un factor limitante de la aplicación del material plástico o una propiedad que le da valor añadido al envase (Gnanasekharan y Floros, 1997; Caner, 2011). La Figura 3 describe dichos procesos de transferencia que se establecen entre el sistema entorno-envase-alimento. Este intercambio, puede dar lugar en ocasiones, a procesos no deseables como alteraciones de las características sensoriales y nutritivas de los alimentos, o la incorporación de sustancias tóxicas en el mismo, así como cambios en las características físico-químicas y mecánicas del envase (Tehrany y Desobry, 2004).

Sin embargo, estos fenómenos de permeación, sorción y migración, pueden también ser empleados con el objetivo de mejorar la calidad, aceptabilidad y vida útil del alimento mediante los nuevos sistemas conocidos como envases activos e inteligentes (Vermeiren y col., 1999).

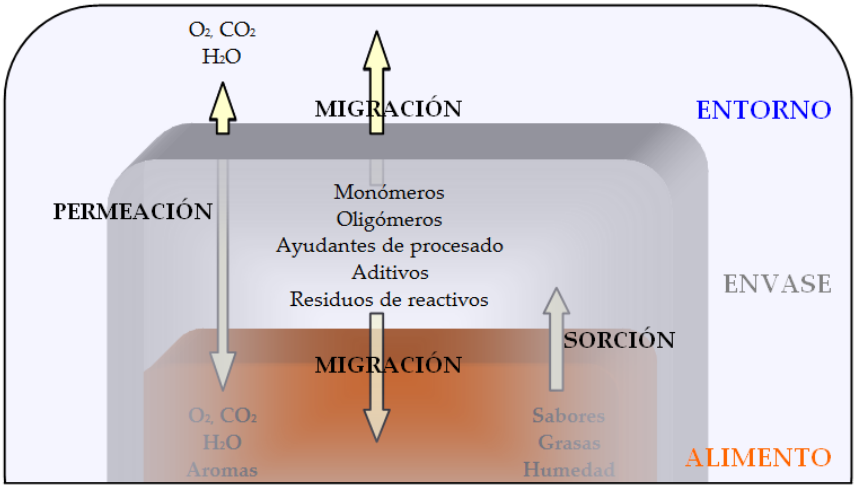


Figura 3. Interacciones entorno-envase-alimento.

1.2. ADITIVOS EN ENVASES ALIMENTARIOS: ANTIOXIDANTES.

El polímero obtenido como resultado de la polimerización no tiene, en la mayoría de los casos, una utilidad práctica por descomponerse al calentarlo, o por ser excesivamente rígido, o muy blando, o por no tener un color atractivo o simplemente por no responder a las características necesarias para su uso concreto. A fin de conseguir las mejores características de los polímeros en cada una de sus aplicaciones, la industria ha recurrido a la modificación del polímero mediante la adición de sustancias, que modifiquen sus propiedades, favoreciendo su transformación y uso posterior, los denominados aditivos.

El término aditivo se define como "aquella sustancia que se incorpora a los polímeros a fin de conseguir un efecto técnico en el producto final, siendo una parte esencial del mismo". Los aditivos pueden ser, en relación a su naturaleza química, especies orgánicas (alquilfenoles, hidroxibenzofenonas, por ejemplo), inorgánicas (sales, óxidos,…) u organometálicas (complejos de

níquel, aceleradores de cinc, entre otros) (Bart, 2005). Entre las características que debe cumplir un aditivo destacan (Brydson, 1975):

- Ser eficientes en su acción.
- Estables en las condiciones de procesado.
- Estables en las condiciones de uso.
- No sufrir exudado o sangrado.
- No ser tóxicos ni dar lugar a olor o sabor.
- Ser económicos.
- No afectar negativamente a las propiedades del polímero.

Numerosas sustancias han sido empleadas para mejorar las características de los polímeros. En la Tabla 1 se recogen los tipos más comunes de aditivos empleados y las funciones básicas de los mismos (Avendaño, 1992; Maier y Calafut, 1998).

El efecto producido por el aditivo en el plástico depende tanto de la composición química del polímero, de la presencia de otros aditivos y de un posible efecto sinérgico con los mismos, así como de la cantidad de aditivo incorporada. En la Figura 4 se muestra como sólo añadiendo el aditivo en la proporción necesaria éste va a realizar su función en toda su extensión y con la mayor efectividad posible. Un exceso de aditivo no va a dar lugar a mejoras en el resultado y un porcentaje inferior al efectivo no va a provocar el efecto deseado.

Tabla 1. Tipos de aditivos más comúnmente empleados en las formulaciones de polímeros.

Tipo de aditivo	Función
Antioxidantes	Inhibir las reacciones de termooxidación
Estabilizantes a la luz	Evitar el deterioro del polímero causado por la exposición a la luz UV (agrietamiento, decoloración, pérdida de brillo, transparencia y fragilidad)
Plastificantes	Aumentar la flexibilidad y la resistencia al impacto
Agentes antiestáticos	Proteger al polímero frente a las descargas electrostáticas
Captadores de ácido o antiácidos	Neutralizar residuos ácidos catalíticos
Desactivadores metálicos	Desactivar residuos metálicos presentes en la formulación de las poliolefinas
Agentes nucleantes	Mejorar la claridad, transparencia y propiedades mecánicas
Retardadores de llama	Alterar el proceso de combustión originado por un importante flujo térmico
Colorantes	Proporcionar color al polímero
Agentes "antiblocking"	Evitar la adhesión entre films como consecuencia del frío o de la electricidad estática
Lubricantes	Disminuir la viscosidad de flujo y evitar el pegado del polímero a las superficies metálicas
Agentes "slip"	Impedir el pegado entre films mediante lubricación y reducir la carga electrostática
Agentes espumantes o de soplado	Disminuir la densidad del polímero por formación de gas en el procesado del polímero

El uso de antioxidantes tiene un interés especial en los materiales poliméricos destinados a envases de alimentos. En general, la formulación de las poliolefinas incluye la adición de antioxidantes para evitar la degradación generada como consecuencia de las reacciones de oxidación, que producen una pérdida de sus propiedades mecánicas: resistencia al impacto, a la tensión, a la elongación, a la flexión; cambio de color, especialmente amarilleo, así como una apariencia superficial endurecida, con manchas,

grietas y menor brillo; reflejándose todo ello en una apariencia envejecida del envase (Sánchez López, 1992; Zweifel, 2001).

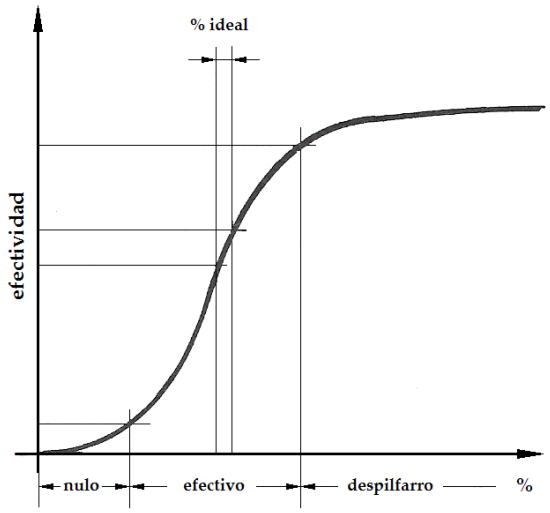


Figura 4. Efectividad del aditivo en función del porcentaje añadido al plástico (Avendaño, 1992).

La oxidación puede tener lugar en cualquier momento del ciclo de vida de la poliolefina, ya sea durante la fabricación y almacenamiento de la granza, durante su procesado o durante el tiempo de uso del envase una vez fabricado.

Los efectos de la oxidación dependen de ciertas propiedades como la cristalinidad del polímero, estructura, residuos de catalizador, temperatura e irradiación. Así, por ejemplo, el efecto estructural de la oxidación sobre un polímero puede ser la ruptura de las cadenas o el entrecruzamiento de las mismas. En el caso concreto de las poliolefinas, la primera de las situaciones se produce en el propileno debido a su alta susceptibilidad a la oxidación

dando lugar a un descenso en su masa molecular, en su resistencia y en general, en todas sus propiedades mecánicas, manifestándose también con un aumento del índice de fluidez. En el caso del polietileno, el entrecruzamiento de las cadenas como resultado de la oxidación provoca un aumento de sus propiedades mecánicas y un descenso del índice de fluidez (Wang, 2000).

Además, en los polímeros utilizados en envases para alimentos hay que tener en cuenta que el alimento también es susceptible de sufrir procesos de alteración por oxidación y que una de las funciones del envase es proteger al alimento de este tipo de degradación.

La oxidación lipídica constituye también una de las formas más comunes de deterioro de los alimentos, principalmente de aquellos de largo tiempo de almacenamiento y sólo por detrás de las alteraciones producidas por los microorganismos en productos con corto tiempo de almacenamiento (Gunstone, 2003). Los lípidos son uno de los mayores constituyentes de los alimentos y junto con los aceites, son particularmente susceptibles a la oxidación lipídica produciendo deterioros en la calidad del alimento al dar lugar a la degradación de los nutrientes, pérdida de vitaminas y ácidos grasos poliinsaturados, aparición de olores anómalos y cambios en la textura y el color. Además, algunos de los productos resultantes del proceso de oxidación pueden resultar tóxicos, pudiendo contribuir al desarrollo de ciertas enfermedades (Gotoh y col., 2006).

1.2.1. Reacciones de oxidación

Los procesos de degradación oxidativa del polímero han sido descritos como reacciones de radicales libres (R·) formados por efecto del calor, luz o

esfuerzos mecánicos, los cuales combinados con el oxígeno forman radicales peróxido (ROO·) capaces de extraer un átomo de hidrógeno de las cadenas poliméricas para formar un hidroperóxido y otro radical libre, repitiéndose el ciclo. Los hidroperóxidos, a su vez, se descomponen en más radicales libres capaces de continuar la reacción. La secuencia de reacciones que tienen lugar durante la oxidación de las poliolefinas se muestra en la Tabla 2 (Brydson, 1975; Sánchez López, 1992).

Tabla 2. Reacciones de oxidación de polímeros.

Periodo de inducción	
- Formación de radicales	$RH \text{ ó } RR \xrightarrow{Energía} R\cdot$
- Acción del oxígeno sobre los radicales	$R\cdot + O_2 \rightarrow ROO\cdot$ $ROO\cdot + RH \rightarrow ROOH + R\cdot$
- Rotura de los enlaces de los hidroperóxidos	$ROOH \xrightarrow{Energía} RO\cdot + \cdot OH$ $2ROOH \rightarrow RO\cdot + ROO\cdot + H_2O$
Propagación	
- Acción de los radicales alcoxi y radicales hidroxilo en el polímero	$RO\cdot + RH \rightarrow ROH + R\cdot$ $\cdot OH + RH \rightarrow H_2O + R\cdot$
- Reacciones secundarias	$R\cdot + R_1R_2C = CR_3R_4 \rightarrow H_2O + RR_1R_2C - CR_3R_4\cdot$ $R_1CH_2CH_2 \rightarrow R_1\cdot + H_2C = CH_2$
Terminación	
- Formación de especies no reactivas	$R\cdot + ROO\cdot \rightarrow ROOR$ $2R\cdot \rightarrow RR$ $R\cdot + RO\cdot \rightarrow ROR$

La oxidación lipídica está basada en una reacción en cadena de radicales libres siguiendo el mismo mecanismo en cuatro etapas descrito en la Tabla 2 para la oxidación de los polímeros con abstracción de átomo de hidrógeno del

ácido graso. En el caso de los lípidos dicho mecanismo constituye una sobre simplificación del proceso real en el cual la oxidación puede transcurrir a través de muchas posibles reacciones químicas bajo diferentes condiciones, siendo así un proceso dinámico con continuo cambio de los productos de oxidación (Choe y Min, 2005; Schaich, 2005).

1.2.2. Antioxidantes

Con el objetivo de lograr una mayor vida útil, tanto del propio polímero como del alimento envasado, así como minimizar el efecto peligroso de los productos de oxidación en la calidad del alimento y en la salud del consumidor, es necesario retardar el proceso de oxidación.

Una de las técnicas más efectivas es el uso de antioxidantes tanto en la formulación de los materiales de envasado como en el propio alimento contenido.

1.2.2.1. Clasificación de antioxidantes

Los antioxidantes son compuestos químicos capaces de reducir la oxidación de un sustrato al interceptar y reaccionar con los radicales libres a una velocidad mayor que el sustrato oxidable. La estructura química de los antioxidantes condiciona su mecanismo de acción en las poliolefinas, pudiendo clasificarse en antioxidantes primarios y secundarios, aunque algunos de ellos pueden exhibir más de un mecanismo de actuación (Sánchez-López, 1992; Maier y Calafut, 1998).

- *Antioxidantes primarios*: también llamados terminadores de radical libre o rompedores de cadena, inhiben la reacción de oxidación por combinación con radicales libres R· o con radicales peroxi (ROO·). Se trata principalmente de compuestos fenólicos con impedimentos estéricos y de aminas aromáticas secundarias. En este caso, el grupo reactivo OH de los fenoles o el NH de las aminas dona un hidrógeno al radical carbono, oxígeno o peroxi (ROO·) dando lugar a un producto estable e inactivo, quedando ellos mismos, como un nuevo radical. La forma aromática del anillo así como los posibles sustituyentes alquilo existentes proceden a estabilizar ese radical libre, el cual no es capaz de abstraer un nuevo hidrógeno para proceder a la propagación de la reacción, sin embargo, si puede reaccionar y combinarse con otro radical libre peroxi para dar lugar a un nuevo producto inactivo. Por lo tanto, al mismo tiempo que el antioxidante primario es capaz de interrumpir la reacción oxidativa pierde su actividad como antioxidante (Gramza y Korzack, 2005).

- Antioxidante fenólico: $ROO\cdot + Ar-OH \rightarrow ROOH + Ar-O\cdot$

$$Ar-O\cdot + ROO\cdot \rightarrow ROO-Ar=O$$

- Antioxidante amina: $ROO\cdot + Ar-NH \rightarrow ROOH + Ar-N\cdot$

$$Ar-N\cdot + ROO\cdot \rightarrow Ar-N-OOR$$

Dado el tipo de protección a la que dan lugar este tipo de antioxidantes son empleados para proteger a la poliolefina durante las etapas posteriores de su vida útil: almacenamiento y uso.

Los antioxidantes fenólicos permiten, generalmente, evitar el manchado y decoloración de los polímeros así como reducir el sangrado. Sin embargo su

oxidación da lugar a la formación de estructuras quinonas con dobles enlaces conjugados, las cuales son cromóforas y absorbentes de luz dando lugar al amarillamiento de la poliolefina. Este efecto se puede reducir al usar compuestos fenólicos de alta masa molecular y con sustituyentes voluminosos que, interfiriendo estéricamente en la reacción de oxidación, evitan con facilidad que se produzca este fenómeno. Coaditivos, tales como ciertos compuestos de fósforo y antiácidos son también comúnmente empleados con el fin de evitar el amarillamiento. En el caso de las aminas aromáticas la decoloración se produce fácilmente, lo cual limita su aplicación a casos en los cuales la decoloración se ve enmascarada por el efecto de otras sustancias, como, por ejemplo, con pigmentos o con negro de humo (Brydson, 1975).

- *Antioxidantes secundarios*: o destructores de peróxido inhiben la oxidación de la poliolefina por descomposición de los grupos hidroperóxidos (ROOH). Se trata de compuestos de azufre (éteres y ésteres del ácido tiodipropiónico) y fosfitos (triésteres de ácidos fosforosos), los cuales reducen los hidroperóxidos a especies de alcohol estables en una reacción de oxidación-reducción en la cual el fosfito o el tioéter se oxidan a fosfato o sulfóxido, respectivamente:

  - Antioxidante fosfito: $(RO)_3P + ROOH \rightarrow (RO)_3P = O + ROH$

  - Antioxidante tioéter: $ROOH + SR_1R_2 \rightarrow ROH + OSR_1R_2$

Este tipo de antioxidantes se encarga de proteger a la poliolefina frente a la degradación, principalmente, durante la etapa de procesado.

Habitualmente, los fosfitos son los antioxidantes secundarios más empleados dado que no producen decoloración y son bastante compatibles con los polímeros como consecuencia de su baja volatilidad. Sin embargo, son susceptibles a la hidrólisis dando lugar a especies ácidas por reacción con el agua lo que hace necesario un embalaje y almacenamiento especial (Habicher y col., 2005).

Antioxidantes primarios y secundarios suelen emplearse combinados dando lugar a un efecto sinérgico con un efecto antioxidante final mayor que los efectos antioxidantes individuales (Ray e Isenhart, 1975). Del mismo modo, los *antioxidantes multifuncionales* incluyen en su estructura grupos capaces de actuar como antioxidantes primarios y grupos capaces de actuar como antioxidantes secundarios englobando en una sola molécula las capacidades antioxidantes anteriormente proporcionada por dos compuestos individuales (www.specialchem4adhesives.com, 2013; Zhang y col., 2006).

Los antioxidantes que se añaden directamente al alimento actúan de acuerdo al mismo mecanismo que los antioxidantes empleados en poliolefinas y su clasificación es equivalente. Así, es frecuente el uso de compuestos fenólicos y de agentes reductores como los ácidos orgánicos o compuestos sulfurados. En este caso, el papel del antioxidante no es realzar o mejorar la calidad del alimento, sino mantener su calidad y extender su vida útil (Laguerre y col., 2007).

1.2.2.2. Antioxidantes sintéticos

De todos los antioxidantes sintéticos existentes en el mercado, sólo una parte han podido ser empleados en el campo alimentario dada la necesidad

de comprobar la ausencia de toxicidad y actividad carcinogénica de sus formas oxidadas y de sus productos de reacción con los constituyentes del alimento.

La capacidad de migrar desde el envase al alimento de algunos de estos antioxidantes sintéticos ha causado también cierta preocupación en cuanto a su uso continuo en los materiales de envasado (Dopico-García y col., 2007).

Si bien estos antioxidantes sintéticos son altamente efectivos y económicos (Shahidi, 2000), su potencial efecto tóxico sobre la salud del consumidor, así como la necesidad de que en ocasiones el producto permanezca envasado durante un largo periodo de tiempo, ha llevado a la búsqueda de nuevas sustancias naturales que reemplacen a los antioxidantes comerciales (Ito y col., 1986; Botterwek y col., 2000).

1.2.2.3. Antioxidantes naturales

En general, como antioxidantes naturales se pueden considerar aquellas sustancias que siendo extraídas de fuentes de origen natural (animales o vegetales) son capaces de actuar de forma efectiva frente a los procesos de degradación oxidativa.

Los antioxidantes naturales son primordialmente compuestos fenólicos (Shahidi y col., 1992). En general, los antioxidantes fenólicos se pueden dividir en varios grupos diferentes en función de su estructura básica. En la Tabla 3 se recogen ejemplos de algunos de los más comúnmente encontrados en plantas (Herrero y col., 2005; Pokorný, 2007).

Tabla 3. Antioxidantes de origen natural más comunes en plantas.

Clase de antioxidante	Ejemplos
Ácidos fenólicos	Ácido gálico, ácido salicílico
Ácidos hidroxibenzoicos	Ácido vanillinico
Ácidos hidroxicinámicos	Ácido ferúlico, ácido clorogénico
Flavonoides	Quercetina, catequina, rutina
Antocianinas	Delfinidina
Taninos	Procianidina, ácido tánico
Lignanos	Sesamol
Estilbenos	Resveratrol
Coumarinas	Orto-coumarina
Aceites esenciales	S-carvona

De entre los antioxidantes naturales, el ácido ascórbico (vitamina C) y sus derivados (ascorbato sódico, ascorbato cálcico, palmitato de ascorbilo), los tocoferoles, los ácidos fenólicos y los flavonoides son los antioxidantes naturales más comúnmente utilizados en aplicaciones alimentarias. Otros compuestos presentes de forma natural con actividad antioxidante son compuestos nitrogenados como los alcaloides, aminoácidos y aminas o los carotenoides y ciertas proteínas (Velioglu y col., 1998).

El **ácido ascórbico** ha sido ampliamente utilizado en la industria alimentaria, no sólo como antioxidante sino también como prooxidante, quelatante de metales, agente reductor o secuestrador de oxígeno, dependiendo de las condiciones. Su aplicación como antioxidante único no suele ser muy efectiva y habitualmente es empleado junto con otros antioxidantes, especialmente tocoferol, dado el gran efecto sinérgico entre ambos, produciéndose la regeneración del tocoferol a partir del radical

intermedio tocoferilo mediante un mecanismo de oxidación-reducción (Frankel, 1996). Sin embargo, su fácil degradación en presencia de oxígeno, metales, luz o calor se presenta como su mayor inconveniente (Kaur y Kapoor, 2001).

Los **tocoferoles** constituyen unos de los antioxidantes naturales más ampliamente empleados no sólo en aplicaciones directas en alimentos, sino también en envase alimentario. Están considerados como sustancias "generalmente reconocidas como seguras" (GRAS) por la FDA y sus productos de degradación, principalmente quinonas de tocoferol, son inocuas (Laermer y Zambetti, 1992). Los tocoferoles se presentan en varias formas isoméricas (α , β, δ, γ) con diferente actividad. Su capacidad antioxidante se debe a su habilidad para actuar como antioxidantes primarios atrapando radicales libres y donando un átomo de hidrógeno formándose el radical tocoferilo. Y esta capacidad antioxidante es altamente dependiente de la concentración y de la temperatura. Aunque generalmente el orden de capacidad antioxidante suele considerarse como $\delta>\beta>\gamma>\alpha$, a bajas concentraciones el α-tocoferol es más antioxidante que el γ-tocoferol, invirtiéndose el orden a altas concentraciones (Sahidi, 2000). Aplicado en protección del envase alimentario, actúa como un buen estabilizador durante el procesado del polímero dada su alta estabilidad durante esta etapa, así como su alta solubilidad en poliolefinas (Al-Malaika y col., 1999). Sin embargo, su bajo rendimiento durante esta etapa de procesado, así como una cierta actividad prooxidante a altas concentraciones (Frankel, 1996), su relativo alto coste en relación a su efectividad y la posibilidad de producir efectos colaterales en las propiedades sensoriales de los alimentos como

resultado de la liberación de sustancias altamente aromáticas (López de Dicastillo y col., 2011), se presentan como sus mayores desventajas.

Los **flavonoides** representan el grupo más común y ampliamente usado de compuestos fenólicos de origen natural utilizados como antioxidantes. Su esqueleto básico está formado por una estructura común de difenilpropano (C_6-C_3-C_6) con dos anillos aromáticos (A y B) unidos a través de tres carbonos formando un heterociclo oxigenado (C). La Figura 5 representa la estructura general de los flavonoides.

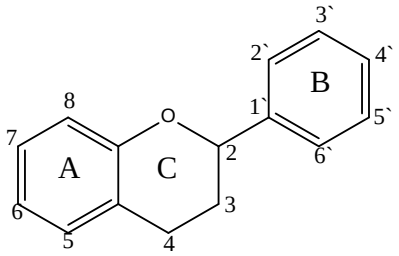
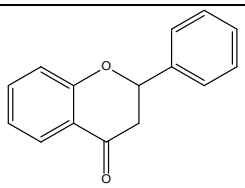
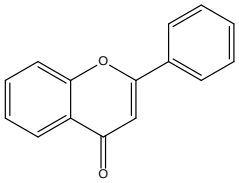
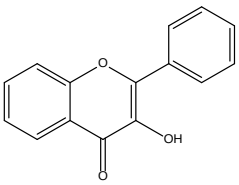
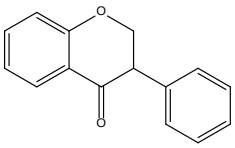
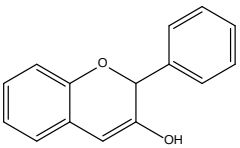
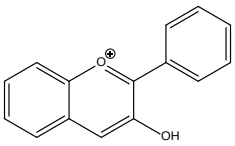


Figura 5. Estructura general de los flavonoides.

Se han caracterizado más de 4000 especies de flavonoides encuadradas en diferentes grupos en función de su estructura particular: variación en número y posición de grupos hidroxilos y naturaleza y extensión de alquilación y/o glicosilación de esos grupos (Rice Evans y col., 1996). En la Tabla 4 se recopilan los principales grupos de flavonoides así como ejemplos representativos de los mismos (van Acker y col., 1996; Bravo, 1998). El número de monómeros y la presencia/ausencia de grupos funcionales en diferentes posiciones marcan los diferentes compuestos encuadrados dentro de cada grupo.

Tabla 4. Principales familias de flavonoides y ejemplos de los mismos.

Flavonoide	Estructura	Ejemplos
Flavanonas		Naringenina Naringina Hesperitina Taxifolina
Flavonas		Apigenina Luteolina Diosmetina
Flavonoles		Quercetina Rutina Miricetina Kaemferol
Isoflavonas		Genisteína Genistina
Flavanoles		Catequinas
Antocianinas		Cianidina Malvidina Pelargonidina

Principalmente los flavonoides actúan como antioxidantes primarios y su capacidad antioxidante es distinta entre los diferentes grupos de flavonoides y entre los compuestos de un grupo dependiendo del número de monómeros que conforman su estructura, así como de la presencia de distintas modificaciones o grupos funcionales y de su posición: presencia del grupo hidroxilo en la posición 3 del anillo C, doble enlace entre C_2 y C_3 del anillo C,

el grupo carbonilo en el átomo C_4 o el número de grupos hidroxilos (Cook y Sammman, 1996; Harborne y Williams, 2000).

Se han estudiado los efectos positivos de estos compuestos sobre la salud humana. Así, se conoce que su capacidad antioxidante, así como su capacidad para inhibir ciertas enzimas induce efectos antiarterioescleróticos, antiinflamatorios, antitumurales, antitrombóticos, antiosteoporóticos y antivirales, entre otros (Nijveldt y col., 2001).

Entre los flavonoides, flavonoles como la quercetina y flavanoles como la familia de las catequinas presentan una elevada capacidad antioxidante, comparable e incluso mayor que la presentada por alguno de los antioxidantes sintéticos más utilizados, como el dibutilhidroxitolueno (BHT) o el tercbutilhidroxianisol (BHA) o por otros antioxidantes naturales como la vitamina C o los tocoferoles (Pérez Mateos y col., 2005, Yilmaz, 2006).

La **quercetina** es uno de los flavonoides más efectivos frente a la degradación oxidativa y el más común entre los flavonoles. Su potencial antioxidante es debido a su capacidad para secuestrar radicales libres (Vinson y col., 1995). Sin embargo, la degradación oxidativa de la molécula de quercetina da lugar a cambios inusuales en su capacidad antioxidante dependiendo de la temperatura y del disolvente (Pinelo y col., 2004).

Las **catequinas** son potentes antioxidantes capaces de secuestrar diferentes radicales reactivos de oxígeno en virtud de las propiedades reductoras de grupos hidroxilos unidos en los anillos aromáticos. Su actividad antioxidante varía en función de la relación estructural entre las diferentes partes de la estructura química y de la propiedades redox de los grupos hidroxifenólicos (RiceEvans y col., 1996). En base a su capacidad antioxidante, las distintas catequinas son consideradas como prometedores

antioxidantes naturales para ser empleadas como aditivos en alimentos. Además, se les han atribuido importantes propiedades beneficiosas para la salud del consumidor como antimutagénicas, antiobesidad, antidiabéticas, antibacterianas, entre otras (Fukumoto y Mazza, 2000; Zaveri, 2006). La catequina (C) y su isómero la epicatequina (EC), la galocatequina (GC) y su isómero epigalocatequina (EGC), y los ésteres con ácido gálico en posición 3 como las epigalocatequina galato (EGCG), epicatequina galato (ECG), catequina galato (CG) o galocatequina galato (GCG) son las formas predominantes. Las formas glicosiladas son menos frecuentes. La posible actividad prooxidante de las catequinas a elevadas concentraciones y bajo condiciones fotooxidativas se presenta como el mayor inconveniente de las catequinas (Gramza y Korczak, 2005).

1.2.2.4. Principales fuentes de antioxidantes naturales

Los antioxidantes naturales se encuentran presentes en numerosas fuentes naturales del reino vegetal, siendo frutas y vegetales las dos más importantes. En la Tabla 5 se recogen las principales fuentes naturales de antioxidantes fenólicos (Lindberg-Madsen y Bertelsen, 1995; Harbowy y col., 1997; Shahidi, 2000; Gramza y Korczak, 2005; Dimitrios, 2006; Yanishlieva y col., 2006; Pokorný, 2007; Brewer, 2011).

Materiales lignocelulósicos provenientes de residuos agroalimentarios y forestales pueden ser también considerados como fuentes naturales de este tipo de antioxidantes a pesar de tratarse de matrices previamente procesadas (Garrote y col., 2004).

Tabla 5. Principales fuentes de antioxidantes de origen natural.

Fuentes	Ejemplos	Antioxidantes
Frutas	Bayas	Flavanoles de ácido hidroxicinámico, ácido hidroxibenzoico, antocianinas
	Cerezas	Ácidos hidroxicinámicos
	Frutas cítricas	Flavanonas, flavonoles, ácidos fenólicos
	Ciruelas,manzanas, peras, kiwi	Ácidos hidroxicinámicos, catequinas
Vegetales	Berenjenas	Antocianinas, ácidos hidroxicinámicos
	Escarola, alcachofa	Ácidos hidroxicinámicos
	Ruibarbo	Antocianinas
	Hojas de patatas	Flavonoles, flavones
	Cebollas, perejil, col, judías	Flavonoles
	Espinacas	Flavonoides, ácido p-cumárico
Harinas	Avena, trigo, arroz, maíz	Cafeína, ácido ferúlico, tocoferoles
Tes	Negro, verde	Flavanoles, flavonoles
Bebidas alcohólicas	Vino tinto	Flava-3-ol, flavonoles, antocianinas
	Sidra	Ácidos hidroxicinámicos
Otras bebidas	Zumo de naranja	Flavanoles
	Café	Ácidos hidroxicinámicos
Hierbas y especies	Romero	Ácido carnósico, carnosol, ácido rosmarínico
	Salvia	Carnosol, ácido carnósico, lateolin, rosmanul, ácido rosmarínico
	Orégano	Ácido rosmarínico, ácidos fenólicos, flavonoides
	Tomillo	Timol, carvacol, flavonoides, lubeolin
Otros	Chocolate	Flavanoles

Entre todas las matrices fuente de antioxidantes naturales, los tés constituyen una de las fuentes más importantes, no sólo en función del número de antioxidantes presentes en los mismos, principalmente compuestos polifenólicos de la familia de las catequinas, sino también por la capacidad antioxidante de estos, así como por ser la segunda bebida más consumida en el mundo, sólo detrás del agua (Harbowy y col., 1997).

1.3. PURIFICACIÓN DE EXTRACTOS NATURALES: POLÍMEROS DE IMPRESIÓN MOLECULAR.

El conocimiento detallado de la cantidad de cada uno de los antioxidantes presentes en los extractos de las distintas matrices es de gran interés para seleccionar la fuente natural de antioxidantes más adecuada para cada aplicación: suplementos dietéticos o nutricionales, ingredientes alimenticios, aplicaciones farmacéuticas o cosméticas o en envases alimentarios.

El objetivo básico de las etapas de extracción y purificación es lograr la preparación de un extracto de la muestra, uniformemente enriquecido en todos los compuestos de interés y libre de interferencias procedentes de la matriz.

Las extracciones con disolventes son la base de los procedimientos más comúnmente empleados para preparar extractos de muestras naturales, dada su facilidad de uso, eficiencia y amplia aplicabilidad. Disolvente empleado, tiempo, temperatura, proporción muestra-disolvente así como la composición química y las características físicas de la matriz constituyen los principales factores condicionantes de la eficiencia de la separación (Naczk y Shahidi, 2006; Dai y Mumper, 2010).

Los disolventes orgánicos tales como metanol, etanol, acetona, etil acetato, dimetilformamida y las combinaciones entre ellos o con agua, son los más comúnmente empleados para la extracción de compuestos fenólicos. Entre todas estas combinaciones, el metanol o el etanol y las mezclas de acetona-agua suelen ser los disolventes de elección, para la extracción de compuestos polifenólicos de baja y alta masa molecular, respectivamente. La elección del disolvente más adecuado y la solubilidad de los antioxidantes (gobernada por la naturaleza química del antioxidante y la de la matriz), claramente

determinarán la cantidad de compuesto extraído así como la velocidad del proceso de extracción (Xu y Chang, 2007; Hurtado-Fernández y col., 2010). En la actualidad, en base a la preocupación medioambiental y los principios de la química verde, se ha tendido hacia la búsqueda de métodos de extracción que empleen disolventes polares en base acuosa (Raynie, 2006; Ratnasooruya y Rupasinghe, 2012).

El tiempo de extracción y la temperatura también juegan un papel importante en la recuperación de los antioxidantes desde las matrices naturales. La solubilidad de los antioxidantes de interés puede verse aumentada con el incremento de la temperatura de extracción al aumentar tanto la solubilidad del analito, como disminuir la viscosidad del disolvente, lo cual lleva, a su vez, a un aumento en la velocidad del proceso. Sin embargo, largos tiempos de extracción o altas temperaturas incrementan las posibilidades de hidrólisis y oxidación de los propios antioxidantes lo que lleva a una disminución de la cantidad de compuesto extraído (Naczk y Shahidi, 2006; Dai y Mumper, 2010).

1.3.1. Purificación y concentración del extracto: polímeros de impresión molecular.

Los extractos directamente obtenidos de las plantas suelen contener relativamente pequeñas cantidades de los compuestos de interés y grandes cantidades de otros compuestos presentes en la matriz tales como carbohidratos y/o material lipídico. Por lo tanto, suele ser necesario, previo al análisis, incluir etapas de preconcentración y limpieza. La técnica a emplear, nuevamente depende tanto de la naturaleza y concentración de los analitos, como de la muestra. Las técnicas más conocidas son la extracción líquido-

líquido, extracción en fase sólida (SPE), adsorción selectiva, cromatografía de líquidos preparativa, cromatografía iónica, cromatografía de exclusión molecular o microextracción en fase sólida. Entre estas opciones, el método clásico de extracción líquido-líquido cada vez es menos usado por tratarse de un método que requiere mucho tiempo y consumo de disolvente; la extracción en fase sólida, ha suscitado un mayor interés por su rapidez, sensibilidad y bajo consumo de disolvente (Romanik y col., 2007). Por ello, la búsqueda de nuevos materiales adsorbentes que permitan una extracción más específica y selectiva de los analitos de interés está atrayendo cada vez más atención. Entre estos nuevos materiales empleados como adsorbentes destacan los **polímeros de impresión molecular** (MIPs).

La tecnología de impresión molecular se basa en la síntesis de polímeros altamente entrecruzados y muy estables, con la propiedad de que pueden ser diseñados con capacidad de reconocimiento molecular selectivo para un compuesto o familia de compuestos estructuralmente muy relacionados. Este reconocimiento molecular es proporcionado a través de los sitios de enlace generados en la matriz polimérica y que son complementarios, en forma, tamaño y en funcionalidad con el analito o familia de analitos para los cuales fue generado, de forma similar a como ocurre en los sistemas antígeno-anticuerpo presentes en la naturaleza (Steinke y col., 1994; Ye y Mosbach, 2008).

1.3.1.1. Síntesis de MIPs

La especificidad y selectividad de la matriz polimérica porosa del MIP se alcanza durante la síntesis del polímero, la cual consta de tres etapas básicas que aparecen esquematizadas en la Figura 6.

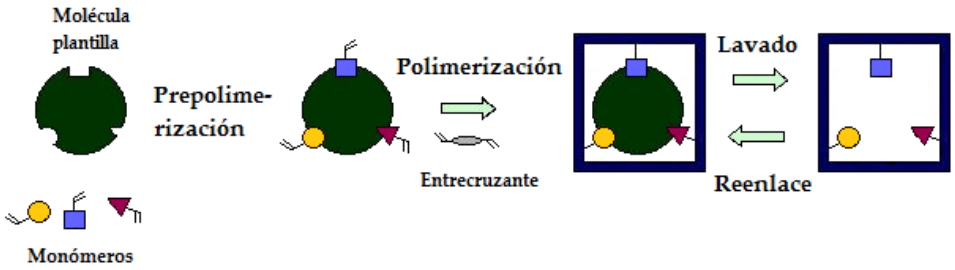


Figura 6. Representación esquemática de las etapas de síntesis de un MIP.

Durante la primera de las etapas se ponen en contacto el analito objeto de estudio (molécula plantilla) y el o los monómeros funcionales, junto con el disolvente, dando lugar a la formación de un complejo de prepolimerización. En la segunda de las etapas tiene lugar la polimerización, en la cual el complejo monómero-molécula plantilla es puesto en contacto con un entrecruzador y un iniciador de la reacción que en presencia de una fuente de energía, ya sea térmica o fotoquímica va a dar lugar al proceso de polimerización. Este proceso suele ser de naturaleza radicalaria. Una vez concluido el proceso de polimerización, en la tercera etapa (etapa de lavado) se procede a la extracción de la molécula plantilla de la matriz polimérica mediante un disolvente o mezcla de disolventes, capaz de anular las interacciones específicas formadas. De este modo, se liberan las cavidades creadas en la matriz polimérica, que son complementarias en tamaño, forma y funcionalidad química a la molécula plantilla. La presencia de estas cavidades hace que el polímero impreso sea capaz de reconocer de forma específica y selectiva únicamente al analito de interés, o la familia de compuestos con estructuras análogas al analito de interés, pudiendo así ser empleado para la

futura extracción de estos analitos presentes en mezclas complejas (etapa de reenlace) (Cormack y Zurutuza-Elorza, 2004; Yan y Row, 2006).

En función del tipo de interacciones formadas entre monómero-molécula plantilla tanto durante la etapa de prepolimerización, como en la posterior etapa de reenlace, los MIPs pueden ser clasificados en: polímeros covalentes, donde ambas interacciones son de tipo covalente reversible lo que confiere a los MIPs una elevada selectividad; polímeros semi-covalentes, en los que la interacción inicial entre monómero y molécula plantilla es de tipo covalente, mientras que la interacción en el reenlace es de tipo no covalente; y polímeros no covalentes con interacciones monómero-molécula plantilla a través de enlaces intermoleculares débiles del tipo de puentes de hidrógeno, fuerzas de Van der Waals, interacciones π–π, dipolo-dipolo, electroestáticas o hidrofóbicas (Piletska y col., 2009).

La evaluación de la especificidad de los sitios de enlace creados se efectúa por comparación con un polímero de similares características pero sin cavidades específicas de enlace. Paralelamente a la síntesis del MIP se lleva a cabo la síntesis de un polímero no impreso molecularmente (NIP), el cual lleva los mismos componentes de síntesis que el MIP a excepción de la molécula plantilla. Las etapas de síntesis son las mismas que las anteriormente descritas para el MIP.

1.3.1.2. Variables que influyen en el proceso de síntesis del MIP

Las características de enlace (especificidad, selectividad, afinidad, capacidad) y las propiedades físicas (porosidad, rigidez, área superficial) del MIP obtenido están directamente relacionadas con la estructura y los grupos

funcionales de los compuestos de partida y de las condiciones de síntesis. A continuación se describen brevemente los principales factores que influyen en el proceso de reconocimiento de los MIPs (Steinke y col., 1994; Sellergren y Hall, 2000).

- **Molécula plantilla.** Al tratarse de la molécula objetivo para la cual se van a crear los sitios específicos de enlace, es la encargada de dirigir la organización de los grupos funcionales de los monómeros durante el proceso de prepolimerización.

- **Monómero funcional.** Es el responsable de las interacciones con la molécula plantilla formando un complejo de prepolimerización estable con la misma, de modo que se den interacciones complementarias que aumenten la afinidad y la selectividad de las cavidades generadas en la matriz polimérica. Por ello, la selección del tipo de monómero más adecuado dependerá de las características y grupos funcionales de dicha molécula plantilla. Normalmente, se requiere el empleo de un exceso de monómero funcional, generalmente en una relación 4:1 (monómero funcional-molécula plantilla) para favorecer la formación del complejo de prepolimerización. Sin embargo, el exceso de monómero queda libre en la red polimérica pudiendo dar lugar a la formación de interacciones no específicas no deseadas. La naturaleza de los monómeros varía en función de la naturaleza de la molécula plantilla, pudiendo ser ácidos, básicos o neutros. Un ejemplo de cada uno de estos tipos de monómeros funcionales son el ácido metacrílico (MAA), la 4-vinilpiridina (4-Vp) y la acrilamida (AA), respectivamente.

- **Entrecruzador.** Su función consiste en interaccionar con el complejo de prepolimerización a fin de preservarlo durante la síntesis del polímero. Por lo tanto, es el principal responsable de la morfología de la matriz polimérica al estabilizar los sitios de unión específica, confiriéndole al polímero sintetizado estabilidad mecánica y térmica y la porosidad necesaria para garantizar la accesibilidad del analito a las cavidades generadas. El etilenglicol dimetacrilato (EGDMA), el divinilbenceno (DVB) y el trimetilpropano trimetacrilato (TRIM) son los tres entrecruzadores más comúnmente empleados.

- **Porogen.** Tiene una doble función, como disolvente clásico y como medio de la polimerización. Las propiedades físicas y químicas del polímero obtenido van también a depender de la naturaleza y cantidad del porogen, al presentar influencia sobre la estabilidad de los enlaces formados entre monómero y molécula plantilla. Su elección dependerá, por lo tanto, del tipo de interacciones formadas. Porogenes apróticos o de baja constante dieléctrica como tolueno o acetonitrilo serán adecuados para estabilizar complejos formados por interacciones de enlace de hidrógeno o fuerzas de Van del Waals; mientras que disolventes próticos como el agua o metanol serán adecuados para interacciones de tipo hidrofóbico (Cormack y Zurutuza-Elorza, 2004).

- **Iniciador.** Es el responsable del comienzo de la polimerización al introducir radicales en el sistema. Su elección depende del método de iniciación empleado. El 2,2'-azo-bis-isobutironitrilo (AIBN) suele ser el iniciador más empleado.

- **Modo de activación.** La radiación ultravioleta o la acción del calor constituyen los métodos fundamentales de iniciación de la reacción de polimerización. Las condiciones de temperatura y tiempo de polimerización gobiernan el proceso de reacción. La temperatura se elige en función de la estabilidad del iniciador que debe fragmentarse y afecta al equilibrio entre la molécula plantilla y el monómero funcional. Evitando temperaturas elevadas que puedan afectar a la estabilidad de la molécula plantilla, 60 ºC suele ser la temperatura más empleada. En lo que al tiempo se refiere, la descomposición de los iniciadores tipo azoico suele ser más rápida que en el caso de la iniciación fotoquímica. El tiempo seleccionado para la mayoría de las polimerizaciones suele ser de 24 horas. La eliminación del posible oxígeno presente constituye también un paso fundamental dado que su presencia retarda el proceso de polimerización.

- **Método de polimerización.** Influye sobre la morfología del polímero obtenido (partículas esféricas mono o polidispersas, partículas de tamaño de poro controlado, agregados aleatorios de partículas, bloques, etc.) y sobre el reconocimiento de los analitos por parte del polímero. Los métodos de polimerización más comunes son: polimerización en bloque (obtención de un monolito de polímero insoluble que necesita ser triturado y tamizado para obtener partículas micrométricas amorfas); por precipitación (obtención de partículas esféricas con alto rendimiento de polimerización y que no necesitan molienda y/o tamizado); por suspensión (obtención de partículas de tamaño controlado); por hinchamiento en dos etapas (obtención de partículas monodispersas de tamaños comprendidos entre 2 y 50 μm) y en partículas de sílice (obtención de partículas esféricas). El empleo de una u otra técnica

depende fundamentalmente de la aplicación a la que se vaya a destinar el polímero.

1.3.1.3. Caracterización y evaluación de los MIPs

El polímero impreso resultante tras la etapa de polimerización se puede presentar como un sólido amorfo de gran diversidad estructural, químicamente heterogéneo e insoluble. Para su estudio se realizan la caracterización química, caracterización morfológica y la evaluación de los parámetros de enlace.

- **Caracterización química.** Dada su naturaleza sólida y su baja solubilidad, es necesario emplear técnicas de caracterización compatibles con muestras en estado sólido como el análisis elemental, el espectroscópico o la resonancia magnética nuclear. La primera de las técnicas proporciona información sobre el porcentaje en masa de elementos como carbono, oxígeno, hidrógeno, nitrógeno, etc., dando una idea de la cantidad de monómero incorporada en la matriz polimérica. Mediante análisis espectroscópico, ya sea por espectroscopia infrarroja (FTIR) o por ultravioleta-visible (UV-Vis), así como mediante resonancia magnética nuclear (RMN) se obtiene información acerca de los grupos funcionales presentes, el grado de polimerización, la formación de enlaces o la relación estequiométrica monómero-molécula plantilla. Finalmente, tanto FTIR como RMN permiten evaluar el grado de curado por medio de la determinación del número de dobles enlaces que quedan sin reaccionar en el polímero (Cormack y Zurutuza-Elorza, 2004; Cela-Pérez y col., 2013).

- **Caracterización morfológica.** La microscopía electrónica de barrido (SEM) permite evaluar la superficie del polímero al proporcionar una imagen superficial de las partículas del polímero y los posibles macroporos superciales. La microscopía de fuerza atómica (ATM) proporciona un perfil tridimensional cuantitativo de la superficie. Por otro lado, técnicas basadas en intrusión/extrusión de mercurio y adsorción/desorción de nitrógeno (BET) permiten determinar la porosidad del polímero y, por lo tanto, la accesibilidad de los analitos a los puntos de unión presentes en el interior de la matriz polimérica (Cormack y Zurutuza-Elorza, 2004; Spivak, 2005; Lok y Son, 2009).

- **Evaluación de los parámetros de enlace.** Mediante el empleo de ensayos de reenlace en discontinuo y modelos matemáticos, pueden evaluarse la especificadad y la selectividad del MIP, la constante de afinidad y la densidad de los sitios de unión, así como la cinética del proceso de unión.

La afinidad del polímero impreso por el o los analitos de interés se evalúa a través de experimentos de saturación, en los cuales se determina la cantidad de analito capaz de reenlazarse en el polímero al realizar incubaciones con una cantidad determinada de polímero y cantidades crecientes de analito durante un periodo de tiempo determinado a una temperatura fija: **isotermas de adsorción**. La evaluación de los datos experimentales se realiza mediante alguno de los modelos matemáticos propuestos, en función de la heterogeneidad del MIP (Tabla 6).

Cinética del proceso de adsorción. La cinética de adsorción describe la velocidad de enlace del analito de interés en el MIP y controla el tiempo de equilibrio. Varios pueden ser los mecanismos controladores del proceso de adsorción: reacción química, difusión o transferencia de masa. A fin de examinar cual de ellos es el mecanismo más significativo, se emplean diversos modelos cinéticos para ajustar los datos experimentales. Para ello, se realizan incubaciones con una única y conocida cantidad del compuesto a enlazar con el polímero impreso, durante intervalos crecientes de tiempo. Los modelos de pseudo-primer orden, pseudo-segundo orden y Elovich son los más empleados para el estudio de la cinética del proceso de adsorción. El mecanismo del sistema de adsorción-difusión se evalúa principalmente mediante los modelos de difusión intrapartícula, difusión externa de film y modelo de Boyd. Las ecuaciones y características de cada uno de los modelos se recogen en la Tabla 7 (Reichenberg, 1953; Wang y col., 2008; Zhang y col., 2008; Tan y col., 2009; Liu y col., 2010; Pan y col., 2010).

1.3.1.4. Aplicaciones de los MIPs

Los polímeros de impresión molecular, como resultado de su capacidad de adsorción selectiva se emplean en numerosos campos (sensores, en catálisis, análisis medioambiental, análisis clínico, ciencia de los alimentos y de los materiales o biotecnología (Mayes, 1997; Haupt, 2001; Ramström y col., 2001; Sellergren y Allender, 2005)).

Tabla 6. Ecuaciones y consideraciones de las isotermas de adsorción.

Isoterma	Ecuación linealizada		Consideraciones
Langmuir	$\frac{C_e}{q_e} = \frac{1}{q_m K_L} + \frac{1}{q_m} C_e$ o: Scatchard Plot $\frac{q_e}{C_e} = q_m K_L - K_L q_e$	C_e (mg L^{-1}): concentración del soluto en condiciones de equilibrio en la disolución q_e (mg g^{-1}): cantidad del soluto adsorbido en condiciones de equilibrio q_m (mg g^{-1}):máxima capacidad de adsorción del adsorbente, lo que representa el número total de sitios de enlace K_L (L mg^{-1}): constante relacionada con la energía libre de adsorción	Esta isoterma está basada en tres supuestos: - la adsorción no puede proceder más allá de la cubierta de la monocapa - equivalencia entre todos los sitios de enlace de la superficie, acomodando, al menos, un substrato por sitio - la habilidad de la molécula plantilla para enlazarse a un sitio de enlace dado no es dependiente de la ocupación de sitios de enlace vecinos Por lo tanto, se asumen sitios de enlace homogéneos en el polímero
	$R_L = \frac{1}{1 + K_L C_0}$	R_L: factor de separación o parámetro de equilibrio C_0 (mg L^{-1}): concentración inicial de soluto	R_L: predicción de la favorabilidad de un sistema de adsorción Si: R_L>1, isoterma no favorable R_L=1, isoterma lineal 0<R_L<1, isoterma favorable R_L=0, isoterma irreversible

Tabla 6. (Continuación I).

Isoterma	Ecuación linealizada		Consideraciones
Freundlich	$\log q_e = m\log C_e + \log K_F$	K_F (mg^{1-m} L^m g^{-1}): capacidad de adsorción del adsorbente m: intensidad de adsorción o heterogeneidad de superficie	Basado en la adsorción en superficies heterogéneas o en superficies con sitios de diferentes afinidades - Los sitios de enlace más fuertes son los primeros en ser ocupados - La fuerza del enlace decrece a medida que aumenta el grado de sitios ocupados 0≤m≤1, más cercano a 1 a medida que la heterogeneidad decrece. Si m=1: sistema homogéneo
		$N(K) = 2.303am\left(a - m^2\right)e^{-2.303m\log K}$ $K_{\min} = 1/C_{\max} \quad K_{\max} = 1/C_{\min} \quad a = K_F$ $N_{K1-K2} = a(1-m^2)(K_1^{-m} - K_2^{-m})$ $K_{K1-K2} = (m/(m-1))\left[\left(K_1^{1-m} - K_2^{1-m}\right)/\left(K_1^{-m} - K_2^{-m}\right)\right]$	Modificación de la isoterma de Freundlich para obtener una ecuación de la distribución de afinidades, siendo N_{K1-K2} el número aparente de sitios y K_{K1-K2} la media aparente de afinidades. Valores calculados entre los límites K_{min} y K_{max}.
Álostérica	$Ln\left(\frac{q_m}{q_e} - 1\right) = LnK_m - nLnC_e$	K_m: constante de enlace n: relacionado con el número de capas de enlace (normalmente n≥1)	Basado en la asunción de una adsorción en un sistema multicapa debido a la alteración de sitios de enlace por efecto de interacciones. Siendo ocupados algunos de los sitios de enlace, la estero-estructura podría verse alterada. La afinidad de enlace podría posiblemente tener efecto y por lo tanto se podría obtener una multicapa de enlace Si n=1 el modelo se reduce al modelo de Langmuir

Tabla 6. (Continuación II)

Isoterma	Ecuación linealizada		Consideraciones
Dubinin-Radushkevich	$Lnq_e = Lnq_m - K_{DR}\varepsilon^2$ $\varepsilon = RTLn\left(1+\frac{1}{C_e}\right)$ $E = (2K_{DR})^{-1/2}$	K_{DR}: relacionado con la energía libre(E, kJ mol) de adsorción por molécula de adsorbato cuando es transferida a la superficie del sólido desde el infinito en la disolución R: constante de los gases ideales (8,314 J mol^{-1} K^{-1}) T (K): temperatura absoluta	Para análisis de isotermas con alto grado de rectangularidad. Valores de $K_{DR} < 1$ representan una superficie rugosa con muchas cavidades E >40 kJ mol^{-1} expresa la quimisorción entre adsorbente y adsorbato

Ref.: Rampey y col., 2004; Spivak, 2005; Syu y Nian, 2005; García-Calzón y Díaz-García, 2007; Subramanyam y Das, 2009; Tan y col., 2009; Pan y col., 2010.

Tabla 7. Ecuaciones y consideraciones de la cinética de adsorción y modelos de mecanismos de adsorción.

Modelo	Ecuación		Consideraciones
Cinética del proceso de adsorción			
Pseudo-primer orden	$\log(q_e - q_t) = \log q_e - \frac{k_1}{2{,}303} t$ $h_1 = k_1 q_e$	q_e y q_t (mg g^{-1}): cantidades de adsorbato adsorbidas a tiempo de equilibrio y a cualquier tiempo, t(min), respectivamente. k_1 (min^{-1}): contante de velocidad de pseudo-primer orden h_1 (mg g^{-1} min^{-1}): velocidad inicial de adsorción	En ambos modelos, todos los pasos de adsorción tales como la difusión externa, la difusión interna y la adsorción son agrupados conjuntamente. La diferencia entre concentración media en la fase sólida y la concentración en el equilibrio son consideradas como estapas clave para la adsorción. Estos modelos son aplicables si log(q_e-q_t) vs t ó t/q_t vs t , son representaciones lineales.
Pseudo-segundo orden	$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$ $h_2 = k_2 q_e^2$ $t_{1/2} = \frac{1}{k_2 q_e}$	k_2(g mg^{-1} min^{-1}): contante de velocidad de pseudo-segundo orden h_2(mg g^{-1} min^{-1}): velocidad inicial de adsorción $t_{1/2}$: tiempo requerido para la adsorción de al menos la mitad del compuesto adsorbido en el equilibrio	
Elovich	$q_t = \frac{1}{\beta} Ln(\alpha\beta) + \frac{1}{\beta} Lnt$	α (mg g^{-1} min^{-1}): velocidad inicial de adsorción β (g mg^{-1}): relacionado con la extensión de cobertura de la superficie y con la energía de activación para quimisorción	Descripción del proceso de quimisorción Aplicable cuando q_t vs Lnt representa una línea recta

Tabla 7. (Continuación I)

Modelo	Ecuación		Consideraciones
	Mecanismos de adsorción		
Difusión externa	$Ln\ \frac{C_t}{C_0} = -k_{ext}\ t$	C_0, C_t (mg L^{-1}): concentración de soluto en la solución inicial y en fase líquida, respectivamente, a tiempo t k_{ext} (min^{-1}): parámetro de la velocidad de difusión para el modelo de difusión	Si la ecuación de la difusión externa es aplicable, la representación de $ln(C_t/C_0)$ frente a t debería dar una línea recta que pasase por el origen de coordenadas.
Difusión intra-partícula	$q_t = k_i \times t^{0,5} + IP$	k_i (mg g^{-1} $min^{-0,5}$): contante de velocidad por el modelo de difusión intra-partícula IP (mg g^{-1}): efecto del grosor de la capa limítrofe	Grandes valores de IP indican un mayor efecto del grosor de la capa limítrofe La difusión intra-partícula es el único paso limitante si la representación gráfica de q_t vs $t^{0.5}$ es una línea recta que pasa por el origen. Si no, algún otro mecanismo junto con la difusión intra-partícula está también implicado en el proceso de adsorción
Boyd	- Si 0<F<0,85 $Bt = 2\pi - \frac{\pi^2}{3} - 2\pi\left(1 - \frac{\pi F}{3}\right)^{1/2}$ - Si 0,86<F<1 $Bt = -0.49770 - Ln(1 - F)$	B_t: parámetro de Boyd F: representación de la fracción de soluto adsorbido a cualquier tiempo t, calculado usando: $F=q_t/q_e$	Si la representación de Bt vs t es una línea recta pasando por el origen, la etapa limitante en el proceso de adsorción es la difusión intra-partícula

De entre esas aplicaciones de los MIPs, ya se ha indicado previamente que una de las más importantes radica en el empleo de los mismos como adsorbentes en procesos de extracción en fase sólida para la limpieza y/o preconcentración de muestras (Tamayo y col., 2007; Turiel y Martín-Esteban, 2010).

Los procesos de extracción en fase sólida sobre MIPs (MISPE) constan de las mismas etapas básicas que cualquier otro proceso de extracción en fase sólida (Figura 7).

Una porción del polímero impreso lavado (generalmente entre 50 y 200 mg) con un tamaño de partícula homogéneo, se introduce en un cartucho de SPE y se somete a las etapas características del proceso de SPE tal y como se describen en la Figura 7.

La primera de las etapas consiste en el **acondicionamiento** del polímero con el disolvente en el que se va a cargar la muestra. La segunda, la **carga de la muestra** sobre el polímero y fijación selectiva de los analitos en las cavidades del mismo. La elección del disolvente más adecuado vendrá condicionada no sólo por la solubilidad de los analitos, sino también por las interacciones analito-polímero-porogen. En este caso se deberá seleccionar aquel disolvente que favorezca las interacciones entre los analitos y los polímeros, normalmente interacciones por puentes de hidrógeno. Habitualmente el propio porogen es el más empleado.

La tercera de las etapas consiste en la **eliminación de los compuestos interferentes** que hayan podido quedar retenidos en la matriz de forma no específica empleando un disolvente que rompa esas interacciones no específicas pero sin provocar la pérdida de los analitos de interés. La cuarta y última etapa consistirá en la **elución de los analitos** de interés con un

disolvente adecuado, el cual será seleccionado en función de su capacidad para romper las interacciones específicas formadas entre los analitos y el MIP.

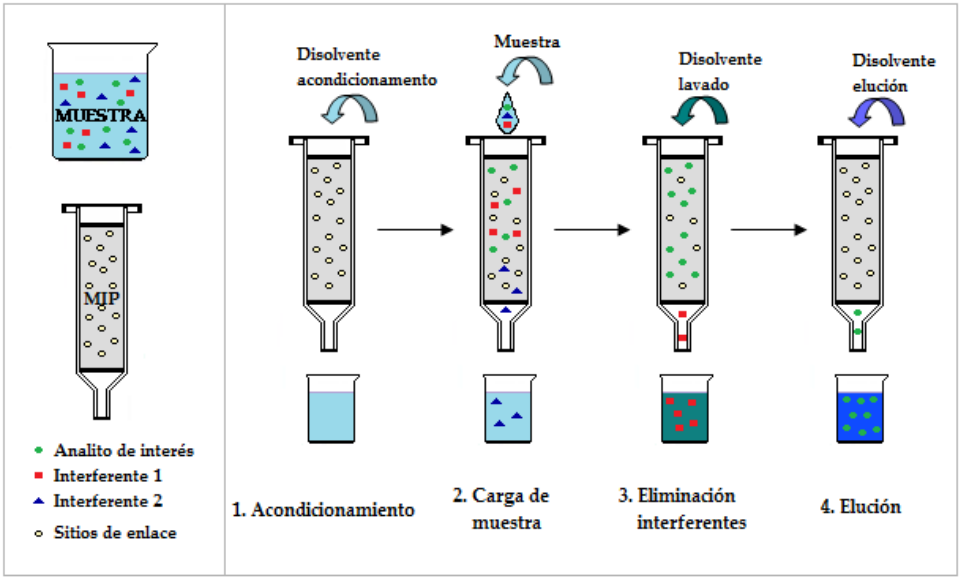


Figura 7. Etapas de un proceso de MISPE

La etapa de elución es determinante en el tiempo de vida útil del polímero, al poderse producir variaciones en la morfología de los MIPs como resultado de cambios en la polaridad y/o naturaleza de los disolventes empleados; cambios que pueden afectar a la accesibilidad de los sitios de unión (Sellergren y Shea, 1993).

Otro de los problemas que pueden aparecer al usar MIPs en extracción en fase sólida es el sangrado de los polímeros, considerándose éste como la elución de moléculas plantilla no totalmente eliminadas durante la etapa de lavado. Para evitar este problema, se pueden utilizar sistemas de limpieza con disolventes más agresivos o calentar el MIP a elevadas temperaturas, aunque,

cuando posible, resulta más adecuada la preparación de polímeros utilizando como molécula plantilla un análogo en estructura y funcionalidad del analito de interés, de tal forma que la posible elución de la molécula plantilla no afecte a la determinación del analito (Jodlbauer y col., 2002; Urraca y col., 2006).

1.4. MÉTODOS DE ANÁLISIS DE ANTIOXIDANTES

Para la determinación de los flavonoides y más concretamente de las catequinas y la quercetina, se han empleado distintos métodos analíticos.

Entre los métodos más antiguos se puede destacar aquellos basados en **medidas espectrofotométricas**. Su simplicidad y rapidez han convertido a la espectrofotometría en una técnica ampliamente usada para la medida de cantidad total de catequinas. Sin embargo, tradicionalmente, la imposibilidad de diferenciar compuestos de interés de interferentes ha dado lugar a sobreestimaciones de las cantidades medidas. El uso de técnicas quimiométricas de análisis de espectros, tales como mínimos cuadrados parciales o análisis de componentes principales han ayudado a solventar dichos problemas (Mölnár-Perl y Füzfai, 2005; Stalikas, 2007).

Por ello, para el análisis y cuantificación de catequinas y quercetina en muestras naturales, el mayor número de aplicaciones analíticas se han basado en el uso de **técnicas de separación.**

La **cromatografía líquida de alta resolución (HPLC)** en fase reversa es la técnica más usada. La mayoría de los métodos emplean fases estacionarias de sílice en fase enlazada con octadecilsílice (C18) y fases móviles polares en gradiente, principalmente mezclas de agua con metanol o acetonitrilo.

Normalmente se ha considerado que la adición de un modificador ácido a la fase móvil (ácido clorhídrico, fórmico, acético, fosfórico o trifluoroacético) era indispensable para lograr la completa resolución de los picos cromatográficos de las catequinas (Dalluge y col., 1998). Sin embargo, la adición de un medio ácido puede contribuir al deterioro de la columna cromatográfica con recubrimiento de sílice dada su baja estabilidad en condiciones de pH extremadamente ácido (Lipper y col., 2007). Entre los detectores y, dado que tanto las catequinas como las quercetina absorben en la región ultravioleta, el más empleado ha sido el de red de diodos. Sin embargo, en ocasiones, la necesidad de interpretar y resolver algunas mezclas complejas, especialmente en el análisis de muestras naturales, ha obligado el uso de otros detectores más selectivos como el de fluorescencia, el electroquímico o el espectrómetro de masas que permite la identificación inequívoca de los compuestos en estudio (Valls y col., 2009; Molnár-Perl y Füzfai, 2005).

La cromatografía de líquidos de ultra resolución (UHPLC), ha sido considerada como una nueva opción en el campo de la cromatografía líquida al permitir trabajar con columnas rellenas de partículas de tamaño mucho más pequeño, presiones más elevadas y flujos más altos; lo cual ha posibilitado análisis más rápidos con mayor resolución y sensibilidad (Spáčil y col., 2008). Sin embargo, esta metodología se ha aplicado en pocas ocasiones al estudio de las catequinas y de la quercetina.

Electroforesis capilar, tanto en su modalidad de electroforesis capilar de zona (CZE) como de cromatografía electrocinética micelar (MEKC) se ha empleado para la resolución y cuantificación de catequinas. Su rapidez, eficiencia, bajo consumo de muestra y disolventes y su elevada tolerancia a las interferencias provenientes de la matriz de la muestra se presentan como

sus mayores ventajas frente, tal vez, a su baja precisión y sensibilidad (Pacáková y col., 1999). En los métodos desarrollados para catequinas las condiciones de análisis más habituales emplean un capilar de sílice, polaridad positiva o negativa dependiendo del tampón empleado, inyección hidrodinámica, detección ultravioleta-visible, tampón borato o borato modificado, 25-30 kV y 20-30ºC (Horie y Kohata, 2000; Dalluge y Nelson, 2000).

También existen referencias empleando la **cromatografía de gases** acoplada a detector de masas; sin embargo, en este caso es necesario emplear pasos intermedios de derivatización a fin de convertir las catequinas en compuestos volátiles, por lo que no se utiliza con mucha frecuencia.

Otras técnicas como cromatografía en capa fina o en papel, quimioluminiscencia, biosensores o resonancia magnética nuclear también han sido empleados en alguna ocasión (Horie y Kohata, 2000; Dalluge y Nelson, 2000; Valls y col., 2009; Molnár-Perl y Füzfai, 2005).

1.5. ENVASES ACTIVOS ANTIOXIDANTES

Como se ha comentado con anterioridad, a fin de lograr una mayor vida útil, tanto del alimento como del propio polímero que lo contiene, así como minimizar los efectos no deseados de los productos de oxidación en la calidad del alimento y en la salud del consumidor, es necesario retardar el proceso de oxidación.

En el alimento, tradicionalmente se adiciona el antioxidante de forma directa, lo que confiere una protección durante un corto periodo de tiempo, dado que el antioxidante es inmediatamente consumido tras su adición. Al

mismo tiempo, no es aconsejable la adición directa de altas cantidades de antioxidante a los alimentos porque puede llevar asociado un efecto prooxidante (Shahidi, 2000).

Otras estrategias como el envasado y procesado en condiciones asépticas y libres de oxígeno (envasado al vacío, o el envasado bajo atmósferas modificadas), a pesar de ser efectivas en algunos casos, pueden resultar de complicada aplicación, caras o no apropiadas para ciertos productos como los alimentos frescos. Además, no se suele alcanzar la eliminación total del oxígeno. En ocasiones se ha empleado la inclusión de absorbentes de oxígeno en formato de pequeños sacos, pero puede provocar un efecto de rechazo por parte del consumidor, además de comportar un posible riesgo derivado de un potencial consumo accidental (Vermeiren y col., 1999; Mastromatteo y col., 2010; Restuccia y col., 2010).

En cuanto al polímero, la concentración de los antioxidantes incorporados en la formulación del envase para protegerlo frente a la degradación y, por lo tanto, frente a la pérdida de propiedades, decrece durante la etapa de almacenamiento debido al transcurso de la propia reacción de oxidación así como por difusión a través del polímero hacia la superficie (Garde y col., 2001).

Aprovechando esta propiedad de difusión, una alternativa para el control de la oxidación es el uso de **envases activos antioxidantes**, en los cuales las sustancias activas antioxidantes son incorporadas en la propia composición del material de envasado, con la intención de que sean liberados de forma controlada hacia el alimento sobre el que ejercerán su actividad antioxidante (Vermeiren y col., 1999; Ozdemir y Floros, 2004). De este modo, se produce un continuo reemplazo de aditivo en el alimento, y no la adición

del mismo en una única etapa inicial, además de ser liberado en la superficie del alimento donde es más necesario, lográndose así la protección frente a la degradación durante un periodo de tiempo más largo; además de lograrse la protección del propio polímero en el cual el antioxidante es incorporado.

1.5.1. Antioxidantes naturales en envases activos.

Distintos autores han empleado varios antioxidantes sintéticos como agentes activos en las formulaciones de envases, en estos estudios se analizó el resultado de incorporar BHT, BHA, ter butil-hidroquinona (TBHQ,) o galato de propilo (PG) en matrices de polietileno de baja densidad o ácido poliláctico, para la protección de productos tan diversos como quesos, leche o cereales (Wessling y col., 2000; Soto-Cantú y col., 2008; Granda-Restrepo y col., 2009; Jamshidina y col., 2012). Sin embargo, no parecía sensato el incorporar algunos de estos aditivos, que presentan restricciones en alimentos debido a su toxicidad, por lo que se han buscado otras alternativas enfocadas hacia la incorporación de antioxidantes naturales. Entre estos, los que más interés están suscitando son los tocoferoles, los extractos de orégano y romero y los flavonoides quercetina y catequinas. En la Tabla 8 se recogen ejemplos de antioxidantes naturales empleados en formulaciones de envases activos, la matriz polimérica a la cual han sido incorporados y el tipo de alimento o simulante alimentario objeto de su aplicación.

Hasta el momento, los desarrollos de envases activos se han realizado empleando PEBD, polímeros hidrofílicos y polímeros biodegradables; sin embargo, casi ninguna aplicación ha considerado el uso de PP como matriz base para el desarrollo de envases activos, a pesar de continuar siendo una de las matrices más ampliamente usada en el sector del envase alimentario

(Wessling y col., 1999). El uso de PP se ha visto condicionado, principalmente, por la baja o nula liberación de los antioxidantes incorporados al mismo hacia el alimento contenido, lo cual ha limitado en gran medida su uso en envases activos (Wessling y col., 1998, 1999; Heirlings y col., 2004).

Tabla 8. Empleo de antioxidantes naturales en envases activos.

Antioxidante	Matriz	Aplicación	Referencia
α-tocopherol	PEBD	Simulante graso	Wessling y col., 1998
		Simulante graso, vino, agua, zumo de naranja, leche, nata	Wessling y col., 1999
		Harina de avena	Wessling y col., 2000
		Simulante graso	Heirlings y col., 2004
		Leche entera en polvo	Granda-Restrepo y col., 2009
		Filetes de atún	Torrieri y col., 2011
		Salmón	Barbosa-Pereira y col., 2013
	PEAD	Leche entera en polvo	Granda-Restrepo y col., 2009
	PEBDL	No especificado	Koontz y col., 2010
	PEBD con CD	Simulante graso	Siró y col., 2006
	PEBD:sílica mesoporosa	Simulante graso	Gargiulo y col., 2013
	PEBDL:PEAD, PEBDL:PP, PEBDL:PS	Simulante graso	Schaich y col., 2008
	PP	Simulante graso, vino, agua, zumo de naranja, leche, nata, mayonesa.	Wessling y col., 1999
	PET	Carne cocinada	Contini y col., 2011
	EVOH	Leche entera en polvo	Granda-Restrepo y col., 2009
	EVA	Simulante graso	Heirlings y col., 2004
	PLA	Aceite de soja	Manzanarez-López y col., 2011
Ácido carnosínico, carnosol (extractos de romero y orégano)	PE	No especificado	Bentayeb y col., 2007
	PEBD	Carne de pollo	Bolumar y col., 2011
	PP	Filetes de ternera	Nerín y col., 2006, 2008
	Films de quitosano	No especificado	Abdollahi y col., 2012
	No especificado (bajo patente)	Cordero	Camo y col., 2008

Tabla 8. (Continuación I)

Antioxidante	Matriz	Aplicación	Referencia
Ácido ascórbico y L-tirosina	Acetato de celulosa	Agua destilada	Gemili y col., 2010
Astaxantina (crustáceos y salmónidos)	PEBD	Simulante graso	Sanches-Silva y col., 2012
Carvacrol	PEAD	Simulantes acuoso y graso	Peltzer, 2009
	PEBDL:zeína de maiz	Hamburguesa de vacuno	Park y col., 2012
Extractos de canela, clavo, jengibre y tomillo	Films en base celulósica	Aceite vegetal	Phoopuritham y col., 2012
Extracto cáscara de cebada	PEBD	Salmón, bacalao y otros pescados	Pereira de Abreu y col., 2010, 2012a, 2012b
Extracto de frutas cítricas	PET	Carne cocinada	Contini y col., 2011
Flavonoides:			
Catequinas	PET	Simulantes acuoso y graso	Colón y Nerín, 2012 Aznar y col., 2012
	EVOH	Simulantes acuoso y graso, sardinas en salmuera	López de Dicastillo y col., 2010, 2011, 2012a
	PLA	Simulantes acuoso y graso, soja	Iñiguez-Franco y col., 2012
	Otros polímeros biodegradables: en base celulósica, polivinil alcohol, almidón	Aceite vegetal, simulantes acuoso y graso, carne roja	Phoopuritham y col., 2012 Wu y col., 2010 Corrales y col., 2009
Quercetina	PEBDL	Aceite de coco	Koontz y col., 2010
	EVOH	Simulantes acuoso y graso, cacahuetes fritos, sardinas en salmuera.	López de Dicastillo y col., 2010, 2012a, 2012b

1.5.2. Sistemas para el control de la cesión en envases activos

Tal y como se ha indicado, la alta, prácticamente total, retención de antioxidantes de origen natural como los tocoferoles y las catequinas en matrices de PP ha limitado su uso en envases activos. Por lo tanto, es precisa una modificación adecuada en la formulación del PP para dotar a éste de las propiedades deseadas. Hoy en día, existen dos alternativas que se están empleando en diferentes aplicaciones como son el uso de extensores de cadena (o plastificantes) y la extrusión reactiva.

1.5.2.1. Extensores de cadena polimérica/plastificantes.

Hasta el momento, la incorporación de extensores de cadena en matrices poliméricas ha constituido una vía de aumento del tamaño de las cadenas poliméricas con el objeto de reparar cadenas rotas durante las etapas de procesado, post-procesado o reciclado de los polímeros. Extensores de cadena y materiales de bajo peso molecular reaccionan a través de grupos reactivos, tales como grupos carboxilo y/o hidroxilo terminales para reenlazar cadenas rotas. El aumento del tamaño de las cadenas confiere a las matrices poliméricas una mejora de sus propiedades físico-químicas, principalmente de su establilidad, viscosidad y de sus propiedades mecánicas. Aplicaciones en matrices de ácido poliláctico, poliuretanos o polietilentereftalatos constituyen los principales casos de uso de extensores de cadena con este fin al tratarse de matrices con cierto carácter polar lo que facilita su reacción con los extensores (Skarja y col., 2000; Semsaezadeh y col., 2008; Zheng y col., 2011).

Sin embargo, en polímeros termoestables, el empleo de extensores de cadena ha sido aprovechado en matrices de poliuretano, para conferir a las mismas una mayor permeabilidad frente a ciertas sustancias (Semsaezadeh y col., 2008). Esta propiedad podría, por lo tanto, ser aprovechada con objeto de conferir mayor capacidad de liberación de antioxidantes desde la poliolefina al alimento.

El tipo, naturaleza, tamaño, cantidad de extensor y grado de compatibilidad con la matriz polimérica, constituyen los factores claves a tener en cuenta en la elección de los extensores de cadena a emplear en cada aplicación concreta.

1.5.2.2. Extrusión reactiva

En el caso de la liberación de los antioxidantes desde los envases activos hacia el alimento, la mayoría de las aplicaciones se basan en procesos de transporte de masa por sorción, migración y permeación, por lo que la liberación del agente activo está gobernada por factores tales como el tipo de polímero o de alimento, entre otras (Vermeiren y col., 1999). Ello hace necesario la búsqueda de nuevos sistemas de liberación controlada de forma que se produzca un continuo reemplazo del antioxidante en la superficie del alimento y en cantidad suficiente para que ejerza su acción antioxidante. Para ello, una posibilidad consiste en actuar sobre la propia matriz.

Entre las técnicas de modificación de los polímeros se está prestando especial atención a la incorporación de injertos de cadena (grafting), que faciliten la compatibilidad entre matrices apolares tales como las poliolefinas y matrices y/o compuestos polares, como los antioxidantes. La incorporación

de estas cadenas se lleva a cabo en la superficie del polímero, de modo que no afecta a las propiedades del resto del material, por medio de la denominada extrusión reactiva.

Poliolefinas injertadas con anhídrido maleico, ésteres de fumarato o maleato, ésteres de metacrilato y ácido metacrílico representan los ejemplos más empleados. Dentro de éstas, comercialmente, las poliolefinas modificadas con anhídrido maleico han sido las más empleadas dado su bajo coste, alta actividad y buena procesabilidad (Moad, 1999; Goddard y Hotchkiss, 2007). La presencia de los grupos anhidro confiere esa alta reactividad dada su tendencia a sufrir una gran variedad de reacciones tales como la hidrólisis, esterificación o aminación (Xing y col., 2005).

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CAPÍTULO II. OBJETIVOS

Desarrollo de polímeros activos antioxidantes para contacto con alimentos mediante adición de antioxidantes naturales y modificación química de la matriz polimérica.

A continuación se enumeran los objetivos específicos cuyo desarrollo secuencial nos conducirá a alcanzar el objetivo general:

- **Determinación y cuantificación de antioxidantes naturales**
  - Desarrollo y comparación de diversos métodos cromatográficos y electroforéticos rápidos y eficientes para la separación, identificación y cuantificación simultánea de antioxidantes naturales del grupo de los flavonoides (catequinas y quercetina) en análisis rutinarios.
  - Aplicación de los métodos desarrollados en la cuantificación de los antioxidantes estudiados en muestras vegetales y en el estudio de la liberación de estos antioxidantes desde films activos en simulantes alimentarios.

- **Purificación de extractos naturales**
  - Síntesis y caracterización de un polímero impreso molecularmente para el reenlace selectivo de catequinas.
  - Aplicación del polímero impreso molecularmente en sistemas de extracción en fase sólida para la preconcentración y limpieza de catequinas en extractos naturales.

- **Adición de antioxidantes naturales a muestras poliolefínicas**

  - Obtención de películas de polipropileno con incorporación de antioxidantes naturales individuales y extractos de matrices vegetales.

  - Caracterización de la protección proporcionada por los antioxidantes naturales para limitar y controlar la degradación oxidativa de las polipropileno.

- **Desarrollo de materiales capaces de realizar una cesión controlada de antioxidantes**

  - Modificación de la capacidad de liberación de los antioxidantes naturales desde la matriz de polipropileno mediante la incorporación de extensores de cadena/plastificantes. Impacto en las propiedades físico-químicas.

  - Modificación superficial de la matriz de polipropileno mediante extrusión reactiva y evaluación de la reactividad entre el polipropileno modificado y el antioxidante. Impacto en las propiedades físico-químicas.

  - Caracterización y evaluación de la liberación de los antioxidantes naturales desde los materiales desarrollados mediante estudios de liberación hacia simulantes alimentarios.

  - Evaluación de la actividad antioxidante de los antioxidantes liberados desde el film a los simulantes alimentarios.

CAPÍTULO III. RESULTADOS Y DISCUSIÓN

El presente capítulo III denominado "Resultados y discusión" engloba un total de siete artículos científicos y una solicitud de patente enmarcados en cuatro subapartados.

A través de los ocho trabajos científicos, se aborda el estudio de la adición de antioxidantes naturales del grupo de los flavonoides a diferentes poliolefinas. El desarrollo de sistemas de separación, cuantificación y purificación necesarios para su estudio, así como la modificación química de la matriz poliolefínica para su aplicación en envases activos.

Los flavonoides y concretamente el grupo de las catequinas y la quercetina destacan por su alto potencial antioxidante lo que les confiere ciertas propiedades beneficiosas para la salud humana. Presentes en numerosas fuentes de origen natural tales como frutas, vegetales o bebidas como el té, su uso en diversas aplicaciones se deriva no sólo de su alta capacidad antioxidante, sino también de la preocupación del consumidor hacia el empleo de productos y aditivos que cumplan el binomio calidad nutricional-seguridad alimentaria.

A fin de ser empleados en las formulaciones de los envases, es necesario disponer de técnicas adecuadas de identificación y cuantificación de estos compuestos en sus matrices vegetales. El conocimiento del contenido de las matrices naturales en estos compuestos antioxidantes permitirá seleccionar aquellos extractos con un mayor contenido y, por lo tanto, con un mayor potencial antioxidante y más adecuados para cada una de las aplicaciones concretas.

Por este motivo, en el **primer subapartado** (3.1. Determinación y cuantificación de antioxidantes naturales) del presente capítulo de esta memoria, se aborda el desarrollo, comparación y aplicación de diferentes técnicas separativas para una determinación simultánea de catequinas y quercetina.

La necesidad de desarrollo de nuevos métodos de separación y cuantificación de catequinas y quercetina, obedece a la búsqueda de sistemas más eficientes y rápidos. La cromatografía líquida en fase reversa constituye uno de los métodos más ampliamente usados para la determinación de este tipo de compuestos. Actualmente, en la bibliografía, se recogen desarrollos cromatográficos para la separación de alguno/s de estos compuestos. Sin embargo, son pocas las metodologías que abordan la determinación conjunta de todos estos antioxidantes de interés o lo hacen por aplicación de métodos con características no idóneas de cara a una aplicación rutinaria (altos flujos y/o tiempos de análisis, complejas fases móviles y/o acidificadas que pueden dañar la columna cromatográfica, etc.). En el primer artículo "*Art.1. Analytical determination of flavonoids aimed to analysis of natural samples and active packaging applications*", se presenta el desarrollo de varias metodologías basadas en cromatografía de líquidos: HPLC-PDA-FL; HPLC-PDA-LTQ *Orbitrap* MS y UPLC-PDA para la identificación y cuantificación de siete catequinas y quercetina. Una primera comparativa de las diferentes metodologías se abordó por evaluación de la capacidad de cada una para detectar/cuantificar los compuestos de interés, siendo posteriormente completada con la evaluación de sus parámetros de calidad: rangos de linealidad, coeficientes de correlación, sensibilidad, límites de detección (LOD) y cuantificación (LOQ) y precisión de los métodos.

Siguiendo pautas de química verde con reducción de disolventes orgánicos tóxicos, se centró el esfuerzo en el empleo de fases móviles consistentes en mezclas en gradiente metanol-agua, realizando la separación con columnas cromatográficas C18. De este modo, se desarrollaron métodos de separación simples y rápidos, con los que se logró la determinación y resolución conjunta de todos los compuestos objeto de estudio, tanto mediante HPLC como UPLC, tal y como se observa en la Figura 1.1 del mencionado artículo ($t_{análisis}$<10min por HPLC; <1,80 min por UPLC). A diferencia de lo recogido en la bibliografía, se realizó una ligera acidificación en el disolvente de patrones y muestras con la que se logró una separación similar a aquella resultante de la acidificación de la fase móvil evitándose tanto la posible degradación de la columna cromatográfica como un elevado consumo de ácido.

Para la cuantificación, se usaron sistemas de detección comunes en aplicaciones rutinarias como son el detector de red de diodos (PDA) y el de fluorescencia (FL). La identificación se confirmó con el uso de un detector de espectrometría de masas con tecnología LTQ Orbitrap MS. Todos los detectores acoplados a las respectivas técnicas cromatográficas mostraron parámetros de calidad adecuados; destacando que el detector de FL mostró una sensibilidad muy elevada con unos LODs y LOQs 3 veces menores que los respectivos obtenidos con HPLC-PDA, lo que aconseja el uso de HPLC-FL para el análisis de trazas. El empleo del sistema de UPLC permitió también reducir los límites de detección y cuantificación en valores que oscilan entre 2 y 6 veces los obtenidos con HPLC-PDA. Aunque en este trabajo, la espectrometría de masas se usó como una técnica confirmatoria, los bajos LODs y LOQs (entre 2-7 veces menores que para HPLC-PDA; 1-4 veces

menores que UPLC-PDA) permite su aplicación en el estudio de trazas de los compuestos y en el de muestras complejas.

Por lo tanto, las catequinas y la quercetina fueron identificadas y cuantificadas mostrando linealidades, límites de detección y cuantificación, así como sensibilidades adecuadas para la aplicación de los métodos desarrollados en análisis de rutina.

En la segunda parte del trabajo se abordó la aplicación de las metodologías analíticas desarrolladas al estudio de los antioxidantes seleccionados en muestras reales sometidas a un mínimo proceso de extracción. El análisis de los extractos permitió, por un lado, elucidar la diferente aplicabilidad de cada una de las técnicas desarrolladas y, por otro, conocer el contenido de cada una de las muestras en los antioxidantes en estudio para una aplicación posterior de las mismas. Con respecto a la primera de las premisas, no todos los métodos mostraron igual fiabilidad. Con el empleo de HPLC-PDA se observaron ciertos problemas en la cuantificación de algún compuesto derivados de las interferencias presentes en la matriz. Dada la alta sensibilidad del detector de FL acoplado a HPLC sí que resultó posible cuantificar todos los compuestos con fluorescencia intrínsica objeto de estudio. Por otro lado, el detector de masas no sólo permitió confirmar la identificación realizada con los otros detectores, sino también detectar otros compuestos de interés, tales como catequinas metiladas o compuestos determinantes de la calidad del té como la teobromina o xantinas metiladas.

En cuanto a la segunda de las premisas, la aplicación de los métodos desarrollados permitió cuantificar las diferentes catequinas en las muestras

analizadas, confirmando el mayor contenido ya esperado en las muestras de té, principalmente en el té verde.

En la última parte del trabajo se seleccionó la muestra con mayor contenido en catequinas y quercetina (té verde), una catequina individual y la metodología más común en los laboratorios de análisis (HPLC-PDA) y se estudió la aplicabilidad del método al estudio de la liberación de las catequinas desde films activos con matriz de polipropileno dopados por extrusión con catequina y con té verde. Como se puede observar en la Figura 1.3. del artículo, el método desarrollado permitió la cuantificación de las cantidades liberadas en los distintos simulantes de alimentos.

Otra de las metodologías analíticas que destaca por su posible aplicación a los compuestos de interés en el presente trabajo es la electroforesis capilar. El auge de esta técnica se ha debido principalmente a sus características de simplicidad, alta velocidad, requerimientos de bajas cantidades de muestra y reactivos, bajo coste y bajos tiempos de análisis. Sin embargo, su baja precisión se ha presentado como su mayor inconveniente.

En el segundo de los trabajos de esta memoria, "*Art.2. Development, validation and application of Micellar Electrokinetic Capillary Chromatography method for routine analysis of catechins, quercetion and thymol in natural samples*", se completó el desarrollo de métodos analíticos del artículo 1 con el desarrollo de métodos de electroforesis capilar para la identificación y cuantificación de catequinas y quercetinas. Al igual que en el artículo anterior, la validación del método se llevó a cabo por estudio de los parámetros de calidad y se

comprobó su aplicabilidad en el estudio de los antioxidantes seleccionados en algunas muestras vegetales.

En este trabajo se estudiaron los diferentes parámetros condicionantes de la separación y resolución: tipo, concentración y pH del tampón electroforético, pH de la muestra, voltaje y temperatura. El método final permitió la identificación y separación de los compuestos en estudio en menos de 10 minutos (Figura 2.2. del artículo), con parámetros de calidad: linealidad, LODs, LOQs y sensibilidad adecuados para su aplicación rutinaria (Tabla 2.3. del artículo). Con el fin de evitar una de las mayores limitaciones de la técnica, su baja precisión, en este trabajo se ha establecido un riguroso procedimiento que contempla: el control exhaustivo de la concentración y pH del tampón electroforético, cambio diario de viales, realización de acondicionamientos y métodos con viales diferentes y el intercambio de viales de entrada y salida entre inyecciones sucesivas.

Comparado con los métodos desarrollados en el primer artículo, la metodología que emplea la electroforesis capilar presenta menor sensibilidad y mayores LODs y LOQs. Pese a que estos límites son superiores a los obtenidos en los métodos cromatográficos, siguen siendo adecuados para la determinación de los compuestos objeto de estudio en las muestras vegetales de interés.

La aplicabilidad del método en el estudio de muestras naturales se completó en este trabajo con estudios de recuperación, comparación entre calibración externa y adiciones estándar y aplicación de tratamientos estadísticos de los datos. Esta metodología presentó resultados adecuados en la cuantificación de muestras con alto contenido en catequinas y quercetina,

sin embargo, presentó ciertos problemas, posiblemente debidos a interferencias de la matriz en muestras con bajos contenidos, como por ejemplo en té rojo.

Por lo tanto, de los dos primeros trabajos desarrollados y englobados en el subapartado 3.1. se concluye que todos los métodos desarrollados muestran características adecuadas para su aplicación en análisis de rutina (métodos sencillos y rápidos con adecuados parámetros de calidad) y la selección de uno u otro va a depender de la aplicación concreta a desarrollar y de los requerimientos que de ella se deriven.

Aunque las catequinas, tal y como se ha mencionado con anterioridad, se encuentran presentes en numerosas fuentes naturales, sólo se encuentran en concentraciones relativamente altas en la muestras de té. Ello, unido a la complejidad de las matrices naturales en cuanto a la presencia de otras sustancias interferentes, hace necesario, cuando se pretende un uso industrial de ellas, el desarrollo de metodologías de purificación y limpieza. En el **segundo subapartado** del capítulo III de la presente memoria (3.2. Purificación de extractos naturales: polímeros de impresión molecular") en el artículo "*Art. 3. Preparation, evaluation and characterization of quercetin-molecularly imprinted polymer for preconcentration and clean-up of catechins*", se aborda el desarrollo de un polímero impreso molecularmente (MIP), para su aplicación en sistemas de extracción en fase sólida para la preconcentración y limpieza de catequinas en extractos naturales.

Los polímeros generados por impresión molecular se caracterizan por su alta especificidad, selectividad, afinidad y simplicidad de obtención y

aplicación, por lo que estos materiales se han considerado, en principio, idóneos para la consecución del objetivo planteado.

La primera de las etapas ha consistido en el estudio y desarrollo del MIP más adecuado para el fin buscado. Se ha empleado quercetina (análogo estuctural de las catequinas) como molécula plantilla, lo que ha permitido no sólo la aplicación de los MIPs desarrollados a la purificación y limpieza de quercetina sino también, y, principalmente, de catequinas evitando además, en este último caso, uno de los problemas más habituales cuando se emplean MIPs, las interferencias por el sangrado de la molécula plantilla. Se llevo a cabo una síntesis de MIPs no covalentes por el método de precipitación. El empleo de este tipo de polimerización da lugar a un polímero con un tamaño y forma más regulares y que no es necesario someter a etapas de molienda o tamizado, lo que a su vez implica menores tiempos de tratamiento. La selección de las condiciones de síntesis se llevó a cabo por estudio de los principales factores condicionantes de los resultados: tipo de monómero funcional, entrecruzador y porogen; relación molécula plantilla:monómero funcional:entrecruzador; método de polimerización y condiciones de tiempo y temperatura de polimerización. La evaluación de estos parámetros y la selección de las condiciones finales se realizó en base a los parámetros de la cantidad de polímero obtenido y de la cantidad inicial de molécula plantilla capaz de formar interacciones específicas y, por lo tanto, de crear sitios específicos de enlace en el MIP. A fin de elucidar la especificidad del MIP, paralelamente se sintetizaron polímeros no impresos (NIPs) en ausencia del analito plantilla. En el caso de la síntesis de MIPs no covalentes, el polímero más representativo de las interacciones no específicas es el NIP. Tal y como se muestra en la Tabla 3.1. del artículo, los MIPs que dieron lugar a los mejores

polímeros impresos fueron aquellos sintetizados con 4-vinilpiridina (4-Vpy) como monómero funcional y etilenglicol dimetacrilato (EGDMA) como entrecruzador, en una relación 1:4:20 molécula plantilla:monómero funcional:entrecruzador; acetona/acetonitrilo 3:1 como porogen, con un curado realizado a una temperatura de 60 ºC durante 24 horas.

Una vez sintetizados el MIP y el NIP se evaluaron las mejores condiciones de carga, lavado y elución para su uso en una aplicación de extracción en fase sólida (MISPE). La selección debe hacerse para generar interacciones específicas entre el MIP y la molécula objetivo, buscando así la máxima recuperación, especificidad y selectividad en el MIP y la mínima en el NIP. Para ello, se ha tenido en cuenta tanto la naturaleza de los disolventes empleados en cada una de las etapas como la de las moléculas objeto de estudio, dado que el carácter polar de quercetina y catequinas indica la formación de interacciones con los grupos funcionales finales del polímero mediante la formación de, principalmente, enlaces por puentes de hidrógeno.

La especificidad se evaluó por uso de la quercetina y la selectividad por uso de quercetina, de sus análogos estructurales de la familia de las catequinas (catequina, epicatequina y epigallocatequina gallato) y de α-tocoferol (estructuralmente diferente a quercetina y catequinas) como contraste. La comparación del funcionamiento de los polímeros impreso y no impreso demostró una clara diferencia entre ambos, lo que se traduce en una alta especificidad del MIP. Los resultados obtenidos (Tabla 3.2. del artículo) mostraron la alta selectividad del MIP tanto para la molécula plantilla (quercetina) como para las otras moléculas objeto de interés (especialmente catequina y epicatequina) y frente a otras moléculas como la

epigallocatequina gallato y el α-tocoferol principalmente como resultado de sus diferencias estructurales. Los resultados obtenidos al cargar cada catequina por separado se han comparado con los obtenidos al cargarlas como una mezcla, demostrando la existencia de competencia entre las distintas catequinas por los puntos de unión al polímero.

Finalmente se realizó una caracterización físico-química y morfológica de los MIP y NIP seleccionados. La caracterización físico-química de los sitios de unión se ha llevado a cabo mediante el estudio de las isotermas de adsorción y de la cinética del proceso. Considerando que el objetivo último del MIP es su aplicación en la purificación y concentración de catequinas, se usaron catequina, epicatequina y epigallocatequina gallato como representantes de las moléculas objetivo para la realización de este tipo de caracterización.

El primer paso para la realización de los estudios de adsorción es garantizar que el sistema se encuentra en equilibrio termodinámico. La evaluación del tiempo mínimo necesario para obtener una recuperación estable de las diferentes catequinas, la cinética del proceso y el mecanismo implicado se evaluaron mediante los estudios de cinética. Estos estudios permitieron concluir que la difusión externa de film fue el paso controlador del mecanismo, observándose una instantánea y rápida adsorción en los sitios más disponibles en la superficie externa del MIP y una adsorción más gradual adscrita al proceso de difusión intrapartícula, lo que estimuló, a su vez, una mayor migración de más compuesto desde la disolución hacia la superficie interna. Los estudios de las isotermas de adsorción y la modelización matemática de los datos, mostraron que la interacción de las catequinas con el polímero impreso está gobernada por la existencia de sitios de

reconocimiento específico homogéneos, que la adsorción se produce sólo en una monocapa y a través de un proceso que se podría asemejar a la quimisorción.

La caracterización morfológica de los polímeros impreso y no impreso, llevada a cabo mediante microscopía electrónica de barrido, y determinación del área superficial y la porosidad por el método de adsorción-desorción con nitrógeno, permitió concluir la existencia de ciertas cavidades presentes en el MIP (frente a una superficie lisa en el NIP) y una estructura macroporosa en el mismo. Por espectroscopía infrarroja con transformada de Fourier se determinó un grado de polimerización oscilando entre 70-80%.

Los resultados obtenidos en los estudios de cinética, de las isotermas de adsorción y en el proceso MISPE con una mezcla de estándares, revelaron que el MIP sintetizado es capaz de retener selectivamente las catequinas, por lo que se presupuso como un material adecuado para la extracción, purificación y concentración de estos compuestos en matrices naturales, seleccionándose varias variedades de té, el cacao o residuos de la producción de uvas. Tal y como se describió en apartados anteriores, la preparación de este tipo de muestras se realiza principalmente en agua, acorde a su modo de consumo habitual. El empleo de agua como disolvente en el proceso de carga reduce ostensiblemente la retención de las catequinas, lo cual se atribuye a la naturaleza de la interacción catequina-polímero que tiene lugar preferentemente mediante la formación de enlaces por puente de hidrógeno y que, por lo tanto, están desfavorecidos en un medio polar. Este hecho obligó a plantear alternativas consistentes en la introducción de etapas intermedias con hexano y/o diclorometano lográndose la eliminación de componentes de

la matriz y una redistribución de los analitos enlazados no específicamente hacia los sitios de unión específica. En estas condiciones, se han logrado recuperaciones de las catequinas en el MIP entre 90 y 100% para algunas de las muestras frente a 16-22% en el NIP. En el caso de otros compuestos no análogos empleados como contraste como la cafeína, apenas se han observado diferencias entre MIP y NIP.

Finalmente, con el objetivo de poder elevar los factores de preconcentración para la determinación de niveles traza de los compuestos de interés y de cara a una aplicación industrial de los MIPs, se evaluó el máximo volumen de muestra que puede ser cargado en el polímero sin pérdida de eficacia, es decir, el volumen de ruptura, siendo este superior a 40 mL por cada 0,2 gramos de polímero.

Todo ello demuestra que el método de extracción MISPE así desarrollado es apto para su aplicación exitosa en la limpieza y preconcentración de catequinas en muestras naturales complejas, permitiendo eliminar en una única etapa de lavado la mayor parte de los compontentes de la matriz que podrían interferir en el análisis de las catequinas, a la vez que se produce la concentración de estas, lo que hace posible su cuantificación a niveles traza.

Tal y como se ha planteado anteriormente, uno de los principales objetivos de la presente tesis doctoral es el estudio de las posibilidades que ofrece el uso de los antioxidantes naturales en formulaciones de polímeros. En este caso el fin es doble: por un lado, se ha buscado la sustitución de antioxidantes sintéticos empleados para proteger al polímero y, por otro, su uso como principio activo, capaz de ser liberado desde el propio polímero al

alimento en contacto, para que ejerza su acción antioxidante sobre éste, capacidad de interés en envases activos.

Hasta el momento, tal y como se ha comentado en el capítulo I de introducción, las poliolefinas constituyen unos de los polímeros más empleados en el sector del envase y embalaje. Por otro lado, los desarrollos de envases activos con incorporación de antioxidantes naturales se han realizado, principalmente, con una base de PEBD, polímeros hidrofílicos, sintéticos como el EVOH y biodegradables tales como el PLA o films en base celulósica. Sin embargo, en la bibliografía de referencian pocos estudios en los que se emplee el polipropileno como matriz para el desarrollo de envases activos, y esto pese a que es una de las matrices más ampliamente usada en el sector del envase alimentario.

Por todo ello, en el **tercer subapartado** del capítulo III (3.3. Empleo de antioxidantes naturales en muestras poliolefínicas) en el artículo denominado "*Art. 4. Natural extracts as potential source of antioxidants to stabilize polyolefins*", se analiza la eficacia de catequina y epicatequina como antioxidante para la protección de polipropileno. También se emplean en este trabajo extractos de diferentes especies vegetales con el mismo objetivo. En base a la cuantificación de las muestras llevada a cabo en la primera parte de este trabajo experimental, se seleccionaron varios extractos naturales considerando su contenido en catequinas: dos con alta concentración (té verde y té negro) y dos con baja concentración (*Lippia citriodora* (Lemon verbena) e *Hypericum androsaemun* (tutsan)). Los estudios anteriores de determinación del perfil fenólico, se completaron con el estudio de su actividad antioxidante. En base a su alto contenido fenólico y su alta

capacidad antioxidante, se seleccionó el té verde como el extracto con un mayor potencial y se incorporó en formulaciones de polipropileno.

La estabilidad proporcionada por la incorporación de té verde en la matriz de PP se comparó con la resultante de incorporar catequina y epicatequina como compuestos individuales. Frente a ellos, y como contraste, también se empleó una mezcla de antioxidantes sintéticos cuya capacidad ya había sido previamente estudiada en trabajos anteriores del mismo grupo de investigación (Irgafos 168+Irganox 1076), así como con el PP sin aditivos.

De este modo se pudo observar que en los materiales dopados con antioxidantes naturales, a partir de medidas de índice de fluidez y del tiempo de inducción a la oxidación se concluye que la adición de los mismos proporciona una mayor protección al PP retrasando la degradación oxidativa (Figuras 4.5 y 4.6 del artículo). En la bibliografía ya se habían propuesto varios mecanismos de la acción de los antioxidantes naturales.

Una vez comprobada la capacidad de los extractos naturales de proteger al polímero frente a su propia degradación, en esta tesis doctoral se abordó el desarrollo de sistemas de envasado activo en los cuales el agente activo fuese liberado de una forma controlada desde el envase para el control de los procesos de oxidación en alimentos envasados.

El empleo de PP en sistemas activos se ha visto limitado por la baja o nula liberación de los antioxidantes incorporados al polímero hacia el alimento contenido. Por ello, en el **cuarto subapartado** de la presente tesis doctoral (3.4. Desarrollo de materiales para el control de la liberación de antioxidantes:

envases activos y extrusión reactiva) se han propuesto modificaciones de la matriz de PP para dotar a éste de las propiedades deseadas. Dos han sido las alternativas abordadas en la presente tesis doctoral: uso de extensores de cadena y extrusión reactiva.

Además de estudiar el efecto de la adición de los agentes en las propiedades funcionales de los materiales, es necesario conocer la cinética de liberación de los agentes y los factores que influyen en dicho proceso exponiendo los films a diversos medios simulantes de alimentos. La liberación del agente activo es un proceso complejo consecuencia de los procesos de transferencia de masa en polímeros, caracterizado por el equilibrio de reparto del agente activo entre envase y alimento. La extensión de la liberación se puede ver condicionada tanto por factores del propio material (estructura, polaridad,…) o del agente activo (concentración inicial, solubilidad en el alimento, polaridad,…) como por factores externos del medio o alimento hacia el cual se produce la liberación (tipo de medio, temperatura, tiempo).

En este cuarto subapartado, se engloban tres artículos y una solicitud de patente que presentan el desarrollo de las dos modificaciones introducidas en la matriz de PP para alterar su capacidad liberadora.

Tal y como se ha comentado en los desarrollos previos, los extractos naturales de té son ricos en flavonoides y presentan una alta actividad antioxidante, derivada principalmente del alto contenido en catequinas y quercetina de los mismos. Estos flavonoides, comercializados ya individualmente, además de una alta actividad antioxidante, presentan un importante carácter no volátil y un perfil aromático bajo, por lo que su

liberación no debería conllevar un cambio significativo de las propiedades sensoriales del alimento envasado. Sin embargo, hoy en día individualmente no son considerados como aditivos alimentarios, por lo que su uso a nivel industrial en envases activos sería complejo. El empleo de extractos completos de té en lugar de los flavonoides individuales que lo constituyen sí poseen el estatus de aditivo alimentario. A mayores, a fin de comparar los extractos completos de té con el empleo de antioxidantes con un origen natural pero que se encuentren comercializados individualmente y, que se puedan usar como aditivos de alimentos, en este trabajo se ha propuesto el uso de tocoferoles, antioxidantes de origen natural empleados tanto en aplicaciones directas sobre alimentos, como en envases alimentarios y que se encuentran incluidos en las listas de aditivos alimentarios permitidos actualmente en la Unión Europea.

El primero de los artículos ("*Art. 5. Effect of PPG-PEG-PPG on the tocopherol-controlled release from films intended for food packaging applications*) y, la solicitud de patente derivada del mismo estudio (*Art. 6/patente. Procedimiento de obtención de un material polimérico*"), se basan en el uso de preparados naturales con diferentes porcentajes de tocoferol como agente activo. Se estudió la liberación de los tocoferoles a diferentes simulantes alimentarios y bajo varias condiciones de concentración de antioxidante y extensor de cadena, tipo de extensor, tiempo y temperatura. También se estudió la influencia del extensor en las propiedades del film. En los materiales de PP, el empleo de extensores de cadena durante el proceso de extrusión produjo pequeñas variaciones en la procesabilidad del material determinando las condiciones de procesado a emplear. Los estudios de liberación de tocoferol pusieron de manifiesto que el empleo de extensores de

cadena en las formulaciones poliméricas conduce a un aumento del porcentaje de compuesto liberado, y a una liberación controlada del mismo a lo largo del tiempo, tal y como se observa en la Figura 5.2 del Art. 5. Una mayor liberación cuando el extensor está presente en la matriz polimérica parece indicar una clara alteración de la permeabilidad de la matriz por la presencia del mismo. Esta liberación se ve claramente influenciada tanto por la cantidad inicial de compuesto aditivado como por la cantidad y tipo de extensor. El tipo de simulante también modificó la capacidad de liberación de los tocoferoles. La liberación hacia los simulantes más acuosos (simulante A, 10% etanol vs simulante D_1, 50% etanol) se ve limitada por la reducida solubilidad de los tocoferoles en este medio. Además, tanto el tiempo como la temperatura juegan papeles clave en la regulación de la liberación. El estudio de la migración del propio extensor confirmaron que no se produce una migración relevante del mismo que pueda dar lugar a una contaminación del alimento envasado.

En el tercero de los artículos de este bloque ("*Art. 7. Improving the capacity of polypropylene to be used in antioxidant active films: incorporation of plasticizer and natural antioxidants*"), se mantuvo tanto la forma de procesado (extrusión) como el material polimérico base (PP), se seleccionó el extensor de cadena que mejores resultados arrojó en el estudio anterior (PPG-PEG-PPG) y se sustituyeron los tocoferoles por un extracto de té verde rico en catequinas y quercetina. La comparativa con el efecto producido por la adición de una sola catequina se abordó con la incorporación de catequina al film. Como se observó tanto en los materiales con catequina como con té verde (Tabla 7.1 del artículo), la adición de ambos antioxidantes modificó de forma relevante la estabilidad del polímero, retrasando su degradación térmica. Al igual que en

el caso del tocoferol, mediante ensayos de liberación hacia simulantes alimenticios variando tanto la concentración del extensor como la de los agentes activos o el tiempo, así como el tipo de simulante, se observó una mejora de la capacidad de liberación controlada en el tiempo al emplear el extensor como modificador de la cadena polimérica. En el caso del té verde se realizó el seguimiento de la liberación de 7 catequinas, ácido gálico, cafeína y quercetina. La incorporación de té verde en la formulación de los films condujo a la liberación de menores cantidades de agente activo en el simulante alimentario que en el caso de films dopados únicamente con catequina (Figuras 7.1 a 7.3 del artículo). Este hecho se atribuye a que el porcentaje de cada uno de los compuestos adicionados en el extracto total de té verde es mucho menor que el porcentaje adicionado cuando se realiza en formato de compuesto individual. La capacidad de liberación no sólo se caracterizó en base a la cantidad de compuesto liberado sino también mediante la estimación de los correspondientes coeficientes de difusión (D) y a la medición de la capacidad antioxidante de los simulantes una vez concluido el tiempo de contacto film-simulante. Los tres estudios confirmaron que se produce liberación controlada, y que ésta a su vez realiza una actividad antioxidante en los simulantes alimentarios expuestos a los films, provocando un efecto protector frente a la oxidación.

Otra de las modificaciones de la matriz polimérica que se abordó en la presente tesis doctoral a fin de alterar la liberación de antioxidantes ha sido la extrusión reactiva. En algunas aplicaciones industriales, en lugar de una liberación inmediata desde el envase hacia el alimento lo que interesa es que

el material que va a ser sometido a una serie de tratamientos previos como, por ejemplo, tratamientos térmicos de esterilización, retenga en un primer momento esos compuestos aditivados para que la liberación no se produzca en los primeros días o incluso horas, ya que para alargar la vida útil del alimento resulta más interesante que esa cesión sea lenta y progresiva. Por lo tanto, se busca una inmovilización del agente activo lo que implica una menor liberación inicial, lo que a su vez provoca que su actividad antioxidante dure un mayor tiempo. Para lograr este fin, en el último artículo recogido en esta memoria ("*Art. 8. Interaction and release of catechin from anhidride maleic grafted polypropylene films*"), se aborda la inmovilización de catequina en la matriz de polipropileno a través de la incorporación de polipropileno modificado superficialmente con anhídrido maleico.

Las poliolefinas en general y el polipropileno en particular, como la mayoría de los polímeros sintéticos, presentan una superficie hidrofóbica e inerte. Consecuencia de ello, presentan una baja capacidad de reacción e incompatibilidad con sustancias hidrofílicas, entre otros problemas. Entre las técnicas empleadas para modificar los polímeros, destaca la acción de injertar monómeros polares en la superficie de las poliolefinas a través de un proceso de extrusión reactiva. La extrusión reactiva consiste en efectuar una reacción química en la extrusora bajo los efectos combinados de temperatura, presión y cizallamiento. En el trabajo recogido en la presente memoria, se empleó un polipropileno modificado con anhídrido maleico (MAH) dado su bajo coste, alta actividad y buena procesabilidad para utilizarlo en mezclas con polipropileno. La presencia de los grupos anhídridos le confiere cierta actividad siendo capaz de sufrir una amplia variedad de reacciones orgánicas. Esta reactividad se plantea como una opción viable para la reacción entre el

polipropileno modificado y los grupos reactivos de los antioxidantes de cara a su inmovilización en la matriz polimérica. En la bibliografía se han encontrado estudios sobre la modificación de las propiedades mecánicas como resultado de la incorporación de PPMAH, sin embargo no se conocen estudios sobre la interacción con antioxidantes. En este estudio se selecciona la catequina como representante de todos los antioxidantes estudiados y dos PP modificados con diferente porcentaje de anhídrido maleico. La caracterización física de la interacción entre los diferentes PPMAH y la catequina se abordó por FTIR (Figuras 8.1 y 8.2 del artículo) poniendo de manifiesto una clara interacción entre el anhídrido maleico y los grupos hidroxilo de las catequinas, tal y como se observa en la Figura 8.3 del artículo. Además, se observó que se retrasa la degradación térmica del polímero.

Finalmente, a la par que los estudios de caracterización física de los nuevos materiales, los estudios de liberación hacia simulantes alimentarios y de actividad antioxidante (caracterizada a través de los valores de los coeficientes de reparto (K) y de difusión (D) de cada antioxidante), revelaron que el uso de PP modificado cambió notablemente la capacidad del polímero de liberar catequina de modo que se produce una cesión más moderada y controlada en el tiempo (Figura 8.5 del artículo) y altamente influenciada por el grado de modificación del polipropileno, por el tipo de simulante, la temperatura y el tiempo de contacto.

De este modo, se puede observar en estos cuatros artículos, que tanto el uso de extensores de cadena, como la extrusión reactiva sobre matrices de PP, ha dado lugar a matrices en las que es posible regular la capacidad de cesión

de los antioxidantes naturales. Es por ello que en estas matrices podemos indicar que se ha conseguido una liberación controlada, lo que puede tener aplicación en un gran número de usos industriales como sistemas de envasado activo con capacidad antioxidante.

A continuación se presentan los 8 artículos científicos que conforman la memoria de esta tesis doctoral estructurados en los cuatro subapartados anteriormente comentados.

3.1. DETERMINACIÓN Y CUANTIFICACIÓN DE ANTIOXIDANTES NATURALES

Artículo 1: Analytical determination of flavonoids aimed to analysis of natural samples and active packaging applications.

Artículo 2: Development, validation and application of Micellar Electrokinetic Capillary Chromatography method for routine analysis of catechins, quercetin and thymol in natural samples.

CAPÍTULO III. RESULTADOS Y DISCUSIÓN

3.1. DETERMINACIÓN Y CUANTIFICACIÓN DE ANTIOXIDANTES NATURALES

Artículo 1

ANALYTICAL DETERMINATION OF FLAVONOIDS AIMED TO ANALYSIS OF NATURAL SAMPLES AND ACTIVE PACKAGING APPLICATIONS

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ABSTRACT

Several HPLC and UHPLC developed methods were compared to analyze the natural antioxidants catechins and quercetin used in active packaging and functional foods. Photodiode array detector coupled with a fluorescence detector and compared with LTQ-Orbitrap-MS was used. UHPLC was investigated as quick alternative without compromising the separation, analysis time shortened up to 6-fold. The feasibility of the four developed methods was compared. Linearity up to 0.9995, low detection limits (between 0.02 and 0.7 for HPLC-PDA, 2 to 7-fold lower for HPLC- LTQ-Orbitrap-MS and from 0.2 to 2 mg L^{-1} for UHPLC-PDA) and good precision parameters (RSD lower than 0.06 %) were obtained. All methods were successfully applied to natural samples. LTQ-Orbitrap-MS allowed to identify other analytes of interest too. Good feasibility of the methods was also concluded from the analysis of catechin and quercetin release from new active packaging materials based on polypropylene added with catechins and green tea.

Keywords: Natural antioxidants, catechins, quercetin, chromatography, comparison, active packaging.

1. INTRODUCTION

Oxidative stress and the consequent release of free oxygen radicals seem to be associated with many diseases, such as cancer, cardiovascular malfunction or inflammatory disorders (Malestic et al., 2008). Due to their increasing incidence, considerably attention has been paid to natural substances with antioxidant activity; which inhibit or delay the reaction of oxidation.

Natural antioxidants, and specially the flavonoids catechins and quercetin have attracted considerable attention showing an important antioxidant activity (Dopico-García et al., 2011). Tea, and especially green tea (*Camellia sinensis*), is one of the most widely consumed beverages worldwide, second only to water, and is a natural source of those antioxidant compounds, which confers tea with important antioxidant and thus, health effects (Gramza & Korczak, 2005). The strong antioxidant capacity of catechins has been reported to have a protective and beneficial health effect related to anti-mutagenic, anti-diabetic, anti-inflamatory, anti-bacterial and anti-viral qualities and prevention against several kinds of cancer. Moreover, they seemed to be effective in against heart and liver diseases, slowing aging and neurodegenerative processes and enhancing weight loss, among others (Shi & Schlegel, 2012; Braicu et al., 2013). Quercetin also provide tea with antioxidant and mood-cognitive-enhancing properties (Nijveldt, R. et al., 2001).

Resulting from a response to trends in consumer preferences towards mildly preserved, fresh, tasty and convenient food products with a prolonged shelf-life, their use in active packaging and functional foods has become increasingly significant. Those active agents provide active antioxidant

functions to the packaging that allow the shelf-life of the packaged food to be extended or to improve the safety or sensory properties while maintaining the quality of the food (Vermeiren et al., 1999). Due to that importance on current consumer demands and market trends, it is fundamental to determine release levels in food matrices using suitable analytical methods.

Therefore, efficiency, speed and cost of analysis have become of a great importance, especially, aimed to routine analysis where it is important to increase throughput and reduce analysis costs. Ways to improve resolution, speed and sensitivity of chromatographic methods have still continued under investigation.

The most developed methods for catechins determination have been established based upon reversed-phase HPLC coupled to PDA (Dalluge y Nelson, 2000; Molnár-Perl y Füzfai, 2005). The desire for higher resolution of catechins has led to consider the use of acidic mobile phases as essential. Column degradation resulting from the lack of stability of the silica-based bonded reversed-phase columns used under low pH conditions could be risky, though (Lipper et al., 2007). High flow values (~1 $mLmin^{-1}$), high analysis times (20-90 min), complex mobile phases, determination of only some catechins or rather scarce simultaneous determination of them, and/or unsatisfactory quantifications limits and resolutions also make those methodologies little useful aimed both to routine analysis and real samples analysis (Dalluge y Nelson, 2000; Molnár-Perl y Füzfai, 2005; Wei et al. 2012; Wu et al., 2012; El-Shahawi et al., 2012; Wang et al., 2012; Qin et al., 2012; Samanidou et al., 2012; Scoparo et al., 2012).

The difficulty in interpreting some complex sample chromatograms also makes necessary to optimise the detection stage (Schieber et al., 2001). To this end, other detectors have been proposed. Fluorescence (FL) in order to avoid interferences with other compounds discriminating between fluorescent and non-fluorescent overlapping peaks; electrochemical detectors (ECD), proving to be a useful completion technique to the analysis of phenolic compounds providing special selectivity related with the diversities in electro active substituents or mass spectrometer (MS) frequently used to obtain structural information after chromatographic separation, are examples of the detectors used (Molnár-Perl & Füzfai, 2005; Novak et al., 2008; Novak et al., 2010; Porgali & Büyüktuncel, 2012; Qin et al., 2012; Wu et al., 2012; Díaz-García et al., 2013).

Ultra-high performance liquid chromatography (UHPLC) has also been considered as a new direction of liquid chromatography (Spáčil et al., 2010). Nevertheless, the very few reports targeted on the determination of catechins lacked in its application to the analysis of real samples in terms of peak resolution, sample matrix effect or detection of some aimed compounds.

Determination of quercetin and catechins in the same run has been very rarely reported, despite being present together and with a similar content and antioxidant capacity in several natural matrices (Dimitrios, 2006).

Therefore, several chromatographic methods have already been reported for a detailed characterization of the antioxidant content of some extracts. Nevertheless, the aim of the present work deal more with studying and selecting the most useful and profitable chromatographic method to evaluate the most outstanding contribution of some extracts on both their daily-intake

or their use as active additives in active packaging and in functional foods. Due to their reported antioxidant capacity (Dopico-García et al., 2011; Gramza y Korczak, 2005) catechin and green tea extract, with high content of catechins, were selected in this work as active agents.

Thus, the feasibility of HPLC-PDA-FL, HPLC-PDA-LTQ Orbitrap MS and UHPLC-PDA methodologies for the routine simultaneous determination of major and minor catechins and quercetin was compared in terms of best resolution and highest sensitivity of detection. The proposed methodologies were also evaluated assaying the catechins and quercetin content in natural samples and the release levels form the active antioxidant films developed.

2. EXPERIMENTAL

2.1. Chemicals and standards

Methanol (> 99.8%) and ethanol (> 99.9%) HPLC-gradient for instrumental analysis were supplied by Merck (Darmstadt, Germany). Formic acid 98-100% puriss-p.a. was from Sigma-Aldrich (Steinheim, Germany). Water was purified using a Milli-Q Ultrapure water-purification system (Millipore, Bedford, MA, USA). (-)-Epigallocatechin (EGC) (>95%), (-)-Epigallocatechin gallate (EGCG) (80%), (-)-Epicatechin gallate (ECG) (>98%), (-)-Epicatechin (EC) (>98%), (+)-Catechin hydrate (C) (>98%), (-)-Gallocatechin Gallate (GCG) (>98%), (-)-Catechin Gallate (CG) (>98%), quercetin (Quer) (>95%) and Irgafos 168 (Tris(2,4-di-tert-butylphenyl)phosphate; I168) (purity not specified) standards were purchased from Sigma-Aldrich.

2.2. Standard preparation

Individual stock standard solutions (1000 mg L^{-1}) were prepared in an aqueous solution of formic acid (pH 1.5) for catechins and ethanol for quercetin. Work standard solution containing all compounds (20 mg L^{-1} for catechins and 5 mg L^{-1} for quercetin) was prepared from individual stock standard solutions in aqueous-formic acid solution at pH 1.5. Work standard solution at concentration of 5 mg L^{-1} of catechins and 0.5 mg L^{-1} for quercetin was prepared for HPLC-MS analysis. Stability of each stock and work standard solution was tested and corroborated trough a period of time of 60 days.

2.3. Sample preparation

The following natural samples were tested. Red and white tea commercialized in infusion bag, green and black tea commercialized in bulk and cocoa, were purchased in local supermarkets. Tutsan and lemon verbena were kindly donated by Serviço de Farmacognosia, Faculdade de Farmácia, University of Porto, Portugal. Grape residues, barley shell and chestnut hedgehog were kindly obtained from Department of Chemical Engineering, University of Vigo, Spain. Hop was obtained from a local farming, and residue of beer fabrication were kindly donated by Estrella de Galicia beer company, Galicia, Spain. Samples were extracted in Milli-Q water (1 g of sample: 25 mL of water) under magnetic stirring for 10 minutes accordingly to common daily consumption way. Formic acid was then added (50 μL of formic acid: 1 mL of extracted sample) to each extract. The final samples were

filtered through an Acrodisc^{R} PTFE CR 13 mm, 0.2 μm filters (Waters, Mildford, MA, USA) and transferred into HPLC vials.

2.4. Preparation of antioxidant films

PP-catechin-containing films and PP-green tea-containing films were obtained by extrusion. Catechin and green tea were incorporated at two levels of concentration: 2 and 5% (w/w). Irgafos 168 at 0.2% (w/w) was added to protect films during manufacture process. Extrusion was carried out using a miniextruder equipped with twin conical co-rotating screws and a capacity of 7 cm^3 (Minilab Haake Rheomex CTW5 (Thermo Scientific)). Screw rotation rate of 40 rpm, temperature of 180 ºC and 1 minute of residence time were used.

2.5. Antioxidant release tests

A study of the release of the active compounds from the films was carried out by determining the specific migration from the polymer into food simulants A (10% ethanol) and D_1 (50% ethanol) as specified in European Commission Regulation Nº 10/2011 (The European Commission, 2011). Total immersion of rectangular strips film pieces (80x0.4x0.17 mm) in 10 mL of food simulant at 40 ºC and 5 and 10 days of storage were the conditions selected. After the contact period, an aliquot of food simulant was filtered and analyzed by means of HPLC.

2.6. Chromatographic conditions

2.6.1. HPLC-PDA-FL analysis

A Waters 2695 (Waters) system was used for HPLC analysis. SunFire™ C_{18} (150 mm x 3.0 mm, 3.5 μm) (Waters) kept at 35 ºC, photodiode array detector (PDA, model 996 UV) set in the range of 200 to 400 nm (output signals of 277 and 380 nm for catechins and quercetin, respectively), and a fluorescence detector (FL, model 2475) (Waters) with $\lambda_{excitation}$ 280 nm and $\lambda_{emission}$ 310 nm were used. A two solvent gradient elution was performed, with flow rate of 0.5 mL min^{-1} and injection volume of 20 μL. Mobile phase was composed by water (A) and methanol (B) under the following gradient: 25% B maintained for 0.5 min, linearly increased to 40% B in 4.5 min, 60% B in 1 min and 100% B in 2 min and finally maintained for 3 min and brought back to the initial conditions.

2.6.2. LC-PDA-LTQ FT *Orbitrap* MS analysis

LC-PDA-LTQ FT *Orbitrap* system consists of an Accela Autosampler, high speed pump, PDA detector set from 200 to 400 nm, and LTQ-FT Orbitrap mass spectrometer (Thermo Electron GmbH, Bremen, Germany), Luna™ C_{18} (150 mm x 4.6 mm, 5 μm) (Phenomenex, Macclesfield, UK) kept at 30 ºC and Water/1% of formic acid (A)-methanol (B) at 0.5 mL min^{-1} under the following gradient were used: 25% B maintained for 0.5 min and linearly increased to 40% B in 4.5 min, 60% B in 3 min, 100% B in 2 min, maintained for 3 min and brought back to the initial conditions. 20 μL were injected. The linear ion trap (LTQ) of the hybrid MS system was equipped with elestrospray ionization

(ESI) probe and operated in positive ion mode. Factors affecting the optimal operating of the ESI interface were tested. Ionspray voltage 5.03 kV, heated capillary temperature 250ºC, capillary voltage 31 kV, tube lens 70V, 40 units of nitrogen sheath gas and 5 units of nitrogen auxiliary gas were selected. Full-scan accurate mass spectra were obtained at high resolution (100,000-200,000 FWHM) by transferring the produced ions to the Orbitrap detector. Data were acquired from 145 to 500 Daltons.

2.6.3. UHPLC-PDA analysis

Waters Acquity Ultra Performance LC System, BEH (Bridge ethylene hybrid) C_{18} column (50 mm x 2.1 mm, 1.7 μm) kept at 35 ºC, needle-over-fill injection mode and photodiode array detector (Waters) set in the range of 200 to 400 nm (277 nm for catechins and 380 nm for quercetin as output PDA signals) were used. A two solvent gradient elution consisted of water (A) and methanol (B) at flow rate of 0.5 mL min^{-1}, injection volume of 3 μL, under the following gradient: 20% B was maintained for 0.24 min and subsequently linear increases to 40% B in 0.86 min, 60% B in 0.17 min, 100% B in 0.75 min and brought back to initial conditions.

2.7. Quality parameters

Performance of the developed methods through linearity range, correlation coefficients, standard deviations of intercepts and slopes, detection and quantification limits and precision was evaluated for each analyte.

Selectivity of the methods was assessed by the absence of interfering peaks at the elution times of the catechins and quercetin. Linearity was evaluated building five point calibration curves plotted through the concentration range of two orders of magnitude for each studied compounds using standards. Sensitivity was assessed through the comparison of the slope values and by determining the detection (LOD) and quantification (LOQ) limits calculated according to Shabir (Shabir, 2003) for each compound and method using the calibration graphs, being LOD=yB+3xSB and LOQ=yB+10xSB. Representing yB (blank signal) = a (intercept of the calibration graph) and SB (standard deviation of the blank) = $S_{y/x}$. Efficiency of the chromatographic columns was evaluated by the resolution (Rs) between each pair of peaks. Precision in area and retention time was also evaluated through repeatability and intermediate repeatability by eight time-injection of a catechin-quercetin work standard solution under every chromatographic developed method in one day (repeatability) and throughout five consecutive days (intermediate repeatability) and expressed as %RSD, describing the closeness of agreement between series of measurements.

3. RESULTS AND DISCUSSION

In performing series of routine analysis, speed, sensitivity, resolution, cost and also column maintenance should be considered. Thus, aimed both to study and overcome previous reported methodologies drawbacks, in the first part of the study, several chromatographic methods were tested and compared in the determination of catechins and quercetin. Their separation efficiencies and quality parameters were also compared. In the next step, the

developed methodologies were applied to detect and quantify catechins and quercetin in several natural samples.

3.1. Degradation of separation column: pH of the mobile phase

pKa values of catechins (8-9.5) indicate that chromatographic resolution should be improved with pHs just under eight; however, catechins have been reported as relatively stable only at pH<4. Therefore, high emphasis used to be on using acidic mobile phases with relatively low pH values (0.3 to 1.4) to achieve their complete resolution (Wei et al., 2012; Viñas et al., 2000; Bravo et al., 2006; Zhu et al., 1997). However, at pH lower pH than 3 over relatively short periods of time, silica-based bonded reversed-phase columns have been reported as non-stable. Column degradation can then occur as a result of the hydrolysis of the bonded phase, resulting in shortened column life and, therefore, bleeding of the stationary phase, gradual decrease in the retention and poor peak symmetry (Lipper et al., 2007).

SunFire™ C-18 column with range stability from 2 to 8 was used in the present work with HPLC-PDA-FL system. Instead of acidic mobile phase, samples were acidified at pH 1.5. Compared with using acidic mobile phase and differing from previous reports, acidic mobile phase did not enhance resolution or retention times. Standard deviations between the resolutions of each peak calculated according to IUPAC recommendations (Ettre, 1993) showed values from 0.001 to 0.06 for catechins and 0.2 for quercetin. Therefore, non-acid mobile phase was selected as to increase chromatography column life.

Nevertheless, ESI requirements in mass spectrometry led to include an acid modifier in the mobile phase as to enhance the formation of molecular ions (Rauha et al., 2001). Therefore, Luna™ C18 was used due to its higher pH range stability (ranged from 1.5 to 10).

3.2. Simultaneous determination of catechins and quercetin: separation

The retention of an analyte on a chromatography column is dependent on the chemical nature of the stationary phase and the mobile phase composition. The optimum conditions (section 2.4) for the routine analysis of the seven catechins and quercetin in one run were investigated by changing mobile phase compositions, gradient profiles, flow-rate programs and column temperatures. During the first part of the study, the analysis times and the performance of the separation using HPLC and UHPLC were compared and tested through resolution (Rs) between each pair of peaks (data not shown).

Figure 1.1 shows representative chromatograms of standard mixtures by all the studied methods.

3.2.1. HPLC. HPLC has provided catechins and quercetin baseline resolution in a short analysis time (10 minutes) by a simple methanol-water elution gradient without using any acid in the mobile phase, which constitutes one of the most relevant characteristic of this HPLC method. As indicated in section 3.1, two C18 columns were used. A conventional endcapped C18 column SunFire™ (150 x 3.0 mm, 3.5 μm) was used for HPLC-PDA-FL analysis. The smaller the particle size of this column compared to

those of recent published papers (Wei et al., 2012; Wu et al., 2012; El-Shahawi et al., 2012; Wang et al., 2012; Qin et al., 2012; Samanidou et al., 2012; Scoparo et al., 2012), the more efficient the separation, but the greater the operating pressure. However, pressure values less than 4000 psi has perfectly allowed to operate. As analytes have MW < 3000, the pore size of the column (< 100 Å) should also be enough to ensure the best efficiency and peak shape. Its comparable or even less surface area and carbon load than those previously reported as well as its shorter length have also contributed to the decrease in the analysis time: 2-10-fold reduction in total analysis time of catechins from existing analytical methods (18-80 minutes).

Moreover, low flow rate and analysis time (0.5 mL min^{-1}-10 minutes) has meant at least 50% of solvent saved per run when compared with reported methods (Wei et al., 2012; Wu et al., 2012; El-Shahawi et al., 2012; Wang et al., 2012; Qin et al., 2012; Samanidou et al., 2012; Scoparo et al., 2012), meaning a more environmental and economic friendly method.

3.2.2. HPLC-PDA-LTQ Orbitrap MS. Due to the ESI acid modifier requirements (Rauha et al., 2001), Luna™ C_{18} column with a broader pH range (1.5 to 10) was the other column used in this work. Adding formic acid in the mobile phase, the retention time of some phenolic compounds decreased since it is believed to interact with hydroxyl groups of the analytes via hydrogen-bond formation, increasing the virtual polarity of the polyphenols (Nicoletti et al., 2008). To get high resolution, minor changes were done to the final step of the gradient (section 2.4).

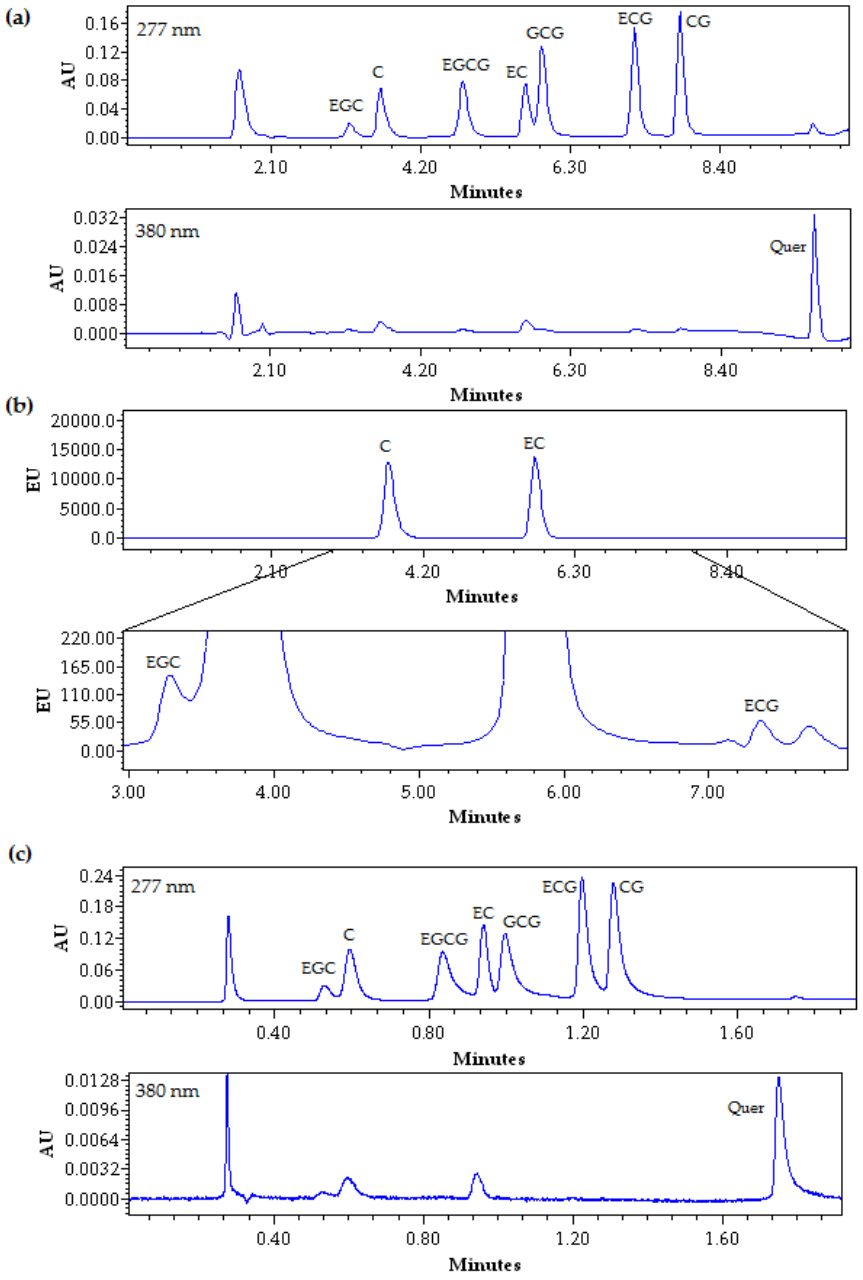

Figure 1.1. Chromatograms of a mixture of standard catechins (5 mg L^{-1}) and quercetin (0.5 mg L^{-1}) obtained by: a) HPLC-PDA; b) HPLC-FL and its amplification; c) UHPLC-PDA; d) LC-LTQ- FT Orbitrap MS (including the mass spectrum of each peak).

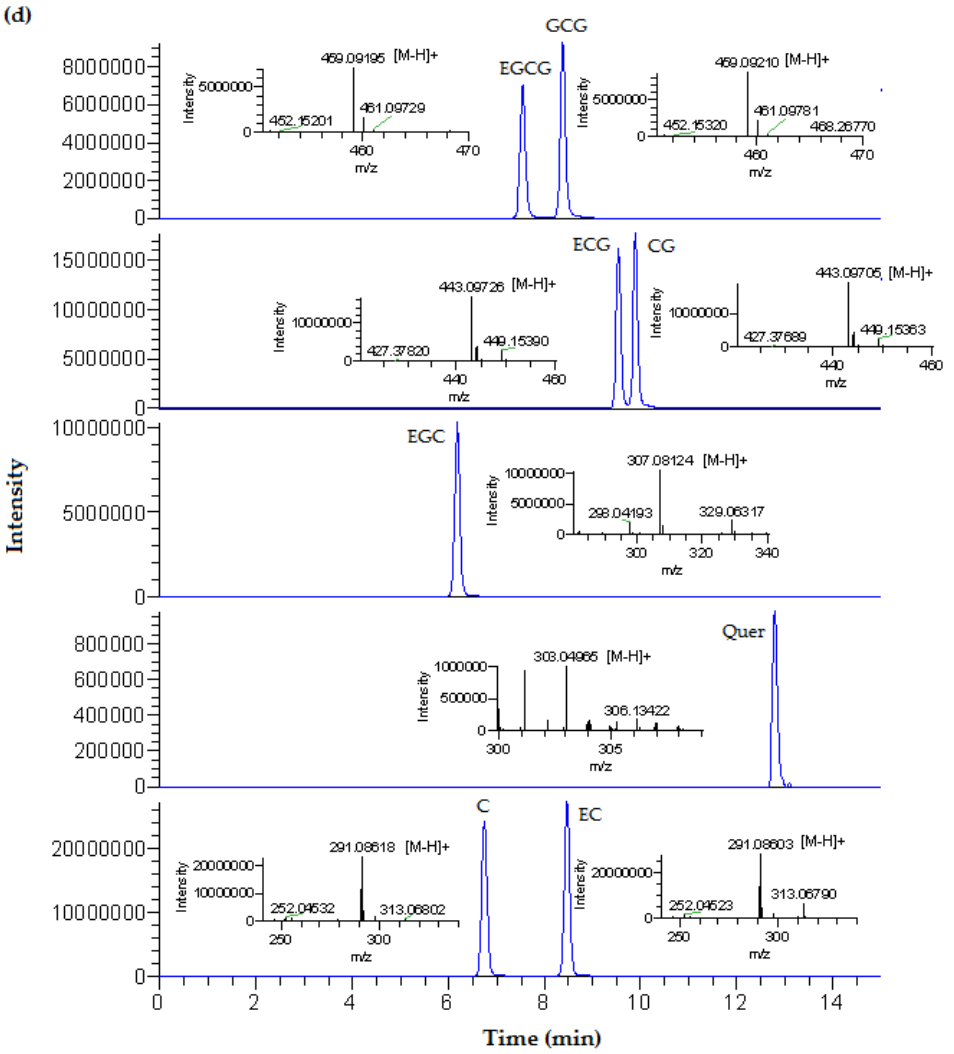

Figure 1.1. (Continuation I).

Resolution of epicatechin and catechin gallatechin gallate was particularly inefficient presumable because Luna™ C_{18} higher diameter, surface area and percentage of carbon load compared to SunFire™ C_{18}, which influences the efficiency of the separation. It was not considered as problematic though as they are aimed to be detected by a mass spectrometer.

3.2.3. UHPLC. Regarding low analysis time and solvent consumption, UHPLC was also tested. Very few attempts in the analysis of catechins and quercetin by UHPLC have been reported (Scoparo et al., 2012; Spáčil et al., 2010; Nováková et al., 2010; Zhao, Chen et al., 2011). Hybrid BEH C_{18} packed column with 1.7 μm particles, able to tolerate pressures up to 15,000 psi and with pH operational range from 1.5 to 10 was selected. Elution gradient was selected after minor changes to the method obtained by starting from the HPLC-PDA-FL method aided by Acquity UHPLC performance calculator software (section 2.4).

Analysis time was then shortened up to 6-fold compared to HPLC-PDA-FL method (Figure 1.1). This analysis time also compares very reasonably and even shortens (up to 2 to 6-fold) with recently UHPLC reported methodologies (Scoparo et al., 2012; Spáčil et al., 2010; Nováková et al., 2010; Zhao et al., 2011). As it decreases, solvent consumption was also reduced. Moreover, UHPLC method showed good sensitivity allowing the injection of 3 μl versus the 20 μl of HPLC method.

Although exact comparison of HPLC and UHPLC system cannot be done, owing to the different chemistry and particle size of the columns, UHPLC method showed general advantages over HPLC in terms of time saving, solvent saving, high performance and high efficiency. Nevertheless, both of them showed good performance to be applied to routine analysis of catechins and quercetin.

Satisfactory resolutions (Rs~1) were achieved for all the peaks by every developed method, except for the pair EC-GCG by HPLC. Its resolution was highly improved by UHPLC.

3.3. Determination of catechins and quercetin: detection

Detection significantly affects the selectivity, sensitivity and calibration parameters.

Different detectors were used in this study aimed to both detection and quantification purpose. Performance of the developed methods was evaluated through quality parameters (linearity range, correlation coefficients, standard deviations of intercepts and slopes, detection and quantification limits and precision (results listed in Tables 1.1 and 1.2)) and through their application to the analysis of the 12 natural samples (white, green, red and black teas; grape residues, cocoa, tutsan, lemon verbena, barley shell, chestnut hedgehog, hop and residue of beer fabrication (Figure 1.2)).

3.3.1. PDA detection. Based on the spectral characteristics of catechins and quercetin, their identification and quantification by PDA has provided both higher certainty and selectivity assessed by the absence of interference in the same chromatographic windows in both standards and most of the studied samples.

HPLC-PDA and UHPLC-PDA methods have both provided linear concentration ranges over several orders of magnitude with linearity over 0.9997 and 0.9995, respectively (Table 1.3). High sensitivity with slope values up to 1.0E4 except for the pair EGC-EGCG (5.2E3) for HPLC and up to 1.1E3 for UHPLC was found (Table 1.1). As well, low detection and quantification limits (Table 1.1) ranging between 0.05 and 0.8 mg L-1 and from 0.16 mg L^{-1} and 2.5 mg L^{-1}, respectively, were found by HPLC for catechins and quercetin.

These LODs and LOQs were between 2 to 6-fold times lowered by UHPLC method.

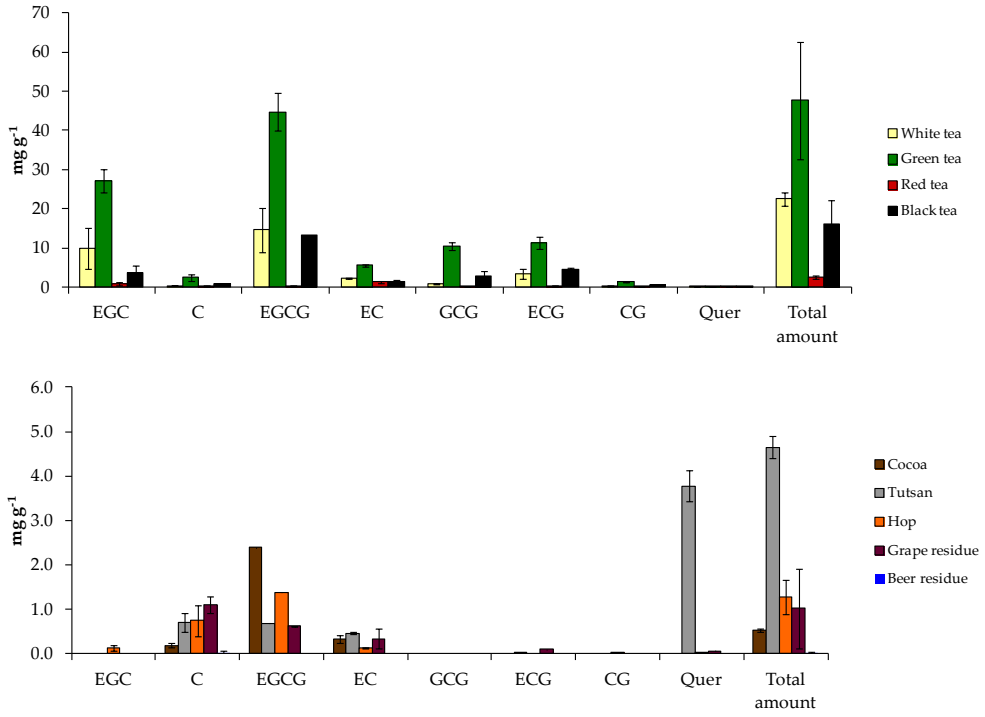

Figure 1.2. Catechins and quercetin content in real samples quantified by HPLC-PDA, UHPLC-PDA, HPLC-FL and HPLC- LC-LTQ FT *Orbitrap* MS and expressed as average concentration. Lemon verbena, barley shell and chestnut hedgedog data not included since none of the studied compounds was detected.

Compared to recently reported methods where a relatively high number of catechins was also studied, the LOD and LOQ achieved with the present HPLC-PDA and UPLC-PDA are between 1 and 8-fold lower than that (Nováková et al., 2010; Samanidou et al., 2012). However, low LODs and LOQ

are achieved when only two or three catechins are studied (El-Shahawi et al., 2012; Porgali & Büyüktuncel, 2012).

Table 1.1. Sensitivity (slope values±standard error (%)), LOD and LOQ of the proposed methods for catechins and quercetin.

	Slope values±standard error (%)			
	HPLC-PDA	**UHPLC-PDA**	**HPLC-Orbitrap**	**HPLC-FL**
EGC	4.3E3±1.3E1	9.5E2±2.3E0	1.5E7±8.0E4	8.6E5±1.7E3
C	1.2E4±8.9E1	3.8E3±2.2E1	4.6E7±2.0E5	6.4E7±1.7E5
EGCG	6.1E3±4.8E1	1.3E3±3.1E1	1.1E7±4.0E4	--
EC	1.4E4±4.8E1	4.3E3±1.9E1	4.6E7±3.4E5	7.0E7±2.3E5
GCG	2.2E4±3.2E2	1.3E3±5.2E1	2.6E7±3.1E5	--
ECG	2.6E4±5.0E2	4.3E3±3.8E1	2.2E7±1.8E5	1.8E5±7.8E2
CG	2.6E4±5.1E2	4.4E3±3.6E1	2.1E7±2.4E5	--
Quer	3.8E4±1.4E3	2.7E3±7.6E1	3.6E7±1.5E5	--
	LOD (mg L^{-1})			
EGC	0.137	0.0966	0.104	0.0399
C	0.328	0.216	0.0565	0.111
EGCG	0.105	0.079	0.0729	--
EC	0.171	0.172	0.128	0.100
GCG	0.586	0.167	0.0857	--
ECG	0.747	0.141	0.123	0.172
CG	0.762	0.126	0.140	--
Quer	0.0496	0.0435	1.63E-3	--
	LOQ (mg L^{-1})			
EGC	0.457	0.322	0.345	0.133
C	1.09	0.719	0.188	0.371
EGCG	0.349	0.623	0.243	--
EC	0.569	0.575	0.427	0.335
GCG	1.95	0.474	0.286	--
ECG	2.49	0.471	0.412	0.575
CG	2.54	0.419	0.467	--
Quer	0.165	0.145	5.45E-3	--

Table 1.2. Precision (repeatability and intermediate repeatability) of the proposed methods (% RSD, number of replicates: n=8).

	Repeatability – tr (min)			
	HPLC-PDA	**UHPLC-PDA**	**HPLC-Orbitrap**	**HPLC-FL**
EGC	0.197	0.174	0.000	0.213
C	0.235	0.161	0.000	0.245
EGCG	0.275	0.130	0.000	--
EC	0.201	0.104	0.0681	0.205
GCG	0.216	0.116	0.0688	--
ECG	0.171	0.0966	0.0605	0.177
CG	0.157	0.0955	0.0583	--
Quer	0.0398	0.0237	0.0782	--
	Repeatability - Area			
EGC	1.38	1.10	1.08	3.98
C	1.33	1.28	0.426	1.14
EGCG	2.33	1.78	0.890	--
EC	1.03	1.36	0.406	0.630
GCG	1.60	1.39	0.238	--
ECG	0.608	0.882	0.238	2.28
CG	0.408	0.969	0.641	--
Quer	0.873	2.20	3.33	--
	Intermediate repeatability – tr (min)			
EGC	0.377	0.412	0.191	0.376
C	0.353	0.506	0.0701	0.351
EGCG	0.320	0.721	0.220	--
EC	0.209	0.361	0.111	0.210
GCG	0.217	0.582	0.141	--
ECG	0.210	0.323	0.124	0.202
CG	0.190	0.339	2.45	--
Quer	0.0563	0.158	0.129	--
	Intermediate repeatability - Area			
EGC	1.54	2.27	2.29	4.61
C	1.12	1.43	8.56	1.37
EGCG	2.35	9.96	1.94	--
EC	2.04	3.15	3.10	1.23
GCG	3.94	8.69	1.18	--
ECG	1.15	5.50	7.14	2.99
CG	0.729	4.48	8.44	--
Quer	5.09	7.66	10.9	--

Table 1.3. Calibration ranges used for quantification and correlation coefficients for catechins and quercetin obtained by the proposed methods.

	Calibration range (mg L^{-1})			
	HPLC-PDA	**UHPLC-PDA**	**HPLC-Orbitrap**	**HPLC-FL**
EGC	0.4-10	0.5-10	0.05-5	0.1-10
C	0.4-10	0.5-10	0.05-3	0.2-10
EGCG	0.4-4	0.1-2	0.05-5	--
EC	0.4-10	0.5-10	0.05-5	0.2-10
GCG	1-10	0.1-2	0.05-2	--
ECG	1-10	0.5-5	0.05-5	0.2-10
CG	1-10	0.5-5	0.05-3	--
Quer	0.05-0.5	0.1-0.6	0.05-3	--
	Correlation coefficient (r)			
EGC	1.000	1.000	1.000	1.000
C	0.999	0.999	1.000	1.000
EGCG	1.000	0.999	1.000	--
EC	1.000	1.000	0.999	1.000
GCG	0.998	0.997	0.999	--
ECG	0.996	0.999	0.999	1.000
CG	0.996	0.999	0.999	--
Quer	0.992	0.995	1.000	--

Thus, although slight differences between HPLC and UHPLC, validation data were within acceptable range, indicating their suitability for the evaluation of complex samples with variable amounts of catechins and quercetin. When applied to natural samples, HPLC-PDA has failed to quantify some compounds such as EGC, which can be probably attributed to some matrix interferences. Nevertheless, these methods were especially useful as they allow some compounds which usually regards as being in too low concentration to detection, such as CG, to be as well quantitatively detected.

UHPLC-PDA has also allowed to detect and quantified EGCG and GCG in cocoa and GCG and CG in hop, not able to be detected with other studied methodologies.

3.3.2. FL detection. Special attention was drawn in this work on the native/self fluorescence of the studied compounds. Excitation and emission spectra scans showed natural fluorescence for C, EC, EGCG, EGC and ECG. CG, GCG and Quer did not show any fluorescence.

The excellent linearity (R≈1.000), low LODs and LOQs (between 1.7 and 4.3-fold lower than with PDA, depending on the compound considered) and slope values between 2 to 3 orders of magnitude higher (Table 1.1) also confirmed the highest sensitivity of the HPLC-FL method which allows its application to the analysis of traces. It also allowed to identify and quantify EGC and ECG in natural samples which remained as not quantified by previous methodologies reported in bibliography.

3.3.3. MS detection. HPLC-PDA was coupled with MS detection as confirmatory technique. EGC, GCG and CG, highly affected by matrix interferences could be unequivocal confirmed and quantify. Maximum structural information and peak confirmation was obtained (Figure 1.1). Good linearity (R-values > 0.999), low LODs and LOQs (1.3 to 7-fold lower than HPLC-PDA and 1 to 10-fold lower than UHPLC-PDA for catechins and almost 30-fold lower for quercetin), and high sensitivity (between 3 and 4 orders of magnitude higher than HPLC-PDA, depending on the compound

considered) (Table 1.1) were found. Therefore, HPLC-PDA-LTQ Orbitrap MS developed method shows a potential range of applications to the analysis of natural samples both to detect traces and to confirm their identity. Thus, EGCG and GCG in cocoa and GCG and CG in hop (only been detected by UHPLC) were also detected by HPLC-PDA-MS. ECG could be also detected and quantify in tutsan. Nevertheless, the higher qualification of the analyst required is shown as its great drawback.

Other compounds of interest could be also detected due to the high mass accuracy proportionate by the LTQ Orbitrap MS. Since catechins have two asymmetric carbon atoms in the C ring, four epimerization products and their methylated forms are possible. *O*-methylated derivates of EGCG and ECG: (-)-epigallocatechin-3-*O*-(3-*O*-methyl)gallate, (-)-epigallocatechin-3-*O*-(4-*O*-methyl)gallate and (-)-4′-methyl epigallocatechin-3-*O*-(4-*O*-methyl)gallate and (-)-epigallo-3-*O*-(3-*O*-methyl)catechin which exhibit important anti-allergic properties (Yoshino et al., 2004) were successfully detected in tea. Caffeine and theobromine, main methyl xanthines that constitute tea alkaloids and important factors to determine the tea quality, were also detected in tea and cocoa. Two further catechins, gallocatechin and gallic acid were also detected. Regarding gallic acid, as being a degradation product of catechin derivates, its presence in higher quantity in fermented tea than in non-fermented (up to 70%) was confirmed. Its presence in cocoa and hop was also detected.

Precision of all the developed methods in area and in retention time was also evaluated through repeatability and intermediate repeatability. The relative standard deviation levels (RSD) (Table 1.2) revealed HPLC-LTQ FT *Orbitrap* MS and UHPLC-PDA methods as providing better repeatability

(RSD lower than 1% in area and 0.06% in time). All the developed methodologies have shown similar intermediate repeatability. Costs of each technique were also evaluated aiming to routine analysis. In this sense, although UHPLC is a more expensive instrumentation than HPLC the lower time of analysis, and amount of sample injected could mean a less price per sample, which could compensate the initial cost of the instrumentation. However, in the case of HPLC-LTQ Orbitrap MS, the high initial cost of the instrumentation could not be compensated by other savings. Nevertheless, it may be extremely useful when a high level of confirmation is necessary.

3.4. Determination of catechins and quercetin: quantitative measurement of samples, sample clean-up

The developed methods when used for the analysis of white, green, red and black teas; grape residues, cocoa, tutsan, lemon verbena, barley shell, chestnut hedgehog, hop and residue of beer fabrication, allowed to detect and quantify catechins and quercetin (Figure 1.2).

Contrary to previously reported methods (Guillarme et al., 2010), our methods have shown enough resolving power to easily discriminate catechins in complex samples without complex pre-treatment and clean up, by simple aqueous extraction.

As expected, tea samples showed higher catechins and quercetin content, followed by tutsan, cocoa, hop and grape residue.

Regarding to the individual polyphenols (Figure 1.2), tea also present the highest quantitative amounts of every studied catechin being EGC, EGCG, EC

and ECG the predominant. The amount of each catechin was found to be higher in both green and white tea than in black and red. It could be attributed to the fermentation process suffered by the latter, where some catechins can undergo enzymatic oxidation to produce a range of other polyphenols (Watanabe et al., 1998).

C, EC and EGCG were the most frequently occurring catechins in the other studied samples, except in lemon verbena, barley shell, chestnut hedgehog in which none of the studied compounds were detected.

Low amounts of quercetin were quantified in the studied samples, except in tutsan were quercetin is the predominant compound.

As previously reported, other compounds of interest such as methylated catechins, caffeine or theobromine were also detected.

3.5. Antioxidant release from the films

The main mechanism of action of the active films studied is by the release of the antioxidants into the food simulant. A suitable analytical method should provide performance enough to be used to elucidate the release of those active agents and thus to help in the assessment of active food packaging functions. Catechin and green tea extract were selected as representative individual antioxidant and natural sample, respectively in the present work due to their high antioxidant potential (Gramza y Korczak, 2005).

Therefore, the release of catechins and quercetin from catechin-containing and green tea-containing films into simulant A and D_1 were evaluated at 40

ºC during 10 days by means of HPLC-PDA. Figure 1.3 shows the release of catechin from catechin-containing films and catechins and quercetin from green tea-containing films. HPLC was selected as a representative analytical methodology to follow the release of catechins and quercetin, due to its previously proven applicability to analysis of catechins and quercetin, being, at the same time, one of the most conventional analytical techniques used in laboratories.

As some epimerization of catechin in epicatechin was observed, data of catechin release from catechin-containing films was expressed as sum of catechin and epicatechin released levels.

As can be seen, the analytical method developed was suitable enough to be applied to the detection and quantification of the release of catechins and quercetin both from the two types of active films developed, although some low levels of release were observed (percentages of compound migrated vs the correspondent amount of compound extruded ranging from 0.05 and almost 50%). Release of catechins and quercetin could alse be simultaneously determined despite the difference in the amounts released (between 50 and 250-fold for catechins) which indicate the performance of the analytical method to be used for the analysis of the release of several active compounds of different chemical nature and abundance in the same run.

According to release data (Figure 1.3), percentage of active agent onto the films formulation, food simulant nature and time have noticeable influence on released levels. Thus, increasing levels of release from both catechin- and green tea-containing films were observed as did the percentage of active agent and time.

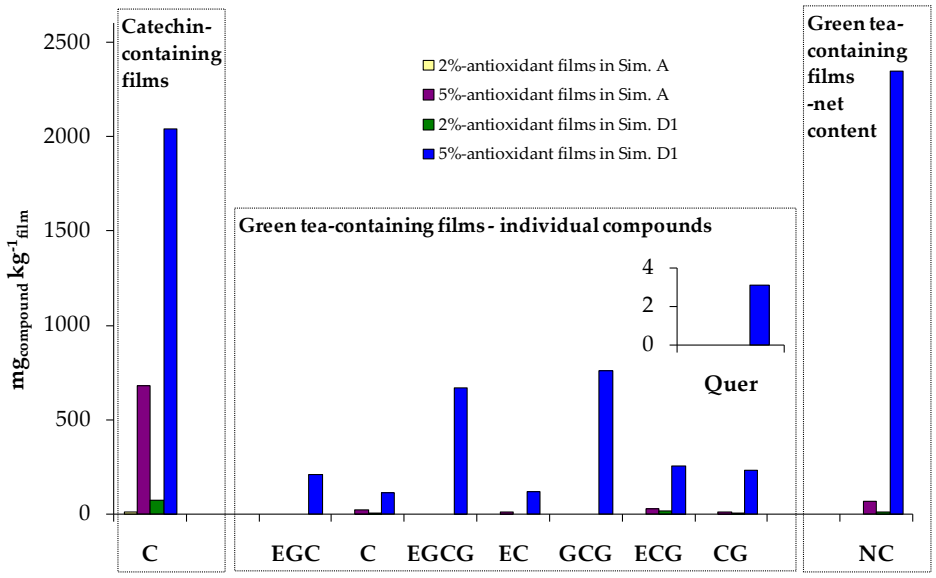

Figure 1.3. Release of catechin from catechin-containing films, expressed as sum of catechin and epicatechin; release of catechins and quercetin from green tea-containing films expressed as individual compounds and net content (NC) in catechins and quercetin. Food simulants A and D_1, 40 ºC, 10 days.

The higher solubility of the studied compounds in ethanol resulted in a higher release into simulants with high ethanolic contain (simulant D_1).

Comparing the nature of the active agent, differences into the migration profiles were observed when catechin or green tea extract are incorporated into the film formulation. Considering net content, similar amount of antioxidant were released from both catechin- and green tea-containing films into simulant D_1. However, higher differences were observed into simulant A, which could be attributed to the difference in molecular weight among catechin and the rest of catechins which also contribute to the total net content in the latter films. Release of individual catechins from green tea-containing

films showed to be correlated with their content in green tea sample, being EGCG, the major component in the green tea sample, which showed the higher levels of release.

4. CONCLUSIONS

Four chromatographic methodologies were developed, improve and compare developing well-defined and reliable systems for the efficient routine simultaneous identification, separation and quantification of major and minor catechins and quercetin in natural samples aimed to active packaging and functional foods. Good performance in terms of chromatographic efficiency, complete separation of all analytes, short analysis times (10 min for HPLC, being shorten up to 6-fold by UHPLC), low consumption of solvent and the ability to analyze complex natural samples without any pre-treatment were obtained, overcoming previously reported methods.

The use of acidic mobile phases that could damage silica-based columns was avoided without damaging the efficiency of the separation.

The four developed methods provide the selectivity and sensitivity well suited to both the rapid, routine analysis of many compounds in complex natural matrices without any sample pre-treatment, and to study the release of those compounds from active packaging formulations.

Furthermore, two catechins, EGC and ECG which regarded as non-quantified could be now quantified by the developed HPLC-FL method.

Other compounds of interest such as caffeine, theobromine or several methylated catechins could also be detected by LC-PDA-FT Orbitrap MS.

Therefore, the developed methodology could be considered as potential candidates to be applied to the analysis of both new compounds and new natural samples in active packaging and functional foods.

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Artículo 2

DEVELOPMENT, VALIDATION AND APPLICATION OF MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY METHOD FOR ROUTINE ANALYSIS OF CATECHINS, QUERCETIN AND THYMOL IN NATURAL SAMPLES.

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ABSTRACT

Natural matrices such as several vegetables, fruits or beverages are the origin of different natural antioxidants with great interest to be used instead of synthetic antioxidants in several applications, as a result of their health-promoting and disease-preventing properties. In this work, a capillary electrophoresis method capable of separating and quantifying some of these antioxidants (seven catechins with the same or similar molecular weight and relation q/z) is showed. Short analysis time and precision parameters equivalent to those obtained by liquid chromatography were obtained. This method was developed using the micellar electrokinetic chromatography (MEKC) mode with ultraviolet-visible detection. Quality parameters were established, obtaining low instrumental detection and quantification limits (0.6-2.0 mg L^{-1} and 2.0-6.5 mg L^{-1}, respectively), good precision (relative standard deviation in the intermediate repeatability lower than 7 % for every compound) and short analysis time. The developed method also showed good performance for the determination of two more natural antioxidants, quercetin and thymol. The applicability of the method to the analysis of catehins in several natural samples was tested.

Keywords: Antioxidants, Catechins, MEKC, Natural matrices, Polyphenolic compounds

1. INTRODUCTION

In recent years, trade growth of functional foods has increased the interest in compounds with health-promoting or disease-preventing properties, such as cancer-fighting properties. Due to their important antioxidant capacity neutralizing the damage caused by free radicals, the interest in polyphenolic compounds, especially those of natural origin, has been increased (Shahidi et al., 1992; El-Hady and El-Maali, 2008). Their possible use instead of synthetic antioxidants, mainly as a consequence of the toxicity of some of these ones (Rababah et al., 2004; Dopico-García et al., 2005; Ito et al., 1986; Botterweck et al., 2000), has revealed their possible application in several fields, such as in the pharmaceutical industry (Dreosti, 2000; Boudet, 2007; Zaveri, 2006), active and intelligent food packaging (Al-Malaika et al., 1999; Ozdemir and Floros, 2004; López-de-Dicastillo et al., 2010; Dopico-García et al., 2011) or as additives in functional foods (Herrero-Martínez et al., 2005).

The strong antioxidant capacity of catechins has been reported to have a protective and beneficial health effect related to antimutagenic, antidiabetic, anti-inflamatory qualities and prevention against several kinds of cancer (Fukumoto and Mazza, 2000; Cabrera et al., 2003; Hsu, 2005; Zaveri, 2006). Several biological matrices such as tea (Wörth et al., 2000), cocoa (Dreosti, 2000), fruits (berries, citrus fruits, apples, pears), vegetables (aubergine, chicory, onion, potato, parsley) (Dimitrios, 2006), alcoholic drinks (wine (de Sousas Dias et al., 2010), beer (Cortacero-Ramírez et al., 2004)), juices (Dimitrios, 2006)) and herbs and spices (Shahidi, 2000) present considerable concentration of these phenolic compounds. Among them, tea samples, especially green and white varieties, contain high concentration of major and

minor catechins. The amount present in red and black tea has been reported as being lower as a result of fermentation that occurs in their manufacture (Dreosti, 2000; Shahidi, 2000; Dimitrios, 2006). During this procedure some catechins undergo enzymic oxidation producing a range of polyphenolic compounds which include theaflavins, bisflavanols and several pigments of thearubigen class, which results in the degradation of those catechins (Larger et al., 1998; Lin et al., 1998).

Interest in exploitation of natural extracts to obtain and purify natural antioxidants depends on net and relative content of these compounds with different antioxidant activities.

This interest has led to the development of new analytical procedures able to handle even the most complex matrices in which these compounds are detected. Several techniques have been reported in the literature for the determination of catechins. High performance liquid chromatography has been the most used on the basis of its simple mode of operation, high applicability to trace analysis and good selectivity, detection and quantification limits (Dalluge et al., 1998; Aucamp et al., 2000). Gas chromatography coupled to a mass selective detector or near infrared spectroscopic has also been used. Nevertheless, each technique has often suffered from diverse disadvantages with regard to cost and selectivity, previous sample preparation procedures or long analysis time (Dalluge et al., 1998; Schulz et al., 1999; Chen et al., 2006).

The possibility of capillary electrophoresis as a tool for separation and quantification of these natural antioxidants has been evaluated according to its simplicity, high speed, low amount of sample and volume of eluent

required, low cost and short analysis time (Alnajjar et al., 2007; Zhang et al., 2008). In spite of these advantages, the main CE drawback is the need for careful operation to avoid low precision, which can cause changes in migration time, turning difficult the compounds identification (Fritz and Steiner, 2001; Mayer, 2001). Among the different electrophoretic modes, the best selective separation was provided by CZE and MEKC modes. Several bibliographic methods were focused on the determination of catechins (Komatsu et al., 1993; Horie et al., 1997; Zhu et al., 1997; Nelson et al., 1998; Watanabe et al., 1998; Barroso and van de Werken, 1999; Chen et al., 2001; Teshima et al., 2001; Bonoli et al., 2003; Huang et al., 2005; Kartsova and Ganzha, 2006). The developed methodologies do not seem to be satisfactory for routine analysis, because of the complicated buffers mixtures or high analysis time employed when a large number of catechins are analysed. They not even include all the catechins in a single run due to the difficulty of separating them (Zhang et al., 2008).

Therefore, the aim of this work is to develop a fast and easy CE method for simultaneous determination of seven catechins in routine analysis. An improvement in the migration time by optimization of buffer characteristics (type, concentration and pH) is sought. Due to the requirement of better precision to achieve optimum routine determinations of catechins, several guidelines are also developed. The performance of the developed method will be evaluated studying quality parameters such as linearity range, correlation coefficients, standard deviations of intercepts and slopes, detection and quantification limits. The applicability of the developed method to determine other natural antioxidants such as quercetin or thymol, which has not been

previously determined by CE, is tested. The applicability of the method to the analysis of catehins in several natural samples is also carried out.

2. MATERIALS AND METHODS

2.1. Reagents and solvents

(-)-Epigallocatechin gallate (EGCG), (-)-Epigallocatechin (EGC), (-)-Epicatechin gallate (ECG), (-)-Epicatechin (EC), (+)-Catechin hydrate (C), (-)-Gallocatechin Gallate (GCG), (-)-Catechin Gallate (CG), Quercetin (Quer) and Thymol (Thy) were obtained from Sigma-Aldrich (Steinheim, Germany). Their structures and some properties are compiled in Table 2.1. Borax buffer and sodium hydroxide (NaOH) solution were supplied by Agilent Technologies (Waldbronn, Germany). Sodium dodecyl sulphate (SDS) and formic acid were supplied from Sigma-Aldrich (Steinheim, Germany). Ethanol, potasium phosphate and hydrochloric acid were supplied from Merck (Darmstadt, Germany), Fluka, (Steinheim, Germany) and Panreac (Barcelona, Spain) respectively. Water was purified using a Milli-Q Ultrapure water-purification system (Millipore, Bedford, MA, USA).

The running buffer solution was prepared by mixing 3 mL of 50 mM borax solution, 1.75 mL of 400 mM SDS solution and 0.17 mL of 300 mM potasium phosphate solution, diluting to 10 mL and adjusting the pH value to 8.0 with a 1 M hydrochloric acid solution. Before use, the running buffer was filtered through an AcrodiscR PTFE CR 13 mm, 0.2 μm filter (Waters, Mildford, MA, USA), degassed in an ultrasonic bath for about 2 min and transferred into EC vials.

Table 2.1. Chemical information about studied antioxidants.

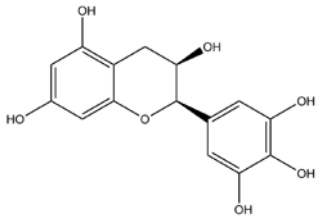
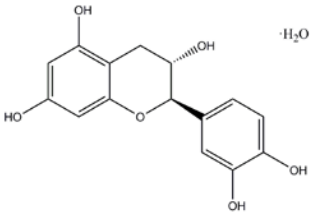
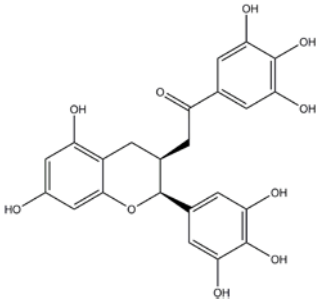
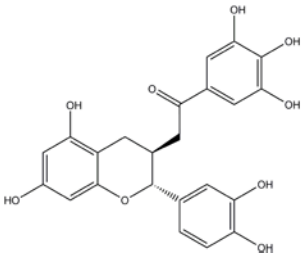
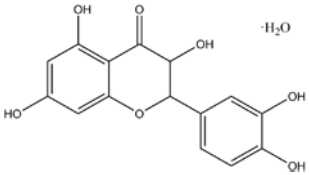
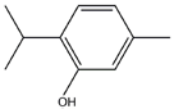
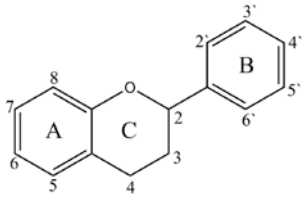
Components	Purity (%)	CAS no.	Formula	Structure	M.W. (u)	λ_{max} (nm)	pKa
(-)-Epigallocatechin (EGC)	98	970-74-1	$C_{15}H_{14}O_7$		306.3	206	8.99
(+)-Catechin hydrate (C)	98	154-23-4	$C_{15}H_{14}O_6 . xH_2O$		290.3	204	9.50
(-)-Epigallocatechin gallate (EGCG)	80	989-51-5	$C_{22}H_{18}O_{11}$		458.4	206	8.38

Table 2.1. (Continuation I).

Components	Purity (%)	CAS no.	Formula	Structure	M.W. (u)	λ_{max} (nm)	pKa
(-)-Epicatechin (EC)	90	490-46-0	$C_{15}H_{14}O_6$		290.3	204	9.50
(-)-Gallocatechin gallate (GCG)	98	4233-96-9	$C_{22}H_{18}O_{11}$		458.4	206	8
(-)-Epicatechin gallate (ECG)	98	1257-08-5	$C_{22}H_{18}O_{10}$		442.4	204	8.39

Table 2.1. (Continuation II).

Components	Purity (%)	CAS no.	Formula	Structure	M.W. (u)	λ_{max} (nm)	pKa
(-)-Catechin gallate (CG)	98	130405-40-2	$C_{22}H_{18}O_{10}$		442.4	206	8
(-)-Quercetin hydrate (Quer)	95	849061-97-8	$C_{15}H_{10}O_7 . xH_2O$		302.2	203	9.2
Thymol crystalline (Thy)	99.5	89-83-8	$C_{10}H_{14}O$		150.2	204	10.6
[a]Experimental value [b] (Barroso and van de Werken, 1999; Ozdemir and Floros, 2004)		General flavan-3-ols structure					

Individual stock standard solutions (1000 mg L^{-1}) were prepared in an aqueous solution of formic acid at pH 2 for each catechin. Work standard solution (25 mg L^{-1}) containing all catechins was prepared from individual stock standard solutions (1000 mg L^{-1}) by dilution with aqueous solution of formic acid at pH 2.

2.2. Sample preparation

Several natural matrices were selected for application purpose: four tea samples (red and white marketed in infusion bags, and green and black marketed in bulk), grape residues, cocoa, *Hypericum androsaemum*, *Lippia citriodora*, barley shell, chestnut hedgehog, hop and a residue of beer production. Samples were extracted in Milli-Q water (1 g of sample: 25 mL of water) under magnetic stirring for 10 minutes. Formic acid was added (17 μL of formic acid: 1 mL of extracted sample) to each extract. The final samples were filtered through an AcrodiscR PTFE CR 13 mm, 0.2 μm filter.

2.3. Capillary electrophoresis

MEKC was carried out using the G1600A capillary electrophoresis system from Agilent Technologies equipped with a UV-Vis detector. Data acquisition and processing were accomplished by a PC equipped with a software HPCE version G1601A from Agilent Technologies. Uncoated fused silica capillary with extended light path, 50 μm of i.d., 64.5 cm of total length and 56 cm of effective length was used for the separation. The capillary was thermostated at 25 ºC, the applied voltage was kept at 30 kV and normal polarity was used.

Hydrodynamic injection (50 mbar, 4 s) was carried out. In order to obtain good precision, the capillary was conditioned at different stages (Table 2.2). Running buffer characteristics (type, concentration and pH value) were investigated. Detection was performed at 210 nm.

Table 2.2. Steps of conditioning procedure.

Conditioning	Solvent	Time (min)*
New capillary	1. H_2O	10
	2. NaOH 1 M	20
	3. H_2O	5
	4. NaOH 0.1 M	10
	5. Buffer	10
Every day before sample analysis	1. NaOH 0.1 M	10
	2. Buffer	10
Between injections	1. H_2O	3
	2. Buffer	3
Final washing, after all sample analysis	1. H_2O	30
	2. Air	30

** Wait of 0.50 min between every step.*

3. RESULTS AND DISCUSSION

3.1. Method development

First experiments for separation of the studied catechins were carried out employing capillary zone electrophoresis. Since catechins are weak bases (pKa ranged from 8 to 10), two weak basic alkaline buffers were tested: acetate and borax buffer. Buffer concentrations ranging from 20 to 50 mM and pH value within 7.5 and 8.5 were studied. All separations were performed at 25 ºC by application of 30 kV across the separation capillary. Positive polarity was used due to the ionization state of analytes according with their pKa

values (Table 2.1) and their preparation at pH<4, induced by their reported poor stability in alkaline solutions (Komatsu et al., 1993; Zhu et al., 1997; Chen et al., 2001; Su et al., 2003).

Even though the injection of individual compounds has shown the ability of the CZE method for the detection of each compound individually, no separation could be obtained for all the studied catechins in a single run. Even in the presence of borax 20 mM at pH 8.5, which has provided the best result, the compounds with a gallate group could not be resolved (Figure 2.1). The change in the type of buffer has not led to the achievement of better separation. Raising the borax concentration led to improve peak resolution; nevertheless, longer migration times of the compounds were observed, which could be related to the decrease in the electroosmotic flow. It might be a result of the polyhydroxylated nature of the compounds, being thus able to undergo complexation with tetraborate resulting in a complex with negative charge and higher molecular size (Larger et al., 1998).

Due to the not possible separation in CZE by simple variation of the influencing factors, MEKC was then considered. The high efficiency of CE is thus combined with a chromatographic separation mechanism provided by the introduction of surfactant in the buffer composition, acting as a pseudo-stationary phase.

Although previous papers reporting the applicability of MEKC for catechins analysis (Larger et al., 1998; Nelson et al., 1998; Watanabe et al., 1998; Barroso and van de Werken, 1999; Aucamp et al., 2000; Wörth et al., 2000; Bonoli et al., 2003; Kartsova and Ganzha, 2006) have been published, the long analysis time and the poor resolution achieved, make this methodology

unsuitable for routine analysis (Pomponio et al., 2003). Most of these problems were solved in this work after studying the different key parameters.

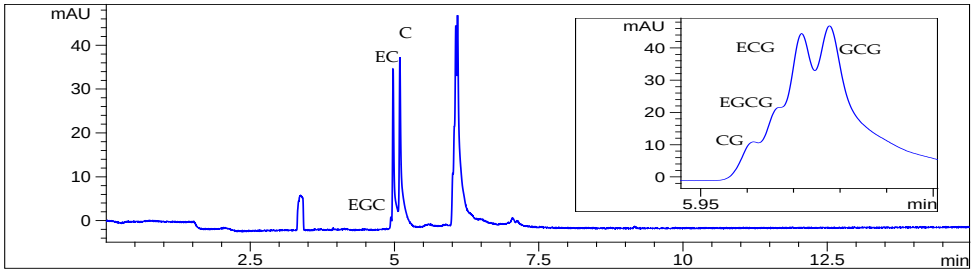


Figure 2.1. Electropherogram of a work standard solution of seven catechins (25 mg L^{-1}) by CZE with borax buffer at pH 8.5, 30 kV, 25 ºC, UV detection (210 nm) and uncoated fused-silica capillary, 50 μm i.d., 64.5 of total length and 56 cm of effective length.

3.1.1. Running buffer: type and concentration

The most usual MEKC buffer components used are borax and potassium phosphate containing surfactants above their critical micellar concentration (CMC). In the present work, sodium dodecyl sulphate (SDS) was selected as the surfactant to generate the hydrophobic micellar phase. Since catechins are charged under the studied conditions, the separation is achieved on the basis of their own electrophoretic mobility and their interaction with the micelles.

The performance achieved with two types of buffer was tested: borax-SDS and borax-SDS-phosphate buffer. After several trials modifying buffer concentration and pH, the less promising results were obtained for the borax-SDS buffer, being, therefore, refused.

To improve the separation, the effect of borate-SDS-potassium phosphate buffer concentration was investigated.

Since catechins can undergo complexation with borate the increased size of the borax-polyhidroxylated complexes has resulted in a bigger non polar zone facilitating their partitioning into the micellar phase. Nevertheless, as a result of the electrostatic repulsion between negative complexes and the micellar phase, this effect has been opposed to some degree (Larger et al., 1998; Bonoli et al., 2003). Thus, raising the borax buffer concentration has resulted in longer migration times with improvement of peak resolution. The use of high levels of SDS has contributed to increase the probability of interactions between the analyte and the micelles. The different interaction analyte-micelle was showed by the resolution enhancement of close migrating solutes. As a result of the insignificant binding capacity of SDS micelles with the wall of an uncoated capillary (Huang et al., 2005), the mobility of the electroosmotic flow was almost not altered by the change in the SDS concentration. A decrease in phosphate concentration led to improve peak resolution of the first compounds increasing their migration time. However, peak shape was worsened and the overlapping of the last compounds peaks was produced, taking place impossible baseline separation of the peaks. Concentrations of 15 mM for borax, 70 mM for SDS and 5 mM for potassium phosphate were selected as optimal since these values maintain good peak shape, suitable baseline separation of all the compounds with the shortest analysis time and the best peak resolutions for all the studied antioxidants.

3.1.2. Buffer pH

The effect of the buffer pH in the separation within 7 and 9.3 was tested. Hydrochloric acid (1 M) was used as acidified agent. Even though pH 7

provided the best separation, not appropriate intra-assay precision was observed. It can be associated with the use of a silica capilar, in which high electroosmotic flow mobility changes are produced with small variations of pH close to 7 (Heiger, 2000). High pH running buffer provided a decrease in the migration time of the compounds, resulting in the overlapping of the peaks without baseline resolution. Therefore, pH 8 was chosen as the pH that provides a suitable compromise between short analysis time and resolution.

3.1.3. Sample pH

Standards and samples were prepared under acidified conditions due to the instability of catechins associated to isomerisation and epimerization reactions at pH higher than 4 (Su et al., 2003; Zhu et al., 1997; Chen et al., 2001; Komatsu et al., 1993). Raising sample pH from 1.8 to 2.5, decreases in migration time and resolution were observed. The best compromise in reasonable migration time, without loss of resolution was achieved employing pH 2.

3.1.4. Voltage and temperature

The effect of voltage and temperature on the separation of analytes, sensitivity and analysis time was investigated within 10 and 30 kV and 20 and 30 ºC, respectively. Migration times of the analytes were significantly shortened (from 50 to 15 minutes) as the separation voltage was increased. Temperature changes have not shown significant effects. Thus, the best resolution with the shortest analysis time was achieved at 30 kV and 25 ºC.

The electropherograms obtained by the developed MEKC method are shown in Figure 2.2.

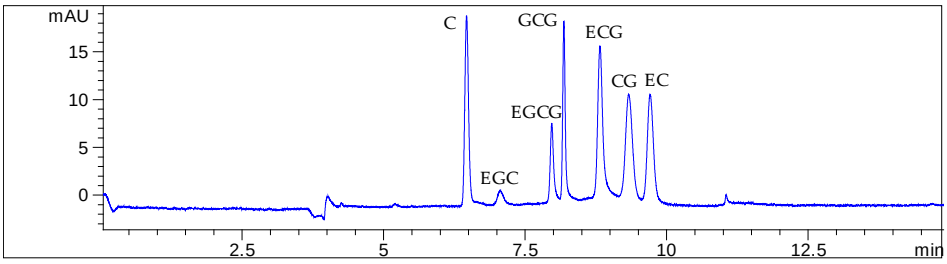


Figure 2.2. Electropherogram of a work standard solution of seven catechins (25 mg L^{-1}) by MEKC with 15:70:5 mM borax-SDS-phosphate buffer at pH 8, 30 kV, 25 ºC, UV detection (210 nm) and uncoated fused-silica capillary, 50 µm i.d., 64.5 cm of total length and 56 cm of effective length.

All the catechins were successfully separated in a short interval of time (3.5 min between the first and the last detected compound). Even though the separation of catechins under the studied conditions can be mainly associated with their own electrophoretic mobility and their interaction with the micelles, the migration order achieved, also reflects some influence from the degree substitution of every compound on the migration time. According to Pomponio et al (Pomponio et al., 2003), the hydroxylation of the C2 atom leads to the increase of migration time (faster migration for catechin than epigallocatechin). The substitution of the C3 atom with a galloyl ester (gallato) also leads to an increase in the migration time, being associated with a higher affinity of these compounds to the SDS micelles (epigallocatechin faster than gallato catechins).

3.2. Validation studies

The performance of the MEKC method was evaluated studying quality parameters such as linearity range, correlation coefficients, standard deviations of intercepts and slopes, detection and quantification limits and precision. The applicability of the developed method to the analysis of other natural antioxidants was also tested. The analytical characteristics studied are compiled in Tables 2.3 and 2.4.

The linearity of the MEKC method was tested plotting the calibration curve for each compound with concentration levels ranging from 5/10 to 50 mg L^{-1}. Each point of the calibration graph corresponds to the mean value obtained from three independent area measurements. Satisfactory results in terms of linearity were obtained for all the compounds according to the linear correlation coefficients for the calibration curves of every antioxidant ($r > 0.999$).

Detection (LODs) and quantification (LOQ) limits were calculated according to Shabir, 2003, using the calibration graphs, being LOD=yB+3xSB and LOQ=yB+10xSB. Representing yB (blank signal) = a (intercept of the calibration graph) and SB (standard deviation of the blank) = Sy/x. The LODs and LOQs obtained (Table 2.3) support the suitability of the proposed MEKC method for its application to real samples in a routine analysis.

The sensitivity of the developed method for every compound was tested through the comparison of the slope values (Table 2.3). The lowest sensitivity was shown by EGC due to the lowest slope value (one order of magnitude lower than the one achieved for other antioxidants).

Table 2.3. Analytical quality parameters of the proposed MEKC method.

	Calibration range/mg L^{-1}	Correlation coefficient (r)	Intercept ± SD	Slope ± SD	LOD (mg L^{-1})	LOQ (mg L^{-1})
C	5 - 50	0.9998	-7.88 ±8.27E-01	4.62 ±3.10E-02	0.908	3.03
EGC	10 - 50	0.9997	-5.80 ±1.70E-01	9.69E-01 ±8.00E-03	0.618	2.06
EGCG	10 - 50	0.9989	-11.8 ±8.24E-01	2.25 ±3.40E-02	1.68	5.61
GCG	10 - 50	0.9993	-19.8±1.23	3.71 ±4.60E-02	1.23	4.10
ECG	10 - 50	0.9985	-33.7±2.69	5.71 ±1.04E-01	1.93	6.43
CG	10 - 50	0.9998	-24.9± 8.96E-01	5.52 ±3.60E-02	0.747	2.49
EC	5 - 50	0.9993	-13.6±1.39	4.76 ±5.50E-02	1.44	4.81

Table 2.4. Precision parameters of the proposed MEKC method.

	Area		Migration time (t_R)	
	Repeatability RSD (%)	Intermediate repeatability RSD (%)	Repeatability RSD (%)	Intermediate repeatability RSD (%)
C	2.33	3.22	0.603	2.43
EGC	1.09	2.16	0.593	4.59
EGCG	2.97	6.69	0.792	2.29
GCG	3.03	5.02	0.817	3.13
CG	0.889	1.72	0.843	4.38
ECG	1.08	3.46	0.820	4.96
EC	1.27	3.79	0.846	4.09

To achieve good precision parameters, several guidelines are proposed to avoid the variation of electroosmotic flow mobility and the necessity of using an internal standard. Therefore, to prevent the poor precision usually attributed to CE methodologies, especially when uncoated capillaries are

used (Wörth et al., 2000; Mayer, 2001), is needed: (i) a thorough preparation of the buffer with an comprehensive control of the concentrations and the pH value; (ii) removal of vials every day; (iii) capillary conditionings and separations methods carried out with different vials; (iv) exchange of the buffer vials (inlet and outlet) positions in consecutives injections. These premises were carried out to avoid a siphoning effect and, so that, a change in electroosmotic flow mobility related to the different vials levels. With these premises repeatability and intermediate repeatability, based on area and migration time were calculated carrying out eight replicate injections of the work standard solutions in one and five consecutive days, respectively. In Table 2.4 are shown relative standard deviations less than 3 % and 0.9 % for repeatability and 7 % and 5% for intermediate repeatability, in terms of area and migration time, respectively.

As the antioxidants thymol and quercetin are simultaneously present with catechins in a great number of natural matrices, the developed method was also tested to the analysis of these compounds. Work standard solutions were prepared in the same way as for catechins except for quercetin whose insolubility in aqueous solution makes necessary its preparation in ethanol. The achieved electropherograms (Figure 2.3) show the applicability of the MEKC method, being only necessary to increase the analysis time for the thymol resolution.

3.3. Application to natural samples

In order to assess the feasibility of the developed MEKC-UV/Vis method, it was applied to assaying catechins in commercial samples of red, white,

green and black tea (where a high amount of the studied compounds was expected) and grape residues, cocoa, *Hypericum androsaemum*, *Lippia citriodora*, barley shell, chestnut hedgehog, hop and residue of beer fabrication (where the possible presence of the studied compounds was studied).

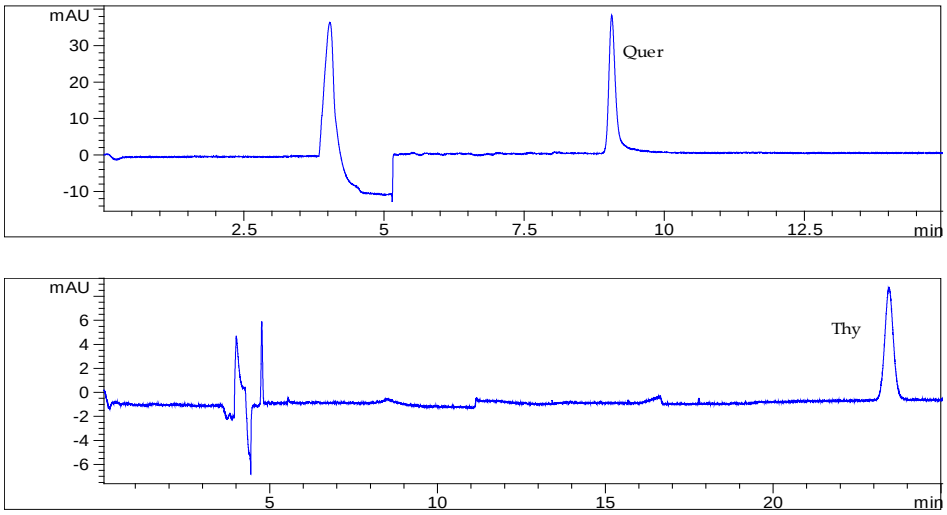


Figure 2.3. Electropherograms of work standard solutions of quercetin (60 mg L^{-1}) and thymol (25mg L^{-1}) by the developed MEKC-UV/Vis method at 210 nm.

Since the most common way of consumption of these natural samples is in aqueous media, extraction was carried out using simply water. The extracted samples were acidified, filtrated and directly injected under the developed MEKC-UV/Vis method.

Recovery test was performed to evaluate the accuracy of the developed analytical method. Tea samples were selected as representatives for this purpose. Samples were spiked with standard solution of the studied catechins at three levels of concentration, ranging from 10 to 50 mg L^{-1} and injected by

triplicate. The results have shown recovery values ranging from 76 to 120% for the tea matrices. It was indicated from the recovery test that the developed method was accurate and reliable in the simultaneous determination of the catechins present in real samples.

Aiming to validate the procedure, two methods of quantitative evaluation of the analysis were compared by statistical treatment and direct comparison: external calibration and standard additions. External calibration curves were plotted as described above. Standard addition curves were plotted preparing duplicate base ´zero` samples and standard addition samples of 10, 30, 50, 70 and 100 mg L^{-1}, being taken through the same analysis procedure described above. Statistical treatment applying the t-test for the slopes of the calibration curves has shown matrix effect in the case of EGC, EGCG and GCG in tea samples. The use of the method of standard additions to the quantification of these compounds in the studied samples was thus preferred. For the rest of the compounds, external calibration method was found to be suitable since it yields entirely reliable results, showing no significant statistical differences between both methods. Figure 2.4 shows the graphical comparison between the method of standard addition and external calibration for EC (as representative of the non-matrix effect) and EGCG (as representative of matrix effect) in white tea samples. Similar results were achieved for the rest of the studied matrices.

The antioxidants in these samples were identified by comparison of migration times and UV spectra with standards. The results showed that the studied compounds were detected in tea samples, cocoa and hop (electropherograms in Figure 2.5). Worst baseline definition was observed for

black and red tea, being attributed to the more complex matrix present in fermented varieties.

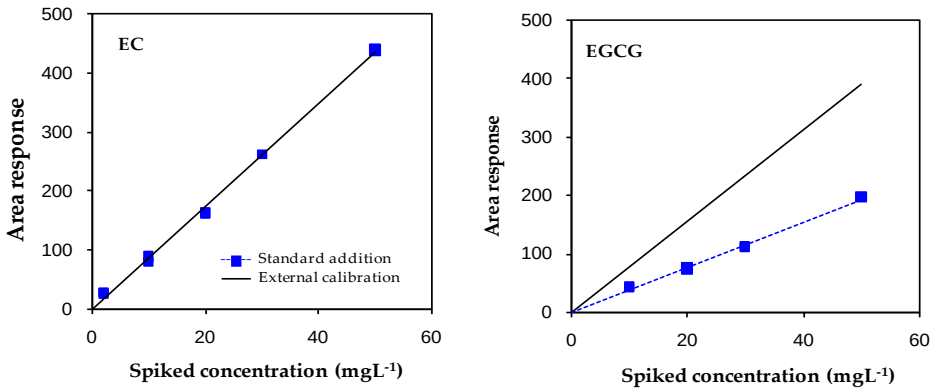


Figure 2.4. Graphical comparison between the curves obtained by the method of standard addition and external calibration for EGCG and EC as representative of the absence and the presence of matrix effect, respectively in green tea samples.

Quantitative results (Table 2.5) showed higher content both in terms of number and amount of catechins, in tea samples. All the studied catechins were identified in green and white tea, being EGCG, EGC, ECG, C, EC the most abundant. Lower catechin content was observed in black and red tea.

According to Gramza and Korczak, 2005, some investigations on the tea flavanols efficiency as antioxidants has showed that green tea components possess about 40% higher antioxidant potential than black tea. It can be related with their total and net catechin contain. According to the results showed in Table 2.5, green tea contain both higher total catechin amount and higher net content of EGCG, EGC and ECG, which are related with its higher efficiency as antioxidant. Black, white and red tea has showed fewer values.

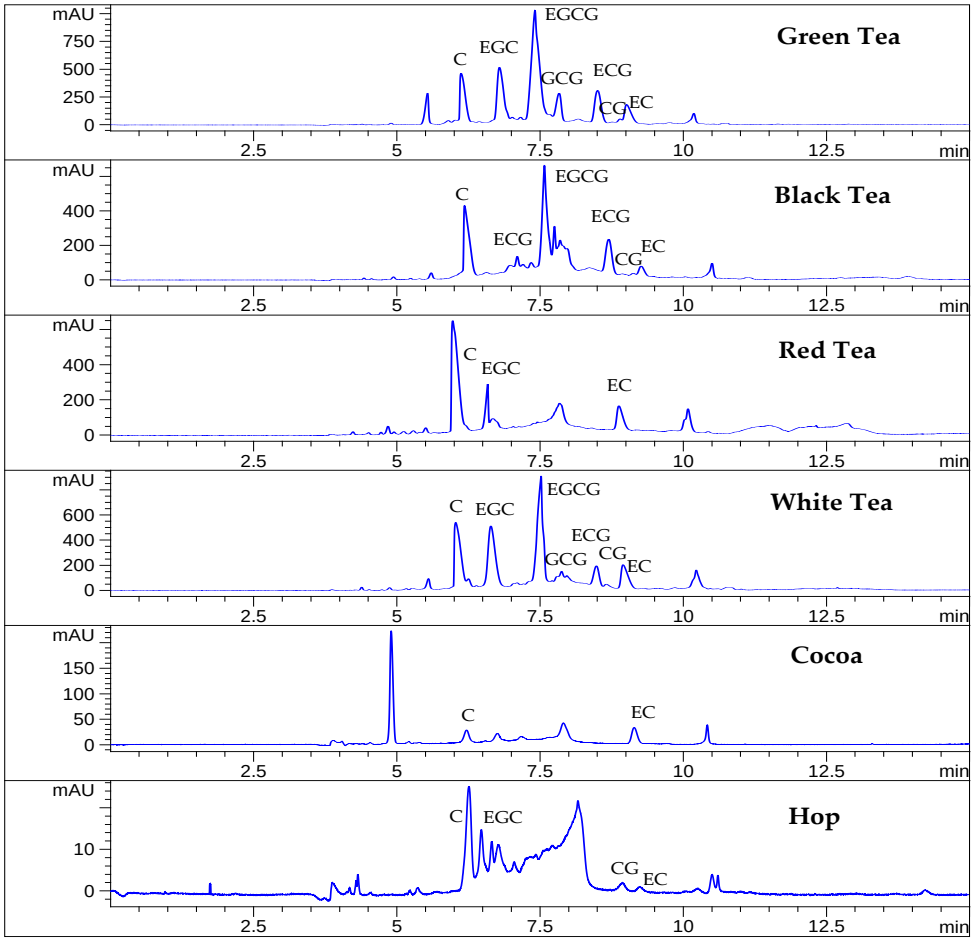


Figure 2.5. Obtained electropherograms at 210 nm of analyzed samples by the developed MEKC-UV/Vis method.

Thus, the developed method seems to be adequate to the determination of the studied compounds in these samples, particularly in tea samples, even considering their complex chemical composition, which reveals the performance of the developed method for the analysis of catechins in complex real samples.

Table 2.5. Quantification of natural samples employing the developed MEKC method.

	Concentration $mg_{component}\ g^{-1}_{sample}$							
	C	**EGC**	**EGCG**	**GCG**	**ECG**	**CG**	**EC**	**Total antioxidant content**
Red tea	0.577	6.02	ND	ND	ND	ND	1.45	8.05
White tea	0.380	7.95	11.1	0.240	3.19	0.170	2.16	25.2
Green tea	1.90	36.2	20.7	9.65	10.5	0.680	7.98	87.6
Black tea	0.311	ND	35.2	ND	5.55	0.34	1.93	43.3
Cocoa	0.272	ND	ND	ND	ND	ND	0.384	0.656
Hop	0.110	0.0812	ND	ND	ND	0.0202	0.0103	0.222

ND not detected

4. CONCLUDING REMARKS

The present work has proposed a rapid and efficient method for the simultaneous determination and quantification of seven catechins and quercetin by micellar electrokinetic capillary chromatography. CZE performance was also studied. In the present work besides being developed a rapid method (within 10 minutes per run) a simple buffer has been used. The developed method could greatly contribute to the analysis of these compounds in natural matrices such as tea, hop or cocoa, proving to be adequate for catechins and quercetin quantification without being necessary any especial sample clean-up step. Moreover, the developed method could also be used to the determination of thymol, which, as far as our knowledge, has never been determined by MEKC. Several separation parameters were also tested to enhance the method precision and the qualitative analysis of compounds. Despite the difficulties to obtain a good precision in capillary electrophoresis, with both the guidelines and the easy and quickly

methodology proposed, good repeatability and intermediate repeatability were achieved. Adequate correlation coefficients, detection and quantification limits and recovery values were also achieved with the proposed method, demonstrating to be simple and fast while reliable and sensitive. The low sample and solvent consumption as well as the low residues that are produced, also raise the capillary electrophoresis methodology as a good alternative to the traditional liquid chromatographic techniques.

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3.2. PURIFICACIÓN DE EXTRACTOS NATURALES: POLÍMEROS DE IMPRESIÓN MOLECULAR

Artículo 3: Preparation, evaluation and characterization of quercetin-molecularly imprinted polymer for preconcentration and clean-up of catechins.

3.2. PURIFICACIÓN DE EXTRACTOS NATURALES: POLÍMEROS DE IMPRESIÓN MOLECULAR

Artículo 3

PREPARATION, EVALUATION AND CHARACTERIZATION OF QUERCETIN-MOLECULARLY IMPRINTED POLYMER FOR PRECONCENTRATION AND CLEAN-UP OF CATECHINS

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ABSTRACT

Molecularly imprinted polymer (MIPs) for solid extraction and preconcentration of catechins have been successfully prepared by a thermal polymerization method using quercetin as template, 4-vinylpyridine as functional monomer and ethylene glycol dimethacrylate as crosslinker. A solution mixture of acetone and acetonitrile was used as porogen. Systematic investigations of the influence of monomer, cross-linker, porogen, as well as polymerization conditions on the properties of the MIPs were carried out. The quercetin MIPs were evaluated according to their selective recognition properties for quercetin, structurally related compounds (catechin, epigallocatechin gallate and epicatechin) and a unrelated compound of similar molecular size (alpha-tocopherol). Good binding was observed for quercetin, catechin and epigallocatechin gallate with an optimized MIP in a solid phase extraction system. Adsorption and kinetic characteristics were evaluated for catechins which indicated that the synthesized polymer had high adsorption capacity and contained homogeneous binding sites. Chemical and morphological characterization of the MIP was investigated by FTIR, SEM and BET, which confirmed a high degree of polymerization. Finally, the MIP was successfully applied to the clean-up and preconcentration of catechins from several natural samples.

Keywords: molecularly imprinted polymer, quercetin, catechins, isotherm, kinetics, physical characterization, natural samples.

1. INTRODUCTION

Over the last years, molecularly imprinted polymers (MIPs) have been used as chromatographic media, sensors, artificial antibodies, catalysts and specially in solid phase extraction (SPE) due to their high affinity and selectivity towards a specified analyte (Tamayo et al., 2007; Haginaka, 2008). Low cost, storage stability, high mechanical strength, robustness, resistance to a wide range of pH, solvents and temperatures are shown as their main advantages. Therefore, their use for the treatment of complex matrices, environmental samples or food samples has rapidly become widespread (Qiao et al., 2006; Tamayo et al., 2007; Dopico-García et al., 2011).

MIPs are synthetic receptors with several generated recognition sites which are able to specifically rebind a particular target molecule (template). These recognition sites are created in situ by a copolymerization process among functional monomer, an excess of crosslinker and template molecule. Prior to polymerization, self-assembly between the template and the functional groups of the appropriate functional monomer takes place in solution. Depending on the type of interactions involved both during pre-polymerization and template-functional monomers rebind processes, molecular imprinting can be classified as covalent, non-covalent or semi-covalent, involving a pre-organized or a self-assembly approach, respectively (García-Calzón and Díaz-García, 2007; Tamayo et al., 2007).

After polymerization, template molecules are removed from the polymer network, leaving complementary sites in terms of size, shape and functionality. Therefore, the final network should exhibit significantly higher affinity and selectivity for the template than for closely related structures.

Although bulk represents the conventional and the most popular approach to synthesize the MIP, crushing, grinding, sieving and time-consuming as well as the irregular size and shape obtained, have led to develop alternative methods. Multi-step swelling methods, suspension polymerization or precipitation polymerization have been recently developed as alternatives (Qiao et al., 2006).

Flavonoids, especially the group of catechins, have been reported as responsible for several beneficial health effects due to their strong antioxidant capacity, such as antimutagenic, antidiabetic, anti-inflamatory qualities, and prevention against several kinds of cancer (Fukumoto and Mazza, 2000; Cabrera et al., 2003). Catechins are present in several species of the plant kingdom such as tea, cocoa, fruits and vegetables (Dimitrios, 2006). Although their pharmaceutical properties have been widely studied, new efforts concerning purity and selectivity are still under progress. Although catechins are widely used natural antioxidants, the concentration of these antioxidants usually found in natural matrices has been reported as high only in tea samples. Therefore, and because of the complexity of natural matrices, using catechins from these samples needs techniques for sample enrichment and purification.

Owing to its previously reported high selectivity, affinity and simplicity, SPE involving a molecular imprinted polymer (MISPE) has been applied as a selective sorbent material for the clean up and preconcentration of several target compounds from biological and environmental samples (Xie et al., 2001).

In the present work, a selective MIP intended to clean up and pre-concentration of several catechins from natural samples was prepared. MIP and the corresponding NIP were prepared by precipitation polymerization technique. To avoid inherent bleeding (main drawback of MISPE in trace analysis (Sellergren, 2001; Tamayo et al., 2007)), quercetin was used as template. Different polymerization conditions, porogens, monomers and Template: Monomer: Crosslinker (T:M:Cr) ratios were tested. Specificity of the MIP binding sites and its selectivity towards different antioxidants (quercetin, catechin, epicatechin, epigallocatechin and α-tocopherol) were also tested. MIP was physical characterized by scanning electron microscope (SEM) and Brunauer–Emmett–Teller (BET) method.

The equilibrium and kinetic data of the adsorption process were then analyzed to study the adsorption isotherms, kinetics and adsorption mechanism of catechin, epicatechin and epigallocatechin gallate onto the MIP. Intended to MISPE, breakthrough volumes were calculated. The performance of the developed MISPE for the clean up and preconcentration of catechins from several tea samples, grape residues and cocoa was also tested.

2. EXPERIMENTAL

2.1. Chemicals and standards

Ethylene glycol dimethacrylate (EGDMA, ≥97%), methacrylic acid (MAA, ≥98%) and 2,2`-Azobis(2-methylpropionitrile) (AIBN, ≥98%) were supplied by Fluka (Sigma-Aldrich, Steinheim, Germany). 1,1,1-Tris(hydroxymethyl)-propantrimethacrylat tech. (TRIM) and 4-vinylpyridine (4-Vpy, 95%) were

supplied by Sigma-Aldrich. Standards of (+)-Catechin hydrate (C, >98%), (-)-Epicatechin (EC, >90%), (-)-Epigallocatechin gallate (EGCG, >80%), (-)-Quercetin dihydrate (Quer, >95%), alpha-tocopherol (α-TOCO, >95%) were obtained from Sigma-Aldrich. Their chemical structures are shown in Figure 3.1.

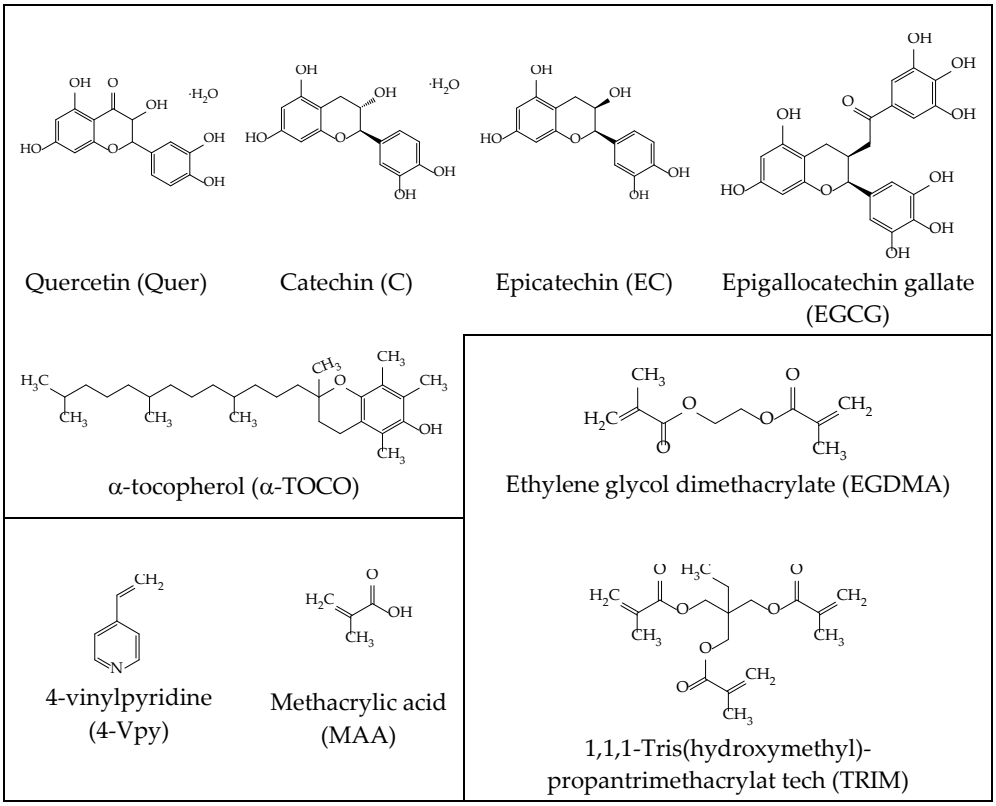

Figure 3.1. Chemical structure of the used compounds.

Methanol, acetonitrile, n-hexane, dichloromethane and tetrahydrofuran HPLC grade were supplied by Merck (Darmstadt, Germany). Acetone puriss

p.a. and formic acid 98-100 % puriss p.a. were from Sigma-Aldrich. Water was purified using a Milli-Q Ultrapure water-purification system (Millipore, Bedford, MA, USA).

Individual stock standard solutions (1000 mg L^{-1}) were prepared in an aqueous solution of formic acid at pH 1.5 for each catechin. Individual stock standard solution of quercetin was prepared in ethanol and mixtures of acetone:acetonitrile. Work standard solution containing all the studied compounds was prepared from individual stock standard solutions (1000 mg L^{-1}) by dilution with aqueous solution of formic acid at pH 1.5. Work standard solutions of quercetin were also prepared in different mixtures of acetone:acetonitrile.

2.2. HPLC-PDA-FL analysis

HPLC analysis were performed using a Waters 2695 (Waters, Mildford, MA, USA) system with gradient pump and automatic injector. Chromatographic experiments were carried out using a stainless steel column packed with SunFire™ C18 (150 x 3.0 mm, 3.5 μm) (Waters) kept at 35 ºC. Detection was carried out using a photodiode array detector (PDA, model 996 UV) set in the range of 200 to 400 nm, and a fluorescence detector (FL, model 2475) (Waters) with $\lambda_{excitation}$ 280 nm and $\lambda_{emission}$ 310 nm. Output signals were monitored and integrated using a personal computer operated under the Empower™ software (Waters). Wavelengths of 277 nm and 380 nm for catechins and quercetin analysis, respectively, were selected as output PDA signals.

A two solvent gradient elution was performed, with flow rate of 0.5 mL min^{-1} and injection volume of 20 μL. Mobile phase was composed by water (A) and methanol (B). Two gradient elution profiles were used. For samples prepared in acidified water: mobile phase composition started at 25% of B, was linearly increased to 40% B in 5 min, followed by a linear increase to 60% B in 1 min and then brought back to the initial conditions. For samples prepared in acetone:acetonitrile mixtures: mobile phase composition started at 55% B, followed by linear increase to 100% B in 0.5 min, being held for an additional 5 min and then brought back to initial conditions in 1 min.

2.3. Preparation of the MIP

MIPs were prepared by non-covalent approach using quercetin as template. Quercetin contains hydroxyl groups which enable the creation of hydrogen-bonding with the functional monomers (Figure 3.1). Several functional monomers, crosslinkers, Template:Monomer:Crosslinker ratios (T:M:Cr), solvents (porogens) and initiation methods were screened to obtain the best MIP for the desired application (Table 3.1).

Briefly, MIPs were prepared according to the following method: The template molecule was placed in a 60 mL glass ampoule with a portion of porogen previously purged with nitrogen for at least 15 min. After template dissolution, functional monomers, crosslinker, azo initiator (AIBN) and remaining porogen (until a total of 40 mL) were added. Mixture was shacked for homogeneity in a sonicating bath (10 min), degassed under nitrogen atmosphere (5 min) and sealed under vacuum. Polymerization was carried out by means of temperature (water bath at 60 ºC). UV source at room

temperature and 4 ºC were also tested as polymerization media. 20 to 72 hours were tested as polymerization trial periods. After polymerization, MIPs were dried and weighted. Precipitates with relatively uniform particle size (determined by scanning electron microscopy (SEM)) were obtained which avoided crushing, grinding and sieving steps.

Table 3.1. Composition of MIPs and NIPs using quercetin as template and AIBN as initiator. Initial Imprinting value, performance of polymers and Imprinting values.

Polymers	T:M:Cr molar ratio	Monomer	Crosslinker	Porogen	Polymer yield/ treatment facility	PreI	I
MIP/NIP1	1:1:5	4-Vpy	EGDMA	Acetone	Poor /difficult	--	--
MIP/NIP2	1:1:5	4-Vpy	EGDMA	Acetone/ ACN 1:1	Poor /difficult	97	--
MIP/NIP3	1:1:5	4-Vpy	EGDMA	Acetone/ ACN 3:1	Poor /difficult	85	--
MIP/NIP4	1:1:5	4-Vpy	EGDMA	ACN	Poor /difficult	--	--
MIP/NIP5	1:1:5	4-Vpy	EGDMA	THF	Poor /difficult	--	--
MIP/NIP6	1:4:20	4-Vpy	EGDMA	Acetone/ ACN 1:1	High/difficult	50	--
MIP/NIP7	1:4:20	4-Vpy	EGDMA	Acetone/ ACN 3:1	High/easy	84	4.6
MIP/NIP8	1:4:20	4-Vpy	TRIM	Acetone/ ACN 3:1	High/easy	60	4.6
MIP/NIP9	1:4:20	MAA	EGDMA	Acetone/ ACN 3:1	High/easy	51	0.7
MIP/NIP10	1:4:20	MAA	TRIM	Acetone/ ACN 3:1	High/easy	52	1.3

Table 3.1. (Continuation I).

Polymers	T:M:Cr molar ratio	Monomer	Crosslinker	Porogen	Polymer yield/ treatment facility	PreI	I
MIP/NIP11	1:5:30	4-Vpy	EGDMA	Acetone/ ACN 1:1	High/difficult	62	--
MIP/NIP12	1:5:30	4-Vpy	EGDMA	Acetone/ ACN 3:1	High/easy	26	--
MIP/NIP13	1:6:30	4-Vpy	EGDMA	Acetone/ ACN 1:1	High/difficult	78	--
MIP/NIP14	1:6:30	4-Vpy	EGDMA	Acetone/ ACN 3:1	High/easy	52	--

Initiation method tested:
- 60 ºC 24h in a water bath at for every MIP/NIP.
The following MIP/NIP were also tested under the following initiation methods:
- MIP/NIP7: 60 ºC 20, 48 and 72 hours.
- MIP/NIP1 to 5: UV at room temperature and 4ºC, 24h.

preI: initial imprinting value for the template during the synthesis step, calculated as indicated in section 2.4.
I: Imprinting factor calculated as indicated by D. A. Spivak, 2005.

Non-imprinted polymers (NIPs) were also prepared and treated in an identical manner to MIPs, although in the absence of the template molecules.

2.4. Binding evaluation parameters by solid phase extraction

200 mg of the dried polymers were packed into 6 mL polypropylene SPE cartridges (Supelco, Bellefonte, PA, USA) and capped with fritted PTFE disks at the bottom and the top of the cartridges. The outlet tips were connected to a vacuum pump (VISIPREP Solid Phase Extraction Vacuum Manifold). First, the MISPE was conditioned with 5 mL of porogen. An initial imprinting value, named as preI, was introduced to estimate the percentage of template initially bound during polymerization. It was calculated as the amount of template bound during the polymerization step compared to the initial amount of template added in the polymerization mixture. The more quercetin is initially retained onto MIPs (higher preI), the more binding sites are formed to act as active sites to bind new target molecules.

The polymer was then washed with porogen-acetic acid (9:1) until the entire template was removed (45 mL). Acetic acid was washed out with acetone-acetonitrile (3:1) (5mL). After washing, the sorbent was dried by means of vacuum, after which it is ready for use.

Molecular recognition behaviour of the synthesized polymers was tested passing 2 mL of work standard solutions of quercetin at different concentration levels through the MISPE and the corresponding blank (NIP). The polymers were previously conditioned with 5 mL of acetone:acetonitrile 3:1. Retained quercetin was subsequently eluted from the column with 4 mL of eluent. Different solvent mixtures were tested ((acetone-acetonitrile (3:1))-acetic acid (9:1), methanol, methanol- 5% formic acid, water-5% formic acid) being methanol selected as providing the best performance. After each step, the eluate was collected and analyzed. The specificity of the polymers was

then estimated by the imprinting factor (I) (Spivak, 2005) being I= KpMIP/ K_{pNIP}, where $K_p = S_b/X_f$; being S_b the amount of substrate bound to the polymer and X_f the concentration of substrate remaining in solution after adsorption to the polymer. Thus, preI and I were used to estimate the most specific MIP for quercetin adsorption.

Selectivity of the most specific MIP towards structurally quercetin-related (C, EC and EGCG) and unrelated (α-TOCO) compounds was tested. 2 mL of a 25 mg L^{-1} solution prepared in acetone-acetonitrile (3:1), individually and as a mixture of the studied compounds were loaded onto the cartridges, eluted, collected and analyzed. Selectivity was evaluated by S (specific selectivity factor) defined as $S=I_1/I_2$ where I_1 and I_2 are the imprinting factors for two different substrates, being I_1 the imprinted factor for quercetin (Spivak, 2005).

The maximum volume that can be loaded onto the MIP cartridges without breakthrough of the analyte was estimated by loading different volumes of two of the studied standard solutions. Increasing volumes (from 2 to 100 mL) of solutions of C and EC containing 0.04 mg of each antioxidant were loaded onto 200 mg of MIP. The data were graphically analyzed.

2.5 Batch adsorption experiments: adsorption binding isotherms, kinetic adsorption curve and adsorption mechanism.

The imprinted polymer that exhibited the best recognition ability (in terms of specificity and selectivity) as assessed by MISPE was subjected to batch rebinding assays. Kinetic adsorption and adsorption binding isotherms tests were carried out placing 20 mg of the selected MIP into glasses. Kinetic

assays were carried out incubating 2 mL of a 10 mg L^{-1} solution into glasses for periods of time ranging from 2 to 1800 min at room temperature. Adsorption binding isotherms were performed incubating the MIP for 24 h at room temperature with 2 mL of a solution of C, EC and EGCG in acidified water at concentrations ranging from 2 to 200 mg L^{-1}. After incubation, supernatants were removed and analyzed by HPLC determining the concentrations of the analytes not bound by the polymer. Adsorbed concentrations were calculated by substracting these free concentrations from the initial concentrations of these compounds. The final data were fitted to different kinetic or isotherm models.

2.6. Polymer physical characterization

Particle diameter and size distribution of MIPs and NIPs were determined with a scanning electron microscope (SEM, JEOL JSM-6400).

An ASAP2020 (Micromeritics) Surface Area and Porosimetry Analyzer was used for the determination of the polymer surface areas from multipoint N_2 adsorption isotherms. Prior to the measurements, MIPs and NIPs were degassed at 25 ºC and 60 ºC under high vacuum for 10 h. The surface areas were evaluated using the Brunauer-Emett-Teller (BET) method.

FTIR spectroscopic measurements were performed in the attenuated reflection mode (ATR) using an OPUS/IR PS 15 spectrometer (Bruker) equipped with a thermostated MK II Golden Gate™ Diamond 45º ATR accessory. Liquid monomer mixtures and the final polymerized MIP were directly poured onto the diamond surface. The spectra were the results of 100

coadded interferograms at 2 cm^{-1} resolution between 400 and 4000 cm^{-1}. In order to avoid interferences with water bands and hydrogen bonding due to water vapor sorption because of the hygroscopic character of these polymers, the study was carried out at room temperature and at 55 ºC. The FTIR peak area corresponding to the absorption at 1637 cm^{-1} (C=C of methacrylate group) as baseline was measured. The percentage of unreacted carbon-carbon double bonds was determined from the ratio of absorbance areas of that peak and internal standard (the asymmetric C-H deformation band at 1460 cm^{-1} and the C=O stretching vibration band at 1717 cm^{-1} of the EGDMA) before and after polymerization. The degree of monomeric conversion (DC) was determined using the following equation (Mendes et al., 2005):

$$DC(\%) = 100 - \frac{\left[abs(C=C)_{1638} / abs(C-H)_{1460}\right]_{polymerized}}{\left[abs(C=C)_{1638} / abs(C-H)_{1460}\right]_{monomer}} \times 100$$

2.7. Natural samples analysis

MISPE was applied to the clean up and preconcentration of catechins and quercetin from several natural samples: red and white tea commercialized in infusion bag, green and black tea commercialized in bulk, grape residues and cocoa.

0.5 mg of each sample was extracted with 50 mL of water under magnetic stirring for 10 minutes. Formic acid was then added (5%).

5 mL of each solution was passed through the MISPE and the corresponding NISPE. Conditioning, binding and elution steps were carried out following the procedure described in the 2.4. section. The performance of

the introduction of a washing step after sample loading in order to avoid non-specific interactions was also studied.

Catechin, epigallocatechin gallate, epicatechin, quercetin and caffeine (Caff) concentrations in each sample were evaluated by the injection of each eluate in the HPLC system under the previous described conditions.

3. RESULTS AND DISCUSSION

3.1. Preparation of MIPs and NIPs

Different quercetin-MIP compositions were tested by studying the influence of the polymerization method, porogen, monomers, crosslinkers and T:M:C molar ratio on the properties of the MIP (Table 3.1) .

3.1.1. Polymerization process

Synthesis of MIPs and NIPs under photochemical (4 ºC and room temperature, 24 and 72h) and thermal (60 ºC, 20 to 72h) conditions were tested. The stability of the template under UV and thermal conditions compared to solutions kept at 4 ºC was confirmed (recoveries between 96 and 106%). Under UV conditions polymerization did not proceed. Quercetin-MIP polymerization under thermal conditions was highly influenced by time. Times lower than 24 h did not lead to complete polymerization. At 48 and 72h the amount of quercetin initially retained during polymerization and thus, the number of binding sites created, was diminishing (preI from 84% at 24 h to 34 and 33% at 48 and 72h, respectively). It may reveal a trade-off between the

extent of polymerization and stabilization of the template-functional monomer complex. Hence, thermal polymerization at 60 ºC for 24h was selected as polymerization method.

3.1.2. Porogen

The strength of non-covalent interactions is highly determined by the porogen, which also influences polymer morphology being responsible for creating the pores in macroporous polymers (Masqué et al., 2001; Cormack and Zurutuza-Elorza, 2004). Several porogens were then tested: acetone, acetonitrile, their 1:1 and 3:1 mixtures and THF. Sufficient solubility of quercetin at the desired level of concentration, as well as high yield and easy polymer post-treatment were only obtained by acetone:acetonitrile 3:1, which was selected as the optimum porogen.

3.1.3. Functional monomer and crosslinker choice

The choice of functional and crosslinker monomers are essential to maintain stable monomer-template complexes during the imprinting process and to control the morphology of the polymer matrix, stabilizing the imprint binding site and imparting mechanical stability to the polymer matrix, respectively. Different functional monomer (4-Vpy or MAA) and crosslinker (EGDMA or TRIM) combinations were tested for MIPs and NIPs synthesis (Table 3.1). Polymers were evaluated through initial imprinting value for the template during the synthesis step and imprinting factor.

It can be seen that between the MIPs prepared using the two different types of functional monomers, 4-Vpy-containing polymers show the highest binding affinity for the template (higher preI values). Since imprinting factor is obtained from the ratio of the partition coefficient of quercetin onto the imprinted and non-imprinted polymer (Cormack and Zurutuza-Elorza, 2004), the higher values also achieved for 4-Vpy-containing polymers (4.6 vs 0.7 or 1.3) indicated better specificity. However, MAA-containing polymers showed lower binding affinity for the template, even though the template has several proton-accepting and hydrogen-bonding functional groups. This effect could be attributed to the formation of more stable interactions template-monomer in the prepolymerization solution when 4-Vpy is used.

Looking at the crosslinker, EGDMA (di-functional methacrylate and more rigid) associated with 4-Vpy turned out to be the best crosslinker with an initial imprinting value and a net binding markedly higher than the achieved for TRIM (tri-functional methacrylate and more flexible).

Therefore, 4-vpy and EGDMA as monomer and crosslinker were selected as enhancing recognition properties of MIPs towards quercetin.

3.1.4. T:M:C molar ratio

Ratio of template to monomer and crosslinker could affect both imprinting result and adsorption properties (Cormack and Zurutuza-Elorza, 2004). 4 relations T:M (1:1, 1:4, 1:5, 1:6) and 3 relations T:C (1:5, 1:20, 1:30) were tested to study the formation of template-functional monomer and template-crosslinker assemblies, respectively. Since the more quercetin is

initially retained onto MIPs (higher preI), the more binding sites are formed to act as active sites to bind new target molecules, binding properties were evaluated through the initial imprinting value (Figure 3.2).

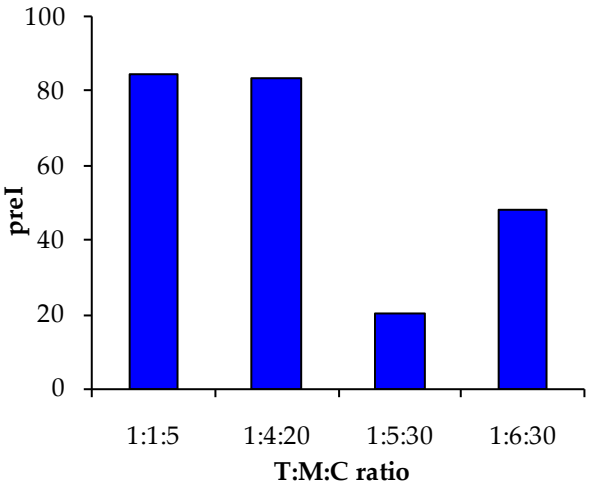

Figure 3.2. Effect of ratio of 4-Vpy and EGDMA on the binding properties of MIPs. (preI: initial imprinting value for the template during the synthesis step, calculated as indicated in section 2.4.).

Increasing both the amount of monomer 4-Vpy and the crosslinker EGDMA, the adsorption properties of MIPs could rapidly decrease the binding capacity of the MIP towards quercetin. Non-excess of monomer (1:1 Quer:4-Vpy ratio) resulted in polymerization becoming difficult (poor polymer yield and difficult post-treatment), which may be detrimental to template–functional monomer interactions. At Quer:EGDMA ratio of 1:5 and 1:20 binding effects were rather similar. 1:20 molar ratio template:crossliker

was selected considering the increased rigidity of the polymer provided by the crosslinker. This can facilitate the formation of stable binding sites and improve the selectivity of MIPs (Cormack and Zurutuza-Elorza, 2004).

Thus, a Quer:4-Vpy:EGDMA (1:4:20) imprinted polymer and its corresponding NIP, synthesized under thermal conditions (60 ºC, 24 h) in acetone:acetonitrile (3:1) were selected for further test.

3.2 Evaluation of the polymers.

3.2.1. Binding specificity and selectivity of the MIPs

Specific and selective adsorption of selected MIP were evaluated by using quercetin, its structurally related compound catechin, epicatechin and epigallocatechin gallate and a unrelated compound of similar molecular size: α-tocopherol, according to the experimental procedure described in section 2.4. Table 3.2 shows the specificity and selectivity values of the tested compounds onto the final selected MIP and the corresponding NIP.

Aimed to the application of MIPs to concentrate and purify catechins from natural samples, imprinting values for the template as well as for all the other studied compounds were calculated. As can be seen from I values, MIP uptakes quercetin with high specificity. The adsorption capacity of the MIP for catechin and epicatechin was even slightly better than the obtained for quercetin (higher I values and S<1). It may be explained by its close structural homology to quercetin. Molecular recognition ability of MIPs is mainly dependent on several factors, such as shape complementarity, functional complementarity and contributions from the surrounding environment (Yan

and Row, 2006). Quercetin molecule contains a δ-carbonyl group not present in catechin, entailing a smaller structure in the latter. Thus, catechin may easily enter the MIP cavity. Both catechin and quercetin have the same number of H-Bond donors, which gives catechin the possibility of forming the same number of hydrogen bonds than quercetin. Thus, a better match of catechin in the cavities induced by quercetin could be then explained. Even though epicatechin and catechin have the same molecular structure and, so that, similar performance should be expect, the different spatial distribution of the γ-phenolic hydroxyl group may carry to a different matching in the cavities of the MIP.

Table 3.2. Specificity and selectivity values of quercetin, catechin, epicatechin, epigallocatechin gallate and α-tocopherol (25 mg L^{-1} in acetone:acetonitrile (3:1)) onto the selected MIP and NIP.

Individual compounds	**I**	**S**
Quercetin	4.6	--
Catechin	7.9	0.59
Epicatechin	11	0.42
Epigallocatechin gallate	3.2	1.3
α-tocopherol	0.47	9.9
As a mixture		
Quercetin	78	--
Catechin	29	2.7
Epicatechin	38	2.0
Epigallocatechin gallate	1.9	40
α-tocopherol	0.55	141

For Epigallocatechin gallate, it would be reasonable to assume that the presence of a large substitution in the β-hydroxyl group may cause a mismatch between the molecule and the specific binding sites in the polymer network. As a result, weak interactions are expected and, therefore, lower specific and selectivity parameters were achieved.

α-tocopherol seemed to show higher binding to the non-imprinted polymer, revealing the weakness or even the absence of specific interactions. α-tocopherol could not be fitted into the specific network induced by quercetin due to both its different structure in comparison with quercetin and the low number of H-bond donors (1 vs 6, respectively). Accordingly, low imprinting factor and high selectivity factors were observed.

Similar performance was achieved when a mixture of compounds was loaded onto both the NIP and the MIP. Nevertheless, higher I values with respect to their individual data has revealed an increase in the non-specific interactions of the compounds onto the NIP when individual compounds were loaded. It may suggest some influence in the number of non-specific interaction formed by a possible competitive effect when several compounds of similar nature are present.

3.2.2. Cartridge capacity test

Determination of breakthrough volume was performed according to the procedure previously described. While breakthrough does not occur, the amount preconcentrated remains constant but when breakthrough occurs, the amount extracted is reduced. As can be observed in Figure 3.3, C and EC

recoveries ranged from 100 to 88 % were obtained for the assays between 2 to 40 mL, suggesting that no significant compound loss (analyte breakthrough) occurred before. Thus, volume samples even of 40 mL can be loaded onto the prepared MIP, because breakthrough does not occur at lower values.

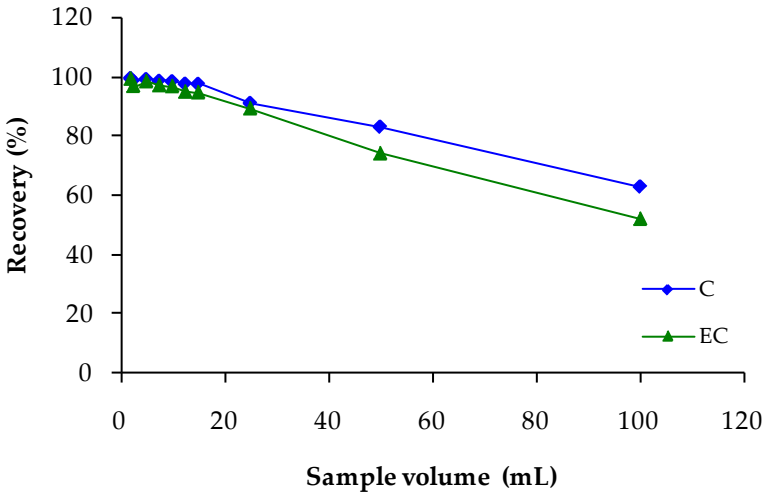

Figure 3.3. Evaluation of the breakthrough volume determined for C and EC on MIP.

3.2.3. Kinetic considerations

Kinetic of adsorption describes the rate of adsorbate uptake on MIP and controls the equilibrium time. To examine the controlling mechanism of sorption process (chemical reaction, diffusion control and mass transfer) several kinetic models were used to test experimental data. Pseudo-first order, pseudo-second order and Elovich kinetic models were applied to study the kinetics of adsorption process. Intraparticle diffusion model, external film

diffusion model and Boyd model were studied to determine the diffusion mechanism adsorption system. Equations and characteristics of the used models are described in Table 3.3.

Table 3.3. Equations and considerations of kinetic adsorption and mechanism models.

Model	Equation		Considerations
• **Kinetic of adsorption process**			
Pseudo-first order	$\log(q_e - q_t) = \log q_e - \frac{k_1}{2.303} t$	q_e and q_t (mg g^{-1}): amounts of adsorbate adsorbed at equilibrium and at any time, t(min), respectively.	In both models, all the steps of adsorption such as external diffusion, internal diffusion and adsorption are lumped together. The difference between the average solid phase concentration and the equilibrium concentration is considered as the driving force for adsorption. These models are applicable if log (q_e-q_t) vs t or t/q_t vs t, respectively gives a linear plot
	$h_1 = k_1 q_e$	k_1 (min^{-1}): pseudo-first order adsorption rate constant h_1 (mg g^{-1} min^{-1}) : initial sorption rate	
Pseudo-second order	$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$	k_2(g mg^{-1} min^{-1}): pseudo-second order adsorption rate constant h_2(mg g^{-1} min^{-1}): initial sorption rate	
	$h_2 = k_2 q_e^2$ $t_{1/2} = \frac{1}{k_2 q_e}$	$t_{1/2}$: Time required for the adsorption to take up half as much compound as its equilibrium value	
Elovich	$q_t = \frac{1}{\beta} Ln(\alpha\beta) + \frac{1}{\beta} Lnt$	α (mg g^{-1} min^{-1}): initial sorption rate β (g mg^{-1}): related to the extended of surface coverage and activation energy for chemisorption	Description of the chemisorption process. Applicable when q_t vs Lnt represents a straight line.

Table 3.3. (Continuation I).

Model	Equation		Considerations
• **Adsorption mechanism**			
External diffusion	$Ln\ \frac{C_t}{C_0} = -k_{ext}\ t$	C_0, C_t (mg L^{-1}): concentration of the solute in the initial solution and in the liquid phase, respectively, at time t k_{ext} (min-1): diffusion rate parameter for film diffusion model	If external film diffusion equation is applicable, the plot of $\ln(C_t/C_0)$ against t should give a straight line with zero intercept
Intra-particle diffusion	$q_t = k_i \times t^{0.5} + IP$	k_i (mg g^{-1} $min^{-0.5}$): intra-particle diffusion rate constant IP (mg g^{-1}): efect of the boundary layer thickness	A larger IP values means a greater effect of the boundary layer. Intra-particle diffusion is the sole rate-limiting step if the plot of q_t vs $t^{0.5}$ is a straight line pasing through the origin. Otherwise, some other mechanism along with intra-particle diffusion is also involved
Boyd	- If 0<F<0.85 $Bt = 2\pi - \frac{\pi^2}{3} - 2\pi\left(1 - \frac{\pi F}{3}\right)^{1/2}$ - If 0.86<F<1 $Bt = -0.49770 - Ln\,(1 - F)$	Bt: Boyd parameter F: represents the fraction of solute adsorbed at any time, t, as calculated using: $F=q_t/q_e$	If the plot of Bt vs t is a straight line passing through the origin, the rate-limiting step in the adsorption process is the intra-particle diffusion.

Table 3.3. (Continuation II).

Model	Equation	Considerations
• **Validation of the kinetic models**		
	- Normalized standard deviation (Δq_t) $\Delta q_t(\%) = 100\sqrt{\frac{\sum\left[\left(q_{t,exp.} - q_{t,cal}\right)/q_{t,exp}\right]^2}{N-1}}$	$q_{t,exp}$ and $q_{t,cal}$ (mg g^{-1}): experimental and calculated adsorption uptake, respectively N: number of data points
	- Relative error (%) $relative\_error = 100\frac{\left\|q_{e,cal} - q_{e,exp}\right\|}{q_{e,exp}}$	$q_{e,exp}$ and $q_{e,cal}$ (mg g^{-1}): experimental and calculated adsorption uptake at the equilibrium, respectively
References: Reichenberg, 1953; Wang et al., 2008; Zhang et al., 2008; Tan et al., 2009; Liu et al., 2010; Pan et al., 2010.		

3.2.3.1. Kinetic of adsorption process. To quantitatively compare the accuracy and validity of the kinetic models, correlation coefficient R^2, normalized standard deviation Δq_t (%) and relative error (%) (Tan et al., 2009) were calculated and listed in Table 3.4.

Table 3.4. Pseudo-first order model, pseudo-second order model and Elovich equation constants and normalized standard deviations and relative error for adsorption of C, EGCG and EC on MIP.

Kinetic models	Parameters	C	EGCG	EC
	$q_{e,exp}$ (mg g^{-1})	0.785	1.27	0.874
Pseudo-first order	$q_{e,cal}$ (mg g^{-1})	0.0889	0.0884	0.305
	k_1 (min^{-1})	3.55E-03	9.10E-04	3.44E-03
	h_1(mg $g^{-1}min^{-1}$)	3.15E-04	8.04E-05	1.05E-03
	R^2	0.584	0.185	0.262
	Δqt	43.4	31.2	33.5
	Relative error (%)	88.7	93.2	65.2
Pseudo-second order	$q_{e,cal}$ (mg g^{-1})	0.807	1.24	0.897
	k_2 (min^{-1})	0.128	0.378	0.0522
	R^2	0.999	0.999	0.998
	h_2 (mg g^{-1} min^{-1})	0.0836	0.584	0.0420
	$t_{1/2}$	9.65	2.13	21.4
	Δqt	7.40	8.24	8.80
	Relative error (%)	2.71	2.33	2.58
Elovich equation	$q_{e,cal}$ (mg g^{-1})	0.815	1.31	0.817
	β (g mg^{-1})	24.9	19.3	17.5
	α (mg g^{-1} min^{-1})	2.36E+04	2.86E+06	1.53E+02
	R^2	0.606	0.500	0.765
	Δqt	20.7	17.6	15.5
	Relative error (%)	3.66	3.13	6.98

Table 3.4 also shows that the pseudo-second order model fits all the kinetic adsorption curves better than the other studied models does for C, EGCG and EC. According to I. A. W. Tan et al, 2009, it may suggest that the overall rate of the adsorption process was controlled by chemisorption. Furthermore, the low R^2 values for the first-order model also suggest the same conclusion. Nevertheless, it has to be taken into account that no

absolutely sharp distinction between chemisorption and physisorption can be made. Intermediate cases such as adsorption involving strong hydrogen bonds or weak charge transfer are possible.

3.2.3.2. Adsorption mechanism. External diffusion model (or film diffusion model) and intra-particle diffusion model were used to determine the rate limiting step (Reichenberg, 1953; Wang et al., 2008; Zhang et al., 2008; Tan et al., 2009; Liu et al., 2010; Pan et al., 2010). Figure 3.4 shows the curves obtained from the experimental data for both models.

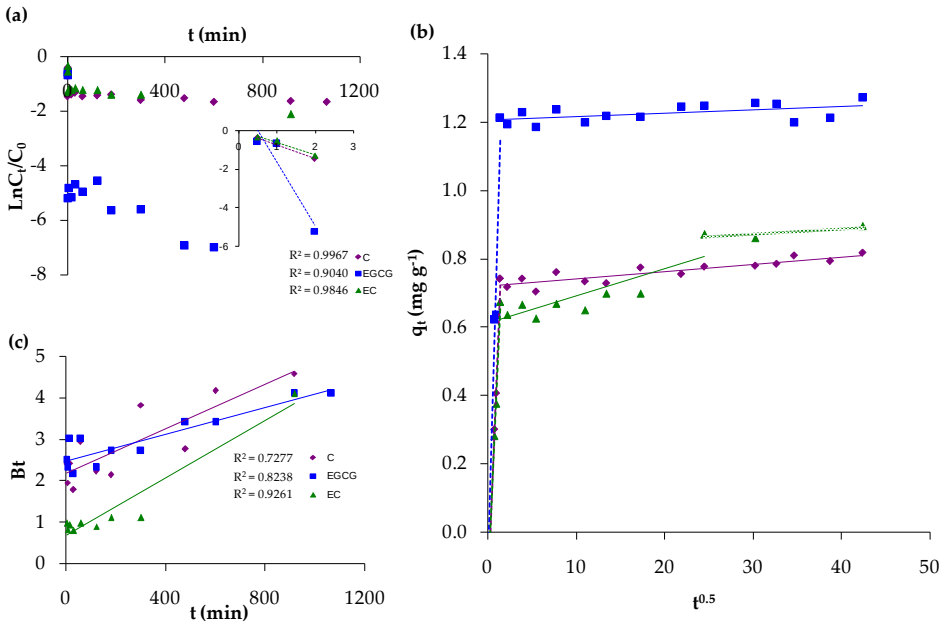

Figure 3.4. Plot of (a) external diffusion model, (b) intra-particle diffusion model and (c) Boyd model for adsorption of C, EGCG and EC onto the MIP.

External diffusion kinetic model: linear relation plot in C, EGCG and EC initial stages (Figure 3.4a enlarged) and small intercepts values suggested external film diffusion as rate-controlling step of the initial fast adsorption of C, EGCG and EC onto the MIP. Rate constants values are given in Table 3.5.

Table 3.5. External diffusion model and intra-particle diffusion model constants for adsorption of C, EGCG and EC on MIP.

Adsorbate	External diffusion model	Intra-particle diffusion model					
	k_{ext} (min^{-1})	**k_{p1}**	**k_{p2}**	**k_{p3}**	**IP_1**	**IP_2**	**IP_3**
		($mg\ g^{-1}min^{-0.5}$)					
C	0.692	0.637	2.14E-03	----	-0.179	0.721	----
EGCG	3.273	0.870	9.77E-04	----	-0.081	1.21	----
EC	0.652	0.567	7.93E-03	1.54E-03	-0.144	0.643	0.827

Intra-particle diffusion model (Figure 3.4b): adsorption of C, EGCG and EC onto MIP has followed two steps: straight line passing through the origin attributed to the instantaneous and extremely fast uptake of the most readily available sites on the adsorbent external surface; and gradual adsorption of the compounds ascribed to the intra-particle diffusion, stimulating further migration of adsorbate from the fluid phase to the adsorbent internal surface. A third region attributed to the final equilibrium state, for which the particle started to slow down due to the extremely low adsorbate concentrations, remain in solution was observed for EC.

k_{pi} and IP_i values (Table 3.5) denote EGCG diffusing faster through external surface while C and EGCG diffuse faster during the second step. It

might be attributed to their different structure, fitting C and EC better in the pores created by quercetin in the MIP than EGCG.

The existence of different regions indicates that both external diffusion and intra-particle diffusion models are simultaneously occurring. Boyd kinetic model was used to determine the slowest step in the overall adsorption process. Plots (Figure 3.4c) not passing through the origin and slightly scattered points, indicated film diffusion as the rate-controlling step.

3.2.4. Adsorption isotherms

Affinity of the imprinted polymer for C, EGCG and EC was investigated through saturation binding experiments. Linearized forms of Langmuir, Freundlich, Allosteric and Dubinin-Radushkevich adsorption isotherm models (Table 3.6) were applied to fit the equilibrium data. Results are shown in Table 3.7.

Binding isotherms of C, EGCG and EC (Figure 3.5a) showed that the amount of C, EGCG and EC bound to the MIP at equilibrium increased with concentration until reach a saturation level. It suggested that the binding to the polymer is then more possibly caused by specific binding to a limited number of binding sites in the polymer network than by non-specific adsorption (Zhu et al., 2002). The linear fitting of the Scatchard plot (Figure 3.5b) suggested that homogeneous recognition sites were formed in the MIPs.

Table 3.6. Equations and considerations of adsorption isotherms

Isotherm	Linearized equation		Considerations
Langmuir	$\frac{C_e}{q_e} = \frac{1}{q_m K_L} + \frac{1}{q_m} C_e$ o: Scatchard Plot $\frac{q_e}{C_e} = q_m K_L - K_L q_e$	C_e (mg L^{-1}): equilibrium concentration of the solute in the bulk solution q_e (mg g^{-1}): amount of solute adsorbed at equilibrium q_m (mg g^{-1}): maximum adsorption capacity of the sorbent, representing the total number of binding sites K_L (L mg^{-1}): constant related to the free energy of adsorption	This isotherm is based in three assumptions: - adsorption cannot proceeded beyond monolayer coverage - equivalency of all surface binding sites accommodating, at most, one substrate per site - ability of the template to bind at a given site not depend on the occupation of the neighbouring sites Thus, homogeneous sites within the polymer are assumed for binding.
	$R_L = \frac{1}{1 + K_L C_0}$	R_L: separation factor or equilibrium parameter C_0 (mg L^{-1}): initial concentration of the solute	R_L: prediction of the favourability of an adsorption system. If: R_L>1, unfavourable isotherm R_L=1, linear isotherm 0<R_L<1, favourable isotherm R_L=0, irreversible isotherm

Table 3.6. (Continuation I).

Isotherm	Linearized equation		Considerations
Freundlich	$\log q_e = m\log C_e + \log K_F$	K_F ($mg^{1-m}\ L^m\ g^{-1}$): adsorption capacity of the sorbent m: adsorption intensity or surface heterogeneity	Based on sorption on a heterogeneous surfaces or surfaces supporting sites of varied affinities. - Stronger binding sites are the first on being occupied - Binding strength decreases with the increasing degree of site occupation 0≤m≤1, closer to 1 as heterogeneity decreases. If m=1: homogeneous system
		$N(K) = 2.303am\left(a - m^2\right)e^{-2.303m\log K}$ $K_{\min} = 1/C_{\max} \quad K_{\max} = 1/C_{\min} \quad a = K_F$ $N_{K1-K2} = a(1-m^2)(K_1^{-m} - K_2^{-m})$ $K_{K1-K2} = (m/(m-1))\left[\left(K_1^{1-m} - K_2^{1-m}\right)/\left(K_1^{-m} - K_2^{-m}\right)\right]$	Modification of Freundlich isotherm in order to obtain an equation for the affinity distribution, being N_{K1-K2} the apparent number of sites and K_{K1-K2} the apparent weighted average affinity. Values calculated within the limits of K_{min} and K_{max}.
Allosteric	$Ln\left(\frac{q_m}{q_e} - 1\right) = LnK_m - nLnC_e$	K_m: binding constant n: correlated to the number of binding layers (normally n≥1)	Based on the assumption of multi-layer adsorption due to the altered binding sites from interaction effect. Being occupied some of the binding sites; the stereo-structure could be altered. The binding affinity could possibly be formed and thus multi-layer binding could be obtained. If n=1 the model is reduced to the Langmuir model

Table 3.6. (Continuation II).

Isotherm	Linearized equation		Considerations
Dubinin-Radushkevich	$Lnq_e = Lnq_m - K_{DR}\varepsilon^2$ $\varepsilon = RTLn\left(1+\frac{1}{C_e}\right)$ $E = (2K_{DR})^{-1/2}$	K_{DR}: related with free energy (E, kJ mol) of adsorption per molecule of the adsorbate when it is transferred to the surface of the solid from infinity in the solution R: gas constant (8.314 J mol^{-1} K^{-1}) T (K): absolute temperature	To analyze isotherms with high degree of rectangularity. Value of K_{DR} < 1 represents the rough surface with many cavities E >40 kJ mol−1 expresses the chemisorption between sorbent and adsorbate.

Rampey et al., 2004; Spivak, 2005; Syu y Nian, 2005; Gracía-calzón y Díaz-García, 2007; Subramanyam y Das, 2009; Tan et al., 2009; Pan et al., 2010

Table 3.7. Adsorption isotherm constants and correlation coefficients for MIP.

Adsorption equations	Parameters	C	EGCG	EC
Langmuir	R^2	0.823	0.968	0.843
	K_L (L mg^{-1})	0.0110	0.0358	0.0094
	q_m (mg g^{-1})	34.1	86.9	28.5
	R_L	$0.3<R_L<1$	$0.1<R_L<0.3$	$0.3<R_L<1$
	Δq_e	5.25	2.71	5.16
Freundlich	R^2	0.985	0.956	0.984
	K_F (mg^{1-m} L^m g^{-1})	0.500	7.55	0.374
	m	0.822	0.512	0.817
	K_{K1-K2} (L mg^{-1})	0.0493	0.0387	0.0410
	N_{K1-K2}	4.01	32.3	3.66
	Δq_e	8.08	5.47	8.67
Allosteric	R^2	0.994	0.992	0.995
	K_m	90.3	28.4	78.9
	n	0.992	0.949	0.991
	Δq_e	5.39	2.72	5.37
Dubinin-Radushkevic	R^2	0.950	0.940	0.872
	K_{RD}	6.24E-06	1.42E-05	4.67E-06
	q_m (mg g^{-1})	8.26	52.0	6.67
	E (kJ mol^{-1})	283	188	327
	Δq_e	36.9	9.72	59.4

Table 3.7 compiled the fitting results for modelling C, EGCG and EC adsorption. Goodness of the fit and adequacy of the studied models are estimated by R^2 and Δ_{qe} (normalized standard deviation), respectively. Adsorption isotherms of C, EGCG and EC on the MIP were better fitted by Freundlich and Allosteric models ($R^2 > 0.95$); although Langmuir isotherm model also showed good agreement between the experimental and observed

data especially for EGCG (correlation coefficient of 0.97). Δ_{qe} < 5% confirmed the adequacy of the models to fit the experimental data.

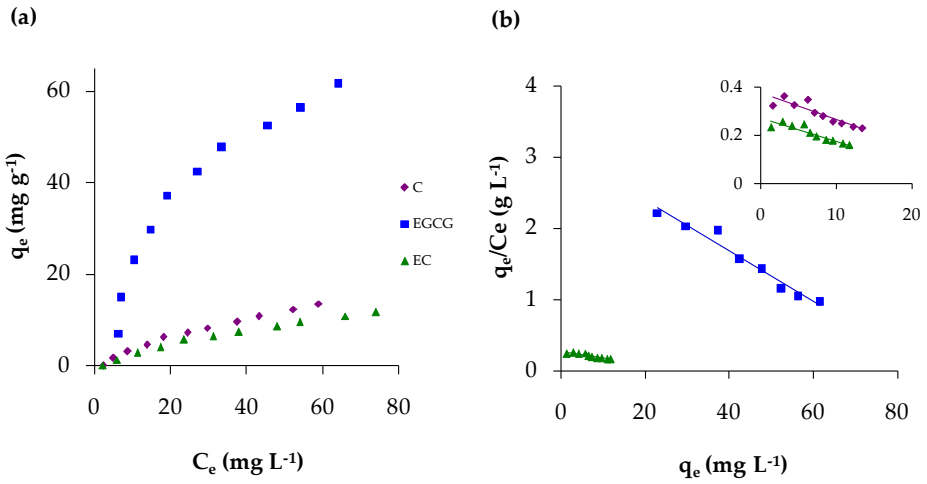

Figure 3.5. (a) Binding isotherm and (b) Scatchard plot analysis of the binding of C, EGCG and EC to imprinted polymer.

While Langmuir model is basically intended for a homogeneous system, Freundlich model is suitable for a highly heterogeneous surface, being the system more heterogeneous as m value is closer to 0. Experimental data (m between 0.5 and 0.9) suggests that, although some degree of heterogeneity is present, a more homogeneous surface can be assumed. R_L values between 0 and 1 also indicated a favourable adsorption of C, EGCG and EC on the MIP at the studied conditions.

Allosteric model fitting suggested C, EGCG and EC adsorption process having place in a mono-layer binding system (n=1).

K_{DR} (Dubinin-Radushkevich model) is related with the free energy (E) of adsorption per molecule of adsorbate when it is transferred to the surface of the solid from infinity in the solution, allowing to predict the type of adsorption. Since E > 40 kJ mol^{-1} (Table 3.7) for C, EGCG and EC, a possible chemisorption process between sorbent and adsorbate could be proposed.

3.3 Characterization of the polymers

MIPs and their corresponding NIPs were physically characterized through chemical and morphological characterization by FTIR, SEM and BET.

3.3.1. Fourier-transform infra-red spectroscopy (FTIR)

FTIR was used as a useful tool for the measurement of functional groups incorporation by the quantification of the degree of polymerization (Spivak, 2005). The study was carried out at two different temperatures, 25 ºC and 55 ºC in order to consider possible interferences with water bands. The DC values for the synthesized MIP were 69 and 63% at 30ºC and 79 and 75% at 55ºC when C-H deformation band and C=O stretching vibration band were used, respectively. DC values suggested that most crosslinker and monomer were cross-linked. Dependency of DC values with temperature confirmed the presence of interferences with water bands.

3.3.2. Microscopy characterization: scanning electron microscopy (SEM)

Particle size and size distribution of the MIP and NIP were determined with a scanning electron microscope. While the NIP displayed a relatively smooth surface, some speckles and cavities were observed within the MIP, indicating that an imprinting was formed within the prepared MIP. SEM size data (Table 3.8) reflect a bigger average size for the MIP particles than for the NIP, suggesting some influence of the template on the particle growth during the precipitation polymerization.

Table 3.8. Average size particles data, surface area and pore volume of the selected MIP and NIP.

	Average particle size (nm)	Surface area ($m^2\ g^{-1}$)		Pore volume ($cm^3\ g^{-1}$)	
		25 ºC	60 ºC	25 ºC	60 ºC
MIP	300	39.6	69,6	-2.52E-04	6.52E-03
NIP	129	154	264	-6.41E-04	1.21E-02

3.3.3. Surface area and porosity

Morphological characterization was performed by nitrogen adsorption-desorption. BET method was applied to determine the specific surface area and the Barret-Joyner-Halenda (BJH) method for the pore diameter estimation. Data of surface area and pore volume are listed in Table 3.8. As is evident, the sample presents an extended macroporous structure. Similar surface area values to the ones typically found in imprinted polymer (ranged between 100 and 400 m^2/g^{-1} (Spivak, 2005)) were obtained in the present

study. Although the molecular cavity of the imprinted matrix should have larger surface area (Brüggemann, 2001; Shi et al., 2007; Chang et al., 2009), it can be seen that the imprinted material appeared to have smaller surface area and pore volume than the non-imprinted polymer. Having into consideration that the MIP should contain more hydroxyl groups than the NIP, according to Chang et al., 2009 it could be assumed that the MIP could absorb a lot more water molecules, which could occupied the imprinted material. Thus, less surface area and pore volume could be observed for the MIP. It may be also confirmed by the higher surface area and pore volume observed when sample was degassed (Table 3.8).

Showing higher specific surface area for the NIP than for the MIP, BET-measurements also allows to discard that the higher commented affinity for the MIP could be simply caused by a higher surface area, and not by a specific imprint.

3.4. Natural samples analysis

Selected MISPE conditions (as described in Section 2.4) were applied for the determination of catechins in real samples. Aqueous samples could be directly loaded onto MIP; nevertheless, retention and selectivity of the MIP is reduced (Weiss et al., 2002). Therefore, after loading the sample solution in the cartridge containing the polymer, a suitable washing step was introduced. Hexane and dichloromethane were selected as they could remove matrix components and re-distribute non-specifically bound analytes to the selective imprints. Considering their immiscibility with water, an exhaustive drying step had to be introduced. To study the performance of both solvents, a

mixture of the aimed compounds, C, EGCG, EC (the most abundant flavonoid compounds present in the aimed samples) and caffeine (Caff) at two levels of concentration (20 and 100 mgL^{-1}) was applied to the MISPE column as sample. The column was then washed with series of aliquots of 2.5 mL of each solvent. Remaining adsorbed material was finally eluted with methanol. Wash and elution fractions were collected and analyzed by HPLC as described in Section 2.2. When hexane was used as washing solvent C, EGCG, EC were completely retained on the MIP (elution percentages ranged from 90 to 103 %).

Dichloromethane has slowly washed out the catechins (between 10 to 20% of the initially retained compound after 15 mL). When Caff is considered, 70 to 80% of the initially retained amount onto the MIP was washed out with the first 5 mL of dichoromethane. Roughly 20% was washed out by hexane. Therefore, dichloromethane was selected as selective washing solvent in the MISPE method since both the best selectivity and good retention was shown.

To demonstrate the applicability of the prepared MIP, real samples were loaded onto the MIP and the corresponding NIP, washed and eluted following the proposed SPE procedure. Percent recoveries (% R) of C, EGCG, EC and Caff from tea, cocoa and grape residues extracts on the MIP and the NIP are presented in Figure 3.6.

Similar results were obtained for catechin, epicatechin and epigallocatechin gallate. It could be attributed to their structural similarity; also being the most structurally-related compounds with the template. Percent recoveries between 90 to 100% were obtained for adsorption and elution of catechins from white tea sample, ranging from 16 to 22% when NIP

is used as SPE. When red, green and black tea, cocoa and grape residue are loaded onto the MIP, % R higher than 50% for catechins were obtained, compared to %R between 14 and 40% onto the NIP. These percent recoveries are the result of the high rates of adsorption of catechins onto the MIP (close to 100%) and high elution percentages (between 60 and 100% depending on the compound and sample considered). In contrast, when NIP is used as sorbent, adsorption values are well below 50% in almost every case. These values confirmed the specificity of the prepared imprinting polymer for the catechins from real samples.

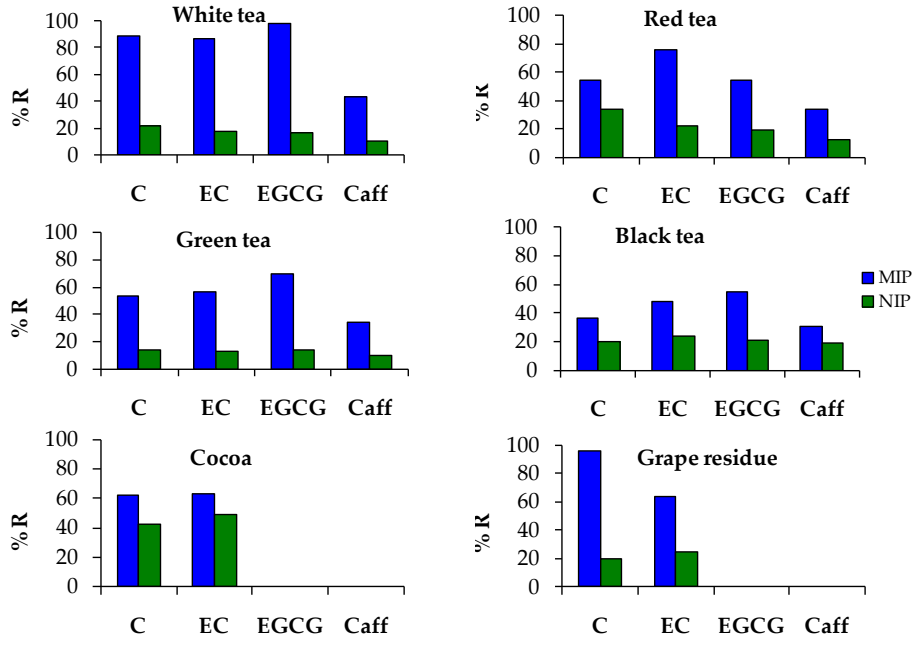

Figure 3.6. Percent recovery of C, EGCG, EC and Caff from white tea, red tea, green tea, black tea, cocoa and grape residues extracts on the MIP and the NIP (% R calculated as (mass of the eluted compound after MISPE/mass of the compound in the initial sample)x100).

Regarding caffeine, no relevant differences between recoveries onto the MIP and the NIP were obtained (around 30% when MIP is used as sorbent versus 20% when caffeine is applied onto the NIP). The different structure of Caff and the different number of H-Bond donor and acceptor (0 and 3 in Caff) with quercetin led to the low capacity of the MIP for the selective retention of this compound. It revealed the efficiency of the MIP to be used as clean-up step. Thus, an effective MIP for clean-up and preconcentration of catechins from real samples has been developed.

4. CONCLUSIONS

The present paper showed that the quercetin-MIP was a promising adsorbent to be used in the enrichment and clean up of catechins from natural matrices. The molecularly imprinted polymer prepared by precipitation technique using quercetin as a template was characterized by testing its molecular selectivity against structurally and non-structurally related compounds in MISPE format and studying its adsorption and kinetic behavior. This was confirmed by FTIR, SEM and BET and applied for the SPE of natural matrices.

The good imprinting parameters, high specificity and selectivity values, as well as the low non-specific binding of other non-interested compounds, such as caffeine on the polymer, have confirmed the performance of the synthesized MIP for catechins enrichment and clean up versus standard non-specific SPE techniques.

Adsorption and kinetic batch studies of the MIP using catechins as adsorbates also indicated that MIP displayed fast adsorption in a homogeneous surface and high adsorption capacity for C and EC.

Based on kinetic modeling (low R^2 pseudo-first order, good pseudo-second order fit) and adsorption isotherms (E values (kJ mol^{-1}) Dubinin-Radushkevich model) the specific adsorption in the imprinted cavities could be described as strong hydrogen bonds comparable to chemisorption process. The External diffusion step seems to be the rate-limiting step, followed by the intra-particle diffusion step. Possibly associated to their smaller molecular size, C and EC diffused faster through the intra-particle, while EGCG has showed faster diffusion velocity through the external surface.

Physical characterization of the prepared imprinted and non-imprinted polymers, such as pore surface area and pore size, as well as the high degree of polymerization were found to be complementary to adsorption and kinetic data.

The development and analysis of this MISPE to bind several catechins with high affinity, thus displaying certain group specificity, may provide a strategy for the development of new imprinted polymers for further antioxidants determination in natural matrices.

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3.3. ADICIÓN DE ANTIOXIDANTES NATURALES A MUESTRAS POLIOLEFÍNICAS

Artículo 4: Natural extracts as potential source of antioxidants to stabilize polyolefins.

3.3. ADICIÓN DE ANTIOXIDANTES NATURALES A MUESTRAS POLIOLEFÍNICAS

Artículo 4

NATURAL EXTRACTS AS POTENTIAL SOURCE OF ANTIOXIDANTS TO STABILIZE POLYOLEFINS

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ABSTRACT

Several natural matrices were investigated as potential sources of antioxidants to be used as plastic additives. Extracts of four matrices obtained under the same experimental conditions were initially considered: green tea, black tea, *Lippia citriodora* and *Hypericum androsaemum*. Both, the antioxidant activity of the extracts and their content in flavanols and quercetin, were compared. The antioxidant activity was determined by DPPH analysis and the phenolic composition by High Performance Liquid Chromatography (HPLC) using ultraviolet (UV) diode array and Fluorescence (FL) detectors. Concentration of the flavanols reduced in the same way as their antioxidant activity starting with green tea, through black tea, *Hypericum androsaemum* and *Lippia citriodora*. The performance of polypropylene samples stabilised with green tea extract, or its individual components catechin and epicatechin, was compared with samples stabilised with a mixture of the synthetic antioxidants Irganox 1076 and Irgafos 168. Each sample was extruded and consecutively reextruded up to four times. The melt flow index (MFI) and the oxidation induction time (OIT) of the samples were measured after each step. The obtained results showed the interest of this natural matrix as a potential source of antioxidants for plastics.

Keywords: antioxidants; green tea; high performance liquid chromatography (HPLC); poly(propylene) (PP); stabilization.

1. INTRODUCTION

Polymers, and especially polyolefins, need the addition of antioxidants in their formulations to provide protection during processing or fabrication into finished product (Chen-Yu Wang, 2000). Chain breaking antioxidants, sometimes referred to as primary antioxidants, interrupt the first degradation cycle by removing the polymer propagating radicals ROO·. Preventive antioxidants, sometimes referred to as secondary antioxidants, interrupt the second oxidative cycle by preventing or inhibiting the generation of free radicals. The most important preventive mechanism is the non-radical hydroperoxide decomposition. Hindered phenols and phosphite esters are important classes of primary and secondary antioxidants, respectively. Because of their complementary antioxidant mechanisms, they are generally used in combination to ensure both highly efficient melt stabilising systems and long term stability at high service temperatures (Pospíšil, 1992; Al-Malaika, 2003).

The antioxidants and other additives that can be used in the manufacture of plastic materials and articles intended to come into contact with foodstuffs are included in a list of additives established by Directive 2002/72/EEC (Commission Directive 2002/72/EEC). During processing or storage additives or their degradation products could migrate from plastic packaging into foodstuffs; therefore, their migration is also regulated by European legislation (Commission Directive 2002/72/EEC) through Specific Migration Limits (SMLs). In the last years, instead of the synthetic antioxidants usually employed natural antioxidants such as α-tocopherol (Al-Malaika et al., 1994; Mallegol et al., 2001; Strandberg and Albertsson, 2005; Peltzer et al., 2007),

carnosic acid (Jipa et al., 2005), oregano, savory, and essential oils (Salmieri and Lacroix, 2006), carvacrol (Peltzer et al., 2007) or hydroxytyrosol (Peltzer and Jimenez, 2009) have started to be investigated in order to reduce the problems associated with the contamination of the food.

In this work, several natural matrices were investigated as potential sources of antioxidants for polyolefins stabilization: green tea, black tea, *Lippia citriodora* and *Hypericum androsaemum*. *Lippia citriodora* is a herbal species mainly used as a spice and medicinal plant and *Hypericum androsaemum* is a medicinal plant species. These matrices were selected considering their high content in flavonoids, compounds whose high antioxidant capacity has been extensively shown (Vinson et al., 1995; Fukumoto and Mazza, 2000).

Finally, the performance of polypropylene stabilised with green tea extract, its individual components catechin or epicatechin, or a mixture of synthetic antioxidants was compared.

The melt flow index (MFI) and oxidation induction time (OIT) of the polypropylene samples was determined after multiple extrusions.

2. EXPERIMENTAL

2.1. Study of the natural plant extracts

2.1.1. Standards and reagents

Methanol (MeOH) was obtained from Merck (Darmstadt, Germany). The water was treated in a Milli-Q water purification system (Millipore, Bedford,

MA). The phenolic compounds used as references were obtained from the following sources: caffeine, catechin gallate, epigallocatechin, epigallocatechin gallate, gallic acid, gallocatechin gallate, myricetin-3-O-rhamnoside, and quercetin-3-O-glucoside from Sigma–Aldrich (Steinheim, Germany); catechin, epicatechin, epicatechin gallate, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside and quercetin-3-O-rutinoside from Extrasynthése (Genay, France); Irgafos 168 (tris(2,4-di-tert-butylphenyl)phosphate) and Irganox 1076 (octadecyl-3-(3,5-di-tertbutyl-4-hydroxyphenyl)-propionate) from Ciba (Basel, Switzerland).

2.1.2. Plant extracts preparation

Aqueous extract. About 3.0 g of each dried powdered sample were boiled for 15 minutes in 300 mL of water and then filtered through a Büchner funnel. The resulting extract was lyophilized in a Labconco 4.5 Freezone apparatus (Kansas City, MO, USA). A yield of 0.9-1.1 g based on dry-ash free basis was obtained.

Methanolic extract. About 3.0 g of dried powdered sample was mixed with 300 mL of methanol under stirring for fifteen minutes at 30ºC and then filtered through a Büchner funnel. The resulting extract was evaporated to dryness under reduced pressure at 30 ºC. A yield of 0.57 g based on dry-ash free basis was obtained.

2.1.3. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The antiradical activity of the extracts was determined spectrophotometrically in an ELX808 IU Ultra Microplate Reader (Bio-Tek Instruments, Inc.), by monitoring the disappearance of DPPH at 515 nm, according to a described procedure (Ferreres et al., 2006). For each extract, a dilution series composed of five different concentrations was prepared in a 96 well plate. The reaction mixtures in the sample wells consisted of 25 μL of aqueous extract and 200 μL of DPPH dissolved in methanol. The plate was incubated for 30 min at room temperature. Three experiments were performed in triplicate.

2.1.4. Chromatographic analysis

Qualitative analysis by HPLC-UV. The extracts were analyzed on an analytical HPLC unit (Gilson) with a photodiode array detector, using a Spherisorb ODS2 column (25.0 cm x 0.46 cm; 5μm particle size Waters, Mildford, MA, USA) with a C_{18} ODS guard column. The system solvent used was a gradient of water/formic acid (19:1) and methanol (Valentão et al., 2007).

Quantitative analysis by HPLC-UV-FL. It was carried out using a Waters Alliance 2695 system equipped with a quaternary pump, autosampler, a Waters 996 photodiode array detector and a Waters 2475 fluorescence detector. Chromatographic separation was performed on a reversed-phase SunFire C18 analytical column (3.0 x 150 mm^2, 3.5 μm Waters). Reverse phase

chromatography with methanol:water was used as mobile phase (Castro-López et al., 2007).

2.2. Study of the use of green tea extract as antioxidant in polypropylene

2.2.1. Green tea extract

Several dried organic green tea samples were accurately weighed (2 g ± 0.0001 g), added in an Erlenmeyer flask and extracted in a sonication system with 20 mL of methanol. The methanolic extracts were mixed, filtered and then, concentrated to dryness under reduced pressure (30ºC, 200 mbar). A viscous liquid was obtained and stored at 4ºC until its use.

2.2.2. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed using a Perkin Elmer TGA-7 microbalance coupled to a 1022 Perkin Elmer microprocessor. The microbalance was calibrated making use of the Curie points of perkalloy and nickel. Dynamic experiments were conducted under oxygen atmosphere. The heating rate was 10ºC min^{-1}. The temperature range of the experiments was from room temperature to 700 ºC. Catechin was heated at 100 ºC during 4 hours prior to the test.

2.2.3. Polymer processing

The polymers containing antioxidants were extruded using a Brabender DSE 20 double screw extruder with five heating zones with the following

zone temperature settings: 200/200/200/200/200/200ºC and a die head temperature of 210ºC; length/diameter (L/D): 40; screw speed: 35 rpm.

The polypropylene used was REPSOL PP044 W3F (commercially stabilised with a little amount of Irgafos 168), an homopolymer with MFI: 3.0 g/10 min (230ºC; 2.16 kg) (ISO 1133).

Polypropylene was mixed with the corresponding concentration of antioxidants according to Table 4.1.

Table 4.1. Properties of the additives used and concentrations added to the PP samples.

Sample	Additive	Melting point (ºC)[a]	Boiling point (ºC)[b]	Thermooxidative stability (ºC)[c]	Concentration (%)
1	Nonstabilized PP				-
2	Green tea extract				0.05
3	Irgafos 168	183-186	594.2 ± 50.0	259	0.1 of each compound
	Irganox 1016	50-55	568.1 ± 45.0	276	
4	(+)-Catechin	175-177	629.2 ± 55.0	227	0.05
5	(-)-Epicatechin	240	629.2 ± 55.0	265	0.05

[a] Melting point values were provided by the corresponding commercial sources.

[b] Available at https://scifinder.cas.org.

[c] Thermooxidative stability was measured at 5% mass loss.

A sample of pellets of the extruded polymer from the first pass was taken out for melt flow index (MFI) and oxidation induction time (OIT) measurements. The remaining polymer sample was re-extruded under the same conditions up to four times with polymer sampling after each pass for further analysis.

2.2.4. Assessment of thermal stability

Melt flow index (MFI) was measured using a CEAST melt flow tester at 230 ºC (2.095 mm x 8 mm die, 2.16 kg). The obtained results are mean of three measurements.

Oxidation induction time (OIT) was measured on a Perkin Elmer serie 7 DSC isothermally at 200 ºC under inert atmosphere, which was subsequently switched to oxygen atmosphere. Analyses were carried out according to EN 728. The showed results are mean of two measurements.

3. RESULTS AND DISCUSSION

3.1. Comparison of antioxidant activity of plants extracts

The antioxidant capacity of extracts obtained from four natural matrixes: green tea, black tea, *Lippia citriodora* and *Hypericum androsaemum* was measured by the DPPH assay. The DPPH assay constitutes a screening method currently used to provide basic information about the antiradical activity of extracts. Reduction of DPPH by antioxidants leads to a loss of absorbance at 515 nm (Fukumoto and Mazza, 2000). The concentration that causes a decrease in the initial DPPH concentration by 50% is defined as IC_{50}. Aqueous extracts of each matrix were prepared under similar experimental conditions. Both tea extracts showed higher antioxidant capacity (IC_{50} = 9 μg mL^{-1}) than *Lippia citriodora* (IC_{50} = 31 μg mL^{-1}) and *Hypericum androsaemum* (IC_{50} = 23 μg mL^{-1}) (Figure 4.1). A methanolic extract of green tea was also prepared showing IC_{50} = 9.6 μg mL^{-1}.

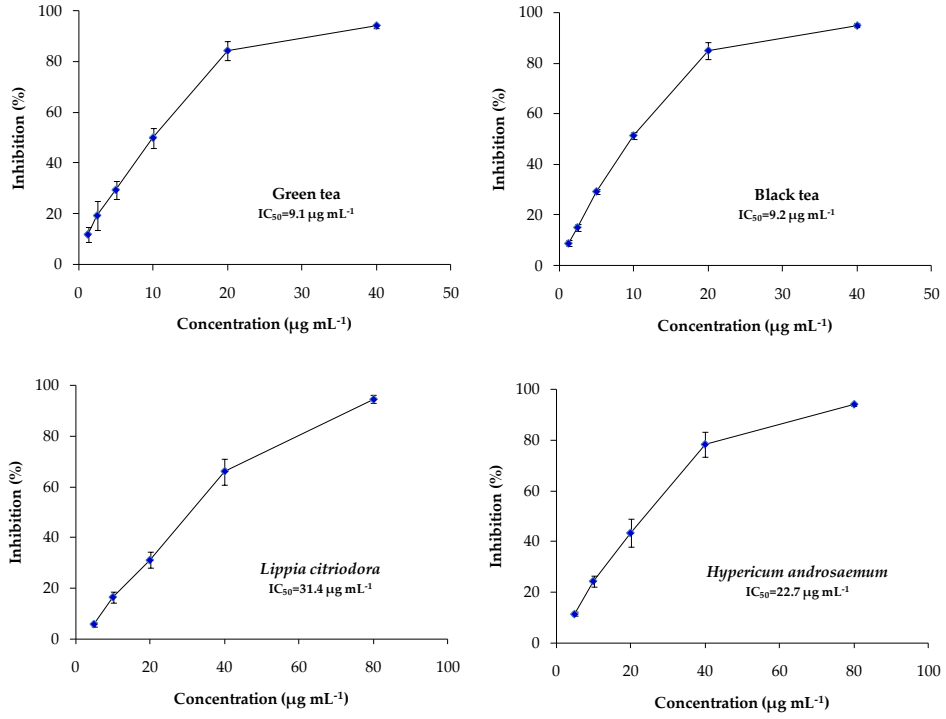

Figure 4.1. Effect of the extract on DPPH reduction. Values show mean ± standard deviation from three experiments performed in triplicate (aqueous extract).

3.2. Analysis of the extracts by HPLC

First, the qualitative phenolic profile of the selected matrices was considered. Phenolic profile of the extracts of *Lippia citriodora* and *Hypericum androsaemum* has been previously reported (Valentão et al., 2002a,b) with high content in flavonols. Both tea extracts, green and black, were analyzed by HPLC with a UV diode array detector. 12 compounds were identified (Figure 4.2 and Table 4.2): gallic acid, caffeine, 5 flavanols (catechin, epigallocatechin, epigallocatechin gallate, epicatechin, epicatechin gallate) and 5 flavonols (myricetin glycoside, quercetin glucoside, quercetin rutinoside, kaempferol

glucoside, kaempferol rutinoside). As it was expected, the most abundant compounds seem to be the flavanols.

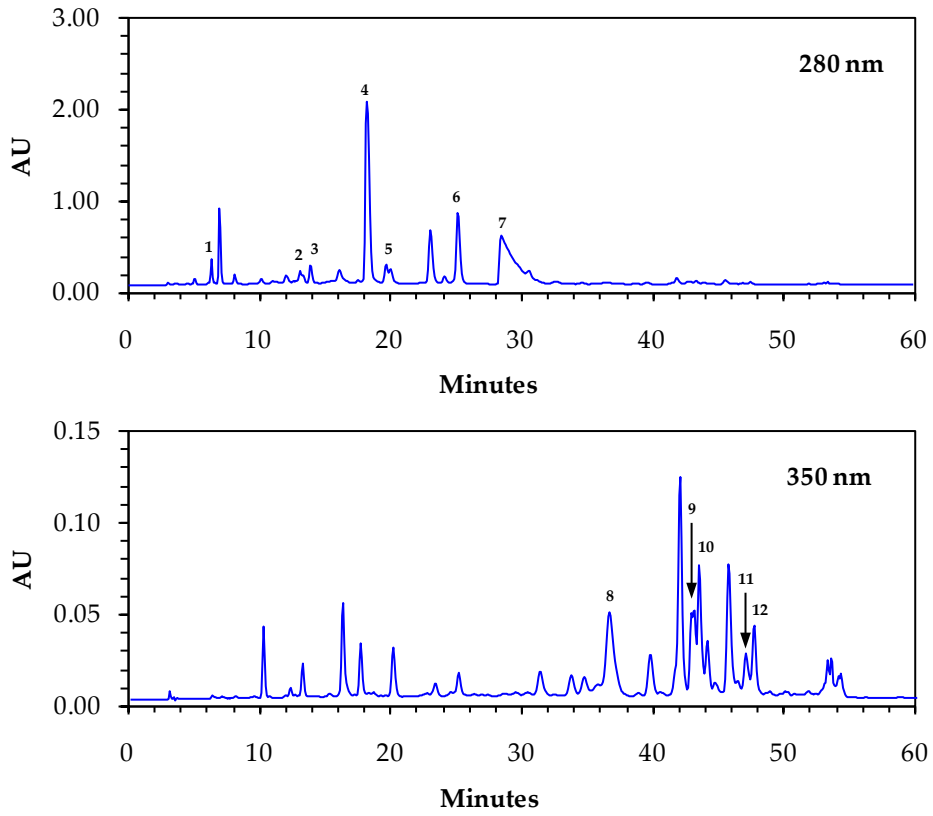

Figure 4.2. Initial identification of the green tea extract by HPLC-UV. Identification of the peaks according to Table 4.2.

According to Vinson et al., 1995, flavanols have higher antioxidant activiy than flavonols, and quercetin is the flavonol that shows to be the best antioxidant. Therefore, the second part of the chromatographic study was focused on the determination of flavanols and quercetin in the considered

extracts using a HPLC method with two detectors, UV diode array (Figure 4.3) and fluorescence (FL) (Figure 4.4). Fluorescence detector allowed determining epigallocatechin free of the interferences that appeared with the UV detector. Moreover, it improved the sensitivity of the method for some compounds such as catechin and epicatechin.

Table 4.2. Identified compounds in tea extracts by HPLC-UV.

Peak	*Compound*	**Wavelength**
1	Gallic acid	280 nm
2	Catechin[a]	
3	Epigallocatechin	
4	Epigallocatechingallate	
5	Epicatechin	
6	Epicatechingallate	
7	Caffeine	
8	Myricetin glycoside	350 nm
9	Quercetin glucoside	
10	Quercetin rutinoside	
11	Kaempferol glucoside	
12	Kaempferol rutinoside	

[a] **Peak is not pure**

Concentration of the flavanols decreased starting with green tea, through black tea, *Hypericum androsaemum* and *Lippia citriodora* (Table 4.3). Concentration of quercetin was much lower and could be only quantified in green tea and Hypericum androsaemum.

Green tea extract was selected to be tested as an antioxidant for polypropylene, considering that it showed both the highest content in

flavanols and the highest antioxidant capacity. Moreover, its individual components catechin and epicatechin were also chosen to be added to polypropylene, due to their higher stability compared with the other flavanols (Zhu et al., 2002; Su et al., 2003).

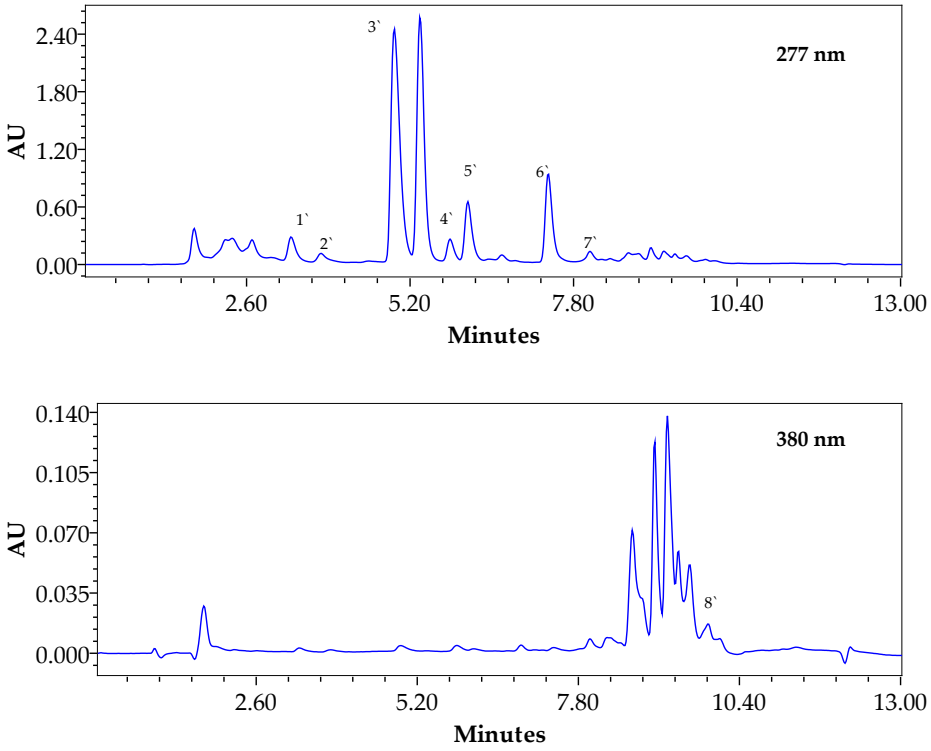

Figure 4.3. Identification and quantification of flavanols and quercetin in the green tea extract by HPLC-UV. Identification of the peaks according to Table 4.3.

3.3. Use of green tea extract as additive of polypropylene

To evaluate the performance of green tea extract as an antioxidant for polypropylene several samples were prepared: stabilised directly with the

green tea extract, with its individual components catechin or epicatechin, and with the mixture of synthetic antioxidants Irganox 1076 and Irgafos 168. They were also compared with a non-stabilized sample (Table 4.1).

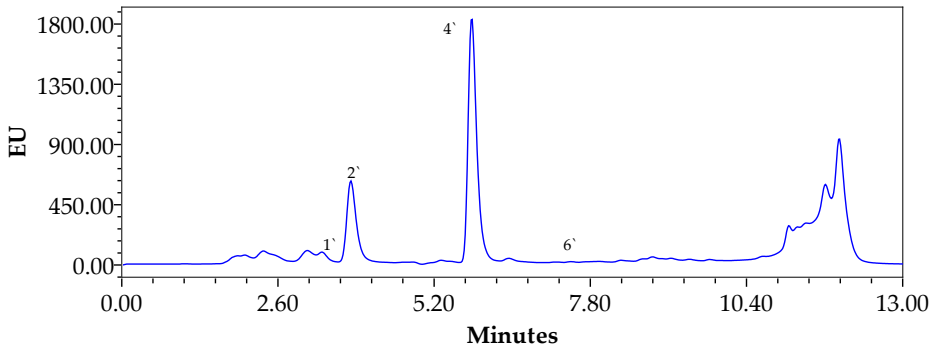

Figure 4.4. Identification and quantification of flavanols in green tea extract by HPLC-FL. Identification of the peaks according to Table 4.3.

To enable the comparison between the samples, the same processing conditions were used for all of them (see above). They were selected considering the properties of the polypropylene and the tested antioxidants (Table 4.1).

As expected, the MFI of each stabilized sample was generally lower than the one corresponding to the nonstabilized PP (Figure 4.5). Initial MFI values were similar for all the samples, but higher number of extrusion passes, higher were the differences observed. By comparing the MFI after four extrusion passes with its initial value for each sample, the highest increase was showed by nonstabilized PP (21%). The rest of the MFI increases provided the following order: Irganox 1076+ Irgafos 168 (12%) > epicatechin

(11%) > tea extract (7.8%)> catechin (3.5%). Therefore, formulations containing natural antioxidants have provided better melt flow property after consecutive processing compared with the commercially tested antioxidants and the nonstabilized PP.

Table 4.3. Concentration of flavanols and quercetin in natural extracts determined by HPLC-UV-FL.

		$mg_{compound}\ g^{-1}_{sample}$							
		Green tea		**Black tea**		***Hypericum androsaemun***		***Lippia citriodora***	
Peak		PDA	FL	PDA	FL	PDA	FL	PDA	FL
1´	(-)-Epigallocatechin	*	29.2	*	4.95	nd	nd	nd	nd
2´	(+)-Catechin	3.47	2.89	0.93	0.67	0.95	0.58	nd	nd
3´	(-)-Epigallocatechin gallate	72.0	nd	13.3	nd	nd	nd	nd	nd
4´	(-)-Epicatechin	5.5	5.23	0.75	0.96	0.49	0.46	nd	nd
5´	(-)-Gallocatechingallate	10.5	nd	1.37	nd	nd	nd	nd	nd
6´	(-)-Epicatechin gallate	13.1	10.3	4.84	4.59	nd	nd	nd	nd
7´	(-)-Catechin gallate	0.98	nd	0.31	nd	nd	nd	nd	nd
	Total flavanols	105	47.6	19.8	11.2	1.45	1.04	nd	nd
8´	Quercetin	0.043	nd	nq	nd	0.71	nd	nd	nd

*: Peak not pure; nd: not detectable; nq: not quantificable

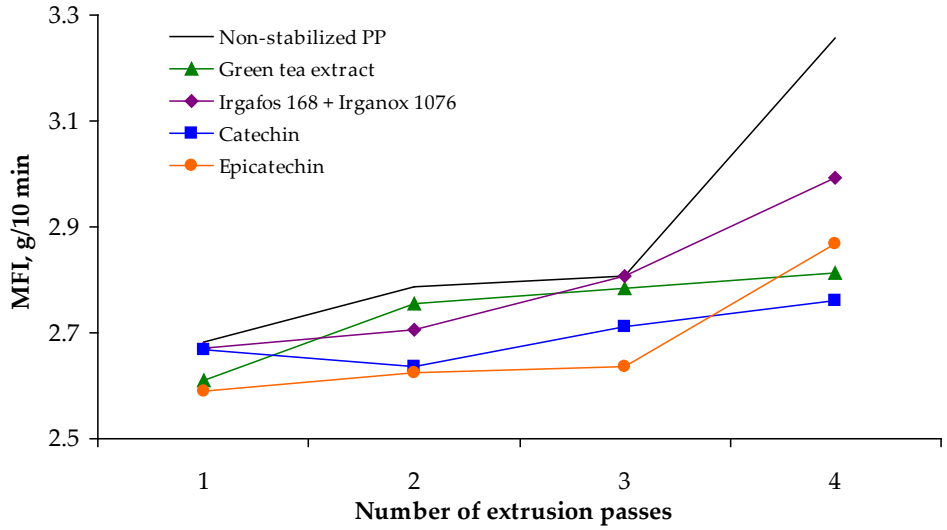

Figure 4.5. Stability of PP with different antioxidants or green tea extract: MFI measurements (n= 3) (López-Vilariño et al., 2006).

To further asses the oxidation stability of the samples, oxidation induction time (OIT) was also measured by DSC (Figure 4.6). The longest OIT obtained for catechin and epicatechin confirmed that these compounds provided polypropylene with stabilization against thermal-oxidation degradation. It is worth to remark that the better performance achieved using natural antioxidants compared with the synthetic ones was obtained using lower amounts of additives (Table 4.1).

The mechanism of action of the synthetic antioxidants used is already known (Figure 4.7) (Al-Malaika et al., 1999; Al-Malaika, 2003). Regarding catechins, although their high antioxidant capacity has been extensively shown, little is known about their antioxidative mechanisms. According to Bors et al., 1990, catechins satisfy one of the criteria for effective radical scavenging: the o-

dihydroxy structure of their B ring (Figure 4.8) which confers higher stability to the radical form and participates in electron delocalization for effective radical scavenging. But in the last years other possibilities to explain the effectiveness of catechins have been proposed.

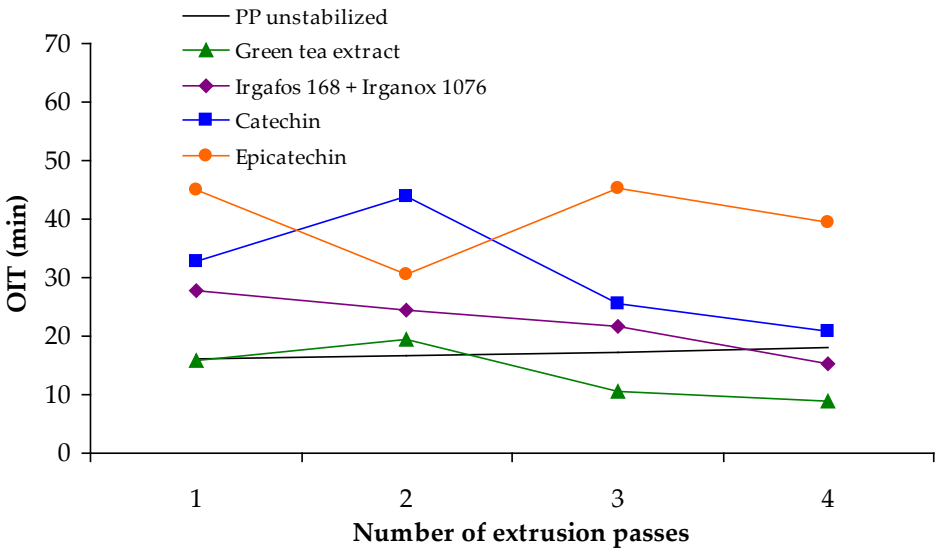

Figure 4.6. OIT of PP containing different antioxidants or green tea extract as measured by DSC (n=2).

Therefore, in a theoretical study about the chemical reactivity properties of (+)-catechin and (-)-epicatechin Mendoza-Wilson and Glossman-Mitnik, 2006, found that the preferential sites for radical attack would be the C6 of the ring A and the H4′ (hydroxyl group) of the ring B . On the other hand, Kondo et al., 1999, carried out a study to try to elucidate the antioxidative mechanisms

of catechins that showed their affectivity in scavenging peroxyl radicals both in a liposomal system and in an aqueous system.

Hindered phenols antioxidants

$ROO^{\bullet} + Ar\text{-}OH \rightarrow ROOH + Ar\text{-}O^{\bullet}$

$ROO^{\bullet} + Ar\text{-}O^{\bullet} \rightarrow$ Inactive products

Aryl phosphite antioxidants

$(OR)_3P + ROOH \rightarrow (OR)_3P{=}O + ROH$

$(OPh)_3P + ROO^{\bullet} \rightarrow [ROOP^{\bullet}(OPh)_3] \rightarrow (OPh)_3P{=}O + RO^{\bullet}$

$(OPh)_3P + RO^{\bullet} \rightarrow [ROP^{\bullet}(OPh)_3] \rightarrow (RO)(OPh)_2P + PhO^{\bullet}$

$PhO^{\bullet} + ROO^{\bullet} \rightarrow$ Inactive products

Figure 4.7. Scheme of the mechanism of action of antioxidants (Al-Malaika et al., 1999; Al-Malaika, 2003).

The authors proposed tentative antioxidative mechanisms of catechins depending on the experimental results and theoretical calculations that suggest that hydrogen at the C-2 position may be abstracted by free radicals (Figure 4.8). Moreover, they found out that the compound produced from epicatechin by radical oxidation can also function as an antioxidant and, as a result, epicatechin has a longer inhibition period. This last one may explain the better performance of the material containing catechin or epicatechin after multiple extrusions.

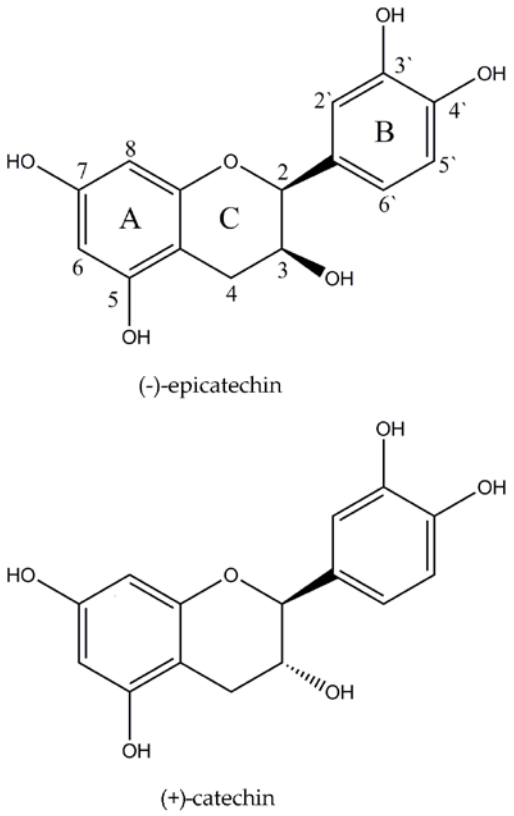

Figure 4.8. Structures of (+)-catechin and (-)-epicatechin.

4. CONCLUSIONS

1. Extracts of green and black tea showed higher antioxidant capacity than other considered plants: *Lippia citriodora* and *Hypericum androsaemum*.

2. Flavanols and quercetin were quantified in the selected extracts by HPLC-UV-FL, considering that these phenolic compounds theoretically show the highest antioxidant capacity. Their content in flavanols decreased in the same order than their antioxidant activity.

3. The possibility of using as antioxidant either an extract of green tea or their individual components catechin or epicatechin in polypropylene was tested. To evaluate the stabilization due to the presence of additives the melt flow index and the oxidation induction time of the samples were measured after multiple extrusions. The stability of the material was comparable to the one stabilised with synthetic antioxidants, showing the interest of this matrix as a potential source of natural antioxidants for plastics.

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3.4. DESARROLLO DE MATERIALES CAPACES DE REALIZAR UNA CESIÓN CONTROLADA DE ANTIOXIDANTES: ENVASES ACTIVOS Y EXTRUSIÓN REACTIVA

Artículo 5: Effect of PPG-PEG-PPG on the tocopherol-controlled release from films intended for food-packaging applications.

Artículo 6 / Patente: Procedimiento de obtención de un material polimérico.

Artículo 7: Improving the capacity of polypropylene to be used in antioxidant active films: incorporation of plasticizer and natural antioxidants.

Artículo 8: Interaction and release of catechin from anhydride maleic grafted polypropylene films.

3.4. DESARROLLO DE MATERIALES CAPACES DE REALIZAR UNA CESIÓN CONTROLADA DE ANTIOXIDANTES: ENVASES ACTIVOS Y EXTRUSIÓN REACTIVA

Artículo 5

EFFECT OF PPG-PEG-PPG ON THE TOCOPHEROL-CONTROLLED RELEASE FROM FILMS INTENDED FOR FOOD-PACKAGING APPLICATIONS

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ABSTRACT

The feasibility of novel controlled release systems for the delivery of active substances from films intended for food packaging was investigated. Being polyolefins highly used for food packaging applications, the reported high retention degree of antioxidants has limited their use for active packaging. Thus, in this study, PP films modified with different chain extenders have been developed to favour and control the release rates of the low molecular weight antioxidant tocopherol. The use of different chain extenders as polymer modifiers (PE-PEG M_w, 575; and PPG-PEG-PPG M_w, 2000) has caused significant changes in tocopherol specific release properties. HPLC coupled to PDA-FL and PDA-MS were used to test tocopherol and chain extender migration, respectively. The release of tocopherol from the prepared films with two chain extenders into two food simulants was studied. Different temperatures and storage times were also tested. Varying the structural features of the films with the incorporation of different levels of PPG-PEG-PPG, the release of tocopherol (food packaging additive) into different ethanolic simulants could be clearly controlled. The effect of the temperature and storage time on the release of the antioxidant has been outstanding as their values increased. The migration of the chain extender, also tested, was well below the limits set by European legislation.

Keywords: PPG-PEG-PPG, tocopherol, controlled release.

1. INTRODUCTION

Traditionally, food packaging provides protection against contamination by external agents such as water, light or odorants; however, increasing demands for greater safety and quality have led to the development of new concepts in food packaging (Vermeiren et al., 1999). Active and intelligent packagings are intended to prevent or retard any deterioration quality of packaged foods by including the concept of the controlled release of active compounds to foodstuffs. Thus, they show a great potential to improve storage stability without adding an excess of additives to food (Vermeiren et al., 1999; LaCoste et al., 2005; López-Rubio et al., 2006), which could also cause neutralization or rapid diffusion into the bulk of food (Mastromatteo et al., 2010).

Polymeric materials react with oxygen producing a chemical aging or degradation of the polymer which may be associated to irreversible changes in their chemical structure such as reduction in molecular weight, increased melt flow index and worsened physical and mechanical properties (Kausch, 2005; Singh and Sharma, 2008). Therefore, the use of stabilizers against mechanical and thermo-oxidative phenomena is a key factor to preserve polymer physical and mechanical properties over time (Garde et al., 2001; Ambrogi et al., 2011).

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or Irganox 1076 has been extensively used (Rababah et al., 2004; Dopico-García et al., 2007). Their low molecular weight also makes suitable their migration from the package into foodstuffs. Nevertheless, there has been an emerging concern regarding their possible

toxicity and carcinogenic potential (Ito et al., 1986; Botterweck et al., 2000; Rababah et al., 2004; Dopico-García et al., 2007).

In short, antioxidants are widely used into plastic formulation both to protect the film from degradation and to improve oxidation stability of food lipids (Laermer, 1990; Sharma et al., 1990). They can prolong food shelf-life since they can be released in a controlled manner from the antioxidant active packaging to the food (López-Rubio et al., 2006).

An increasing interest in the application of natural antioxidants such as tocopherol, carnosic acid, oregano, savory, and essential oils, carvacrol or hydroxytyrosol (Dopico-García et al., 2011) has been recently developed. Besides being effective antioxidants for reducing oxidation in foods, tocopherols are also excellent stabilizers for polymer processing since they have proved to be very stable under processing conditions and very soluble in polyolefins (Al-Malaika et al., 1999; Koontz et al., 2010). Therefore, tocopherols could serve dual functions when added to packaging: as a stabilizer for polymer processing and as an antioxidant in controlled release to reduce oxidation. They are also non-toxic compounds with a positive public perception classified as substances generally recognized as safe (GRAS) for intended to be used in food (Code of Federal Regulations, 2008).

Low-density polyethylene (LDPE) and, specially, polypropylene (PP) are two of the most commonly used polymers in packaging applications involving contact with food (Wessling et al., 1999). The use of antioxidants is especially essential to preserve PP due to its numerous tertiary carbons which are very sensitive to oxidation and radical degradation (Reingruber and Buchberger, 2010; Ambrogi et al., 2011). However, high retention degree of

tocopherols on both LDPE and PP in contact with foodstuffs and food-simulating liquids has been reported being practically total in the latter (Wessling et al., 1998; Wessling et al., 1999; Heirlings et al., 2004). Thus, the use of tocopherols as additives for active PP packaging materials may be limited. In that sense, β-cyclodextrin complexation with antioxidants has been recently used as strategy to try to control antioxidant delivery mainly from LLDPE (Koontz et al., 2010a ,b).

Physical, chemical, mechanical, aesthetic and processing polymer's properties can be enhanced or modified by the incorporation of additives. Chain extenders have been blended into polymer matrixes to modify polymeric chain extension, which lead to enhance physicochemical polymer properties such as stability, degradability or permeability (Skarja and Woodhouse, 2000; Semsaezadeh et al., 2008; Zheng et al., 2011). In this sense, for example, polyethylene glycol has been recently widely used as chain extender in order to improve biocompatibility and degradation rates of polyesters thus modifying their properties (Liu et al., 2012). All those applications have been reported for polymers with certain polar character such as poly(lactic acid), poly(lactic-co-glycolic acid) or polyurethane since the chemical bond between the polymeric matrix and the chain extender should be implemented by the presence of groups able to interact with the hydroxyl groups of the latter. However, little or none applicability in the food packaging field has been reported for them.

Therefore, in this work two block copolymers of polyethylene glycol (M_w 575) and polypropylene glycol (M_w 2000) were tested as chain extenders additives for the preparation of porous PP films via extrusion process. The

effects of type and amount of chain extender used into film's formulation on the release of the antioxidant tocopherol from the polymer were studied. The influence of the type and amount of tocopherol as well as the type of foodstuffs or food simulant in contact, temperature and time conditions were also discussed. Some physical properties of the blend films were also studied.

2. EXPERIMENTAL

2.1. Materials

PP ISPLEN^{R} PP 070 G2M was provided by Repsol YPF. Nutrabiol T90, Tocobiol PV and Nutrabiol T50 PV were supplied by BTSA (Madrid, Spain). Irgafos 168 (Tris(2,4-di-tert-butylphenyl)phosphate; I168), Irganox 1010 (Pentaerythritol tetrakis(3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate, I1010), polyethylene-block-poly(ethylene glycol) (PE-PEG) and poly(propylene glycol)-block-poly(ethylene glycol)-block-poly(propylene glycol) (PPG-PEG-PPG) were supplied by Sigma-Aldrich (Steinheim, Germany).

Methanol and ethanol (EtOH) HPLC-gradient for instrumental analysis were supplied by Merck (Darmstadt, Germany). Formic acid (98-100 % puriss p. a.) was from Sigma-Aldrich. Water was purified using a Milli-Q Ultrapure water-purification system (Millipore, Bedford, MA, USA). Table 5.1 shows the composition of the different antioxidants tested (information provided by the company).

Table 5.1. Composition of the different tocopherol-based antioxidants tested

TOCOBIOL-PV		NUTRABIOL-T50 PV		NUTRABIOL-T90	
Tocobiol	65.0%	Tocopherols	50.3%	Tocopherols	90.2%
Tocopherols	18.6%	α-tocopherol	12.5%	α-tocopherol	15.5%
Sterols	9.7%	γ-, β-tocopherol	59.6%	γ-, β-tocopherol	63.3%
Squalene	4.3%	δ-tocopherol	27.9%	δ-tocopherol	21.1%
Monoglycerides	19.8%	Vegetable oil	9.7%	Vegetable oil	9.8%
Rest: vegetable oil	35.0%	Excipient: silica gel	40.0%		
Excipient: silica gel					

2.2. Polymer samples manufacture

The monolayer compounding packaging were prepared by and subsequently extrusion of PP, commercial antioxidants I168 and I1010, tocopherol based additives Nutrabiol T90, Tocobiol PV and Nutrabiol T50 PV and/or PE-PEG and PPG-PEG-PPG chain extenders at different composition ratios (Table 5.2).

Extrusion was carried out using a miniextruder equipped with twin conical co-rotating screws and a capacity of 7 cm^3 (Minilab Haake Rheomex CTW5 (Thermo Scientific)). Screw rotation rate of 40 rpm, temperature of 180 ºC and 1 minute of residence time were used.

2.3. Release studies

Release tests were performed by total immersion of rectangular strips film pieces (80 mm x 0.4 mm x 0.17 mm) in 9 mL of food simulant contained in a glass-stoppered tubes with PTFE closures. The migration test parameters

were based on the European Commission Regulation Nº 10/2011 (The European Commission, 2011).

Table 5.2. Composition of the prepared film samples and OIT values for stabilized and non-stabilized PP at 200 ºC.

Sample code	Matrix (PP)	Commercial antioxidants		Tocopherol based additives			Extensors		OIT values (min^{-1})
		I168	I1010	Nutrabiol T90	Tocobiol PV	Nutrabiol T50 PV	PE-PEG	PPG-PEG-PPG	
M1	X	--	--	--	--	--	--	--	4.200
M2	X	0.2	--	--	--	--	--	--	4.500
M3	X	--	0.4	--	--	--	--	--	24.00
M4	X	0.2	--	5	--	--	--	--	133.9
M5	X	0.2	0.4	--	--	--	--	--	56.01
M6	X	0.2	0.4	5	--	--	--	--	188.1
M7	X	--	--	5	--	--	--	--	140.4
M8	X	--	--	1	--	--	--	--	53.43
M9	X	--	--	0.5	--	--	--	--	29.85
M10	X	--	--	--	5	--	--	--	28.14
M11	X	--	--	--	1	--	--	--	25.06
M12	X	--	--	--	0.5	--	--	--	15.17

Table 5.2. (Continuation I).

Sample code	Matrix (PP)	Commercial antioxidants		Tocopherol based additives			Extensors		OIT values (min^{-1})
		I168	I1010	Nutrabiol T90	Tocobiol PV	Nutrabiol T50 PV	PE-PEG	PPG-PEG-PPG	
M13	X	--	--	--	--	5	--	--	92.14
M14	X	--	--	--	--	1	--	--	36.63
M15	X	--	--	--	--	0.5	--	--	39.81
M16	X	0.2	--	1	--	--	--	--	51.54
M17	X	0.2	--	1	--	--	1	--	50.82
M18	X	0.2	--	1	--	--	2	--	51.20
M19	X	0.2	--	1	--	--	5	--	45.54
M20	X	0.2	--	1	--	--	--	1	47.00
M21	X	0.2	--	1	--	--	--	2	41.01
M22	X	0.2	--	1	--	--	--	5	32.89

Two food simulants A (10% ethanol) and D_1 (50% ethanol) were selected; the release tests were conducted at 4 ºC and 40 ºC. Samples of all the

treatments were taken at 1, 2, 5 and 10 days of storage. Test materials were also run simultaneously to check for interferences.

After the contact period, the film samples were removed and the simulants were levelled up to 10 mL. An aliquot was filtered through an Acrodisc^{R} PTFE CR 13 mm, 0.2 μm filters (Waters, Mildford, MA, USA) and analyzed by HPLC. As the polymer test pieces were less than 0.5 mm thick (European Committee for Standardization, 2004), the surface area of only one side of the film was taken into account to determine the migration value.

Measurements of the stability of the antioxidants were made in the two selected simulants under selected exposure conditions, by storing a solution of the additive in the simulant in parallel with the migrations tests. Analyses were carried out using the same procedure as for the samples.

The release process is normally described by the kinetic of the diffusion of the antioxidant in the film and is expressed by the diffusion coefficient (*D*). *D* is usually estimated using the Fickian diffusion model (Chen et al., 2012). When release of antioxidant reached equilibrium, eq. 1 is used as the rigorous model for describing the migration controlled by Fickian diffusion in a packaging film:

$$\frac{M_t}{M_{F,\infty}} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[\frac{-D(2n+1)^2 \pi^2 t}{L_P^2}\right] \quad (1)$$

M_t, mass of the migrant in the food at a particular time t(s); $M_{F,\infty}$, mass of migrant in the food at equilibrium; L_P (cm), film thickness; *D* (cm^2 s^{-1}), diffusion coefficient; t(s), time.

Nevertheless, when release is slow and equilibrium is not reached at the end of the experiment, eq. 2 can be used when Mt/MP is < 0.6:

$$\frac{M_t}{M_P} = \frac{4}{L_P}\left(\frac{Dt}{\pi}\right)^{0.5} \quad (2)$$

M_P, initial loading of antioxidants in the film. *D* is estimated from the slope of the plot of M_t/M_P versus $t^{0.5}$.

2.4. Methods

2.4.1. Dynamical rheological properties

The influence of PE-PEG and PPG-PEG-PPG on the dynamic rheological properties of the films was studied using the miniextruder. Measurements of viscosity and shear stress versus share rate were carried out for film samples containing I168 (0.2%), Nutrabiol T90 (1%) and PE-PEG or PPG-PEG-PPG (5%). Test materials without polyethylene glycol copolymers were also tested. Measurements were carried out at 180 ºC in the frequency region from 5 to 200 rpm.

2.4.2. Differential scanning calorimetry

Film samples of the extruded polymers (Table 5.2) were taken out for oxidation induction time (OIT) measurements. OIT was measured on a Perkin–Elmer serie 7 DSC isothermally at 200 ºC under inert atmosphere, which was subsequently switched to oxygen atmosphere. Analyses were carried out according to EN 728:1997 (European Committee for Standardization, 1997). The obtained results are mean of two measurements.

2.4.3. Chromatographic methods conditions

2.4.3.1. HPLC-PDA-FL. A Waters 2695 (Waters, Mildford, MA, USA) high performance liquid chromatography (HPLC) system with a gradient pump and automatic injector was used for the HPLC analysis. The chromatographic experiments were carried out using a stainless steel column 150 mm x 3.0 mm packed with SunFire™ C_{18}, 3.5 μm particle size (Waters) kept at 35 ºC. Detection was carried out using a model 996 UV photodiode array detector (PDA) and a model 2475 fluorescence detector (FL) (Waters). PDA detection was performed in the range between 200 and 500 nm. 295 nm was selected as quantification wavelength. $\lambda_{excitation}$ 295 nm and $\lambda_{emission}$ 325 nm were selected for fluorescence quantification. The output signals were monitored and integrated using a personal computer operated under the Empower™ software (Waters). A two solvent gradient elution was performed, with flow rate of 0.5 mL min^{-1} and injection volume of 20 μL. Mobile phase was composed by water (A) and methanol (B). The following gradient elution profile was used: mobile phase composition started at 30% of B and maintained for 0.5 min. Then, it was linearly increased to 90% B in 2 min, maintained 1 min, and linear increases to 100% B in 0.5 min. Finally, it was maintained for 10.5 min and brought back to the initial conditions.

Each compound was identified by the comparison between its retention time employing spectroscopic and fluorescence detection with corresponding peaks in the standard solution and its UV spectrum. The quantification of the analytes was carried out using a calibration plot of an external standard.

2.4.3.2. HPLC-Mass Spectrometry (MS). An Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) equipped with an on-line degasser, a binary pump delivery system, a high performance SL autosampler, a thermostated column department and on-line coupled to a mass spectrometer detector (MS) was used for analysis. Samples were filtered through a 0.2 μm Acrodisc^{R} PTFE CR (Waters) and injected in Zorbax SB-C18 (50 x 2.1 mm, 1.8 μm) column (Agilent Technologies). A mobile phase system consisting of a mixture of water-0.1% formic acid (A) and methanol (B) under the following gradient system was used: mobile phase initially set at 30% B was linear increased to 100% B in 3 min, and maintained for 13 min. It was then brought back to initial conditions. The mass spectrometer was an Agilent 6410 Triple Quad LC/MS (Agilent Technologies). The column effluent was directly introduced into the triple quadrupole mass detector operated in a positive ionization mode. Ions were formed using electrospray ionization (ESI). The following ESI source parameters were used: temperature of the drying gas (N_2) was set to 350 ºC and flowed at 10 mL min^{-1}. The nebulizing pressure (N_2) was maintained at 35 psi. Capillary voltage was set at 4 kV. Integration and data elaboration were performed using Agilent MassHunter Workstation software, version B03.00 (Agilent-Technology, Santa Clara, USA). The full mass scan range was m/z 50–1000 (1 s/scan) and the target ions generated by PE-PPG and PPG-PEG-PPG were: $(M+H)^+$: m/z 637, 652, 681, 695. Selective ion monitoring (SIM) was used to quantify the target ions. Mass spectral data and retention time were used for peak identification. Quantification of extender was based on an external standard calibration method.

2.4.4. Spectroscopic characterization of the chain extender.

PPG-PEG-PPG chain extender stability was tested through its chemical structure by means of Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR).

FTIR was performed in the transmission mode with an OPUS/IR PS 15 spectrometer (Bruker, Germany). The spectra were the results of 32 coadded interferograms at 2 cm^{-1} resolution between 400 and 4000 cm^{-1}, using a cell specially designed for liquids.

^{1}H NMR spectra were measured at room temperature by a Bruker AVANCE 500 (Bruker) spectrometer. Samples dissolved in D-chloroform ($CDCl_3$) at a concentration of $1*10^{-4}$ M were tested to determinate the chemical structure.

3. RESULTS AND DISCUSSION

Several commercial antioxidants formulated with tocopherol are comercially available. Among them, Nutrabiol T90, Tocobiol PV and Nutrabiol T50 PV were assayed as tocopherol based additives in this paper (Table 5.1). Thermal stability of these commercial tocopherol based antioxidants under processing temperature significantly differed from that of naturally occurring tocopherols. According to Barbosa-Pereira et al., 2013, dynamic experiments conducted under oxidant atmosphere in a thermo balance has indicated that degradation process of that antioxidants did not begin until roughly 250 ºC, well above PP processing temperature (180 ºC).

The most appropriate tocopherol based additive was firstly selected through OIT values.

Aimed at controlled release, migration of tocopherol from processed films into food simulants was then measured. To the desired film application, the factors influencing the migration process where tested considering the film application.

3.1. Assessing chain extender influence on processability

To predict the behaviour during material handling when chain extenders are used in films formulations, dynamic rheological properties of the films were studied. The rheological curves of PP films containing two types of chain extenders: PE-PEG and PPG-PEG-PPG are shown in Figure 5.1 and compared with PP without chain extender. As shear was increased, viscosity decreased. Until roughly $136 s^{-1}$, all the materials showed a similar decrease in viscosity; nevertheless, over it, PP with PPG-PEG-PPG showed a greater decrease in viscosity, which may prevent its processing and comparison with the other formulations under the same conditions. Thus, $136\ s^{-1}$ (roughly 40 rpm) were selected as the most adequate processing conditions.

3.2. Selection of the most suitable additive

As the OIT is a relative measure of the degree or level of stabilization of the material tested, the longer the OIT, the more stable is the material. To assess the oxidation stability of the samples with different proportions of Nutrabiol T90, Tocobiol PV and Nutrabiol T50 PV, OITs of pure and doped

PP were measured by means of DSC. They were also compared with samples doped with synthetic antioxidants, Irganox 1010 and Irgafos 168. The influence of the chain extenders (PE-PEG and PPG-PEG-PPG) on the oxidation stability of the samples was also tested. The obtained results for OIT at 200 ºC are compiled in Table 5.2.

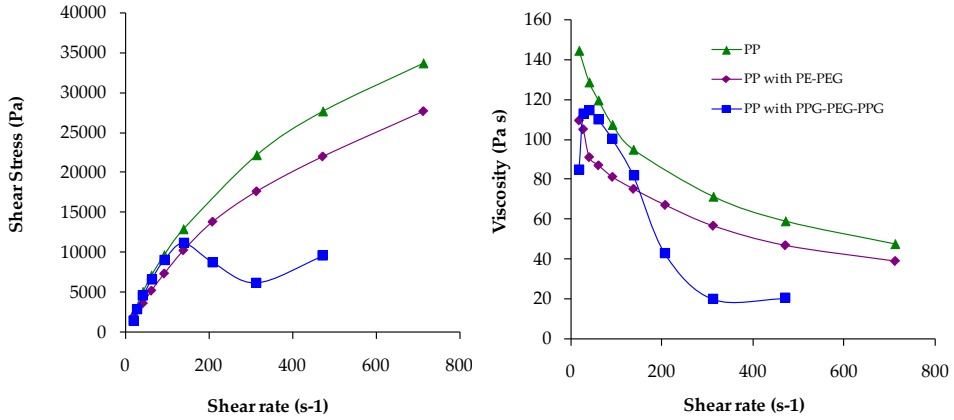

Figure 5.1. Rheological curves of PP films containing two types of chain extenders: PE-PEG and PPG-PEG-PPG.

Tocopherol based additives showed very good performance as stabilizers of PP, as higher values for OIT than those for the control samples were achieved.

As it was expected, there were no important differences in OIT values between the non-doped PP and Irgafos 168 doped PP (M2) samples (4.20 vs 4.50, respectively). It is because Irgafos 168 acts as a decomposer of hydroperoxides and OIT only measures stabilization if the compounds act as

hydrogen atom donor (Cerruti et al., 2009). Doping PP with a mixture of Irgafos 168 and Irganox 1010 led to a higher stabilization. From Table 5.2, it is evident that Nutrabiol T90, Tocobiol PV and Nutrabiol T50 PV increased PP oxidative stability at almost the same extent of Irganox 1010, even though different amounts of Irganox 1010 and tocopherol based additives were used (0.4 % vs 0.5 to 5 % respectively). OIT values for the stabilized PP increased with the concentration for all the tocopherol based additives in agreement with the fact that in polymer matrices containing fairly uniform antioxidant dispersions, the OIT increases approximately linearly with increasing antioxidant concentration (Phease et al., 2000). The addition of 0.5% of antioxidant did show stabilization as OIT was prolonged for more than 20 minutes. It is a clear indication of the good stabilization obtained with the addition of low amounts of antioxidant to PP. Furthermore, at the same concentration level, the maximum onset time was measured for Nutrabiol T90 confirming that in the range tested, the highest content of tocopherol led to the highest PP stabilization (Nutrabiol T90>Nutrabiol T50 PV>Tocobiol PV).

3.3. Release of tocopherol from film into food simulant

The use of tocopherols as additives for active PP packing materials has been limited by their practically total lack of migration into foodstuffs and food simulating liquids (Wessling et al., 1999). To achieve a useful migration level of tocopherol, the possibility of including PE-PEG and PPG-PEG-PPG in PP formulation was tested. The preparation of porous PP films with chain extender additives was via extrusion process. OITs of those pure PP and doped with different proportions of PE-PEG and PPG-PEG-PPG showed an

almost negligible influence of the chain extender onto the oxidation stability of the sample; even though a very slight decrease of OIT is observed by increasing the amount of chain extender in the polymeric formulation.

The effects of film composition as well as thermal and temporal conditions on the antioxidant levels released from the film were tested. 1% of Nutrabiol T90 was selected as the most appropriate concentration for the release studies since some tocopherol exudation from PP doped with 5% of Nutrabiol T90 was observed.

3.3.1. Effect of chain extender on the release of tocopherol.

To determine the effect of the incorporation of chain extender on the release of tocopherol from the film, two block copolymers of polyethylene glycol and polypropylene glycol of different molecular weight: PE-PEG (M_w: 575) and PPG-PEG-PPG (M_w: 2000) at three levels of concentration (1, 2 and 5%) were tested as chain extender additives for the preparation of porous PP films (formulations M17 to M22, Table 5.2). They were brought into contact with 50% ethanol (simulant D_1). The release tests were conducted at 40 ºC. Samples were taken at 1, 2, 5 and 10 days of storage and analysed by means of HPLC-PDA-FL. Test materials (M16, Table 5.2) were also run simultaneously to check for interferences. Figure 5.2 shows the migration of Nutrabiol T90 as delta-tocopherol because this showed to be the most stable isomeric form of tocopherol (coefficient of variation <1% for delta-tocopherol vs CV>10 and 60% for gamma and alpha-tocopherol, respectively, under the same studied conditions). The use of PE-PEG as chain extender has barely changed the ability of the polymer to release tocopherol into the food simulant D_1 in

comparison with the blank samples. Nevertheless, PPG-PEG-PPG showed a significant effect with regard to the antioxidant migration into the food simulant D_1. The maximum levels of tocopherol released from films containing PPG-PEG-PPG were between 2 to 3.6-fold higher than the corresponding films containing PE-PEG.

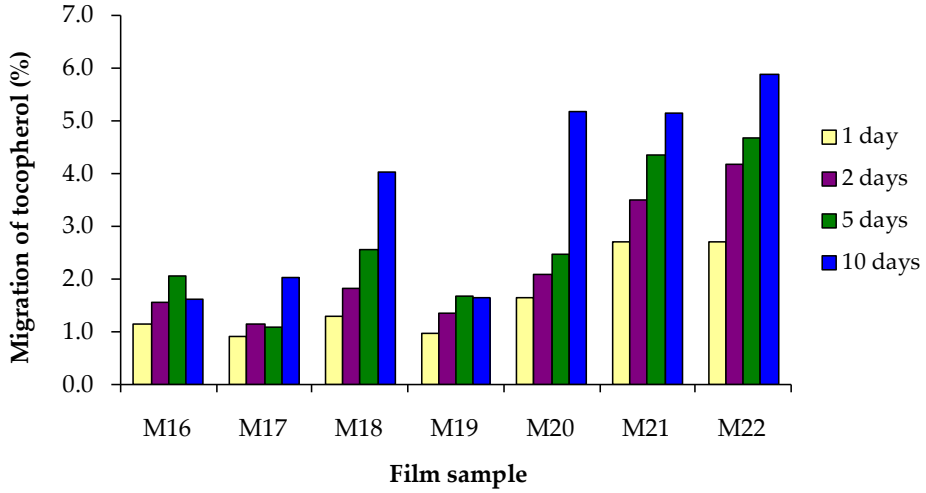

Figure 5.2. Influence of chain extender on release of tocopherol from films M16 to M22 in contact with simulant D_1 at 40ºC, 1 to 10 days.

Moreover, Figure 5.2 shows the gradual increase of antioxidant released from the film as the amount of chain extender is increased (just about 2-fold). It could be showed a slight increase in film porosity when PPG-PEG-PPG is used as chain extender (Zhang et al., 2010). Diffusion coefficients (Table 5.3), estimated by eq. 2 (section 2.3) also showed that the release of tocopherol was accelerated by the presence of the chain extender. In the absence of PPG-PEG-

PPG, *D* (cm^2 s^{-1}) of $2.9x10^{-13}$ at 40 ºC and into simulant D_1 was estimated. When the chain extender is incorporated, D values ranging from $2.4x10^{-12}$ to $2.0x10^{-11}$ cm^2 s^{-1} were calculated. Hence, adding PPG-PEG-PPG increased tocopherol diffusivity between one and two orders of magnitude. Moreover, compared to reported literature diffusion coefficients for release of tocopherol from PP films (Chen et al., 2012), the data from the present work also showed how the incorporation of PPG-PEG-PPG has significantly improved the release of tocopherol from that material (*D* of at least 5 orders of magnitude higher than that reported on literature).

Table 5.3. Estimation of diffusion coefficient (*D*, cm^2 s^{-1}) for the release of tocopherol from the studied films to simulants A and D_1 at 4 and 40ºC.

Sample	**Simulant A**		**Simulant D_1**	
	4 ºC	**40 ºC**	**4 ºC**	**40 ºC**
M16	Not estimated	4.9E-18	7.8E-17	2.9E-13
M20	1.6E-18	5.5E-16	7.2E-15	1.9E-11
M21	1.3E-17	2.4E-18	9.6E-15	2.4E-12
M22	5.2E-15	2.1E-15	1.4E-13	3.1E-12
D estimated by eq. 2, section 2.3.				

3.3.2. Effect of contact time and temperature on the release of tocopherol.

Both, time and temperature during the contact period showed a significant effect with regard to the antioxidant migration of tocopherol from PP+PPG-PEG-PPG systems into food simulant.

Regarding time, release levels of tocopherol were studied over the following range: 1 to 10 days. Samples analysed by means of HPLC-PDA-FL

showed that increasing amounts of tocopherol were released from the films into food simulant over time (Figure 5.2). At the end of the storage period the maximum had not been reached yet. Therefore, the release was expected to continue. A controlled release of antioxidant with time was then proved.

Release tests of tocopherol from PP were also done for two different temperatures: 4 ºC aimed to packaging materials for refrigerated food and 40 ºC to simulate room temperature conditions. The results obtained using PPG-PEG-PPG as chain extender and D_1 as food simulant are reported in Figure 5.3.

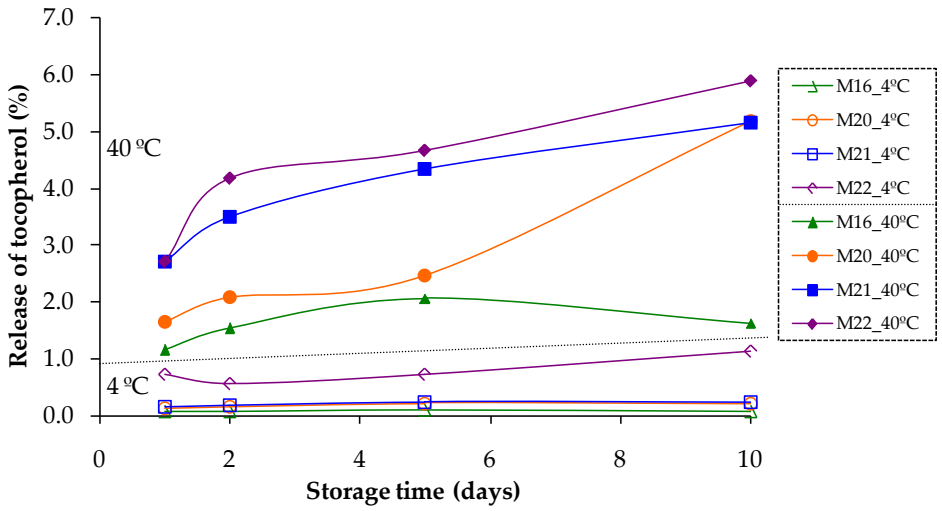

Figure 5.3. Influence of temperature (4 and 40ºC) on release of tocopherol from films with PPG-PEG-PPG in contact with simulant D_1 from 1 to 10 days.

From these results, significant differences were found by effect of temperature when the release of tocopherol from the films at 4 ºC and 40 ºC

were compared. An increase in the amount of released tocopherol can be observed when temperature increases, which can be related to an increase in vibration and motion of polymer chains as temperature increases favouring the migrant movement through the amorphous zones of the polymer (Galotto et al., 2011). In the same way, estimated diffusion coefficients also increase as temperature does, showing *D* values (cm^2 s^{-1}) between 1 to 4 orders of magnitude higher at 40 ºC than at 4 ºC (Table 5.3).

3.3.3. Effect of food simulant on the release of tocopherol.

Specific release tests designed to determine the dependence of tocopherol released with the food simulants were carried out. At this stage the tests were carried out at two different temperatures (4 and 40 ºC), from 1 to 10 days. Two food simulants were selected to cover a wide range of food products: simulant A (10% EtOH) and simulant D_1 (50% EtOH).

Figure 5.4 shows the percentage of tocopherol migrated from the different studied samples into simulants A and D_1.

As expected, simulant A was the simulant presenting the lowest levels of migrant. The non-significant differences found in the migration of tocopherol from the films could be clearly attributed to the polarity difference which does not favour the mass transfer of the antioxidant into the food simulant, which results in the very low water solubility of tocopherol. However, the migration increased with the percentage of EtOH in the simulant (Figure 5.4) because the higher percentage of EtOH in the solution decreases its polarity

favouring the mass transfer of the migrant from the film into the food simulant (Liu et al., 2012).

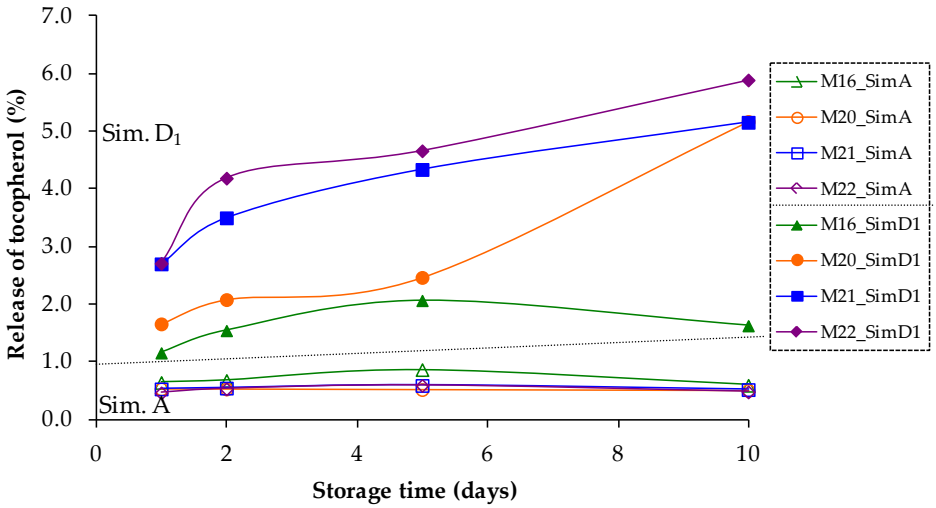

Figure 5.4. Influence of simulant A (10% EtOH) and D_1 (50% EtOH) on release of tocopherol from films at 40 ºC from 1 to 10 days.

Estimated diffusion coefficients of tocopherol from the studied films into the different simulants (Table 5.3) also reinforce that statement, showing *D* values ($cm^2\ s^{-1}$) even 4 orders of magnitude higher as the percentage of ethanol in the simulant is increased.

The different migration values obtained, indicated that the migration of tocopherol from the polymer also depends on the type of food simulant in contact with the plastic film and the solubility of the migrant into the food simulant tested.

3.4. Migration of chain extender from film into food simulant

The introduction of chain extenders into film formulations may be accompanied by the occurrence of their own release from the film into the food or food simulant contained. Since that could have a potential influence into the packed foods properties; their migration was also controlled. However, according to European Regulations (The European Commission, 2011), polymeric substances of a molecular weight of at least 1000 Da comply with the requirements of the Regulation.

To determine the migration of the chain extender from the film, release tests under the most favourable conditions intended to migration and aimed to the biggest consumer's protection were selected: 40 ºC, into simulant D_1, from 1 to 10 days. Samples with 1, 2 and 5% of PPG-PEG-PPG were analysed by means of HPLC-PDA-MS as described in section 2.4.3.

The migration of the chain extender from films was dependent on the storage time and the amount of chain extender in the film formulation. Figure 5.5 shows the percentage of PPG-PEG-PPG migrated from the manufactured films into the food simulant under the tested conditions. The data shows that there is essentially no migration from the film when a low amount (1%) of PPG-PEG-PPG was introduced into the film formulation (sample 20). Higher amounts (> 2%) of PPG-PEG-PPG have meant slight release of PPG-PEG-PPG from the film.

The effect of the time on the migration behaviour can be explained as increased time resulted in higher percentages of PPG-PEG-PPG. This effect is more remarkable, though, the higher is the percentage of extender in the film.

PPG-PEG-PPG as chain extender is not on the Union list of authorised monomers, other starting substances and macromolecules (The European Commission, 2011); moreover, as having a molecular weight higher than 1000 Da and being capable to form the main structural component of the plastic material, it complies with the requirements of the Regulation. Since no specific migration limit (SML) or other restrictions are provided for it, a generic specific migration limit of 60 mg Kg^{-1} shall apply. Migration of PPG-PEG-PPG from all the studied films resulted in migration levels quite lower than SMLs allowed by legislation. Values ranging from 1 to 25 mg Kg^{-1} depending on the type of sample, simulant and release conditions considered were obtained, which represents 50 to 98% less than legislation.

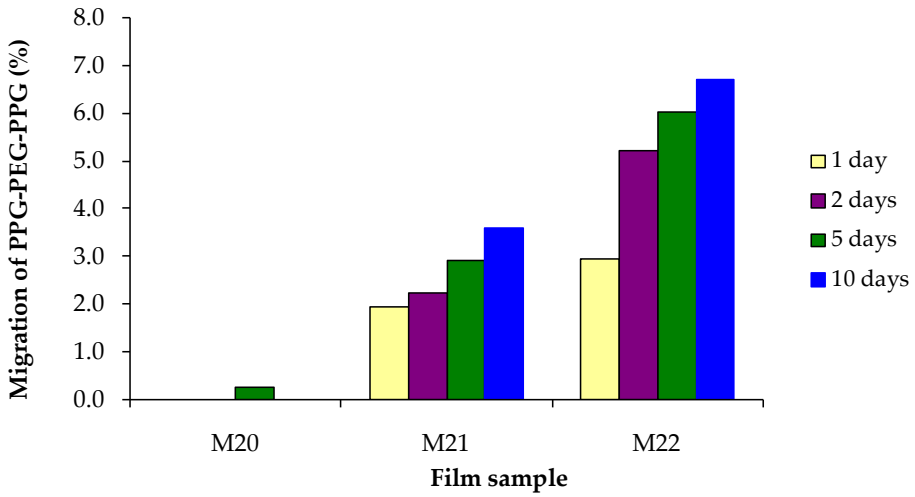

Figure 5.5. Migration of chain extender PPG-PEG-PPG from film to simulant D_1 at 40 ºC from 1 to 10 days.

3.5. PPG-PEG-PPG stability characterization: FTIR, NMR

PPG-PEG-PPG chain extender stability was tested through its chemical structure by means of FTIR and NMR. Pure PPG-PEG-PPG and blended into PP matrix were tested. Spectral FTIR and NMR data are illustrated in Figures 5.6 and 5.7.

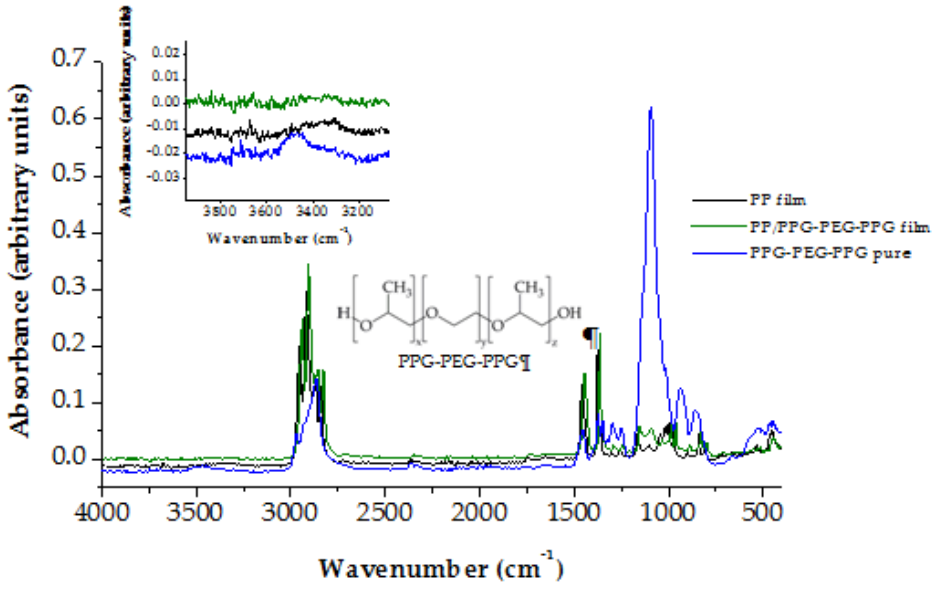

Figure 5.6. FTIR spectra of pure PPG-PEG-PPG vs PP film and PP/PPG-PEG-PPG film.

FTIR spectra showed a characteristic hydroxyl stretching band at 3480 cm^{-1}. Moreover, the band at 1095 cm^{-1} can be assigned to the CH, CO and CC stretch vibrations.

A strong proton signal at δ 3.65 ppm assigned to -OCH2CH2- repeating unit in PEG segments and proton signals at δ 3.45, 3.40 and 1.20 ppm

associated with -O(CH3)CHCH2- repeating unit of PPG were observed by NMR.

Regardless intensity considerations, the presence of such bands into PP+PPG-PEG-PPG extruded samples supported PPG-PEG-PPG no degradation through extrusion process.

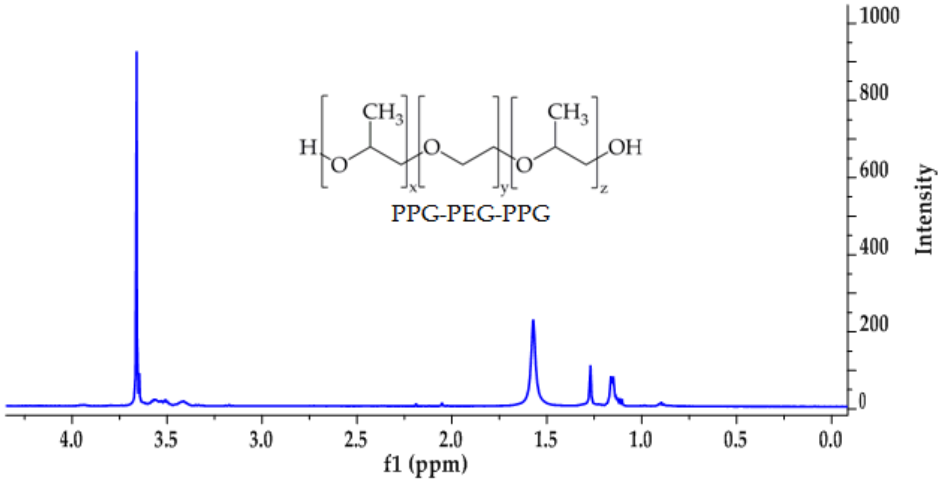

Figure 5.7. ^{1}H NMR spectrum of PPG-PEG-PPG $1*10^{-04}$ sample in $CDCl_3$.

Thus, this study clearly showed the good potential of PPG-PEG-PPG as PP modifier to control the release of tocopherol. Changing the composition of the film by using the chain extender, the desired release rates of tocoferol were obtained. Percentage of PPG-PEG-PPG and tocopherol into the film composition, as well as storage time and temperature were key factors to control the release process. The higher the tocopherol and chain extender concentrations in the films, the higher the amount of antioxidant migrated

from the film into the food simulant. Increasing PPG-PEG-PPG content from 0 to 5% has resulted in an increase of tocopherol released from 50 to 75% depending on the contact time. The storage time and temperature also had a great effect on migration. An increase in the storage temperature from 4 to 40 ºC and in time from 1 to 10 days resulted in a significant increase in the migration (from 70 to 95 and from 20 to 55%, respectively as temperature and time were increased). It could be related to an increase in vibration and motion of polymer chains as temperature increases favouring the migrant movement through the amorphous zones of the polymer.

No significant differences in migration from the films were found towards the simulant A (10% EtOH). Nevertheless, increasing the percentage of ethanol in the simulant D_1 (50% EtOH) higher migration levels were found. 80-90% of increase of tocopherol released depending on the film sample considered.

Although PPG-PEG-PPG was introduced as a new component into the polymer composition, its migration from the polymer into the food simulant has resulted to be well below the limits set by European legislation.

Thus, the PP modified films for controlled release of tocopherol has showed a good potential to assure the availability of the antioxidant to protect the film and the packed product in conditions of storage and commercialization using a material based on PP from which absence of released properties has been previously reported.

ACKNOWLEDGEMENTS

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Artículo 6 / patente

PROCEDIMIENTO DE OBTENCIÓN DE UN MATERIAL POLIMÉRICO

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1. CAMPO DE LA INVENCIÓN

La presente invención describe el uso de extensores de cadena PPG-PEG-PPG en una matriz de poliolefinas para la obtención de un material polimérico. Comprende la inclusión de este extensor de cadena en matrices de poliolefinas con el objetivo de favorecer la liberación controlada de compuestos activos desde el polímero al producto en contacto con él. Tiene aplicación en el desarrollo de plásticos para conservación y embalaje de alimentos perecederos en la industria de alimentación, cosmética o farmacéutica.

2. ANTECEDENTES DE LA INVENCIÓN

Los extensores de cadena han sido incorporados a las mezclas poliméricas con el objetivo de aumentar el tamaño de la cadena polimérica, lo cual lleva asociado una mejora de las propiedades del polímero. Entre estas mejoras, mayor estabilidad de los polímeros, la viscosidad, mayor el peso molecular y en general sus propiedades mecánicas (Li et al., 2010).

Awaja y Pavel, ha descrito el uso de extensores de cadena polimérica en el proceso de reciclado de PET con el objetivo de enlazar cadenas rotas por el uso del material polimérico (Awaja y Pavel, 2005). La solicitud US 2004/0138381 A1 describe el uso de extensores de cadena oligoméricos para el procesado, post-procesado y reciclado de polímeros de condensación, su síntesis, composición y aplicaciones con el objetivo de mejorar las características físicas de dichos polímeros.

Otros autores han demostrado la permeabilidad conferida por el uso de extensores de cadena en matrices de poliuretano. Esta permeabilidad permite mejorar o modificar las propiedades de los polímeros obtenidos con la incorporación de aditivos externos.

En la técnica actual la capacidad de liberación controlada de elementos activos desde el polímero al exterior gracias a la presencia de extensores de cadena como modificadores de la matriz polimérica ha sido únicamente propuesto para polímeros basados en monómeros con cierta polaridad, tales como ácido poliláctico (PLA), acetato de celulosa, ácido poliláctico co-glicólico (PLGA), etc; polímeros que tienen una baja o nula aplicabilidad en el sector del embalaje alimentario debido principalmente a su alta permeabilidad al oxígeno (US 2010/0112055 A1; GB 2408510 A).

La formación del enlace entre la matriz polimérica y el extensor se ve favorecida por la presencia de grupos capaces de interaccionar con los grupos hidroxilos funcionales terminales de este último, de ahí que la técnica actual sólo utilice los extensores para reaccionar con matrices poliméricas de cierta polaridad. Nunca se ha propuesto un sistema similar para polietileno de baja densidad (LDPE) o para polipropileno (PP), empleados en el sector del embalaje alimentario y, basados en monómeros apolares de naturaleza completamente diferente a los anteriores.

El LDPE y PP son los polímeros comúnmente empleados en aplicaciones de embalaje concernientes a uso alimentario. La inclusión de elementos activos en la composición del material de envasado con el doble objetivo de la protección del propio envase y de la liberación controlada de dicho principio

activo al alimento constituye un área del que los inventores no conocen antecedentes.

Respecto al extensor utilizado en la presente invención, el PPG-PEG-PPG, existen en el mercado tres tipos diferenciados principalmente por su peso molecular y por el número de unidades repetitivas presentes en cada uno de ellos. Sus características se recogen en la Tabla 6.1:

Tabla 6.1.

	PPG-PEG-PPG 2000	**PPG-PEG-PPG 2700**	**PPG-PEG-PPG 3300**
Peso molecular	~2000	~2700	~3300
Composición	PEG, 50 wt. %	PEG, 40 wt. %	PEG, 10 wt. %
Índice de refracción	*n*20/D 1,461	*n*20/D 1,459	*n*20/D 1,454
Tensión superficial	51 dinas/cm, 25 °C, 0,1 wt. % en H_2O	44 dinas/cm, 25 °C, 0,1 wt. % en H_2O	34 dinas/cm, 25 °C, 0,1 wt. % en H_2O
Viscosidad	480 cP (77 °C)	600 cP (25 °C)	660 cP (25 °C)
Temperatura de transición	Punto de congelación: 15 °C	Punto de ablandamiento: 18 °C	Punto de ablandamiento: −25 °C
	Punto de turbidez 69 °C (1 wt% solución acuosa)	Punto de turbidez 46 °C (1 wt% solución acuosa)	Punto de turbidez 25 °C (1 wt% solución acuosa)
Densidad	1,058 g/mL a 25 °C	1,048 g/mL a 25 °C	1,018 g/mL a 25 °C
Balance hidrofílico-lipofílico (HLB)	12,0 – 18,0	7,0 – 12,0	2,0 – 7,0

Estos tres tipos de PPG-PEG-PPG han sido comercializados para sus aplicaciones como limpiadores de superficies duros y blandos, antiespumantes en recubrimientos y en tratamientos de agua, lubricante en el trabajo de metales, anti-espumantes y como extensores de cadena en

poliésteres lineales y poliuretanos. A diferencia del PP y PE utilizados en la presente invención, los poliésteres y los poliuretanos se caracterizan por ser polímeros de carácter polar, termoplásticos, lineales y con alta cristalinidad en el caso de los poliestirenos lineales y termoplásticos o termoestables en función de su estructura química, con alta resistencia mecánica y alto poder amortiguador en el caso de los poliuretanos, propiedades todas ellas que determinan su funcionalidad y aplicaciones.

Tekade propone el uso de PEG 400 para mejorar la formación de una lámina polimérica proveniente de fuentes naturales, las semillas de la planta Delonix regia, para reducir la fragilidad de dicha lámina y dotarle de elasticidad con el fin de promover la liberación controlada de fármacos en el colon (Tekade y Gattani, 2010). Este artículo no describe ninguna aplicación relacionada con el sector alimentario ni sugiere su uso para ese fin, del mismo modo que la matriz polimérica no consiste ni en PP ni PE sino en la lámina experimental extraída de las semillas de *Delonix regia*, biodegradable y cuyas propiedades se encuentran bajo fase de estudio.

La solicitud internacional WO 2010/140041 A1 describe el uso de copolímeros de bloque como extensores de cadena y modificadores de superficie. Los copolímeros de bloque, definidos como macromoléculas compuestas por dos o más unidades repetitivas distintas unidas por medio de enlaces químicos con alternancia de largas secuencias de uno y otro monómero, se presentan como atractivos modificadores de polímeros cristalinos al no provocar serios descensos de la Temperatura de Cristalización (Tm), demostrando a su vez propiedades térmicas y mecánicas superiores a la de sus correspondientes copolímeros y homopolímeros. El

extensor de cadena de la presente invención pertenece a esta familia de los copolímeros de bloque así definidos. Sin embargo, la solicitud de referencia no estudia la permeabilidad de los polímeros obtenidos ni aporta ningún dato de la velocidad de liberación de posibles principios activos introducidos en su estructura, y por tanto no sugiere la presente invención ni puede afectar a su patentabilidad.

El problema que plantea la técnica es conseguir modificar las propiedades de polímeros destinados a su uso en alimentación y dotarles de la capacidad de liberación controlada de principios activos. La solución que propone la presente invención es la incorporación del extensor de cadena PPG-PEG-PPG en el polímero poliolefínico que sirve de matriz en el proceso de fabricación del poliuretano.

3. DESCRIPCIÓN DE LA INVENCIÓN

La presente invención es un procedimiento de obtención de un material polimérico, que comprende añadir copolímero de polipropilenglicol-polietilenglicol-polipropilenglicol (PPG-PEG-PPG), preferiblemente con un peso molecular medio de 2000 daltons, a una matriz de polímero poliolefínico seleccionado preferiblemente entre el grupo comprendido por tereftalato de polietileno (PET), polímero de base poliéster, polipropileno (PP), polietileno de alta densidad (HDPE), polietileno de media densidad (MDPE), politetileno de baja densidad (LDPE) o policloruro de vinilo (PVC), previo al conformado de dicho material polimérico.

En una realización más restringida, el procedimiento de la invención consiste en añadir sólo los componentes descritos, previo a su conformado.

La obtención de dicho material polimérico comprende el conformado de la mezcla que comprende el extensor PPG-PEG-PPG y el polímero poliolefínico a una temperatura de entre 150 y 320 ºC, preferiblemente entre 170 y 200 ºC. Esta temperatura es superior a la temperatura de fusión de la matriz poliolefínica y a su vez asegura la estabilidad térmica de las sustancias activas y del resto de aditivos que pueden añadirse a la mezcla. En otra realización preferible más, el conformado se realiza por extrusión, inyección o termoconformado de la granza modificada.

En otra realización preferible, dicho copolímero de PPG-PEG-PPG está presente en un porcentaje entre el 1% y el 5% en peso respecto de dicho polímero poliolefínico.

Mientras los PEG contienen unidades repetidas (-CH_2CH_2O-), los PPG contienen unidades con presencia de grupos hidroxilo primarios y secundarios (-$CH_2(CH_3)CH_2O$-) lo que, junto a la presencia de cadenas metilo laterales, les confiere propiedades físicas y químicas únicas. El número de unidades repetidas de cada uno de los fragmentos del extensor correspondiente es un entero variable en función del peso molecular del copolímero.

Una realización muy preferible de la invención es el material polimérico obtenido por el procedimiento de la invención. Dicho material polimérico se caracteriza por que presenta en el espectro infrarrojo (IR) una banda correspondiente al grupo hidroxilo y otra banda correspondiente al conjunto de grupos CH, CO y CC (Figura 6.1), y por que presenta señales de protón en

Resonancia Magnética Nuclear (RMN) a desplazamientos δ de 3,65 ppm asignada a la unidad repetitiva –OCH_2CH_2- del segmento de PEG, y a δ de 3,45, 3,40 y 1,20 ppm asociadas con la unidad repetitiva $(CH_3)CHCH_2$- del PPG (Figura 6.2) con una tolerancia de ±0,02 ppm.

En el espectro IR, la banda correspondiente a la banda de vibración de alargamiento del grupo hidroxilo de la presente invención es una banda débil característica a 3480 cm^{-1}, y la banda de vibración de los grupos CH, CO o CC es una banda fuerte a 1095 cm^{-1} atribuible por su alta intensidad a las bandas de vibración de los grupos CH, CO y CC.

En una realización preferible, el material polimérico de la invención comprende al menos un agente activo, preferiblemente con un peso molecular entre 150 y 1500 daltons, que más preferiblemente un antioxidante y más preferiblemente aún es el tocoferol. En otra realización preferible dicho antioxidante está presente en un proporción de hasta el 5% en peso respecto del total.

Además, el material polimérico de la presente invención puede ser monocapa o multicapa; de forma que otra realización de la invención es que dicho material se presente en forma de monocapa, y otra realización, en forma de multicapa.

Otra realización muy preferible es un plástico que comprende el material polimérico de la invención. En una realización más restrictiva, dicho plástico consiste en el material polimérico de la invención.

La inclusión por mezclado en fundido del extensor de cadena PPG-PEG-PPG según la invención favorece la liberación controlada de sustancias

activas al producto en contacto con dicho material polimérico, lo cual supone la principal ventaja tecnológica de la invención sobre la técnica. La incorporación del extensor de cadena favorece la migración de los principios activos que se adicionan al material polimérico mejorando así las prestaciones de los materiales activos existentes actualmente para la protección de alimentos, cosméticos o productos farmacéuticos.

Los resultados obtenidos muestran que un mayor porcentaje aditivado del extensor está directamente relacionado con un mayor nivel de migración del agente activo, en el caso de los siguientes ejemplos del tocoferol, de la lámina al simulante alimentario.

4. BREVE DESCRIPCIÓN DE LAS FIGURAS

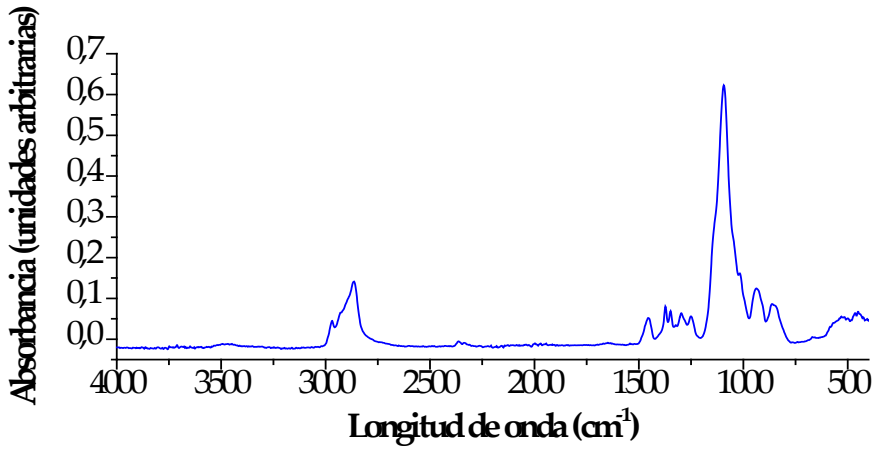

Figura 6.1: muestra el espectro FTIR del extensor empleado en la presente invención: PPG-PEG-PPG.

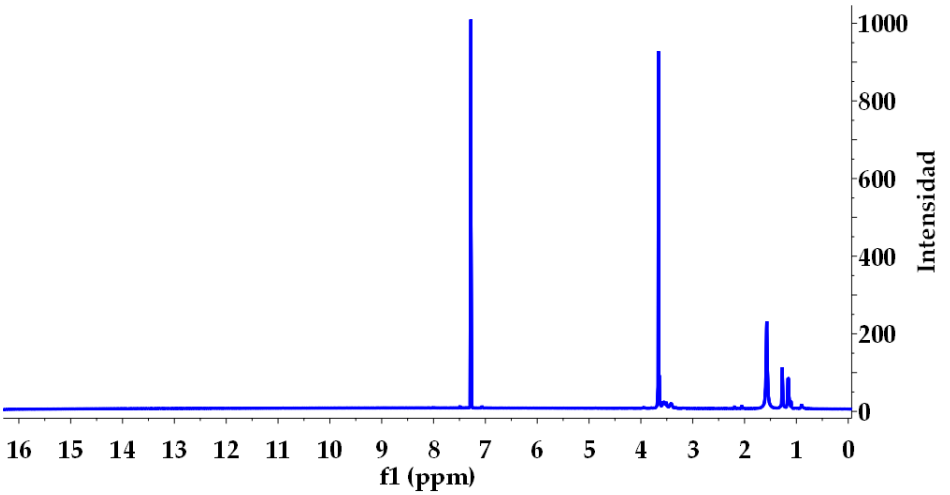

Figura 6.2a: muestra el espectro completo de la estructura química del PPG-PEG-PPG caracterizada por 1H RMN.

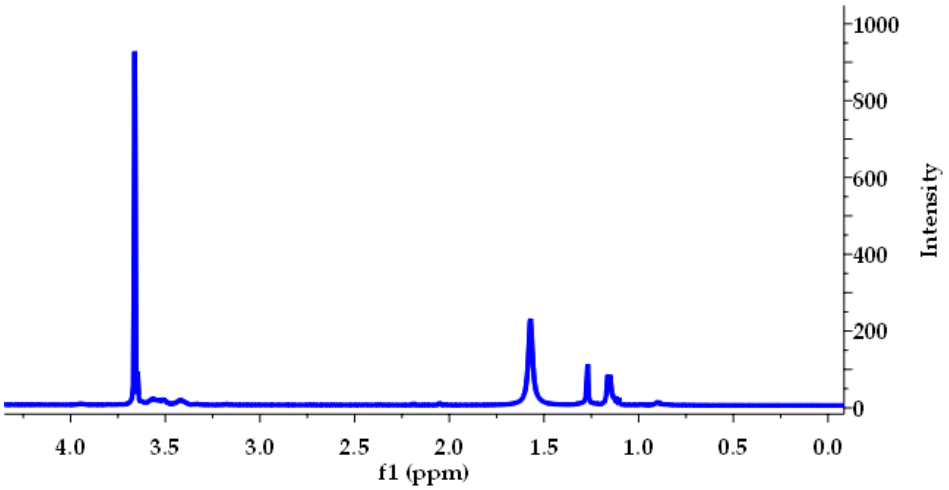

Figura 6.2b: muestra la ampliación de la zona de interés del espectro por 1H RMN.

5. DESCRIPCIÓN DETALLADA DE LA INVENCIÓN

Con la intención de mostrar la presente invención de un modo ilustrativo aunque en ningún modo limitante, se aportan los siguientes ejemplos. Todas las normas o reglamentos que se citan son accesibles y conocidos por el experto medio, y representan el valor de los estándares más usados en la técnica para las mediciones que se indican.

Ejemplo 1: Fabricación del polímero de la invención

Se realizó una mezcla simple de las formulaciones poliméricas correspondientes a la matriz de composiciones según la tabla 6.2:

Tabla 6.2.

	PP (%)	Tocoferol (%)	PPG-PEG-PPG 2000 (%)	Tris(2,4-di-tert-butilfenil) fosfito (%)
F1	97,8	1	1	0,2
F2	96,8	1	2	0,2
F3	93,8	1	5	0,2
F4	93,8	5	1	0,2
F5	92,8	5	2	0,2
F6	89,8	5	5	0,2
		CONTROLES NEGATIVOS		
F7	94,8	5	---	0,2
F8	95	5	---	---
F9	99,8	---	---	0,2
F10	99	1	---	---
F11	99,5	0,5	---	---

El tris(2,4-di-tert-butilfenil) fosfito es un antioxidante secundario añadido con el objetivo de proporcionarle protección al polímero durante la etapa del procesado, evitando así su degradación temprana como consecuencia de la extrusión. Una vez premezcladas se extruyeron para obtener láminas poliméricas empleando una miniextrusora con husillos co-rotantes cónicos y capacidad de 7 cm^3, velocidad de rotación del tornillo de 40 rpm a 180ºC y tiempo de residencia de 1 min. Estas láminas fueron almacenadas bajo condiciones de refrigeración a 4 ºC y oscuridad.

Ejemplo 2: Estabilidad de los polímeros frente a la oxidación proporcionada por los antioxidantes aditivados.

La estabilidad frente a degradación oxidativa de las láminas obtenidas en el Ejemplo 1 (F1 a F11) fue evaluada midiendo el tiempo de inducción a la oxidación mediante Calorimetría Diferencial de Barrido. Se tomó una muestra de cada una de las láminas extruidas para la medida del tiempo de inducción a la oxidación (OIt). Dicha medida se realizó isotérmicamente a 200 ºC bajo atmósfera inerte, que posteriormente se hizo evolucionar a una atmósfera oxidante por empleo de un calorímetro diferencial de barrido (Perkin–Elmer serie 7 DSC). Los análisis se llevaron a cabo de acuerdo con la norma EN 728. Se observó que las nuevas formulaciones poliméricas mejoran en al menos un 50% la estabilidad frente a la oxidación respecto a las poliolefinas aditivadas con la combinación F9 del Ejemplo 1, que por otra parte es la más utilizada en la técnica (valores de OIT<5 min para F9, frente a valores de OIT entre 53 y 140 min obtenidos para el resto de formulaciones (Tabla 6.3)).

Tabla 6.3

	OIT (min)
F7	134
F8	140
F9	<5
F10	53
F11	30

Ejemplo 3: Influencia de la cantidad de extensor de cadena en la migración del antioxidante.

Se realizaron ensayos con la matriz polimérica según el Ejemplo 1 en presencia de diferentes proporciones de extensor PPG-PEG-PPG 2000 (Sigma-Aldrich) en la mezcla de polimerización. Se ensayaron las láminas compuestas por 1 % de tocoferol; 0, 1, 2 ó 5% de PPG-PEG-PPG 2000 según lámina; 0,2% de Tris(2,4-di-tert-butilfenil) fosfito; y PP (F1 a F3, Ejemplo 1). El test de migración fue realizado por inmersión de tiras de lámina rectangulares (8x0,4x0,17mm) en 9 mL de simulante alimentario D_1 (50% etanol, Reglamento (UE) No 10/2011 de La Comisión de 14 de enero de 2011 para materiales plásticos destinados a entrar en contacto con alimentos) contenido en tubos de vidrio con tapón de teflón (PTFE). Los tubos fueron recubiertos con parafilm a fin de evitar evaporaciones del simulante durante el periodo de contacto. El contacto con el simulante alimentario se realizó durante 10 dias y temperatura de contacto de 40 ºC de acuerdo con el posible uso final del envase, tal y como estipula el Reglamento (UE) No. 10/2011. Muestras de todos los tratamientos fueron tomadas tras 1, 2, 5 y 10 días de almacenamiento. Tras el periodo de contacto, las muestras de las láminas

fueron eliminadas y el simulante enrasado hasta un volumen final de 10 mL. Una alícuota del mismo fue filtrada a través de filtros de PTFE (13 mm, 0,2 μm) y analizada por cromatografía de líquidos HPLC. El resultado muestra cómo el incremento de 0 a 5 % en el porcentaje de extensor presente en la mezcla produce un aumento en la liberación de compuesto aditivado (Tabla 6.4).

Tabla 6.4

	mg de tocoferol liberado por Kg de film/mg de tocoferol inicialmente aditivado por Kg de film (%)			
	1 día	**2 días**	**5 días**	**10 días**
F1	30	26	16	69
F2	57	56	52	68
F3	57	63	56	72

Ejemplo 4: Estudio de la liberación controlada del antioxidante – influencia del tipo de alimento contenido

Se realizaron ensayos de migración de tocoferol desde el material polimérico según el Ejemplo 1 conteniendo 1% de tocoferol; 0, 1, 2 ó 5% de PPG-PEG-PPG 2000 según la lámina, 0,2% de Tris(2,4-di-tert-butilfenil) fosfito y PP (F1 a F3, Ejemplo 1) hacia diferentes simulantes alimentarios. Se estipuló el simulante alimentario A (10% etanol) para alimentos tipo azúcares y derivados, frutos de cáscara, hortalizas frescas y en conserva, pescados en todas sus variedades, crustáceos y moluscos, carnes, quesos, alimentos fritos o asados, pizzas, sándwiches, etc.; y simulante D_1 (50% etanol) para bebidas turbias tipo zumos, néctares, chocolate líquido, etc., bebidas alcohólicas,

frutas, hortalizas y carnes en conserva, productos lácteos. Los ensayos fueron realizados bajo las condiciones estipuladas en el Ejemplo 3. Los resultados obtenidos se muestran en la tabla 6.5.

Tabla 6.5.

	mg de tocoferol liberado por Kg de film (%)							
	Sim. D_1				Sim. A			
	1 d.	2 d.	5 d.	10 d.	1 d.	2 d.	5 d.	10 d.
F1	1,7	2,1	2,5	3,6	0,5	0,5	0,5	0,5
F2	2,7	3,5	4,3	3,6	0,5	0,5	0,6	0,5
F3	2,7	4,2	4,7	4,1	0,5	0,5	0,6	0,5

La migración de tocoferol hacia el simulante alimenticio se vio favorecida en condiciones de contacto con simulante D_1 con un mayor porcentaje de fase orgánica (50 % de EtOH frente al 10 % del simulante A).

Ejemplo 5: Estudio de la liberación controlada del antioxidante – influencia del tiempo

Se realizaron ensayos de la influencia de un tiempo de contacto de 1, 2, 5 y 10 días en el perfil de migración de tocoferol desde el material polimérico según el Ejemplo 1 con láminas compuestas por 1 ó 5% de tocoferol; 0, 1, 2 ó 5% de PPG-PEG-PPG 2000 según la lámina, 0,2% de Tris(2,4-di-tert-butilfenil) fosfito y PP (F1 a F6 Ejemplo 1) bajo las condiciones de ensayo estipuladas en el Ejemplo 3. Los resultados obtenidos mostraron que cantidades crecientes de tocoferol son liberadas hacia el simulante alimenticio a lo largo del tiempo,

obteniéndose por tanto una migración controlada. Incrementos de porcentaje de liberación del compuesto aditivado (considerando los datos de mg de tocoferol liberado por Kg de film con respecto a los mg de tocoferol inicialmente aditivado por Kg de film) desde un 30% tras el transcurso de 1 día de contacto al 70% tras el transcurso de 10 días de ensayo (Tabla 6.3).

Ejemplo 6: Obtención de los espectros de IR y H-RMN, disolvente y equipos

Se realizaron ensayos de espectroscopia infrarroja con transformada de Fourier (FTIR) y resonancia magnética nuclear de protón (H-RMN) y con el objetivo de caracterizar el extensor empleado en las formulaciones del Ejemplo 1. Los ensayos de FTIR del PPG-PEG-PPG 2000 fueron realizados en modo transmisión con un espectrofotómetro OPUS/IR PS15 (Bruker) con obtención del espectro entre 4000 y 400 cm^{-1} empleando una celda especial para líquidos (Figura 6.1). Los resultados obtenidos mostraron que bandas IR y desplazamientos característicos para la molécula de PG-PEG-PPG 2000: banda débil característica a 3480 cm^{-1} correspondiente a la banda de vibración de alargamiento del grupo hidroxilo, y otra banda a 1095 cm^{-1} atribuible por su alta intensidad a las bandas de vibración de los grupos CH, CO o CC. Los ensayos de H-RMN fueron llevados a cabo por empleo de un espectrofotómetro de resonancia magnética nuclear Bruker AVANCE 500 (Bruker) sobre disoluciones del extensor PPG-PEG-PPG 2000, $1*10^{-04}$ M preparadas en diclorometano deuterado ($CDCl_3$) (figura 2). Los resultados mostraron señales de protón en Resonancia Magnética Nuclear a desplazamientos δ de 3,65 ppm asignada a la unidad repetitiva $–OCH_2CH_2$-

del segmento de PEG, y a δ de 3,45, 3,40 y 1,20 ppm asociadas con la unidad repetitiva $(CH_3)CHCH_2$- del PPG.

6. REIVINDICACIONES

1. Procedimiento de obtención de un material polimérico, que comprende añadir copolímero de PPG-PEG-PPG a una matriz de polímero poliolefínico previo al conformado de dicho material polimérico.

2. Un procedimiento según la reivindicación 1, en que dicho conformado se realiza por extrusión, inyección o termoconformado.

3. Un procedimiento según una de las reivindicaciones 1 ó 2, en que dicho conformado se realiza a una temperatura de entre 150 y 320 ºC.

4. Un procedimiento según la reivindicación 3, en que dicha temperatura es de entre 170 y 200 ºC

5. Un procedimiento según cualquiera de las reivindicaciones 1 a 4, en que dicho copolímero de PPG-PEG-PPG presenta un peso molecular medio de 2000 Dalton.

6. Un procedimiento según cualquiera de las reivindicaciones 1 a 5, en que dicho copolímero de PPG-PEG-PPG está presente en un porcentaje entre el 1% y el 5% en peso respecto del total de dicho material polimérico.

7. Un procedimiento según cualquiera de las reivindicaciones 1 a 6, en que dicho polímero poliolefínico está seleccionado entre el grupo comprendido por PET, polímero de base poliéster, PP, HDPE, MDPE, LDPE y PVC.

8. Material polimérico obtenido por el procedimiento según cualquiera de las reivindicaciones anteriores.

9. Un material polimérico según la reivindicación 8, que comprende al menos un agente activo.

10. Un material polimérico según una de las reivindicaciones 8 ó 9, en que dicho agente activo presenta un peso molecular entre 150 y 1500 Dalton.

11. Un material polimérico según cualquiera de las reivindicaciones 8 a 10, en que dicho agente activo es un antioxidante.

12. Un material polimérico según la reivindicación 11, en que dicho antioxidante está presente en una proporción de hasta el 5% en peso respecto del total de dicho material polimérico.

13. Un material polimérico según una de las reivindicaciones 11 ó 12, en que dicho antioxidante es tocoferol.

14. Un material polimérico según cualquiera de las reivindicaciones 8 a 13, con estructura de multicapa.

15. Un material polimérico según cualquiera de las reivindicaciones 8 a 14, caracterizado por que presenta en el espectro IR una banda correspondiente al grupo hidroxilo y otra banda correspondiente al conjunto de grupos CH, CO y/o CC, y por que presenta señales de protón en RMN a desplazamientos δ de 3,65, 3,45, 3,40 y 1,20 ppm con una tolerancia de ±0,02 ppm.

16. Plástico que comprende dicho material polimérico.

RESUMEN

La invención describe un procedimiento de obtención de un material polimérico que comprende añadir copolímero de PPG-PEG-PPG como extensor de cadena a una matriz de polímero poliolefínico, previo a su conformado. El material polimérico de la invención puede contener un antioxidante para su liberación controlada al medio con el que está en contacto, lo cual supone la principal ventaja tecnológica de la invención sobre la técnica. La incorporación del extensor de cadena favorece la migración de los principios activos que se adicionan al material polimérico mejorando las prestaciones de los materiales activos existentes actualmente en el mercado en la protección de alimentos, cosméticos o productos farmacéuticos.

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Artículo 7

IMPROVING THE CAPACITY OF POLYPROPYLENE TO BE USED IN ANTIOXIDANT ACTIVE FILMS: INCORPORATION OF PLASTICIZER AND NATURAL ANTIOXIDANTS

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ABSTRACT

Two types of active antioxidant food packages with improved release properties, based on polypropylene as one of the most common polymers used in food packaging applications, were developed. Release of natural antioxidants (catechins, gallic acid, caffeine and quercetin) into various food simulants from that non-polar matrix were improved by blending PPG-PEG-PPG as plasticizer into polymer`s formulation (increased levels of antioxidants even up to 40 times). The amount of both plasticizer and antioxidant, ethanolic content of food simulant and the time highly influenced the release of the antioxidants. The higher their value, the higher the release of green tea components was observed. The efficiency of the antioxidants into the food simulants after release process was also corroborated through antioxidant activity tests. Therefore, the developed PPG-PEG-PPG modified polypropylene resulted in a potential system to be used in active packaging formulations.

Keywords: active packaging, antioxidant, PP, PPG-PEG-PPG, green tea, flavonoids.

1. INTRODUCTION

Oxidative processes and microbial spoilage are primary causes for the deterioration of food quality. Traditionally, besides containment, convenience and communication (Vermeiren et al., 1999), packaging also provides protection against possible contamination caused by external agents such as

water, light or odorants. However, increasing safety and quality demands have led to the development of new alternatives in the food packaging industry. Active packaging with controlled release of active compounds to foodstuffs has emerged as a promising technology (Vermeiren et al., 1999; LaCoste et al., 2005; López-Rubio et al., 2006). Among them, antioxidant active packaging, in which an antioxidant is incorporated into the polymer to be released into the packaged foodstuff providing a continuous antioxidant effect to prevent lipid oxidation avoiding its rapid depletion compared with its direct addition to food (Vermeiren et al., 1999; LaCoste et al., 2005; López-Rubio et al., 2006; Mastromatteo et al., 2010) and extending the own packaging's shelf-life (Laermer, 1990; Sharma et al., 1990).

Synthetic antioxidants such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA) (Wessling et al., 2000; Rababah et al., 2004; Dopico-García et al., 2007) traditionally used in packing, have been replaced by natural preservaties due to safety concerns (Ito et al., 1986; Wessling et al., 1998; Dopico-García et al., 2007). Then, α-tocopherol (Laermer, 1990; Al-Malaika et al., 1999; Wessling et al., 1999; Heirlings et al., 2004; Pereira de Abreu et al., 2010; Koontz et al., 2010a; Koontz et al., 2010b; Dopico-García et al., 2011) carvacrol and aromatic plant extracts such as oregano or barley, among others (Nerín et al., 2006; Bentayeb et al., 2007; Peltzer et al., 2009; Pereira de Abreu et al., 2010) have been used. Nowadays, polyphenols such as catechins have also aroused high interest as natural antioxidants, being present in several species of the plant kingdom, especially tea (Harbowy and Balentine, 1997; Nijveldt et al., 2001; Gramza and Korczak, 2005). Flavonols such as quercetin (Quer) and other compounds such as caffeine (Caff) are, as

well, important constituents of tea also providing it with antioxidant and mood-cognitive-enhancing properties, respectively (Nijveldt et al., 2001).

Accordingly to their molecular weight and their nonvolatile character, those compounds should be likely to be able to diffuse between the packaging material, the food product and/or partitioning at the interface when they are used in active food packaging. Recently, some research related to the incorporation of catechins, quercetin or caffeine as active agents to active polymer packaging has been developed on the basis of polyethylene terephthalate (PET) (Colon and Nerin, 2012), ethylene vinyl alcohol (EVOH) (López de Dicastillo et al., 2010, 2011, 2012) or biodegradable materials such as polylactic acid (PLA) (Corrales et al., 2009; Arcan and Yemenicioglu, 2011; Iñiguez-Franco et al., 2012).

Nevertheless, those reported active packagings with hydrophilic and/or biodegradable polymers are only intended to short shelf-life products, besides not being as widely used as low-density polyethylene (LDPE) and polypropylene (PP) in packaging food applications (Wessling et al., 1999). No important developments of active packaging with those latter polymers have been reported, though; which could be attributed to the few release capacity of catechins or quercetin reported from those polymers despite their high polar nature. This capacity was then more limited towards the release of lower molecular-weight compounds such as caffeine or gallic acid or the release in contact with food simulants of very highly ethanolic content (95%) (Corrales et al. 2009; López de Dicastillo et al., 2010, 2011, 2012; Arcan and Yemenicioglu, 2011; Colon and Nerin, 2012; Iñiguez-Franco et al., 2012). Some additives such as plastizicers can be used to modify polymer properties,

specially workability, flexibility and extensibility of the polymer. PPG, PEG or their copolymers have been reported as potential plasticizers into film formulations (Kulinski et al., 2006; Kowalczyk et al., 2012). Based on their role in drug release (Siepmann et al., 2008) and following our preliminary study on modified films (Castro-López et al., 2012), those plasticizers may also be an alternative to modify polymer properties and, therefore, mass transport of active agents.

Therefore, the aim of this work was to develop a new antioxidant PP active material to improve food protection. Individual catechin and green tea extract, as well as PPG-PEG-PPG were incorporated by extrusion. The new materials were characterized and compared in terms of release capacity of the catechins, gallic acid, quercetin and caffeine. The influence of the type and amount of antioxidant, amount of plasticizer, type of food simulant and the contact time were also studied. Finally, the antioxidant efficiency of the antioxidants in the food simulants after the release process was tested too.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Polypropylene ISPLEN^{R} PP 070 G2M was provided by Repsol YPF (Madrid, Spain). Irgafos 168 (Tris(2,4-di-tert-butylphenyl)phosphate; I168), (-)-Epicatechin (EC), (+)-Catechin hydrate (C), (-)-Epigallocatechin (EGC), (-)-Epigallocatechin gallate (EGCG), (-)-Epicatechin gallate (ECG), (-)-Gallocatechin Gallate (GCG), (-)-Catechin Gallate (CG), quercetin, gallic acid monohydrate (GA), caffeine, 2,2′-azino-bis(3-ethylbenzothiazoline-6-

sulfonic acid) (ABTS) and poly(propylene glycol)-block-poly(ethylene glycol)-block-poly(propylene glycol) (average M_n~2000, PPG-PEG-PPG) were supplied by Sigma-Aldrich (Steinheim, Germany). Green tea extract was kindly donated by the group of Packaging Lab, Instituto de Agroquímica y Tecnología de Alimentos (CSIC, Valencia, Spain). Methanol and ethanol (EtOH) HPLC-gradient for instrumental analysis were supplied by Merck (Darmstadt, Germany). Formic acid 98-100 % puriss p. a. was from Sigma-Aldrich. Water was purified using a Milli-Q Ultrapure water-purification system (Millipore, Bedford, MA, USA).

2.2. Film preparation

Monolayer polypropylene compounding films containing PPG-PEG-PPG (0, 2 or 5%) as plasticizer and individual catechin (2 or 5%) or green tea extract (2 or 5%) as antioxidants were obtained by extrusion. Commercial antioxidant I168 (0.2%) was also added to protect the polymer during the extrusion process. Specific composition of each sample is shown in Table 7.1. Both catechin as individual compound and the green tea extract were incorporated as solids into the compounding mixture before extrusion. Films without plasticizer were also prepared as reference materials.

Extrusion was carried out using a miniextruder equipped with twin conical co-rotating screws and a capacity of 7 cm^3 (Minilab Haake Rheomex CTW5 (Thermo Scientific)). Screw rotation rate of 40 rpm, temperature of 180 ºC and 1 minute of residence time were used. The resulting films presented an average thickness of 1.5±0.14 mm, although the thickness of every sample was

individually measured before tests using an electronic digital micrometer (Comecta S.A., Barcelona, Spain).

Table 7.1. Composition of the prepared film samples and OIT values for stabilized and non-stabilized PP at 200 ºC. OIT data expressed as mean value±standard error of mean (n=3).

Sample code	Matrix (PP)	Commercial antioxidants I168 (%)	Plasticizer PPG-PEG-PPG (%)	Natural antioxidants (%)		OIT values (min^{-1})
				Catechin	Green tea	
M0-A	X	0.2	---	---	---	4.5 ± 1.03^{a}
M0-B	X	0.2	2	---	---	5.82 ± 2.75^{a} A
M1	X	0.2	---	2	---	46.4 ± 5.65^{b}
M2	X	0.2	2	2	---	$65.0\pm7.43^{c,e}$ B
M3	X	0.2	5	2	---	68.5 ± 1.15^{c}
M4	X	0.2	---	5	---	34.8 ± 4.10^{d}
M5	X	0.2	2	5	---	58.0 ± 3.35^{e} B,C
M6	X	0.2	5	5	---	56.4 ± 2.35^{e}
M7	X	0.2	---	---	2	$45.0\pm5.19^{f,g}$
M8	X	0.2	2	---	2	38.1 ± 7.10^{f} C
M9	X	0.2	5	---	2	$50.3\pm6.30^{f,g}$
M10	X	0.2	---	---	5	$46.0\pm4.81^{f,g}$
M11	X	0.2	2	---	5	53.4 ± 6.50^{g} B,C
M12	X	0.2	5	---	5	55.4 ± 5.90^{g}

Different lowercase superscripts within a column (a-g) indicate significant differences between data according to Tukey's test (p=0.1). Different capital superscripts within groups (A-C) indicate significant differences between data according to Tukey's test (p=0.1). Groups: M0, M1-M3 vs M4-M5 vs M7-M8 vs M10-M12.

2.3. Chromatographic study

HPLC coupled to mass detection was used to identify and quantify the natural antioxidants used, catechins, quercetin and caffeine, and the plasticizer, PPG-PEG-PPG.

An Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) equipped with an on-line degasser, a binary pump delivery system, a high performance SL autosampler, a thermostated column department and on-line coupled to a mass spectrometer detector (MS) was used for analysis. Samples were filtered through a 0.2 μm Acrodisc^{R} PTFE CR (Waters) and injected in Zorbax SB-C18 (50 x 2.1 mm, 1.8 μm) column (Agilent Technologies). Two mobile phases systems consisting of mixtures of water-0.1% formic acid (A) and methanol (B) under the following gradient systems were used: mobile phase initially set at 25% B was linear increased to 100% B in 4 min, maintained for 1 min and brought back to initial conditions, for analysis of catechins, gallic acid, caffeine and quercetin. 30% B linearly increased to 100% B in 3 min, and maintained for 13 min, for the determination of the plasticizer. The mass spectrometer was an Agilent 6410 Triple Quad LC/MS (Agilent Technologies). The column effluent was directly introduced into the triple quadrupole mass detector operated in a positive ionization mode. Ions were formed using electrospray ionization (ESI). The following ESI source parameters were used: temperature of the drying gas (N_2) was set to 350 ºC and flowed at 10 mL min^{-1}. The nebulizing pressure (N_2) was maintained at 35 psi. Capillary voltage was set at 4 kV. Integration and data elaboration were performed using Agilent MassHunter Workstation software, version B03.00 (Agilent-Technology, Santa Clara, USA). The full mass scan range m/z 100–1000 (1 s/scan) and the target ions generated by catechins, gallic acid, caffeine, quercetin and PPG-PEG-PPG were corresponding to $[M+H]^+$. Selective ion monitoring (SIM) was used to quantify the target ions. Mass spectral data and retention time were used for

peak identification. Quantification of plasticizer was based on an external standard calibration method.

2.4. Thermal stability

Film thermal stability. Film samples (Table 7.1) were taken out for oxidation induction time (OIT) measurements in order to obtain information on polymer stability and antioxidant effectiveness. OIT was measured on a Perkin–Elmer serie 7 DSC isothermally at 200 ºC under inert atmosphere, which was subsequently switched to oxygen atmosphere. Analyses were carried out according to EN 728:1997 (European Committee for Standardization, 1997). The OIT was measured as the onset point at which the DSC thermogram suffers a sudden drop respect to the instrument baseline. The obtained results are mean of three measurements.

Antioxidant thermal stability. As to epimerization, i.e. the conversion of the catechins to their corresponding isomers can occur under hot conditions at the C-2 position (Wang and Helliwell, 2000) measurements of the stability of the antioxidants were made in the two selected simulants under the set exposure conditions, by storing a solution of the additive in the simulant in parallel with the release tests. Analyses were carried out using the same procedure as for the samples by means of HPLC-QqQ.

2.5. Release studies

Release tests were performed by total immersion of rectangular strips film pieces ((80±0.099) x (3.4±0.26) x (1.5±0.14)mm) in 10 mL of food simulant

contained in glass-stoppered tubes with polytetrafluoroethylene (PTFE) closures. Milli-Q water was deoxygenated by bubbling nitrogen. The migration test parameters were based on the European Commission Regulation Nº 10/2011 (The European Commission, 2011). Two food simulants were selected as to mimic some food usually and/or able to be packed in plastic films: A (10% ethanol), as representing one of the assigned for foods that have a hydrophilic character (such as for sugar and its products, nuts, vegetables, fish, meat, cheese, sauces, sandwiches, etc); and D_1 (50% ethanol) for foods with lipophilic character and an alcoholic content above 20% (preserved fruits, preserved vegetables, milk, yogurt, cream and soup cream, processed cheese, among others) (The European Commission, 2011). Release studies were conducted at 40ºC over 5 and 10 days of storage. Test materials were also run simultaneously to check for interferences.

After the contact period, an aliquot of food simulant was filtered through an Acrodisc^R PTFE CR 13mm, 0.2 μm filters (Waters, Mildford, MA, USA) and analyzed by means of HPLC-QqQ. Release data was corrected with the information obtained from stabilization of the antioxidant under the exposure conditions and expressed as mg of compound released per kilogram of film.

Release process is normally described by the kinetic of the diffusion of the antioxidant in the film and is expressed by the diffusion coefficient (D). D is usually estimated using the Fickian diffusion model (Chen et al., 2012). When release of antioxidant reached equilibrium, equation 1 is used as the rigorous model for describing the migration controlled by Fickian diffusion in a packaging film:

$$\frac{M_t}{M_{F,\infty}} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left[\frac{-D(2n+1)^2 \pi^2 t}{L_P^2}\right] \quad (1)$$

M_t, mass of the migrant in the food at a particular time t (s); $M_{F,\infty}$, mass of migrant in the food at equilibrium; L_P (cm), film thickness; *D* ($cm^2\ s^{-1}$), diffusion coefficient; t (s), time.

Nevertheless, when release is slow and equilibrium is not reached at the end of the experiment, equation 2 can be used when M_t/M_P is < 0.6:

$$\frac{M_t}{M_P} = \frac{4}{L_P}\left(\frac{Dt}{\pi}\right)^{0.5} \quad (2)$$

M_P, initial loading of antioxidants in the film. *D* is estimated from the slope of the plot of M_t/M_P versus $t^{0.5}$.

Diffusion coefficients for samples doped with green tea were calculated as sum of all the studied compounds.

2.6. Antioxidant activity

ABTS was selected to study the radical scavenging behavior of the developed materials after contact period with simulants. The assay is based on the inhibition by antioxidants of the absorbance of the radical cation 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonate), ($ABTS^{\bullet+}$), which has a characteristic wavelength absorption spectrum withshowing a main absorption maxima at 417 nm, and secondary absorption maxima at 660, 734, and 820nm. Radicals $ABTS^{\bullet+}$ are neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (Sánchez-Moreno, 2002; Prior et al., 2005).

When these radicals are neutralized the absorbance decreases. The percentage inhibition values were calculated using equation 3:

$$I\ (\%) = [(Abs\ control - Abs\ sample)/Abs\ control] \times 100 \qquad (3)$$

Using a calibrated curve of gallic acid concentration vs. I (%), the results can easily be expressed as the equivalent gallic acid concentration (López de Dicastillo et al., 2011).

Sample of each migrated simulant, after contact period, was mixed with a solution of radicals $ABTS^{\bullet+}$ of known concentration at a rate of 9:1 (sample:$ABTS^{\bullet+}$ solution). After 15 minutes of timeout, absorbance was measured.

2.7. Statistical analysis

Data were analysed by a one-way analysis of variance (ANOVA) test using the SPSS statistics software (SPSS Inc., Chicago, IL). Significant differences among the different samples were evaluated by using the Tukey's test at an confidence interval of 95%. Data was expressed as the mean ± standard deviation. Box plots were also used to display differences between groups of data.

3. RESULTS AND DISCUSSION

In this work, PP films containing catechin or green tea extract as antioxidants agents and PPG-PEG-PPG as plasticizer were successfully

produced by means of extrusion. Release from those extruded materials (Table 7.1) has been evaluated in this study. Release of 7 catechins, gallic acid, quercetin and caffeine was tested. Not only have the active substances added to the film been evaluated, but also the chain extender PPG-PEG-PPG which could migrate to foodstuffs.

3.1. Antioxidants content in Green Tea

Table 7.2 compiled the quantification data for extracts of green tea in 10% and 50% of ethanol-water by means of HPLC-PDA-QqQ. The content of green tea catechins together with gallic acid, quercetin and caffeine was found to be approximately 71% of its weight. Very similar content was obtained both in 10% and 50% ethanolic extracts.

Table 7.2. Antioxidants content of green tea extract.

	$mg_{compound}\ g^{-1}_{green\ tea}$	
	10% ethanolic extract	50% ethanolic extract
GA	13.7±0.241	ND
EGC	49.4±0.291	10.2±0.932
CATE	17.4±1.18	17.4±1.08
EGCG	303±3.10	235±26.6
EPI	38.7±1.78	40.0±0.908
GCG	73.0±5.36	32.0±7.89
Caff	94.5±1.94	93.5±0.713
ECG	107±0.518	104±2.08
CG	15.0±0.538	12.8±0.262
Quer	ND	ND

Seven catechins were determined being EGCG, ECG and GCG the most abundant in green tea sample, constituting up to 80% of the content of green tea in catechins (without considering the percentage of gallic acid and caffeine). Therefore, the largest percentage of catechins present in green tea exists as gallate forms, which are also the more polar catechins. Caffeine represents 13 % of the total content of green tea in catechins, gallic acid and caffeine while gallic acid was only found to be in less than 2% of the total content, which is in consonance with those studies that claim that high levels of gallic acid in tea samples should be more related with a degradation process of tea sample (López de Dicastillo et al., 2011).

Therefore, those seven catechins, gallic acid, caffeine and quercetin were selected as to study the release of green tea components from active films formulation.

3.2. Thermal analysis

Stability of the film. Table 7.1 shows the OIT values for the studied films. Longer the OIT value shows that material is more stable against oxidation degradation at that temperature. The results of the OIT measurements revealed that addition of plasticizer did not influence polypropylene stability if antioxidant is not added (no significant differences were observed between those values: M0-A and M0-B, Table 7.1). Nevertheless, the longest OIT obtained for polypropylene doped with catechin or green tea (OIT>30min) confirmed that these compounds provided polypropylene with stabilization against thermal-oxidation. These results are also confirmed by statistical analysis. It is worth remarking that catechin and green tea provided

polypropylene with similar stability, especially when the highest amount of both antioxidants is used. However, on the other hand, as the concentration of catechin in films increases from 2 to 5 % OIT decreases. It could be related with the possible loss of effectiveness when the amount of additive employed exceeds the ideal percentage and, thus, the effective rate, and reaches the so-called waste percentage (Avendaño, 1992).

When antioxidant and plasticizer are simultaneously added to film formulations, significant differences were observed with reference to blank samples. Moreover, higher OIT values are observed at higher plasticizers amounts, which could be an indicator of a possible effect of the plasticizer on the fixing of the antioxidant onto the matrix or a possible protective effect against the oxidant reaction.

Antioxidant stability. Catechin and green tea extracts stability through time and with temperature tested through HPLC measurements revealed that extracts were affected by thermal and temporal conditions. Changes in the concentration of some compounds were observed (Table 7.3). Epimerization of catechin to epicatechin was observed at 40 ºC over prolonged contact time. Several catechins concentrations decreased considerably, especially the gallate species. Ethanol has also resulted important since catechins stability increases with increasing the percentage of ethanol.

Stability of green tea antioxidants through extrusion process was also considered. According to López de Dicastillo et al., 2011, thermogravimetric analysis of the green tea sample revealed a broad degradation band that starts at 150 ºC, with a maximum at approximately 200 ºC. Nevertheless, the specific amount of antioxidant lost during that process was not considered in this

work for the following data, since the aim of the present work was to study how the use of plasticizers improved the release of antioxidants and how it could be used for future packaging applications, were losses through the different processes should be assumed.

Table 7.3. Stability of the studied antioxidants under time and temperature conditions. Data expressed as relative standard deviation (RSD).

	Simulant A		Simulant D_1	
	5 days	10 days	5 days	10 days
GA	Nd	Nd	Nd	Nd
EGC	14.6	7.52	3.71	5.88
CATE	2.91	5.02	7.83	8.06
EGCG	65.6	67.1	Nd	Nd
EPI	5.61	4.05	19.5	12.6
GCG	19.0	27.6	1.06	13.3
Caff	9.48	49.0	0.09	15.1
ECG	8.49	8.64	1.77	1.19
CG	15.5	6.00	7.51	Nd
Quer	Nd	Nd	Nd	Nd

3.3. Release of catechins

Different migration profiles were observed when catechin or green tea extract were incorporated into film formulations with increasing amounts of PPG-PEG-PPG as plasticizer (Figures 7.1 and 7.2).

Differences were observed into the release from films extruded with commercial catechin and with green tea, which can be clearly attributed to the different composition of green tea extracts reported beforehand.

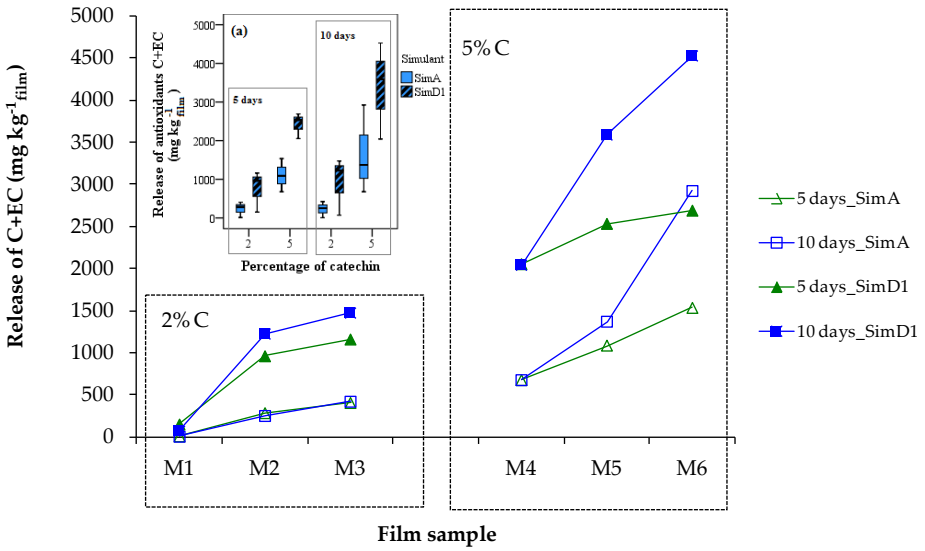


Figure 7.1. Release profiles of catechin from extruded films containing PP, catechin and PPG-PEG-PPG (M1 to M6, Table 7.1) into food simulants A and D_1 at 40ºC over 10 days. Box plots were drawn to graphically represent and compare numerical data sets by using SPSS statistics software and included as graph (a).

The use of a plasticizer into film formulation improved the release capacity of the modified films. In films prepared with commercial catechin (M1 to M6, Table 7.1), the use of PPG-PEG-PPG showed a significant effect with regard to the release of catechin (Figure 7.1). Levels of catechin released from films with 2% of catechin and 2 and 5% of PPG-PEG-PPG (M2 and M3, respectively) between 30 to 40-fold higher than the corresponding films without plasticizer (M1) into simulant A were observed. Increases between 6 and 20-fold were observed into simulant D_1. When considering the samples with 5% of catechin (M5, M6) increasing release values between 2 to 5-fold were observed, though. Adding 5% of catechin into film formulation also has

meant increasing the migration level when compared with those films doped with 2% of catechin (between 3 and 7-fold higher).

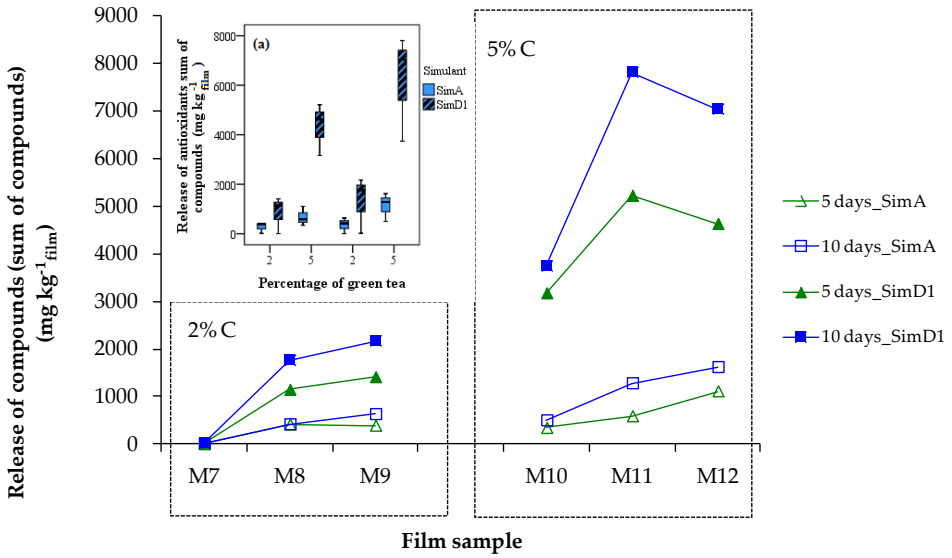


Figure 7.2. Release profiles of the studied compounds from green tea expressed as sum of catechins, gallic acid, caffeine and quercetin (mg kg^{-1}_{film}) from extruded films containing PP, green tea and PPG-PEG-PPG (M7 to M12, Table 7.1) into food simulants A and D_1 at 40ºC over 10 days. Box plots were drawn to graphically represent and compare numerical data sets by using SPSS statistics software and included as graph (a).

In films prepared with green tea (M7 to M12), the use of PPG-PEG-PPG as plasticizer also showed a significant effect in the release of catechins, as well as gallic acid, caffeine and quercetin (Figure 7.2).

Statistical comparison through box plot (Figures 7.1a and 7.2a) also shows differences between release data from films with different percentage of plasticizer.

Individual release data of each catechin, gallic acid, caffeine and quercetin from PP/PPG-PEG-PPG/green tea films (M7 to M12, Table 7.1) are shown in Table 7.4.

As it can be seen, including PPG-PEG-PPG into film formulation generally meant a significant improvement in the amount of compounds released from processed films, specially from film samples doped with 5% of green tea into simulant A, and from samples doped with 2 and 5% of green tea into simulant D_1.

Differences in the release behaviour were observed among the individually studied green tea compounds (Table 7.4). In general, the two main compounds that were released into the simulants were gallic acid and caffeine, followed by catechins, which can be explained by the much smaller molecular size of the former which facilitate their release. That difference is also more evident into simulant A. Moreover, catechins not released from films without plasticizer showed significant levels of migration, from those films into both simulant A and D_1. Average release levels from samples with 2% of green tea between 10 to 20- fold higher than from film samples without plasticizer were then observed. When considering the samples with 5% of green tea (M10 to M12) increased release values between 2 to 12-fold were observed.

Table 7.4. Release of each catechin, gallic acid, caffeine and quercetion from PP/PPG-PEG-PPG/green tea films (M7 to M12, Table 7.1) into simulants A and D_1 at 40ºC after 5 and 10 days of contact. Data expressed as mg of compound per kg of film.

After 5 days

	GA	EGC	C	EGCG	EC	GCG	Caff	ECG	CG	Quer
Simulant A										
M7	13.4	Nd	Nd	Nd	Nd	Nd	0.148	Nd	Nd	Nd
M8	336	Nd	Nd	Nd	Nd	Nd	77.2	Nd	Nd	Nd
M9	223	8.21	5.62	Nd	3.66	Nd	136	11.9	0.731	Nd
M10	211	Nd	17.2	Nd	8.08	0.76	72.8	19.9	10.5	Nd
M11	171	15.7	45.4	17.7	23.8	41.9	163	56.8	50.8	Nd
M12	366	19.2	101	17.6	50.6	39.3	327	109	80.8	Nd
Simulant D_1										
M7	Nd	-7.89	ND	Nd	Nd	Nd	15.4	Nd	-2.26	0.353
M8	904	-7.63	36.2	Nd	10.3	Nd	144	32.5	23.4	0.129
M9	927	-6.66	58.9	Nd	40.3	Nd	255	94.0	38.8	0.612
M10	1014	148	101	507	111	501	404	200	180	2.32
M11	986	328	210	1092	184	944	709	378	380	2.48
M12	1383	137	224	538	189	558	772	452	369	2.25

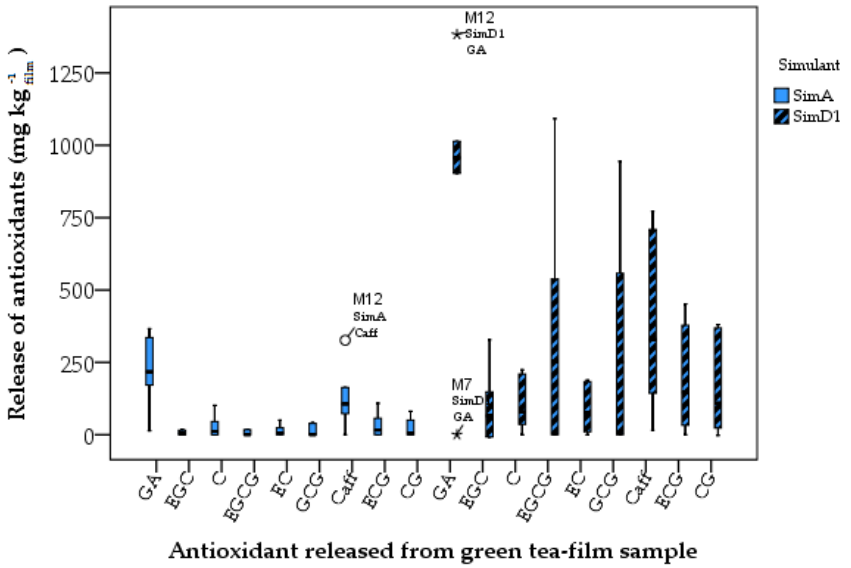
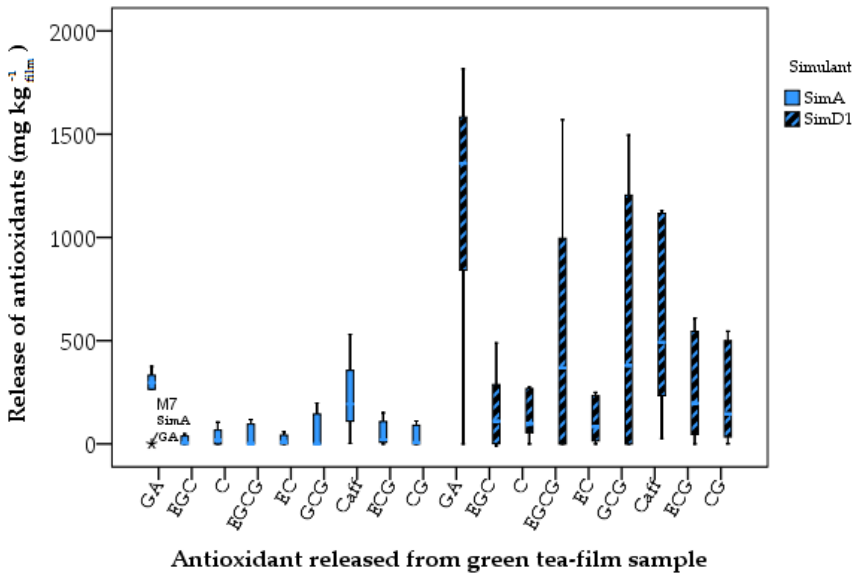


Table 7.4. (Continuation I).

		GA	EGC	C	EGCG	EC	GCG	Caff	ECG	CG	Quer
After 10 days		**Simulant A**									
	M7	Nd	Nd	Nd	Nd	Nd	Nd	2.38	Nd	Nd	Nd
	M8	299	1.76	Nd	Nd	Nd	1.04	110	7.82	Nd	Nd
	M9	333	7.33	16.7	3.31	8.41	Nd	243	16.2	4.73	Nd
	M10	296	Nd	19.9	Nd	9.73	Nd	143	24.5	9.59	Nd
	M11	265	37.9	66.3	118	42.7	197	356	108	89.8	Nd
	M12	375	48.6	106	95.8	58.5	144	530	151	111	Nd
		Simulant D_1									
	M7	Nd	<LOD	Nd	Nd	Nd	Nd	26.0	Nd	0.530	Nd
	M8	1379	1.68	54.2	Nd	15.4	Nd	234	45.5	34.2	Nd
	M9	1338	13.5	83.4	68.36	53.1	Nd	415	139	63.8	0.491
	M10	842	207	111	669	114	760	571	255	227	3.08
	M11	1582	489	268	1569	233	1495	1117	545	546	3.99
	M12	1816	288	273	996	250	1204	1131	609	499	3.51



D estimated by equation 2, section 2.5.; D for M7 to M12, calculated for each individual compound; Nd: not detected. Box plots were drawn to graphically represent and compare numerical data sets by using SPSS statistics software.

However, release of quercetin was only observed into simulant D_1 from both samples doped with 2 and 5% of green tea. The low solubility of quercetin into aqueous media (less than 5 mg L^{-1}) can explain its no release into simulant.

Furthermore, increasing the amount of plasticizer into the film formulation from 2 to 5 has meant an increase into the amount of compound released from 0.6 to 4-fold depending on the film considered.

Due to the extent of the release depends on the compatibility of the active substance with the polymeric matrix and the simulant, the higher the solubility in the simulant, the higher the release. As it could be observed, highest release was displayed from all the films into simulant D_1 than into simulant A (between 2 and 15-fold higher), which could be attributed to the higher solubility of the studied compounds as the higher is the ethanolic content of the simulant. Thus, the higher water solubility of caffeine explains that higher release into simulant A. Regarding gallic acid, the high amount released compared with its low amount in green tea sample, especially when simulant D_1 is the extraction solvent, may also indicate a possible contribution as a result of the degradation of other catechins, in which case the ester bond of the gallates could break during manufacture process, resulting in an increase of gallic acid concentration (López de Dicastillo et al., 2011). Regarding catechins, its release is more evident in simulant D_1 with higher ethanolic content due to their higher solubility in ethanol than in water. According to release data (Table 7.4) and the amount of each compound in green tea sample (Table 7.2), it seems to be a relation between amount released and the sample green tea major components in green tea sample.

Finally, the low solubility of quercetin in water resulted in its non released into simulant A. Nevertheless, the presence of 50% of ethanol into simulant D_1 slightly increased its release. Very low amounts were, however, released (between 100 and 300 times less than catechins).

Statistical comparison between data released into different simulant (Figures 7.1a, 7.2a and Table 7.4) also confirmed those results.

Contact time also influenced release levels. Higher time has led to release of the studied compounds from the film samples.

Diffusion coefficients (Figure 7.3, Table 7.5), estimated by equation 2 (section "2.5. Release studies") and statistical compared through box plot representations, also confirmed that the release of the studied compounds was accelerated by the presence of the plasticizer. PPG-PEG-PPG increased the diffusivity of the studied compounds between 1 and 3 orders of magnitude. Moreover, the diffusivity values of catechin and green tea extract into simulant D_1 were slightly higher than in simulant A, which may be related to their different ethanolic content and its effect over the polymer matrix (López de Dicastillo et al., 2012). Comparing these values of diffusivity with those previously obtained for other compounds, namely tocopherols, from similar polymer matrices (Castro-López et al., 2012), higher D values were obtained for catechins, which may be attributed to their higher solubility in water and, therefore, in simulants A and D_1 than tocopherols. When comparing to the diffusivity of catechins from other matrices, namely EVOH or PLA (Íñiguez-Franco et al., 2012; López de Dicastillo et al., 2011, 2012) with a much more polar nature than PP, the data from the present work showed how the incorporation of PPG-PEG-PPG gives place to a similar diffusivity

than from those polymer matrices (D~1E-10 to 1E-12 vs D~1E-9 to 3E-11 for release of catechin from EVOH matrices into simulant A and D_2 (95% ethanol) (López de Dicastillo et al., 2011, 2012) and D~ 5E-10 from PLA matrices into simulant D2 (95% ethanol) with no release into simulant A or D_1 (Íñiguez-Franco et al., 2012).

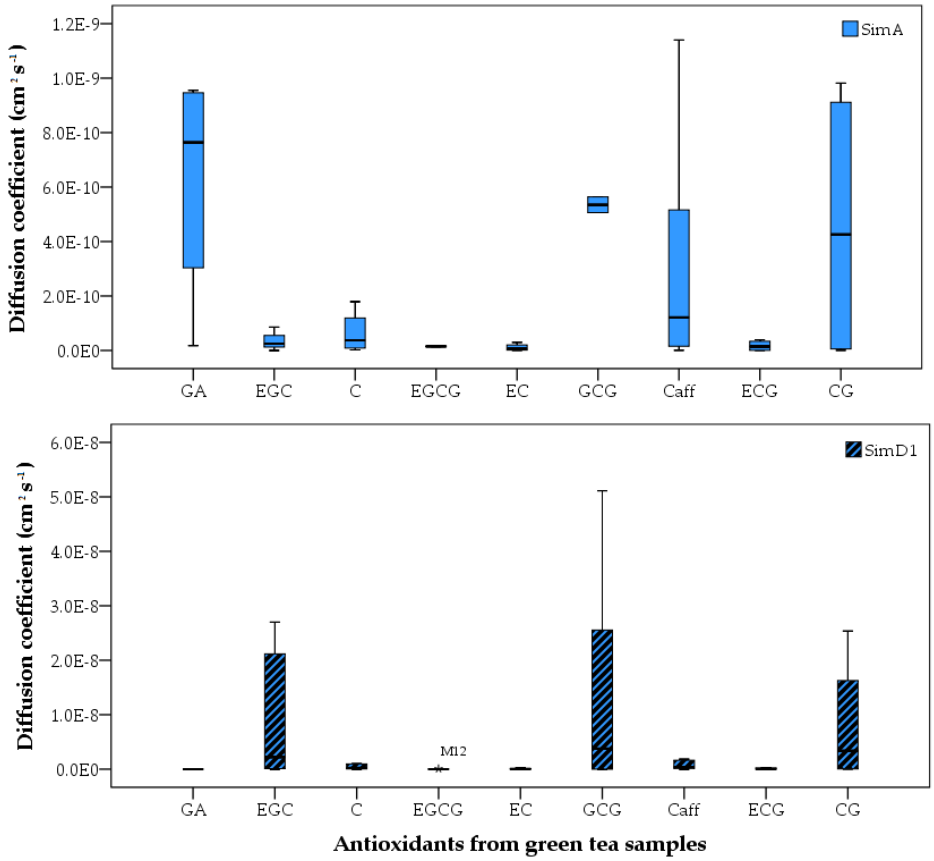
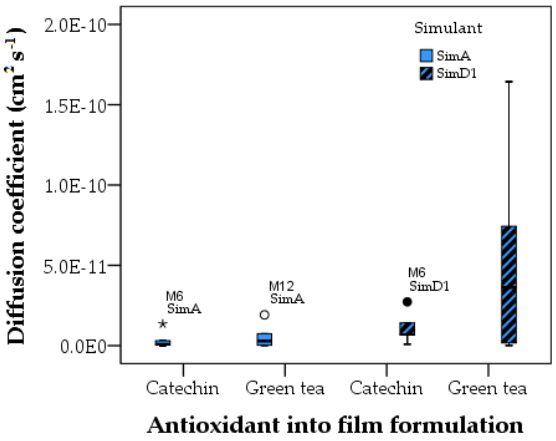


Figure 7.3. Estimation of the diffusion coefficients (D, cm^2s^{-1}) for the release of catechins, gallic acid and caffeine from PP/PPG-PEG-PPG/green tea films into simulants A and D_1 at 40ºC. Data graphically represented and compared as box plot representations.

Table 7.5. Estimation of diffusion coefficient (D, cm^2s^{-1}) for the release of catechin from PP/PPG-PEG-PPG/catechin or green tea films to simulants A and D_1 at 40ºC.

Sample	Simulant A	Simulant D_1
M1	2.3E-14	8.1E-13
M2	3.1E-12	6.8E-12
M3	0.3E-12	6.7E-12
M4	1.2E-12	7.0E-12
M5	0.6E-12	1.4E-11
M6	1.4E-11	2.7E-11
M7	8.4E-14	6.7E-14
M8	6.9E-13	1.3E-11
M9	5.1E-12	5.9E-11
M10	3.0E-13	1.8E-12
M11	7.4E-12	7.4E-11
M12	1.9E-11	1.6E-10



D estimated by equation 2, section 2.5; D for M7 to M12, calculated as sum of catechins, gallic acid, caffeine and quercetin. Box plots were drawn to graphically represent and compare numerical data sets by using SPSS statistics software.

3.4. Migration of PPG-PEG-PPG

Introducing the plasticizer into film formulation also means adding a new potential migrant that can have a potential influence into the food or food simulant in contact with.

Figure 7.4 shows the percentage of PPG-PEG-PPG migrated into simulants A and D_1 at 10 days of contact. The migration of PPG-PEG-PPG depended on its initial amount in the film formulation, on the storage time and on the food simulant in contact with.

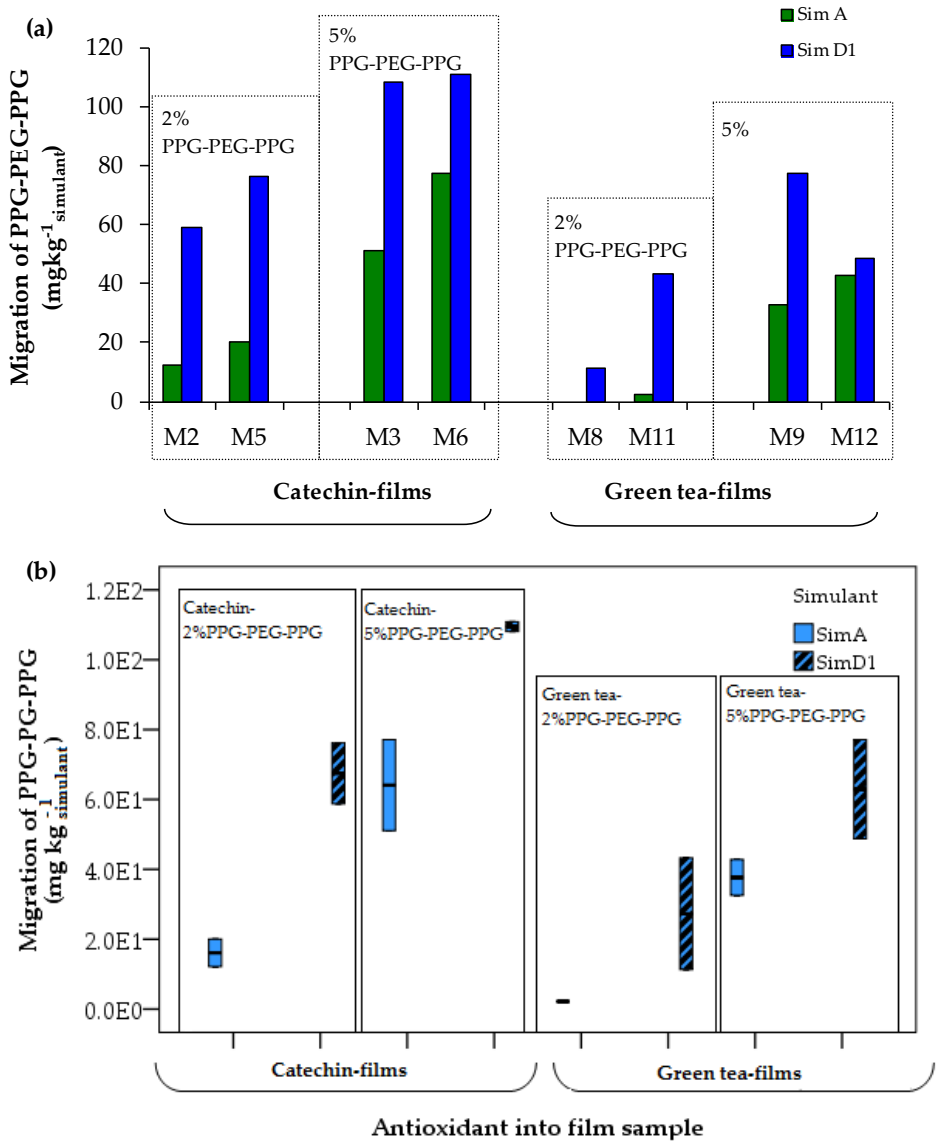


Figure 7.4. (a) Migration of PPG-PEG-PPG from catechin and green tea-containing films into simulant A and D_1 at 40ºC and 10 days. (b) Box plots were drawn to graphically represent and compare numerical data sets by using SPSS statistics software and included as graph.

According to experimental data, higher migration of plasticizer was observed from polymer samples formulated with higher concentration.

Statistical differences in the migration of the plasticizer were also observed among films doped with catechin and green tea (Figure 7.4). Data showed higher migration levels from catechin-film samples.

On the other hand, the effect of the time on the migration behaviour can be explained as increased time resulted in slight higher percentages of PPG-PEG-PPG. Moreover, simulant D_1 was the simulant presenting the higher levels of migration.

Nevertheless, as PPG-PEG-PPG is not included on the Union list of authorized monomers, other starting substances, and macromolecules (The European Commission, 2011), and having a molecular mass higher than 1000 Da and being capable of forming the main structural component of the plastic material, it complies with the requirements of the regulation.

3.5. Antioxidant activity

Figure 7.5 presents the antioxidant activity of food simulants exposed to catechin and green tea-containing films, expressed as equivalent of gallic acid.

Comparing antioxidant activity data with release data (Figures 7.1 and 7.2), the same profile is shown in both studies. Therefore, antioxidant activity was shown to be proportional to the antioxidant concentration in each simulant. Thus, an increase in the initial amount of catechin or green tea and/or the amount of PPG-PEG-PPG lead to higher release and higher antioxidant activity. The effect of the type of simulant was also noticeable (Figure 7.5b),

presenting around an average of 4 times higher antioxidant activity on simulant D_1 than in simulant A, which can be attributed to the highest solubility of the studied compounds in ethanol than in water. Moreover, the effect contact time was also evident.

Likewise in release studies, antioxidant activity data showed a higher antioxidant capacity of the catechin-containing films than the green-tea containing films. It can be associated with the different content of each film in the studied compounds: 2 or 5% of pure catechin or green tea, respectively. However, in the latter, according to green tea extract quantification (Table 7.2), catechins, gallic acid, caffeine and quercetin make up only the 71% of its weight and each compound which results in a less a amount of each compound able to be released than in the catechin-containing films.

Therefore, antioxidant active films, based on polypropylene polymer formulations modified with PPG-PEG-PPG as a plasticizer, and natural antioxidants were successfully developed. Modifying PP matrix with PPG-PEG-PPG, release of catechins, gallic acid, caffeine and quercetin was clearly favoured. Increasing the amount of active agent, the amount of plasticizer, the contact time and/or the ethanolic content of the food simulant, release has been improved.

Moreover, adding an antioxidant as individual or as a component of a natural sample mixed with other antioxidants of different structures and polarities, resulted in a potential system to be used in active packaging likely for a controlled release of those antioxidants to a wide range of foods where they maintain their antioxidant capacity.

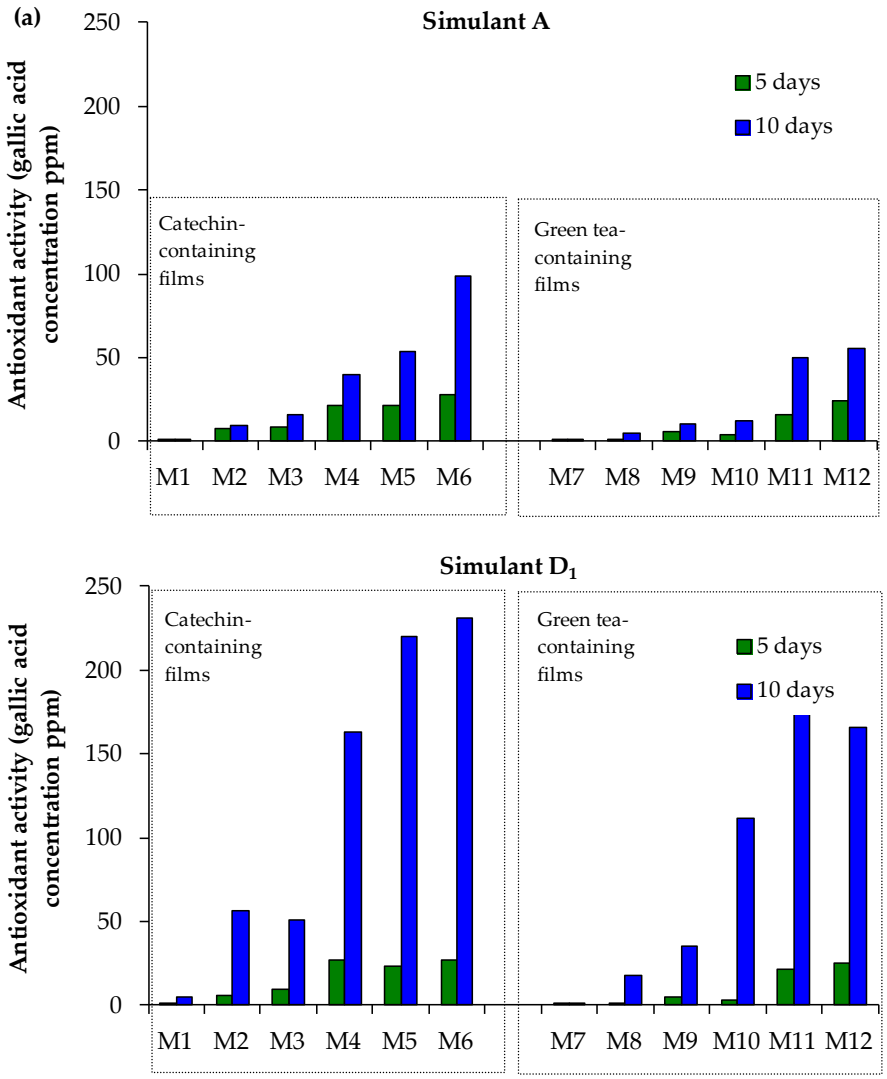


Figure 7.5. (a) Antioxidant activities of simulants A and D_1 in contact with catechin-containing and green tea-containing films (M1 to M12, Table 7.1) over 5 and 10 days of contact at 40ºC. (b) Graphical representation and statistical comparison of antioxidant activity data through box plot (SPPSS statistics software). Data measured by ABTS assay and expressed as gallic acid concentration (ppm).

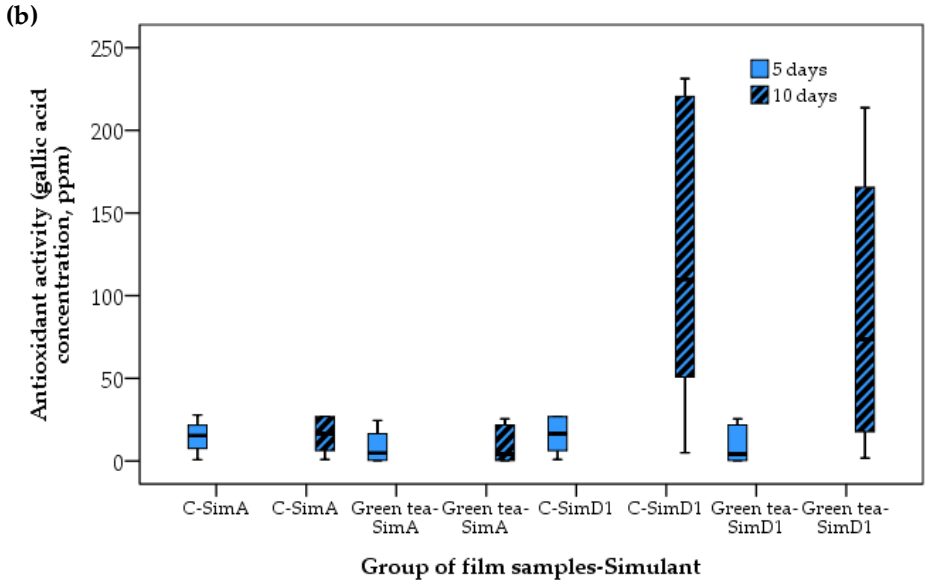


Figure 7.5. Continuation (I).

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Artículo 8

INTERACTION AND RELEASE OF CATECHIN FROM ANHYDRIDE MALEIC GRAFTED POLYPROPYLENE FILMS

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ABSTRACT

In this paper, investigations were carried out on catechin-loaded maleic anhydride (MAH)-modified polypropylenes (PP). Two maleic-modified polypropylenes (PPMAH) with different maleic concentrations have been blended with PP and catechin to obtain composites of improved catechin retention with the aim of studying the possible interactions between these grafted polymers with antioxidants, and a secondary interest in developing an active antioxidant packaging. Composite physicochemical properties were measured by thermal analysis (thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and oxidation induction time (OIT)) and infrared spectroscopy studies. Catechin release profiles into food simulants were obtained by HPLC-PDA-QqQ following European Legislation. Antiradical activity of composites was analyzed by the ABTS and DPPH method. The formation of intermolecular hydrogen bonds between catechin and functionalized PP has been confirmed by fourier transform infrared (FTIR) studies. Besides, a small fraction of ester bonds, formed as a result of a chemical reaction between a fraction of the hydrolyzed anhydride and the catechin hydroxyl groups, is not discarded. OIT results also showed an increase in antioxidant effectiveness caused by the presence of catechin- and maleic-modified PPMAH in the blend formulations. Incorporation of MAH-grafted PP increased substantially the retention rate of catechin, being dependent on the MAH content of the grafted polypropylene. The described interactions between catechin and maleic groups, together with changes in PP morphology in comparison with reference PP explained lower antioxidant

release. Besides formulation, antioxidant release was dependent on the type of food, temperature and time.

Keywords: maleic anhydride grafted polypropylene, natural antioxidants, catechin, active antioxidant packaging, release, immobilization.

1. INTRODUCTION

New polymer formulations are emerging, as far as new applications are considered. Despite the increasing demand for biodegradable materials, polyolefins are still the most common polymers used for food packaging. Polyolefins, similar to most synthetic polymers, have a hydrophobic and chemically inert surface which, therefore, leads to low adsorption of dyes or inks, poor adhesion to coatings or other materials, the generation of static electricity, incompatibility with hydrophillic substances, and many other problems. Among the techniques employed to modify polymers, grafting of polyolefins with polar monomers have received special attention, because of their potential applications, by which a variety of desired graft chains can be introduced onto the polymer surface without changing the bulk properties (Moad, 1999; Xing et al., 2005a; Goddard and Hotchkiss, 2007). There are a wide variety of these materials, such as polyolefins with grafted maleic anhydride (MAH), fumarate and maleate esters, methacrylate esters, or mathacrylic acid. These polymers often serve as precursors of other polyolefin graft copolymers.

Maleic anhydride (MAH)-modified polyolefins are one of the most important class of functionalized polyolefins in commercial applications,

because of their low cost, high activity and good processability. Free radical-induced grafting of MAH onto polyolefin substrates has been carried out in the melt phase in various forms of extruders and batch mixers, in solution, and in the solid state. In all cases, controversy arises concerning the final structure of the functionalized polymer, with respect to the nature of the grafted units and the distribution of the graft sites (Rengarajan et al., 1990; Sclavons et al., 1996; Lu and Chung, 1998; Pang et al., 2000). Maleic anhydride grafting onto polypropylene (PPMAH) has been carried out basically with the objective of achieving compatibility between polar and nonpolar polymers (Pang et al., 2000; Bettini and Agnelli, 2002; Song et al., 2009). In addition, nowadays it is a proved alternative as a compatibilizer between PP and fibers or more hydrophillic materials in order to obtain 'biocomposites`. The latter are composites containing at least one constituent which is derived from renewable resources, offering several advantages such as reducing the cost per unit volume, low density, high strength-to-weight ratio, and nonbrittle fracture. Furthermore, improvements in mechanical properties attributed to fiber/matrix interface enhancement interaction by the addition of PPMAH have been evident (Karmaker and Youngquist, 1996; Stark, 1999). The compounds with anhydride groups are generally active, with tendency to undergo a wide variety of organic reactions such as hydrolysis, esterification, amination, etc. Through these reactions, the graft-modified surface containing anhydride groups are promising to perform extensive post-functionalizations (Xing et al., 2005b).

Moreover, because of the limited stability of polyolefins to high temperatures and ultraviolet light (UV), antioxidants are key ingredients in the formulation of polypropylene (PP) in order to protect the polymer during

package manufacture and use. Most of the common antioxidants are phenolic compounds, secondary arylamines, organophosphates and thioesters of synthetic origin that are approved by the national and international regulations for plastics in contact with food. Nevertheless, migration of these additives and their degradation products into food during storage may change the sensory properties of the product they contain or even lead to toxicity upon consumption. For these reasons, several research studies have focused on the development of alternative polymer formulas with natural antioxidants (Wessling et al., 1999; López de Dicastillo et al., 2010).

In recent years, antioxidants have been incorporated on polymer formulations, not only to protect the polymer during package manufacture and use, but as an active substance to be released on food in order to extend its shelf life. It is the definition of antioxidant active packaging that absorbs radical oxidizing species by the incorporation of an antioxidant into the polymer, being a good choice for many products sensitive to oxidation (Brody et al., 2001; López de Dicastillo et al., 2010; 2012). As mentioned previously, several studies have shown improvements of mechanical properties attributed to enhancement interaction by the addition of PPMAH, but no evidence of interaction between antioxidant substances, incorporated for polymer protection, and functionalized grafted polymer have been studied. In this light, the existence of these interactions is considered interesting, because they can have consequences on the retention of the antioxidant by the polymer matrix.

The objective of this work is to study the interaction of catechin, reported as useful natural antioxidant for PP protection (Dopico-García et al., 2011) within anhydride maleic-modified polypropylenes (PPMAH). Catechin was also selected because it is a flavonoid with multiple biological effects, and it is nonvolatile, reducing the loss during packaging manufacturing that occurs with other compounds such as carvacrol or essential oils. For that, two different maleic anhydride modified polypropylenes (PPMAH) with different MAH concentrations have been blended with polypropylene (PP) and catechin (CAT). Resulting materials were physicochemically characterized, and antioxidant properties were analyzed in comparison with a simple catechin loaded PP as reference material. An important aspect of this study is the characterization of the physical and/or chemical interactions that might have occurred during melt blending between the hydroxyl groups of catechin and grafted PPMAH in order to analyze the influence of these interactions on polymer properties and release profiles in contact with aqueous and fatty food simulants. As well as projecting their future application as food packaging materials, since these systems could extent the shelf life of packaging and packed food products.

2. EXPERIMENTAL SECTION

2.1. Chemicals and reagents

Polypropylene PP 070 G2M (PP) was provided by Repsol YPF (Madrid, Spain). Chemically modified polypropylenes (PPMAH) Fusabond PMD511D and PMZ203D were purchased from DuPont™ (Barcelona, Spain). Both chemically modified polypropylenes have medium and high graft level,

respectively. Specific graft levels of both commercial Fusabond modified polypropylene were reported as confidential by DuPont™. Liu and Konlopoulou, 2006, reported an estimated concentration of maleic anhydride in Fusabond PMD511D between 0.25 and 0.5%. No data was reported for PMZ203D.

Reagent-grade absolute ethanol, methanol, catechin dihydrate (CAT), epicatechin (EPI), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) 95% free radical were purchased from Sigma (Madrid, Spain).

2.2. Materials preparation

Different PP formulations containing catechin as antioxidant agent were obtained in a miniextruder equipped with twin conical co-rotating screws and a capacity of 7 cm^3 (MiniLab Haake Rheomex CTW5, Thermo Scientific). The following parameters were used: a screw rotation rate of 40 rpm, a temperature of 180 ºC, and a residence time of 1 minute.

Ternary mixtures of PP:PPMAH:CAT (4:2:1 w/w/w/) were formulated with chemically modified polypropylenes DuPont™ Fusabond PMD511D and PMZ203D (called PPMAH511CAT and PPMAH203CAT, respectively). Binary blends of PP:CAT (6:1 w/w) were called PPCAT. For comparison, reference samples of PP and modified PPMAH without catechin (PPMAH511 and PPMAH203) were also extruded and submitted to the same characterization. The resulting films were ~ 120 – 130 μm thick.

2.3. Fourier transform infrared spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) was used to characterize the presence of specific chemical groups in the materials. For IR measurements the extruded materials were compression-molded in a hot plate press (IQAP LAP S.L. model PL15-Series 1381, Barcelona, Spain), in order to obtain very thin films (~ 35-45 μm). FTIR was performed in the transmission mode by using an OPUS/IR PS15 spectrometer (Bruker). The spectra were the results of 64 co-added interferograms at 2 cm^{-1} resolution between 4000 to 500 cm^{-1}.

2.4. Thermal characterization

Thermogravimetric analyses (TGA) were carried out using a thermal analyzer (Perkin-Elmer TGA 7). Samples (ca. 10 mg) were heated in 100-μl platinum sample pans from room temperature to 800 ºC under a nitrogen atmosphere at 10 ºC/min, to determine the degradation temperatures of the antioxidant-containing materials.

Differential scanning calorimetry (DSC) measurements (Perkin–Elmer serie 7) were also performed, to analyze the effect of the interaction between the grafted PPMAH and catechin on the morphology and crystallinity of the PP matrix. Crystallinity was also evaluated and contrasted through FTIR data. Thermograms were obtained from -20 to 200 ºC with a heating rate of 10 ºC/min, cooled to -20 ºC, and held at this temperature for 2 min, and a second heating process to 200 ºC was conducted. The melting and crystallization temperatures (T_m and T_c, respectively) and the enthalpies of melting and

crystallization (ΔH_m and ΔH_c, respectively) were calculated from the cooling and the second heating process.

Oxidation induction time (OIT) analyses were conducted to study changes of polymer stability and antioxidant effectiveness caused by the incorporation of PPMAH and catechin in the blend PP formulations. The sample temperature was stabilized for 2 min at 200 ºC under an inert atmosphere, which was subsequently switched to an oxygen atmosphere to start the test. Analyses were carried out according to Standard EN 728 (European Committee for Standardization, 1997).

All thermal analyses were done in triplicates.

2.5. Release studies

A study of the release of the bioactive flavonoid catechin was carried out by determining the specific migration from the polymer into two food simulants specified in European regulations: simulant A (10% ethanol) and simulant D_1 (50% ethanol), as aqueous and fatty food simulants, respectively (The European Parliament and of the council, 2004). Release tests were performed by total immersion of rectangular strips film pieces (8 mm x 0.4 mm x 0.13 mm) in 10 mL of food simulant contained in glass-stoppered tubes with PTFE closures at 40 ºC. Milli-Q water was deoxygenated by bubbling nitrogen, and a final nitrogen flush was done before closing the cells to reduce the oxygen percentage in the cell headspace. Samples were taken after 1, 5, 10 and 20 days of storage. Test materials were also run simultaneously to check for interferences and all samples were performed in triplicate. Legislation just

order migration tests at 40 ºC, but measurements at 25 ºC on day 20 were also performed to observe a possible positive antioxidant release for future active packaging application at room temperature. After the contact period, an aliquot of each simulant was filtered through an Acrodisc^{R} PTFE CR 13-mm, 0.2-μm filters (Waters, Mildford, MA, USA) and analyzed by HPLC-PDA-QqQ, to calculate the released catechin concentration. Release data was expressed as the percentage of catechin released into the food simulants after the contact period: amount of catechin released per kilogram of film with reference to the initial amount of catechin loaded per kilogram of film formulation.

HPLC-QqQ MS conditions. An Agilent 1200 Series Rapid Resolution LC system (Agilent Technologies, Waldbronn, Germany) equipped with an online degasser, binary pump delivery system, high performance SL autosampler, thermostatic column department and is coupled online coupled to a mass spectrometer detector (MS), which was used for analysis. Samples were injected in Zorbax SB-C18 (50 x 2.1 mm, 1.8 μm) column (Agilent Technologies) thermostated at 35 ºC. Binary gradient elution was performed, with flow rate of 0.3 mL min^{-1} and injection volume of 3 μL. Mobile phases were composed by water-1% formic acid (A) and methanol (B). The following gradient elution profile was used: mobile phase composition started at 25% of B, was linearly increased to 40% B in 3 min, followed by a linear increase to 60% B in 0.5 min finally reaching 100% B in other 0.5 min. The column effluent was directly introduced into the triple quadrupole mass detector Agilent 6410 Triple Quad LC/MS (Agilent Technologies) operated in the

positive ionization mode. Ions were formed using electrospray ionization (ESI) with temperature of the drying gas (N_2) set at 350 ºC and flowed at 10 mL min^{-1}. The nebulizing pressure (N_2) was maintained at 35 psi. Capillary voltage was set at 5.5 kV. Selective ion monitoring (SIM) was used to quantify the target ions. Mass spectral data and retention time were used for peak identification. Quantification of catechins was based on an external standard calibration method. Integration and data elaboration were performed using Agilent MassHunter Workstation software, version B03.00 (Agilent-Technology).

2.6. Antiradical activity of materials

Antioxidant activities of catechin-loaded materials were measured by two different antioxidant assays, DPPH and ABTS methods, that measure the antioxidant effectiveness by monitoring the inhibition of their corresponding radicals. The DPPH and ABTS assays are based on the bleaching rate of the free radicals 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), and the radical cation 2,2´-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$), monitored spectroscopically at 517 and 734 nm, respectively. Both radicals, $DPPH^{\bullet}$ and $ABTS^{\bullet+}$, are neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (Prior et al., 2005). These assays were selected, not only because of their simplicity, but also to study the radical scavenging behaviour of the developed materials in different environments, because $DPPH^{\bullet}$ is dissolved in ethanol and $ABTS^{\bullet+}$ in water.

Approximately 30 mg of each material was immersed in 10 mL of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical solutions, and their absorbance was kinetically monitored.

Both radical solutions were obtained as follows: i) DPPH radical scavenging activity was based on the method of Okada and Okada, 1998, with slight modifications (2 mM ethanolic solution of radical DPPH$^{\bullet}$ was diluted to an absorbance value of one at 517nm); ii) ABTS radical cations were produced by reacting 7 mM ABTS in water with 2.45 mM potassium persulfate ($K_2S_2O_8$) and then stored in the dark at room temperature for 16 h. The ABTS radical solution was diluted to give an absorbance value of 1 at 734 nm. All experiments were performed in triplicate.

In both assays, the antioxidant activity is obtained as the percentage inhibition values (I (%)), calculated using this equation:

$$I(\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \right) \times 100$$

To standardize the results, scavenging activities of the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals were expressed as catechin concentration, using a calibrated curve of catechin concentration versus inhibition value (I (%)) of each radical.

Parameters estimation of kinetics of antioxidant activities. Diffusivity (*D*) and partition coefficient (*K*) were estimated based on the experimental results from the antioxidant activities, measured as the percentage inhibition of radicals DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ over time. Because antioxidant activity is based on the antioxidant release to the solvent where radicals are dissolved, through the calibrated curve of catechin concentration versus I (%), results were understood as catechin release. Hence, the extent of the antioxidant activities can be characterized by the partition coefficient, *K*, which defined as

the ratio of the amount of catechin in the polymeric phase, relative to the concentration of released catechin.

The kinetics of the antioxidant activities, translated to catechin released, were also monitored. Several works have demonstrated that the release kinetics of the compounds followed the same profile as antioxidant activity (Pracella et al., 2010; López de Dicastillo et al., 2012). To characterize the kinetics of the mass transport process within the film, it is necessary to solve Fick´s laws considering the boundary conditions of the experiments. In this work, the presence of a partition equilibrium and a limited volume of solvent were considered. Also, the diffusion coefficient (*D*) for the transport of that antioxidant in the film is considered independent of time and position. By integration of Fick´s law with these assumptions, the ratio of the mass of catechin release into the solvent at time t and at equilibrium can be expressed through equation 1:

$$\frac{m(t)}{m_S^f} == 1 - \sum_{n=1}^{\infty} \frac{2\alpha(1+\alpha)}{1+\alpha+\alpha^2 q_n^2} \exp\left(\frac{-4Dq_n^2 t}{l^2}\right) \quad (1)$$

where m(t) is the mass of the migrant in the food at a particular time t (s), M_s^f the mass of migrant in the food at equilibrium, l, the film thickness (cm), *D* the diffusion coefficient t(cm^2 s^{-1}), and t the time (s). The parameter α represents the ratio between the mass of compound in the liquid and that in the polymer at equilibrium ($\alpha=V_S/(KV_P)$, where V_S and V_P are the solution and polymer volumes respectively), and q_n are the positive solutions of the equation 2 (Garde et al., 2001):

$$tg(q_n) = -\alpha q_n \quad (2)$$

3. RESULTS AND DISCUSSION

3.1. Molecular interaction study

Information on the nature of the molecular interactions between catechin and maleic groups within the modified functionalized PP matrix was monitored using infrared spectroscopy. FTIR spectra for PP, MAH-modified polymer (PPMAH203), catechin-loaded PP and catechin-loaded PPMAH203 are shown in Figures 8.1 and 8.2. MAH-modified polymer PPMAH203 was selected because of its higher content of maleic anhydride. The FTIR spectrum of PPMAH203 (Figure 8.1B) exhibits a double absorption band at 1861-1844 cm^{-1}, which is assigned to the asymmetric stretching of the anhydride carbonyl groups, along with a strong broad band centered at 1784 cm^{-1} (the second derivative spectrum reveals that this band is composed of two bands at 1792 and 1779 cm^{-1}), corresponding to the symmetric stretching of the carbonyl groups, with the 1792 cm^{-1} peak being an absorbance characteristic of carbonyl from cyclic anhydrides with five-membered ring (Lu and Chung, 1998; Henry et al., 2008). The band involving the stretching vibration of the C-O-C group appears at 918 cm^{-1} in cyclic five-membered ring acid anhydrides. An extra strong absorption band centered at 1713 cm^{-1} corresponds to the carbonyl stretching (νC=O) of the acid form, indicating that a high proportion of maleic anhydride incorporated into PPMAH203 has been hydrolyzed during the sample processing. The well-defined band at 3640 cm^{-1} (OH stretching of the acid form) corroborates the high level of anhydride hydrolysis (Figure 8.1A).

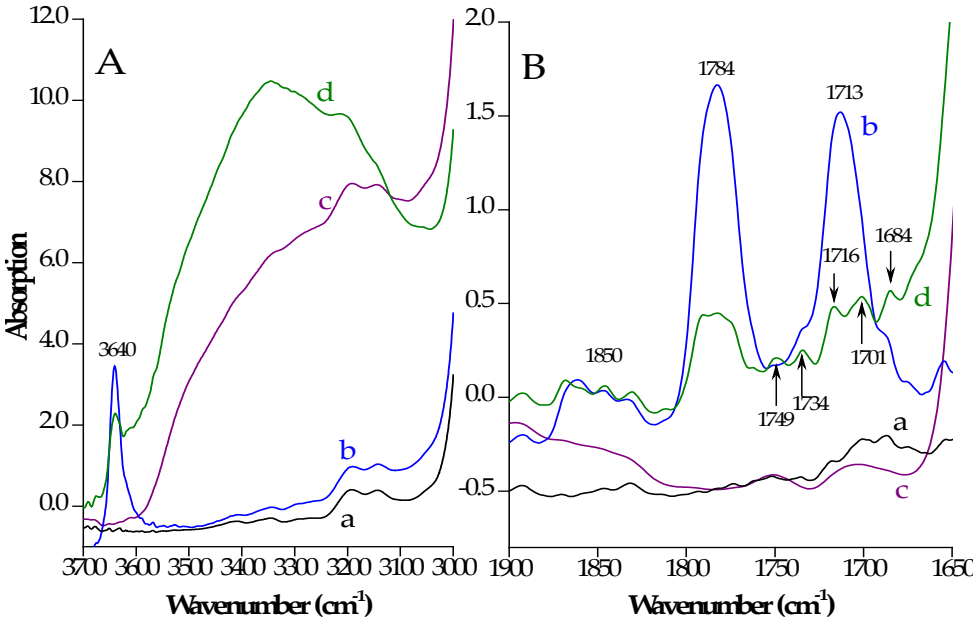

Figure 8.1. FTIR spectra of original PP (spectrum a), MAH-modified polymer (PPMAH203) (spectrum b), catechin-loaded PP (spectrum c) and catechin-loaded PPMAH203 (spectrum d) in (A) the hydroxyl vibration region and (B) the carbonyl vibration region.

For catechin-loaded PP, the FTIR spectrum shows the characteristic catechin bands. Catechin has five hydroxyl groups in one molecule and the most relevant feature of its FTIR spectrum is the broad band centered at 3200 cm^{-1}, attributed to the summation of several contributions corresponding to hydroxyl groups in different situations (intramolecular and intermolecular self-association, non-hydrogen-bonded groups) (Figure 8.1A) (Cesteros et al., 1993). Other bands are the C=C aromatic ring stretching vibrations (1620 and 1517 cm^{-1}), the C–O stretching of the oxygen in the ring (1281 cm^{-1}), the alcohols C-O stretching vibrations (1140, 1108 cm^{-1}) and the aromatic =C-H

out-of-plane deformation vibration (814 cm^{-1}) (see Figures 8.2A and 8.2B) (Chen et al., 2006).

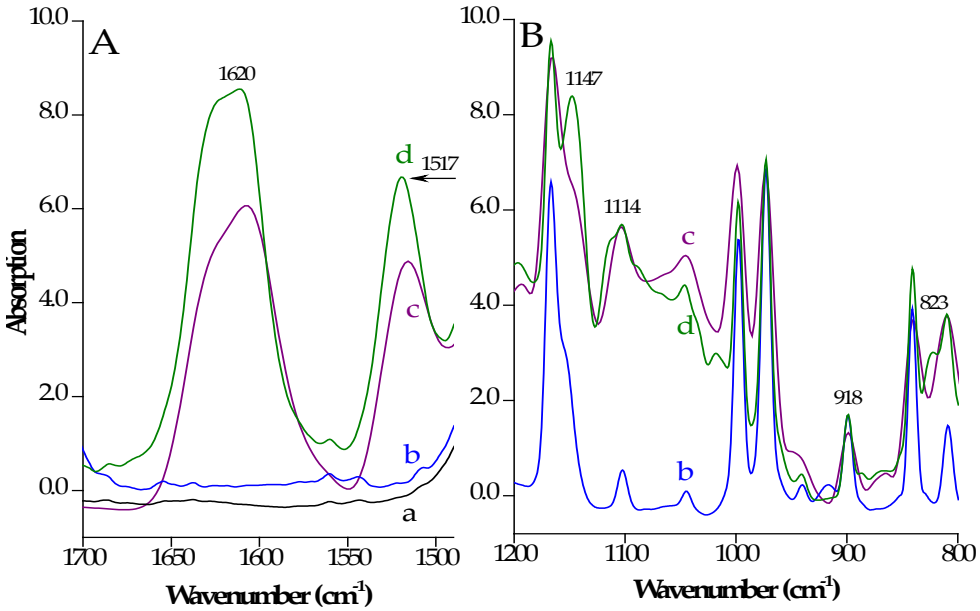

Figure 8.2. FTIR spectra of original PP (spectrum a), MAH-modified polymer (PPMAH203) (spectrum b), catechin-loaded PP (spectrum c) and catechin-loaded PPMAH203 (spectrum d) in (A) the C=C aromatic ring stretching vibration region and (B) the region between 1200 and 800 cm^{-1} (B).

Some differences in the catechin structure as well as in the MAH bands can be observed in the sample that was blended with PPMAH203. The most significant change lies in the hydroxyl vibration region (3600-3000 cm^{-1}). When catechin is blended with PPMAH203, the maximum of the band is shifted toward higher wavenumbers (3345 cm^{-1}) and the band increased in terms of energy absorption. These observations suggest that catechin OH

groups are associated with the carbonyl groups of MAH moieties by hydrogen bonds. The difference between the wavenumber of self-associated hydrogen bond and that of intermolecular hydrogen bonded one could be due to the balance between the number of broken catechin-OH···HO-catechin self-associations and the number of formed catechin-OH···O=C-maleic bonds The fact that the new band lies in the higher wavenumber region indicates that the strength of catechin-maleic acid hydrogen bonds is weaker than that of the catechin-O···HO-catechin ones (Li et al., 2003). Furthermore, there is a significant decrease in the peak intensity at 3640 cm^{-1} corresponding to the OH stretching of the non-hydrogen-bonded maleic acid (Figure 8.1A).

More to the point, as catechin exhibits no absorption in the 1800-1650 cm^{-1} region, any changes observed in the FTIR spectrum in this region should be attributed to those in the chemical environment of maleic anhydride/acid carbonyls such as the formation of covalent bonds (ester bonds) and/or hydrogen bonds. For catechin-loaded PPMAH203, a significant reduction on the intensity of the formerly described anhydride and maleic acid bands is patent, as a result of the PP and catechin dilution effect.

Nevertheless, the appearance of new bands due to the interaction with catechin can be detected, namely, 1749, 1734, 1716 and 1701 (as a result of the splitting of the former peak at 1713 cm^{-1}) and 1684 cm^{-1} (Figure 8.1B). The bands at 1749 and 1734 cm^{-1} could be ascribed to non-hydrogen-bonded and hydrogen-bonded ester bands, respectively, and the bands 1701 and 1684 cm^{-1} could be assigned to hydrogen-bonded carbonyls of the acid form. Apparently, these bands suggest two types of interactions between catechin and maleic moieties. First, a stable ester bond may have been formed as a

result of the chemical reaction between a fraction of the hydrolyzed anhydride and catechin OH groups. Second, hydrogen bonds are formed between the different kind of carbonyl groups present in the polymer mixture (anhydride and acid carbonyls) and the various catechin OH groups. Nevertheless, as the intensities of these bands are very small, near the signal-to-noise ratio, one must be cautious, especially when asserting the presence of ester bands.

Additional changes in the catechin absorption bands lay in the regions of C=C aromatic ring stretching vibrations ~ 1600 cm^{-1} and C-O stretching of alcohol groups (1150-1070 cm^{-1}) (Figures 8.2A and 8.2B). Concerning the region between 1680-1490 cm^{-1}, the narrowing and up-shifting of the two bands assigned to the C=C aromatic ring stretching vibrations (1620 and 1517 cm^{-1}) is detected. Similar changes have been observed in catechin polymer blends upon forming hydrogen bonds between the ester carbonyl group in the polymer (PCL) and the hydroxyl groups in catechin (Kang et al., 2012). Significant modifications in the C-O stretching region are the intensity increase of the bands at 1147 and 1114 cm^{-1}. An increase in absorption is also observed at 823 cm^{-1}, corresponding to the aromatic =C-H out of plane deformation vibration. Concerning maleic anhydride, the band at 918 cm^{-1}, alloted to the stretching vibration of the C-O-C group, can no longer be detected in the catechin-PPMAH203 blends. Changes in catechin bands have been perceived in both catechin-loaded PPMAH203 and PPMAH511 in comparison with the catechin-PP blend, although intensity changes are greater for the catechin-PPMAH203 blends. Conversely, possible modifications in carbonyl anhydride and acid bands have not been clearly observed in the latter mixtures.

From another point of view, the degree of crystallinity of the PP fraction estimated from the isotacticity ratio or band ratio $A_{998/973}$ increases in all catechin-PP blends in comparison with both original and functionalized PPs, as a result of the induction effect of the catechin crystallites on the crystal nucleation of iPP (Table 8.1) (Andreassen, 1999; Pracella et al., 2010). This behavior has been observed in previous works (López de Dicastillo et al., 2010; 2011). In conclusion, the formation of intermolecular hydrogen bonds between catechin and functionalized PP has been confirmed by FTIR analysis in both catechin-loaded PPMAH203 and PPMAH511. Accordingly, the possible molecular interaction between catechin and maleic anhydride-grafted polypropylene is graphically proposed in Figure 8.3.

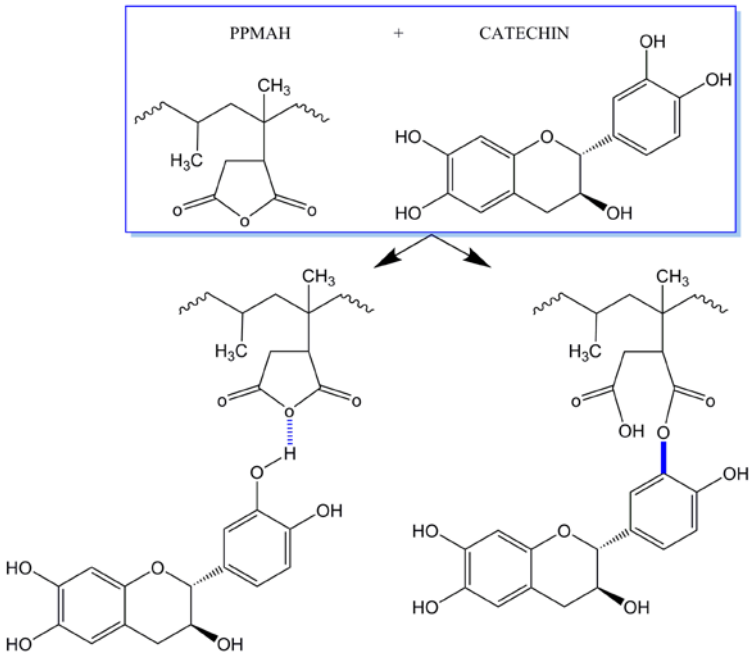

Figure 8.3. Proposed molecular interaction between catechin and maleic anhydride-grafted polypropylene.

Besides, the formation of a small fraction of ester groups as a result of a chemical reaction between a fraction of the hydrolyzed anhydride and catechin OH groups is not discarded, especially in the former blends. Anyway, not only the content but the strength of intermolecular hydrogen bonding between catechin and PPMAH may be lower than expected due to the high crystallinity of i-PP (isotactic polypropylene). Nevertheless, the described interactions, together with changes in the morphology of the polymer matrix, explain the reduction of 'free` available catechin molecules, as shown in the following sections.

3.2. Thermal characterization

The results of TGA analysis revealed that catechin incorporation slightly improved thermal stability of polypropylene whereas when added with maleic anhydride modification significantly increased degradation temperatures. In the derivative weight loss curves of catechin-loaded PP (Figure 8.4), the maximum degradation rate always shifted to higher temperatures, with respect to neat PP (T_{max}= 473 ºC) and the largest increased being observed for the sample with the highest maleic anhydride content. In previous works, it was already described how the incorporation of catechins and green tea extract (natural extract composed basically by catechins) increased the thermal stability of EVOH copolymers considerably, possibly because of the better affinity, which is due to the hydrophillicity of EVOH copolymer (López de Dicastillo et al., 2010; 2011; 2012). PPMAH, normally used as compatibilizer between PP and polar compounds, probably enhanced interaction between catechin and PP matrix, improving thermal stability and

increasing maximum degradation temperatures. Furthermore, the presence of maleic modified polymer did not induce additional degradation process, and values of degradation temperatures for reference materials without catechin were pretty similar to PP (471 and 473 ºC for PPMAH511 and PPMAH203, respectively).

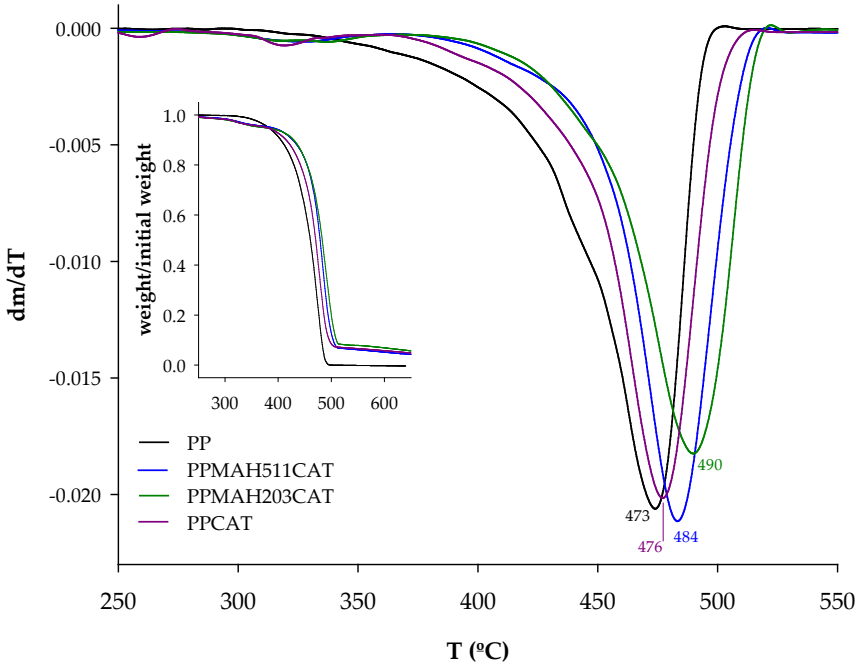

Figure 8.4. Weight loss and derivative of the weight loss of developed materials.

DSC analysis is usually used to provide some evidence of miscibility of polymer blends according to physical phenomena, such as glass transition, melting and crystallization. Table 8.1 summarizes the significant thermal

properties. Differences were not extremely relevant, even though, some small details are presented. First of all, Tc values are increased by both the presence of MAH- grafted-PP and catechin. The highest shift is observed for the composite including the 'highest` maleic content and catechin, PPMAH203CAT, together with a decrease in ΔH_c. These results are in accordance with other studies related to the use of PPMAH as compatibilizer, which higher T_c of the compatibilized systems may result from the enhanced interaction between the two phases (Pang et al., 2000). Besides, during the second heating process, the melting points and enthalpies of ternary blends PPMAH511CAT and PPMAH203CAT, slightly shifted to lower temperatures, which is probably to the fact that functionalized groups interrupted crystallization of the polymer chain.

Table 8.1. Information of developed materials obtained from thermal analysis and FTIR data.

Sample	*Cooling process*		*Second Heating process*		*FTIR data*
	T_c (ºC)	$\Delta H_{c,correg}$ (J/g)	T_m (ºC)	$\Delta H_{m,correg}$ (J/g)	$A_{998/973}$
PP	111.8 ± 2.5	101.4 ± 3.3	166.3 ± 0.3	79.2 ± 1.0	0.82 ± 0.02
PPMAH511	114.7 ± 3.1	107.6 ± 4.9	164.9 ± 0.3	83.2 ± 1.1	0.85 ± 0.03
PPMAH203	114.4 ± 2.9	98.3 ± 2.1	164.3 ± 0.4	78.5 ± 2.0	0.84 ± 0.02
PPCAT	115.6 ± 2.1	101.0 ± 1.7	165.1 ± 0.6	81.5 ± 1.9	0.91 ± 0.05
PPMAH511CAT	114.9 ± 3.5	103.2 ± 0.4	164.5 ± 0.4	79.6 ± 2.8	0.91 ± 0.02
PPMAH203CAT	119.4 ± 2.6	96.3 ± 3.1	163.8 ± 0.4	79.5 ± 1.7	0.89 ± 0.01

Normally, the reduction in melt and crystallization capacities and melting points of blends is attributed to the strong intermolecular interaction between catechin and MAH-grafted PP and the presence of catechin disturbs the crystallization of the polymer. Higher T_c values also indicated that polymer chain movements are more restricted, probably due to hydrogen-bonding interactions between catechin hydroxyls and MAH groups. Nevertheless, no clear increase in crystallinity is evidenced in catechin-loaded PP in relation with reference samples, as occurred in previous works dealing with antioxidant-incorporated polymers (Pracella et al., 2010; López de Dicastillo et al., 2011). These results are not consistent with FTIR data obtained. This inconsistency may arise from the fact that DSC data were calculated from the cooling and the second heating process, which may have changed crystallization conditions, with respect to compression molding.

OIT results (4.20±0.05 min for PP, 8.18±0.02 min for PPMAH511, 7.15±0.27 min for PPMAH203, 39.73±3.08 min for PPMAH511CAT, 33.25±1.17 min for PPMAH203CAT, and 35.16±1.50 min for PPCAT) showed that the incorporation of catechin increased the OIT values for all materials considerably. The presence of maleic anhydride groups in the blend leads to greater protection of the materials, since higher OIT values were observed, compared to neat PP. It may be an indication of catechin that has been retained on polymer matrix being available to serve as protection (Al-Malaika, 2003). The thermal stability of PP doped with synthetic antioxidants was previously measured and the PPMAH203 and PPMAH511 films doped with catechin showed longest OIT values than that PP doped with synthetic antioxidants, which confirmed that these compounds provided polypropylene with highest stabilization against thermal-oxidation (Castro-

López et al., 2012). Moreover, standard deviations of OIT values showed that there are more variations when modified polypropylenes doped with catechin are considered. The clarification of these values is a complex subject, because they are reliant on the appearance of degradation species, according to Thörnblom et al., 2011.

3.3. Catechin release

The migration profiles of the three studied films, expressed as the percentage of catechin released into the food simulant per unit of time, are compared in Figure 8.5.

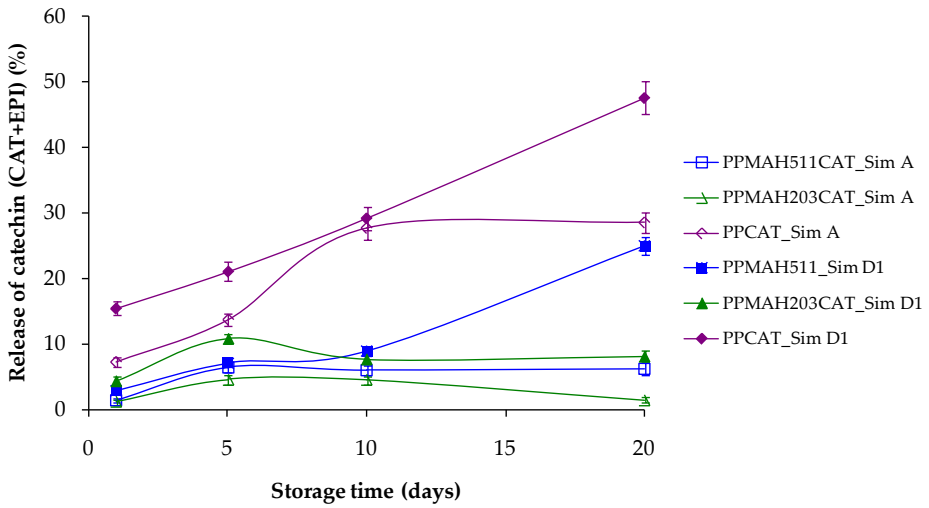

Figure 8.5. Migration of catechin from PP-grafted films (PPMAH511CAT, PPMAH203CAT) compared to pure PP film (PPCAT) at 40 ºC during an analysis period of 20 days.

The initial added amount of catechin was considered to calculate the percentage of catechin and epicatechin released. Although quantification of catechin retained into the films was attempted by means of extraction with both decahydronaphthalene and microwave extraction with different solvents, very low recoveries values were obtained (< 50%). Therefore, quantification data were not considered to be appropriate. It is in concordance with the work of Croptom, 2007, who reported that direct determination of such additives in polymers and their extraction with solvents are not always possible, because of possible spectral interferences from other additives, low relatively molecular weight mass matrix oligomers, or the extraction solvents, among others.

Moreover, released catechin data are expressed as the sum of catechin and epicatechin because epimerization was observed when films were exposed to mild and high temperatures (Wang and Helliwell, 2000).

Large differences in the rate and amount of migration were observed; these differences were dependent on the type of PP used, grafted films versus nongrafted films, and on the type of food simulant. When nongrafted PP films are exposed to 40 ºC, catechin concentration in simulant increased gradually according to its release from the film. After 20 days of storage, the antioxidant concentration in simulant D_1 (50% ethanol) reached 50% of the nominal content, but only 30% in simulant A (10% ethanol). These data mean that half of the incorporated catechin has been released after a short storage time in food simulant D_1. Conversely, the use of grafted PP has notably changed the ability of the polymer to release catechin into the food simulant over time: the amount of catechin released decreased with increasing the

degree of grafted PP. In fact, compared to the migration from PP films, the amount of catechin released from both MAH-grafted-PP films decreased approximately between 2-fold and 5-fold into Simulant D_1, and 2-fold to 20-fold into Simulant A, depending on the contact time.

There was also a large difference in the migration of catechin between the two food simulants A and D_1. As expected from catechin solubility parameters, the release of catechin into aqueous food simulant (simulant A) was significantly lower than the release into fatty food simulant (simulant D_1). At room temperature, catechins are slightly soluble in water (2.26 g L^{-1}) but highly soluble in ethanol (50 g L^{-1}) (Srinivas et al., 2012).

On the other hand, time and temperature also showed a significant effect with regard to the release of catechin from materials into food simulants. In Figure 8.5, the time dependence is clear: the higher the contact times, the broader the difference between grafted and non grafted samples. Regarding the effect of temperature, the antioxidant concentration released into both simulants was significantly smaller at 25 ºC than at 40 ºC (see Figure 8.6). This result can be related to an increase in vibration and motion of polymers chains as temperature increased, favoring the migrant movement through amorphous zones of the polymer (Galotto et al., 2011).

3.4. Antioxidant activities of the materials

The extent of the antioxidant activity can be characterized by the partition coefficient, *K*, which is defined as the ratio of the amount of catechin in the polymeric phase, relative to the concentration of released catechin. The *K*

values, which are displayed in Figure 8.7, show the influence of both the type of solvent where the radicals are immersed and the morphology of the evaluated systems

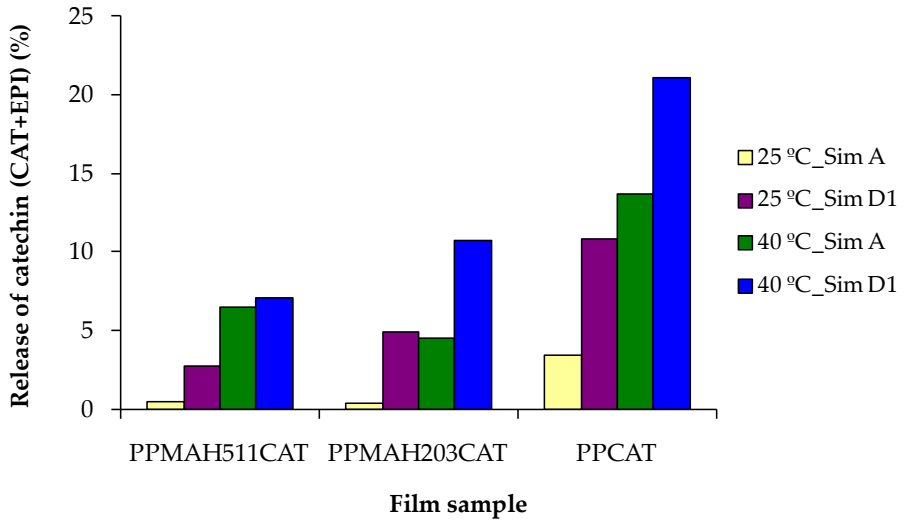

Figure 8.6. Catechin released (expressed as sum of epimers catechin and epicatechin) after 20 days of storage.

As Figure 8.7 shows, PPMAH blends presented higher values of *K*; the immobilization of catechin through the interaction between maleic anhydride groups in the PPMAH polymers inhibited its release, decreasing the antioxidant activity. Differences in the value of *K* values were also noticeable between methods, because of the limited solubility of catechin in water. Antioxidant activity was higher for the DPPH assay ($K < 100$), because of the higher solubility of catechin in ethanol.

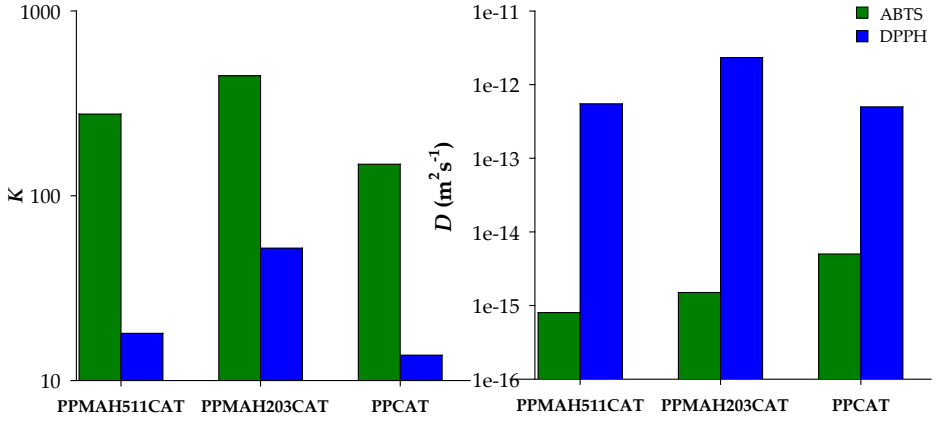

Figure 8.7. Partition and diffusion coefficients of catechin (*K* and *D*, respectively)of developed materials for ABTS and DPPH assays.

The kinetics of the antioxidant activity as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavengers followed similar profiles for the different materials. From a kinetics point of view, the antioxidant activity is dependent on the release of catechin into the radical-containing solution, which is further dependent on the diffusion of the migrant through the polymer matrix and the extent of the process. The diffusion coefficient (*D*), as defined in Fick´s laws, is commonly used to characterize the kinetics of transport in polymeric matrices and reports about material morphologies (Crank, 1975; López de Dicastillo et al., 2011).

The *D* values that best fitted the experimental results for both antioxidant assays in all evaluated systems are plotted in Figure 8.7. *D* values were calculated using the equation that yielded the best fit to experimental data. For the ABTS assay, there was good agreement between the theoretical values and the experimental antioxidant activities applying equation 1. Conversely,

equation 1 did not describe the experimental results obtained from the DPPH assay. The model applicable to the antioxidant activities in an extraction process clearly fit better. Equation 3 is the Fickian solution to this case, in which diffusion is the only variable controlling the process:

$$\frac{m(t)}{m_S^f} = \left[1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\left(\frac{-D(2n+1)^2\pi^2 t}{l^2}\right)\right] \qquad (3)$$

It is obvious from Figure 8.8 that the extraction model described the catechin release from the designed materials to the DPPH solution better than the partition model. Similar behavior was described by Iñiguez-Franco et al., 2012, who reported on diffusion studies of catechin and epicatechin from PLLA. When materials were exposed to 95% ethanol, the systems were considered with an infinite simulant volume, because the release of the antioxidants was almost complete, and equation 3 provided a better fit to obtain *D*. These findings indicated that, in DPPH tests, a large proportion of catechin added to the polymer formulation was extracted during the test; by contrast, the molecules remaining in the polymer were not available for transport. That is to say, they have been immobilized within the polymer matrix. The remaining catechin fraction could only be released during a extraction process in a mixture of dichloromethane and methanol, and even so, not completely. Figure 8.8 shows the curves obtained using the *K* and *D* values determined from Figure 8.7.

Other active films using different antioxidants have been studied in previous papers. The levels of antioxidant added to these films are lower than

catechin levels used in the present study. Therefore, antioxidant activities also are minor (Sonkaew et al., 2012).

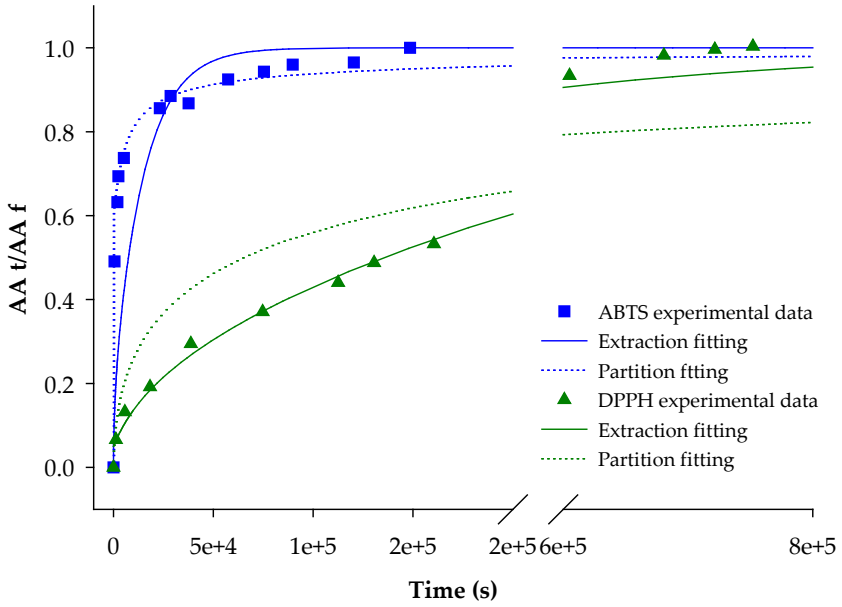

Figure 8.8. Antioxidant activity of catechin from PPMAH511CAT material as ABTS and DPPH radical scavengers: symbols represent experimental data, and lines are values predicted using equation 1, in the case of partition model for ABTS data assay, and equation 3, in the case of extraction model for DPPH method, both calculated with *K* and *D* values indicated in Figure 8.7.

4. CONCLUSIONS

An improvement on thermal stability and a reduction of catechin release levels is shown by films with maleic anhydride (MAH)-modified

polypropylene (PP). This behavior has been related to interactions between catechins and MAH groups.

Therefore, considering real food packaging applications, catechin loaded MAH-grafted PP materials have been proven to be effective systems for controlled release of the antioxidant during longer periods of time, with catechin also being available in the film formulation to protect it from aging (or its own degradation). Besides, with this system, the great release of the active compound during the first days is avoided, with the remaining additive in the polymer being intended for greater protection of both the polymer and food.

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CAPÍTULO IV. CONCLUSIONES

A continuación se recogen las conclusiones más destacables que se pueden deducir de esta tesis doctoral ordenadas de acuerdo a los objetivos específicos:

- **Determinación y cuantificación de antioxidantes naturales**

  - El empleo de muestras aciduladas en lugar de fases móviles aciduladas ha mejorado la identificación, separación y cuantificación simultánea, rápida y eficiente de siete catequinas y quercetina en muestras naturales por empleo de HPLC-PDA-FL, UPLC-PDA y MEKC-PDA como sistemas cromatográficos, salvaguardando, al mismo tiempo, la integridad de la columna C18 cuya degradación podría verse comprometida por el uso de altos volúmenes de ácido en la fase móvil.

  - El empleo de UPLC como técnica cromatográfica gracias a la posibilidad de poder soportar altas presiones debido al uso de rellenos de pequeño tamaño de partícula en la columna cromatográfica, ha permitido reducir 6 veces el tiempo de análisis de catequinas y quercetina con respecto a los tiempos obtenidos con HPLC sin perder resolución y sensibilidad en el método.

  - Cada una de las metodologías analíticas estudiadas, incluyendo HPLC-LTQ Orbitrap, ha resultado adecuada para la detección y cuantificación de las catequinas y quercetina en muestras naturales complejas sin necesidad de la realización de tratamientos previos de purificación y limpieza de los extractos, al

proporcionar sensibilidades y resoluciones adecuadas. El empleo de fluorescencia como sistema de detección ha permitido disminuir hasta 3 veces los límites de detección y cuantificación y aumentar de 2 a 3 órdenes de magnitud la sensibilidad de la metodología, lo que posibilita el análisis de catequinas y quercetina a niveles traza. El uso de HPLC acoplado al detector de masas ha resultado conveniente para la detección de otros compuestos de interés (cafeína, teobrominas, compuestos metilados) en las muestras naturales estudiadas.

- **Purificación de extractos naturales**

  - Se ha desarrollado un polímero impreso molecularmente mediante polimerización por precipitación en disolución, utilizando quercetina como molécula molde, 4-vinilpiridina y etilenglicol dimetacrilato como monómeros funcional y entrecruzador, respectivamente, y acetona/acetonitrilo 3:1 como porogen. Se ha aplicado a la extracción selectiva en fase sólida (limpieza y enriquemiento) de catequinas presentes en matrices vegetales complejas, alcanzándose factores de concentración de hasta 50 veces sin producirse la rotura del MIP, y la eliminación de sustancias que no interesan presentes en diversas muestras vegetales como la cafeína o los tocoferoles. El polímero no impreso, sintetizado de igual modo que el MIP pero en ausencia de molécula plantilla, se ha empleado para demostrar el efecto impronta en el MIP.

- La caracterización físico-química de los sitios de unión mediante el estudio de las isotermas de adsorción y la cinética del proceso han indicado una rápida adsorción en una superficie homogénea del MIP, con una monocapa de adsorción y mediante un proceso que podría ser asimilado a la quimisorción; con la difusión externa de film como paso controlador del mecanismo con una extremadamente rápida adsorción en los sitios más disponibles en la superficie externa del MIP y una adsorción más gradual adscrita al proceso de difusión intrapartícula. La caracterización morfológica ha permitido concluír la existencia de ciertas cavidades presentes en el MIP, frente a una superficie lisa en el NIP, con una estructura macroporosa y un grado de polimerización entre el 70 y el 80%.

- **Adición de antioxidantes naturales a muestras poliolefínicas**

  - Se han obtenido películas extruidas de PP con los antioxidantes naturales: tocoferol, catequinas y extractos de té verde, los cuales han proporcionado una alta protección al polímero frente a la degradación oxidativa (entre 10 y 30 veces mayor resistencia que los polímeros sin antioxidantes naturales y similar protección que la proporcionada por otros antioxidantes comerciales comúnmente empleados en poliolefinas).

- **Desarrollo de materiales capaces de realizar una cesión controlada de antioxidantes**

  - La incorporación de extensores de cadena/plastificantes como modificadores de la matriz polimérica de polipropileno ha permitido modificar las propiedades de cesión de antioxidantes desde las películas modificadas (incrementos de hasta 30 veces de la cantidad de antioxidante liberado). La cesión de los antioxidantes a los diferentes simulantes se desarrolla por un proceso de transferencia de masa, gobernado por el tipo de antioxidante, su concentración y su solubilidad en el simulante alimenticio y por el tipo de extensor de cadena y su concentración, así como por las condiciones de tiempo y temperatura del proceso de contacto. Valores mayores de cantidad de agente activo, extensor, contenido etanólico de simulante, tiempo y temperatura han favorecido la liberación de tocoferoles y, especialmente catequinas desde el polímero (incrementos medios de hasta 20 veces de cantidad de compuesto liberado), indicando la posibilidad del uso de los sistemas desarrollados para la protección durante el tiempo de almacenaje y uso del polímero y del alimento envasado.

  - Se han desarrollado satisfactoriamente películas de mezclas de polipropileno y polipropileno modificado superficialmente con anhídrido maleico, utilizando catequina como agente activo (extrusión reactiva) para el control y retardo de la liberación de antioxidante desde el polímero al alimento. El estudio de las interacciones existentes entre catequina y el polipropileno

funcionalizado así como los ensayos de liberación de catequina demostraron la existencia de una relación directa entre las interacciones de los grupos hidroxilo de la catequina y el anhidrido maleico del polipropileno modificado, con la mayor retención de catequina en los estadíos iniciales del proceso de contacto envase-alimento, quedando así el antioxidante disponible para una mayor protección del polímero y alimento en etapas posteriores de su vida útil.

- El estudio de las propiedades térmicas de los materiales desarrollados revelaron que la incorporación de catequina como agente antioxidante ha mejorado la estabilidad térmica del polímero. Y no se han observado cambios relevantes en la temperatura de transición vítrea, el fundido o la cristalinidad del mismo producidos por las modificaciones químicas de la matriz polimérica.

ANEXOS

Anexo 1: Portada publicaciones.

Anexo 2: Contribuciones a congresos.

Anexo 1

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Development, validation and application of Micellar Electrokinetic Capillary Chromatography method for routine analysis of catechins, quercetin and thymol in natural samples

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ABSTRACT

Natural matrices such as several vegetables, fruits or beverages are the origin of different natural antioxidants with great interest to be used instead of synthetic antioxidants in several applications, as a result of their health-promoting and disease-preventing properties. In this work, a capillary electrophoresis method capable of separating and quantifying some of these antioxidants (seven catechins with the same or similar molecular weight and relation q/z) is showed. Short analysis time and precision parameters equivalent to those obtained by liquid chromatography were obtained. This method was developed using the micellar electrokinetic chromatography (MEKC) mode with ultraviolet-visible detection. Quality parameters were established, obtaining low instrumental detection and quantification limits (0.6–2.0 mg L^{-1} and 2.0–6.5 mg L^{-1}, respectively), good precision (relative standard deviation in the intermediate repeatability lower than 7% for every compound) and short analysis time. The developed method also showed good performance for the determination of two more natural antioxidants, quercetin and thymol. The applicability of the method to the analysis of catechins in several natural samples was tested.

1. Introduction

In recent years, trade growth of functional foods has increased the interest in compounds with health-promoting or disease-preventing properties, such as cancer-fighting properties. Due to their important antioxidant capacity neutralizing the damage caused by free radicals, the interest in polyphenolic compounds, especially those of natural origin, has been increased [1,2]. Their possible use instead of synthetic antioxidants, mainly as a consequence of the toxicity of some of these ones [3–6], has revealed their possible application in several fields, such as in the pharmaceutical industry [7–9], active and intelligent food packaging [10–13] or as additives in functional foods [14].

The strong antioxidant capacity of catechins has been reported to have a protective and beneficial health effect related to antimutagenic, antidiabetic, anti-inflammatory qualities and prevention against several kinds of cancer [8,15–17]. Several biological matrices such as tea [18], cocoa [7], fruits (berries, citrus fruits, apples, pears), vegetables (aubergine, chicory, onion, potato, parsley) [19], alcoholic drinks (wine [20], beer [21]), juices [19]) and herbs and spices [22] present considerable concentration of these phenolic compounds. Among them, tea samples, especially green and white varieties, contain high concentration of major and minor catechins. The amount present in red and black tea has been reported as being lower as a result of fermentation that occurs in their manufacture [7,19,22]. During this procedure some catechins undergo enzymic oxidation producing a range of polyphenolic compounds which include theaflavins, bisflavanols and several pigments of thearubigen class, which results in the degradation of those catechins [23,24].

Interest in exploitation of natural extracts to obtain and purify natural antioxidants depends on net and relative content of these compounds with different antioxidant activities.

This interest has led to the development of new analytical procedures able to handle even the most complex matrices in which these compounds are detected. Several techniques have been reported in the literature for the determination of catechins. High performance liquid chromatography has been the most used on the basis of its simple mode of operation, high applicability to trace

Abbreviations: C, (+)-Catechin hydrate; CG, (−)-Catechin Gallate; CMC, critical micellar concentration; EC, (−)-Epicatechin; ECG, (−)-Epicatechin gallate; EGC, (−)-Epigallocatechin; EGCG, (−)-Epigallocatechin gallate; GCG, (−)-Gallocatechin Gallate; Quer, Quercetin; SB, standard deviation of the blank; Thy, Thymol; yB, blank signal.

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Anexo 2

Natural Extracts as Potential Source of Antioxidants to Stabilize Polyolefins

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ABSTRACT: Several natural matrices were investigated as potential sources of antioxidants to be used as plastic additives. Extracts of four matrices obtained under the same experimental conditions were initially considered: green tea, black tea, *Lippia citriodora* and *Hypericum androsaemum*. Both, the antioxidant activity of the extracts and their content in flavanols and quercetin, were compared. The antioxidant activity was determined by DPPH analysis and the phenolic composition by high performance liquid chromatography (HPLC) using ultraviolet (UV) diode array and fluorescence (FL) detectors. Concentration of the flavanols reduced in the same way as their antioxidant activity starting with green tea, through black tea, *Hypericum androsaemum*, and *Lippia citriodora*. The performance of polypropylene samples stabilized with green tea extract, or its individual components catechin and epicatechin, was compared with samples stabilized with a mixture of the synthetic antioxidants Irganox 1076 and Irgafos 168. Each sample was extruded and consecutively reextruded up to four times. The melt flow index (MFI) and the oxidation induction time (OIT) of the samples were measured after each step. The obtained results showed the interest of this natural matrix as a potential source of antioxidants for plastics. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 3553–3559, 2011

Key words: antioxidants; green tea; high performance liquid chromatography (HPLC); poly(propylene); (PP); stabilization

INTRODUCTION

Polymers, and especially polyolefins, need the addition of antioxidants in their formulations to provide protection during processing or fabrication into finished product.[1] Chain breaking antioxidants, sometimes referred to as primary antioxidants, interrupt the first degradation cycle by removing the polymer propagating radicals ROO•. Preventive antioxidants, sometimes referred to as secondary antioxidants, interrupt the second oxidative cycle by preventing or inhibiting the generation of free radicals. The most important preventive mechanism is the nonradical hydroperoxide decomposition. Hindered phenols and phosphite esters are important classes of primary and secondary antioxidants, respectively. Because of their complementary antioxidant mechanisms, they are generally used in combination to ensure both highly efficient melt stabilizing systems and long term stability at high service temperatures.[2,3]

The antioxidants and other additives that can be used in the manufacture of plastic materials and articles intended to come into contact with foodstuffs are included in a list of additives established by Directive 2002/72/EEC.[4] During processing or storage additives or their degradation products could migrate from plastic packaging into foodstuffs; therefore, their migration is also regulated by European legislation[4] through Specific Migration Limits (SMLs). In the last years, instead of the synthetic antioxidants usually employed natural antioxidants

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Preparation, evaluation and characterization of quercetin-molecularly imprinted polymer for preconcentration and clean-up of catechins

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ABSTRACT

Molecularly imprinted polymer (MIP) for solid extraction and preconcentration of catechins have been successfully prepared by a thermal polymerization method using quercetin as template, 4-vinylpyridine as functional monomer and ethylene glycol dimethacrylate as crosslinker. A solution mixture of acetone and acetonitrile was used as porogen. Systematic investigations of the influence of monomer, crosslinker, porogen, as well as polymerization conditions on the properties of the MIPs were carried out. The quercetin MIPs were evaluated according to their selective recognition properties for quercetin, structurally related compounds (catechin, epigallocatechin gallate and epicatechin) and a unrelated compound of similar molecular size (α-tocopherol). Good binding was observed for quercetin, catechin and epigallocatechin gallate with an optimized MIP in a solid phase extraction system. Adsorption and kinetic characteristics were evaluated for catechins which indicated that the synthesized polymer had high adsorption capacity and contained homogeneous binding sites. Chemical and morphological characterization of the MIP was investigated by FTIR, SEM and BET, which confirmed a high degree of polymerization. Finally, the MIP was successfully applied to the clean-up and preconcentration of catechins from several natural samples.

1. Introduction

Over the last years, molecularly imprinted polymers (MIPs) have been used as chromatographic media, sensors, artificial antibodies, catalysts and specially in solid phase extraction (SPE) due to their high affinity and selectivity towards a specified analyte [1,2]. Low cost, storage stability, high mechanical strength, robustness, resistance to a wide range of pH, solvents and temperatures are shown as their main advantages. Therefore, their use for the treatment of complex matrices, environmental samples or food samples has rapidly become widespread [1,3,4].

MIPs are synthetic receptors with several generated recognition sites which are able to specifically rebind a particular target molecule (template). These recognition sites are created in situ by a copolymerization process among functional monomer, an excess of crosslinker and template molecule. Prior to polymerization, self-assembly between the template and the functional groups of the appropriate functional monomer takes place in solution. Depending on the type of interactions involved both during pre-polymerization and template-functional monomers rebind processes, molecular imprinting can be classified as covalent, non-covalent or semi-covalent, involving a pre-organized or a self-assembly approach, respectively [1,5].

After polymerization, template molecules are removed from the polymer network, leaving complementary sites in terms of size, shape and functionality. Therefore, the final network should exhibit significantly higher affinity and selectivity for the template than for closely related structures.

Although bulk represents the conventional and the most popular approach to synthesize the MIP, crushing, grinding, sieving and time-consuming as well as the irregular size and shape obtained, have led to develop alternative methods. Multi-step swelling methods, suspension polymerization or precipitation polymerization have been recently developed as alternatives [3].

Flavonoids, especially the group of catechins have been reported as responsible for several beneficial health effects due to their

Abbreviations: EC, (−)-epicatechin; EGCG, (−)-epigallocatechin gallate; Quer, (−)-quercetin dihydrate; C, (+)-catechin hydrate; TRIM, 1,1,1-tris(hydroxymethyl)-propantrimethacrylat tech; AIBN, 2,2′-Azobis(2-methylpropionitrile); 4-Vpy, 4-vinylpiridine; α-TOCO, alpha-tocopherol; BJH, Barret–Joyner–Halenda method; BET, Brunauer–Emmett–Teller method; Caff, Caffeine; DC, degree of monomeric conversion; K_d, distribution coefficients; EGDMA, ethylene glycol dimethacrylate; prel, initial imprinting value for the template during the synthesis step; *I*, imprinting factor; MAA, methacrylic acid; MIPs, molecularly imprinted polymers; NIPs, non-imprinted polymers; SEM, scanning electron microscope; S, selectivity factor; SPE, solid phase extraction; MISPE, SPE involving a molecular imprinted polymer; T:M:Cr, template:monomer:crosslinker ratio.

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Effect of PPG-PEG-PPG on the Tocopherol-Controlled Release from Films Intended for Food-Packaging Applications

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Supporting Information

ABSTRACT: The feasibility of novel controlled release systems for the delivery of active substances from films intended for food packaging was investigated. Because polyolefins are used highly for food-packaging applications, the reported high retention degree of antioxidants has limited their use for active packaging. Thus, in this study, PP films modified with different chain extenders have been developed to favor and control the release rates of the low molecular weight antioxidant tocopherol. The use of different chain extenders as polymer modifiers (PE-PEG M_w, 575; and PPG-PEG-PPG M_w, 2000) has caused significant changes in tocopherol-specific release properties. High-performance liquid chromatography coupled to PDA-FL and PDA-MS was used to test tocopherol and chain extender migration, respectively. The release of tocopherol from the prepared films with two chain extenders into two food simulants was studied. Different temperatures and storage times were also tested. Varying the structural features of the films with the incorporation of different levels of PPG-PEG-PPG, the release of tocopherol (food-packaging additive) into different ethanolic simulants could be clearly controlled. The effect of the temperature and storage time on the release of the antioxidant has been outstanding as their values increased. The migration of the chain extender, also tested, was well below the limits set by European legislation.

KEYWORDS: *PPG-PEG-PPG, tocopherol, controlled release*

1. INTRODUCTION

Traditionally, food packaging provides protection against contamination by external agents such as water, light, or odorants; however, increasing demands for greater safety and quality have led to the development of new concepts in food packaging.[1] Active and intelligent packagings are intended to prevent or retard any deterioration quality of packaged foods by including the concept of the controlled release of active compounds to foodstuffs. Thus, they show a great potential to improve storage stability without adding an excess of additives to food,[1−3] which could also cause neutralization or rapid diffusion into the bulk of food.[4]

Polymeric materials react with oxygen, producing chemical aging or degradation of the polymer, which may be associated with irreversible changes in their chemical structure such as reduction in molecular weight, increased melt flow index, and worsened physical and mechanical properties.[5,6] Therefore, the use of stabilizers against mechanical and thermo-oxidative phenomena is a key factor to preserve polymer physical and mechanical properties over time.[7,8]

Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), or Irganox 1076, have been used extensively.[9,10] Their low molecular weight also makes them suitable for migration from the package into foodstuffs. Nevertheless, there has been an emerging concern regarding their possible toxicity and carcinogenic potential.[9−12]

In short, antioxidants are widely used in plastic formulations both to protect the film from degradation and to improve the oxidation stability of food lipids.[13,14] They can prolong the food shelf life since they can be released in a controlled manner from the antioxidant active packaging into the food.[2]

An increasing interest in the application of natural antioxidants such as tocopherol, carnosic acid, oregano, savory, and essential oils, carvacrol or hydroxytyrosol,[15] has been developed recently. Besides being effective antioxidants for reducing oxidation in foods, tocopherols are also excellent stabilizers for polymer processing since they have proved to be very stable under processing conditions and very soluble in polyolefins.[16,17] Therefore, tocopherols could serve dual functions when added to packaging: as a stabilizer for polymer processing and as an antioxidant in controlled release to reduce oxidation.[3] They are also nontoxic compounds with a positive public perception classified as substances generally recognized as safe (GRAS) for intended use in food.[18]

Low-density polyethylene (LDPE) and, especially, polypropylene (PP) are two of the most commonly used polymers in packaging applications involving contact with food.[19] The use of antioxidants is especially essential to preserve PP due to its numerous tertiary carbons, which are very sensitive to oxidation and radical degradation.[7,20] However, a high retention degree of tocopherols in both LDPE and PP in contact with foodstuffs and food-simulating liquids has been reported, being practically total in the latter.[19,21,22] Thus, the use of tocopherols as additives for active PP packaging materials may be limited. In

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Anexo 6

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Improving the Capacity of Polypropylene To Be Used in Antioxidant Active Films: Incorporation of Plasticizer and Natural Antioxidants

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ABSTRACT: Two types of active antioxidant food packages with improved release properties, based on polypropylene (PP) as one of the most common polymers used in food-packaging applications, were developed. Incorporation of catechin and green tea as antioxidant provided PP with 6 times higher stabilization against thermal oxidation. Release of natural antioxidants (catechins, gallic acid, caffeine, and quercetin) into various food simulants from that nonpolar matrix were improved by blending poly(propylene glycol)-*block*-poly(ethylene glycol)-*block*-poly(propylene glycol) (PPG-PEG-PPG) as plasticizer into the polymer formulation. Increasing release levels between 10- and 40-fold into simulant A and between 6 and 20-fold into simulant D1 resulted from the incorporation of catechin and green tea as antioxidants and PPG-PEG-PPG as plasticizer into the film formulation. The efficiency of the antioxidants in the food simulants after the release process was also corroborated through antioxidant activity tests. Therefore, the developed PPG-PEG-PPG-modified polypropylene resulted in a potential system to be used in active packaging.

KEYWORDS: *active packaging, antioxidant, PP, PPG-PEG-PPG, green tea, flavonoids*

INTRODUCTION

Oxidative processes and microbial spoilage are primary causes for the deterioration of food quality. Traditionally, besides containment, convenience, and communication,[1] packaging also provides protection against possible contamination caused by external agents such as water, light, or odorants. However, increasing safety and quality demands have led to the development of new alternatives in the food-packaging industry. Active packaging with controlled release of active compounds to foodstuffs has emerged as a promising technology.[1−3] Among them, antioxidant active packaging, in which an antioxidant is incorporated into the polymer to be released into the packaged foodstuff, provides a continuous antioxidant effect to prevent lipid oxidation and avoids its rapid depletion compared with its direct addition to food,[1−4] extending the packaging's shelf life.[5,6]

Synthetic antioxidants such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA)[7−9] traditionally used in packing have been replaced by natural preservaties due to safety concerns.[7,10,11] α-Tocopherol,[5,12−18] carvacrol, and aromatic plant extracts such as oregano or barley, among others,[13,19−21] have been used. Nowadays, polyphenols such as catechins have also aroused high interest as natural antioxidants, being present in several species of the plant kingdom, especially tea.[22−24] Flavonols such as quercetin (Quer) and other compounds such as caffeine (Caff) are, as well, important constituents of tea, also providing it with antioxidant and mood-cognitive-enhancing properties, respectively.[22]

According to their molecular weight and their nonvolatile character, those compounds should be likely to be able to diffuse between the packaging material and the food product and/or partition at the interface when they are used in active food packaging. Recently, some research related to the incorporation of catechins, quercetin, or caffeine as active agents to active polymer packaging has been developed on the basis of polyethylene terephthalate (PET),[25] ethylene vinyl alcohol (EVOH),[26−28] or biodegradable materials such as polylactic acid (PLA).[29−31] Nevertheless, those reported active packagings with hydrophilic and/or biodegradable polymers are intended only for short shelf life products, besides not being as widely used as low-density polyethylene (LDPE) and polypropylene (PP) in food-packaging applications.[16] No important developments of active packaging with those latter polymers have been reported, though, which could be attributed to the few release capacities of catechins or quercetin reported from those polymers despite their highly polar nature. This capacity was then more limited toward the release of lower molecular weight compounds such as caffeine or gallic acid or the release in contact with food simulants of very highly ethanolic content (95%).[25−32]

Some additives such as plastizicers can be used to modify polymer properties, especially workability, flexibility, and extensibility of the polymer. Plasticizers have been blended into polymer matrices to modify polymeric physical characteristics, which lead to enhanced physicochemical polymer properties such as stability, degradability, or permeability. For example, PPG, PEG, or their copolymers have been reported as potential plasticizers into film formulations providing polymers, specially polyesters, with higher biocompatibility and degradation rates, and, thus, modifying their properties.[33,34] Based on

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Interaction and Release of Catechin from Anhydride Maleic-Grafted Polypropylene Films

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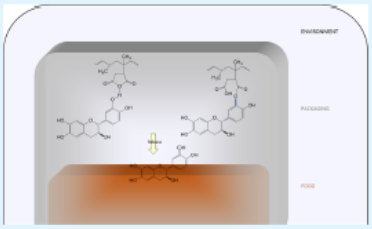
ABSTRACT: In this paper, investigations were carried out on catechin-loaded maleic anhydride (MAH)-modified polypropylenes (PP). Two maleic-modified polypropylenes (PPMAH) with different maleic concentrations have been blended with PP and catechin to obtain composites of improved catechin retention with the aim of studying the possible interactions between these grafted polymers with antioxidants, and a secondary interest in developing an active antioxidant packaging. Composite physicochemical properties were measured by thermal analysis (thermogravimetric analysis (TGA), differential scanning calorimetry (DSC), and oxidation induction time (OIT)) and infrared spectroscopy studies. Catechin release profiles into food simulants were obtained by HPLC-PDA-QqQ, following European legislation. Antiradical activity of composites was analyzed by the ABTS and DPPH method. The formation of intermolecular hydrogen bonds between catechin and functionalized PP has been confirmed by Fourier transform infrared (FTIR) studies. Besides, a small fraction of ester bonds, formed as a result of a chemical reaction between a fraction of the hydrolyzed anhydride and the catechin hydroxyl groups, is not discarded. OIT results also showed an increase in antioxidant effectiveness caused by the presence of catechin- and maleic-modified PPMAH in the blend formulations. Incorporation of MAH-grafted PP increased substantially the retention rate of catechin, being dependent on the MAH content of the grafted polypropylene. The described interactions between catechin and maleic groups, together with changes in PP morphology in comparison with reference PP explained lower antioxidant release. Besides formulation, antioxidant release was dependent on the type of food, the temperature, and the time.



KEYWORDS: *maleic anhydride grafted polypropylene, natural antioxidants, catechin, active antioxidant packaging, release, immobilization*

INTRODUCTION

New polymer formulations are emerging, as far as new applications are considered. Despite the increasing demand for biodegradable materials, polyolefins are still the most common polymers used for food packaging. Polyolefins, similar to most synthetic polymers, have a hydrophobic and chemically inert surface which, therefore, leads to low adsorption of dyes or inks, poor adhesion to coatings or other materials, the generation of static electricity, incompatibility with hydrophilic substances, and many other problems. Among the techniques employed to modify polymers, grafting of polyolefins with polar monomers have received special attention, because of their potential applications, by which a variety of desired graft chains can be introduced onto the polymer surface without changing the bulk properties.[1−3] There are a wide variety of these materials, such as polyolefins with grafted maleic anhydride (MAH), fumarate and maleate esters, methacrylate esters, or methacrylic acid. These polymers often serve as precursors of other polyolefin graft copolymers.

Maleic anhydride (MAH)-modified polyolefins are one of the most important class of functionalized polyolefins in commercial applications, because of their low cost, high activity, and good processability. Free radical-induced grafting of MAH onto polyolefin substrates has been carried out in the melt phase in various forms of extruders and batch mixers, in solution, and in the solid state. In all cases, controversy arises concerning the final structure of the functionalized polymer, with respect to the nature of the grafted units and the distribution of the graft sites.[4−7] Maleic anhydride grafting onto polypropylene (PPMAH) has been carried out basically with the objective of achieving compatibility between polar and nonpolar polymers.[7−9] In addition, nowadays it is a proved alternative as a compatibilizer between PP and fibers or more hydrophilic materials in order to obtain "biocomposites". The

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Anexo 2

CONTRIBUCIONES A CONGRESOS

En contenido de esta tesis doctoral se presentó en formato de comunicación a los siguientes congresos nacionales e internacionales:

- **Determinación y cuantificación de antioxidantes naturales**

  - Autores: Castro-López, M.M.; Carballeira-Amarelo, T.; Noguerol-Cal, R.; López-Vilariño, J.M.; González-Rodríguez, M.V. Development of an HPLC-PDA-FL method for simultaneous determination of nine natural antioxidants. *VII Reunión Científica de la Sociedad Española de Cromatografía y Técnicas Afines*. Granada (España), 17-19 octubre de 2007.

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- **Purificación de extractos naturales**

  - Castro-López, M.M.; Cela-Pérez, M.C; Dopico-García, M.S.; Noguerol-Cal, R.; López-Vilariño, J.M.; Barral-Losada, L.F.; González-Rodríguez, M.V. Molecularly Imprinted Polymer for selective extraction and clean-up of catechins from natural samples. *MACRO2010: 43rd IUPAC World Polymer Congress Polymer Science in the Service of Society*. Glasgow (Reino Unido), 11-16 Julio 2010.

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- **Adición de antioxidantes naturales a muestras poliolefínicas**

  - Castro-López, M.M.; Carballeira-Amarelo, T.; Noguerol-Cal, R.; Dopico-García, M.S.; López-Vilariño, J.M.; González-Rodríguez, M.V. Catechins in polyolephins for food packaging. *4th International Symposium on Food Packaging-Scientific Developments supporting Safety and Quality*. Praga (República Checa), 19-21 de noviembre de 2008.

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polyolefins. *12th Internacional Electronic Conference on Synthetic Organic Chemistry (ECSOC-12)*. www.mdpi.net/ecsoc & www.usc.es/congresos/ecsoc, 1-30 de noviembre de 2008.

- **Desarrollo de materiales capaces de realizar una cesión controlada de antioxidantes**

  - Castro-López, M.M.; López de Dicastillo, C.; López-Vilariño, J.M.; González-Rodríguez, M.V. Effect of chain extension on release properties of polypropylene-tocopherol active food packaging. *5th International Symposium on Food Packaging. Scientific Developments, Supporting Safety and Innovation*. Berlín (Alemania), 14-16 de septiembre de 2012.

  - López de Dicastillo, C.; Castro-López, M.M.; López-Vilariño, J.M.; González-Rodríguez, M.V. Maleic anhydre modified polypropylene: New polymer formulations. *5th International Symposium on Food Packaging. Scientific Developments, Supporting Safety and Innovation*. Berlín (Alemania), 14-16 de septiembre de 2012.